UNIVERSITAT DE BARCELONA

Establecimiento de metodologías analíticas para la separación y caracterización de péptidos y proteínas de interés terapéutico

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Programa de Doctorado:

Química Analítica del Medi Ambient i de la Pol·lució

(Bienio: 1997-1999)

ESTABLECIMIENTO DE METODOLOGÍAS ANALÍTICAS PARA LA SEPARACIÓN Y CARACTERIZACIÓN DE PÉPTIDOS Y PROTEÍNAS DE INTERÉS TERAPÉUTICO

Memoria presenta por Fernando J. Benavente Moreno para optar al grado de Doctor en Ciencias Químicas.

Directores: Dr. José Barbosa Torralbo y Dra. Victoria Sanz Nebot.

Barcelona, Julio 2003

Divide et impera

A mis padres

Hace ya algún tiempo que venía pensando en cuáles serían las palabras que dedicaría a todas aquellas personas que de alguna forma han contribuido para que ahora mismo tú puedas estar leyendo estas palabras. Quizá este es uno de los momentos más agradables de todo el viaje, cuando llega el final y tienes que mirar hacia atrás para agradecer a todos los que te ayudaron a abrir este camino que se iba haciendo camino al andar. A todos ellos quiero darles mil gracias.

En primer lugar, quisiera agradecer al Dr. José Barbosa Torralbo, catedrático y director del departamento de Química Analítica de la Universitat de Barcelona, y a la Dra. Victoria Sanz-Nebot, profesora titular del mismo, el apoyo y los consejos recibidos durante todos estos años, así como la confianza que hace ya algún tiempo depositaron en mi al ofrecerme la oportunidad de formar parte de su grupo de investigación.

Quisiera dar las gracias a Vicki, la segunda persona que ha dedicado mayor esfuerzo a que todo esto llegara a buen puerto. Gracias por echarme una mano siempre que la he necesitado, por tus consejos y por tu apoyo durante todos estos años. Gracias por haberme soportado, realmente ha sido un placer trabajar a tu lado. También quisiera recordar de forma especial a Isabel Toro, Anna Vallverdú y Elvira Balaguer porque sin su duro trabajo esta tesis tampoco habría sido posible. Particularmente quisiera agradecer a éstas dos últimas el haberme acompañado durante buena parte del viaje, y haber compartido las mil historias que fueron ocurriendo, gracias por ser tan perseverantes, trabajadoras e inteligentes, y por vuestra paciencia infinita conmigo. A todas ellas les deseo toda la suerte del mundo. Me gustaría extender ese deseo a Elena que da sus primeros pasos por ese largo camino que tan pronto acaba como luego vuelve a comenzar.

Quisiera agradecer de forma especial a todos aquellos que me cobijaron, cediéndome su casa cuando estaba diluviando ahí afuera. Al Dr. Isidre Casals, siempre dispuesto a brindarnos los Servicios Científico-Técnicos

de la Universitat de Barcelona para nuestros experimentos, por sus geniales consejos y su estoicismo durante todos estos años. Al grupo de péptidos del Departamento de Química Orgánica de la Universitat de Barcelona, gracias por el liofilizador y la hospitalidad. Al grupo del Dr. Mata del Departamento de Ingeniería Química de la Universitat de Barcelona, a Sandra, Eva, Alex y los colombianos, por compartir su Electroforesis Capilar con nosotros, por vuestro sentido del humor del que he disfrutado durante las grandes conversaciones que hemos mantenido juntos. A la Dra. Irene Fernández del Servicio de Espectrometría de Masas de la División III de la Universitat de Barcelona, por su simpatía y asistencia en los trabajos de MALDI-TOF. A la Dra. Maria Josep Cugat del Departamento de Química Analítica de la Universitat Rovira i Virgili, por su interminable sonrisa, qué suerte la de haber podido conocerte y el haber podido compartir algunos momentos de esta historia contigo.

Me considero afortunado, ya que la ciencia también me ha permitido salir a conocer un poquito del mundo que existe ahí afuera. A la gente que conocí durante mis viajes, gracias por haber estado ahí haciendo que cada uno de ellos fuera una experiencia inolvidable.

Al Dr. Norberto A. Guzmán por su infinita hospitalidad durante el tiempo que pasé en el *RWJ Johnson Pharmaceutical Research Institute* en New Jersey. Gracias por tu amistad y por tus sabios consejos durante aquellos meses, y los que han venido después. Realmente ha sido una suerte poder conocerte, espero seguir compartiendo buenos momentos y conversaciones contigo. A la Dra. Cristina Vescina, mi excepcional compañera argentina durante la parada en New Jersey, con la que compartí muchas alegrías y tristezas. Espero poder reencontrarte pronto tras mi visita a Buenos Aires, pero esta vez en Barcelona.

A los miembros del Departamento de Química Analítica, especialmente al resto del grupo de Bioanàlisi y nuestros compañeros de

Interacció Solut-Solvent quisiera darles las gracias por los muchos y buenos momentos que hemos compartido juntos. A la vieja guardia - Albert, Adrián, Deivid, Paquito, Óscar, Jordi y Jorge - quiero agradecerles el haberle dado color y alegría a mi primer año en el mundo de la investigación, y por las reuniones periódicas que espero que sigamos manteniendo. A Sonia e Imma el haber sido la calma y la paz del laboratorio. A Lali el tender siempre su mano cuando la necesitaba. A Fadoua las conversaciones sobre los poderes del té. A Fernando Rived que siempre nos recordara el día en el que vivíamos. A Eli sus sonrisas y las copas que tomamos juntos, ¡ánimo en este tramo final!. A Balbina y Pilar siempre dispuestas a echarme un cable, igual que Sandra, Diana y Piluca. A José Manuel las grandes discusiones científico-terrenales que solemos tener, espero que en algún momento de su ajetreada vida recoja el fruto de tanto sacrificio. A Leo, Chuscris y Charli por las divertidas conversaciones terrenales de los últimos meses. A todos los que vivieron, viven o vivirán en el *Crazy Lab*: Jeroen, Javi, Sabriye, Nurullah, Hale, Alberto, Rebeca, Xavi, Meritxell, Silvia, Eli, Gemma, Óscar,... gracias, y no olvideis dejarlo todo tal y como lo encontrasteis al llegar.

A mis compañeros del Gimnasio de la UPC, por las risas y sudores que hemos compartido juntos y que me han aliviado los duros finales de día. A mis amigos de siempre, a los de toda la vida, que siempre estáis ahí en esos fines de semana tan cortos. Por haber sido mi válvula de escape en muchas ocasiones, por haberme cortocircuitado para que desconectara de una vez, gracias por todo chicos... y chicas, que no me olvido de vosotras. A todos los que compartieron o comparten algún momento de mi vida.

A mi familia, a mis padres sobre todo, porque han trabajado duro toda su vida para que nosotros pudiéramos llegar hasta donde hemos llegado. Gracias por vuestro esfuerzo y apoyo incondicional, por darlo todo sin pedir nada a cambio.

Espero que la memoria no me haya jugado una mala pasada y me haya olvidado de ti, recuerda que allá donde estés nunca olvidaré lo que un día hiciste por mí: MIL GRACIAS.

ABREVIATURAS Y ACRÓNIMOS

aa[a] (*amino acid*): aminoácido.

ALC (*Affinity Liquid Chromatography*): Cromatografía de Líquidos de Afinidad.

CD (*Circular Dicroism*): Dicroísmo Circular.

CE (*Capillary Electrophoresis*): Electroforesis Capilar (en gran parte del texto se asimila CE a CZE).

CEC (*Capillary Electrochromatography*): Electrocromatografía Capilar.

CEC-MS (*Capillary Electrochromatography Mass Spectrometry*): Electrocromatografía Capilar acoplada a la Espectrometría de Masas.

CE-ESI-MS (*Capillary Electrophoresis Electrospray Mass Spectrometry*): Electroforesis Capilar acoplada a la Espectrometría de Masas con Ionización por Electrospray.

CE-LIF (*Capillary Electrophoresis with Laser Induced Fluorescence detection*): Electroforesis Capilar con detección por Fluorescencia Inducida por Láser.

CE-MS (*Capillary Electrophoresis Mass Spectrometry*): Electroforesis Capilar acoplada a la Espectrometría de Masas.

CE-UV (*Capillary Electrophoresis with Ultraviolet Spectrophotometry detection*): Electroforesis Capilar con detección por Espectrofotometría Ultravioleta.

CGE (*Capillary Gel Electrophoresis*): Electroforesis Capilar en Geles.

CIEF (*Capillary Isoelectric Focusing*): Isoelectroenfoque Capilar.

CITP (*Capillary Isotachophoresis*): Isotacoforesis Capilar.

CMC (*Critical Micellar Concentration*): Concentración Micelar Crítica.

CZE (*Capillary Zone* Electrophoresis): Electroforesis Capilar de Zona (en gran parte del texto se asimila CZE a CE).

DE (*Delayed Extraction*): Extracción retardada.

d.i.: Diámetro interno (i.d.: *internal diameter*).

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[a] Los códigos de una y tres letras para cada uno de los aminoácidos proteicos aparecen en la Tabla 1.1 .

EOF (*Electroosmotic Flow*): Flujo Electroosmótico.

EPO (*Erythropoyetin*): Eritropoyetina

ESI (*Electrospray Ionization*): Ionización por Electrospray.

ESI-MS (*Electrospray Mass Spectrometry*): Espectrometría de Masas con Ionización por Electrospray.

ESI-MS-MS (*Electrospray Tandem Mass Spectrometry*): Espectrometría de Masas en tándem con Ionización por Electrospray.

Fmoc (*9-fluorenylmethoxycarbonyl*): 9-fluorenilmetoxicarbonilo.

GC (*Gas Chromatography*): Cromatografía de Gases.

GE (*Gel Electrophoresis*): Electroforesis en Geles.

2D-GE (*2D-Gel Electrophoresis*): Electroforesis bidimensional en Geles.

GnRH (*Gonadotrophin-Releasing Hormone*): Hormona Liberadora de Gonadotropinas. Sinónimo de LHRH.

HFBA (*Heptaflluorobutyric acid*): Ácido Heptafluorobutírico.

HIC (*Hydrophobic Interaction Liquid Chromatography*): Cromatografía de Líquidos de Interacción Hidrofóbica.

IEF (*Isoelectric focusing*): Isoelectroenfoque.

IEC (*Ionic Exchange Liquid Chromatography*): Cromatografía de Líquidos de Intercambio Iónico.

IR Spectroscopy (*Infrared Spectroscopy*): Espectroscopia Infrarroja.

LC (*Liquid Chromatography*): Cromatografía de Líquidos (en gran parte del texto se asimila LC a RPLC).

LC-ESI-MS (*Liquid Chromatography Electrospray Mass Spectrometry*): Cromatografía de Líquidos acoplada a la Espectrometría de Masas con Ionización por Electrospray.

LC-UV (*Liquid Chromatography with Ultraviolet Spectrophotometry detection*): Cromatografía de Líquidos con detección por Espectrofotometría Ultravioleta.

LHRH (*Luteinizing Hormone-Releasing Hormone*): Hormona Liberadora de la Hormona Luteinizante. Sinónimo de GnRH.

LIF (*Laser Induced Fluorescence*): Fluorescencia Inducida por Láser.

LSER (*Linear Solvation Energy Relationships*): Método de Comparación Solvatocrómico.

M (*Molecular Mass*): Masa Molecular.

MALDI (*Matrix Assisted Laser Desorption Ionization*): Ionización por Desorción con Láser Asistida por una Matriz.

MALDI-MS (*Matrix Assisted Laser Desorption Ionization Mass Spectrometry*): Espectrometría de Masas con Ionización por Desorción con Láser Asistida por una Matriz.

MALDI-TOF (*Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry*): Espectrometría de Masas con Ionización por Desorción con Láser Asistida por una Matriz y Analizador de Tiempo de Vuelo.

MeCN (*Acetonitrile*): Acetonitrilo.

MEKC (*Micellar Electrokinetic Chromatography*): Cromatografía Electrocinética Micelar .

MS (*Mass Spectrometry*): Espectrometría de Masas.

MS-MS (*Tandem Mass Spectrometry*): Espectrometría de Masas en tándem.

NESP (*Novel Erythropoiesis Stimulating Protein*): Nueva Proteína Estimuladora de la Eritropoyesis.

NeuNAc (*N-Acetylneuraminic Acid*): Ácido N-acetilneuramínico.

PAGE (*Polyacrylamide gel electrophoresis*): Electroforesis en Geles de Poliacrilamida.

PCR (*Polymerase Chain Reaction*): Reacción en Cadena de la Polimerasa.

rHuEPO (*Recombinant Human Erythropoietin*): Eritropoyetina Humana Recombinante.

RMN: Resonancia Magnética Nuclear (NMR: *Nuclear Magnetic Resonance*).

RPLC (*Reversed Phase Liquid Chromatography*): Cromatografía de Líquidos de Fase Inversa (en gran parte del texto se asimila RPLC a LC).

SEC (*Size Exclusion Liquid Chromatography*): Cromatografía de Líquidos de Exclusión por Tamaños.

SIDA: Síndrome de Immunodeficiencia Adquirida (AIDS (*Acquired Immunodefficiency Syndrome)*).

SPE (*Solid Phase* Extraction): Extracción en Fase Sólida.

SPE-CE (*Solid Phase Extraction coupled on line to Capillary Electrophoresis):* Extracción en Fase Sólida acoplada en línea a la Electroforesis Capilar.

SPE-CE-ESI-MS (*Solid Phase Extraction coupled on line to Capillary Electrophoresis Electrospray Mass Spectrometry):* Extracción en Fase Sólida en línea con la Electroforesis Capilar acoplada a la Espectrometría de Masas con Ionización por Electrospray.

SPPS (*Solid Phase Peptide Synthesis*): Síntesis de Péptidos en Fase Sólida.

TIC (*Total Ion Chromatogram*): Cromatograma Total de Iones.

TFA (*Trifluoroacetic Acid)*: Ácido Trifluoracético.

TOF (*Time-Of-Flight*): Tiempo de Vuelo.

tITP (*transient Isotachophoresis*): Isotacoforesis Transitoria.

UV (*Ultraviolet Spectrophotometry*): Espectrofotometría Ultravioleta.

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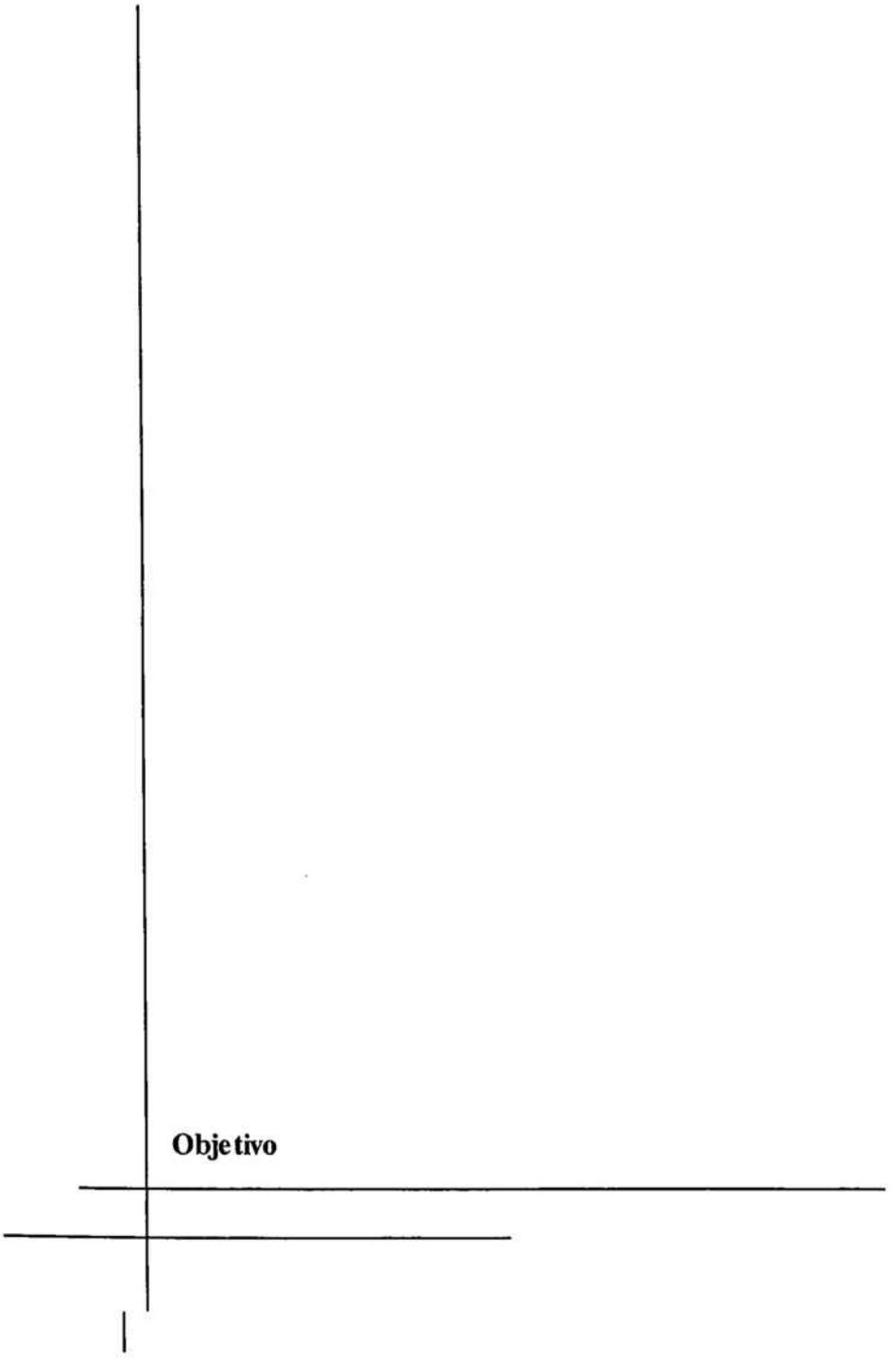

Objetivo

El objetivo final de esta tesis doctoral ha sido el establecimiento de metodologías analíticas para la separación y caracterización de mezclas complejas de péptidos y proteínas de gran interés terapéutico, empleando para ello la Cromatografía de Líquidos (LC) y la Electroforesis Capilar (CE) con detección por Espectrofotometría Ultravioleta (UV) y sus acoplamientos a la Espectrometría de Masas (MS). Este objetivo principal engloba otros más concretos que se detallan a continuación:

- Desarrollar modelos que describan el comportamiento cromatográfico y electroforético de sustancias peptídicas presentes en mezclas complejas, con la finalidad de poder predecir, a partir de un reducido número de medidas experimentales, las condiciones para obtener una separación óptima de estos analitos. Se establecen dos tipos de modelos:
  - Modelos que explican el comportamiento cromatográfico de los analitos en función de la polaridad de la fase móvil.
  - Modelos que explican el comportamiento cromatográfico y electroforético de los analitos ionizables en función del pH de la fase móvil o del electrolito utilizado para la separación electroforética, las constantes de disociación de los analitos estudiados y los coeficientes de actividad.
- Determinar mediante los modelos anteriores los valores de pK_a, en medio acuoso o hidroorgánico, de las sustancias analizadas.
- Utilizar la Cromatografía de Líquidos acoplada a la Espectrometría de Masas con Ionización por Electrospray (LC-ESI-MS) para la separación y caracterización de las sustancias presentes en crudos de síntesis de hormonas peptídicas de gran interés terapéutico. Proponer las condiciones de separación óptimas para una posterior purificación por LC a escala preparativa de la

hormona peptídica sintetizada y para identificar las impurezas presentes en las mezclas.

- Combinar la LC, la LC-ESI-MS y la CE para profundizar en el análisis de crudos de síntesis de hormonas peptídicas de gran interés terapéutico. Utilizar modelos semiempíricos que explican la migración electroforética de las sustancias peptídicas para corroborar asignaciones estructurales.
- Aplicar la Electroforesis Capilar acoplada a la Espectrometría de Masas con Ionización por Electrospray (CE-ESI-MS) para separar y caracterizar mezclas complejas de hormonas peptídicas. Optimizar sistemáticamente las variables relacionadas con este tipo de acoplamiento, que afectan a la sensibilidad y a la selectividad de las separaciones.
- Desarrollar metodologías analíticas basadas en la Extracción en Fase Sólida acoplada en línea a la Electroforesis Capilar (SPE-CE) para mejorar los límites de detección obtenidos por CE. Optimizar sistemáticamente las variables implicadas en la preconcentración de los analitos. Utilizar la metodología SPE-CE desarrollada para preconcentrar, separar y caracterizar mezclas peptídicas en muestras diluidas, empleando su acoplamiento a la Espectrometría de Masas con Ionización por Electrospray (SPE-CE-ESI-MS).
- Separar las glicoformas de la Eritropoyetina Humana Recombinante (rHuEPO) mediante CE, estableciendo una metodología analítica reproducible y potencialmente compatible con el uso de CE-ESI-MS. Optimizar las variables que afectan a la reproducibilidad y selectividad de las separaciones.
- Caracterizar las glicoformas de la rHuEPO empleando diferentes técnicas de MS.

Capítulo 1. Introducción

1. INTRODUCCIÓN

1.1. PÉPTIDOS Y PROTEÍNAS

Los péptidos y las proteínas juegan un papel fundamental en un gran número de procesos biológicos, desempeñando funciones como hormonas, neurotransmisores, toxinas, enzimas, antígenos, citoquinas o factores de crecimiento [1-3]. Los continuos avances que se vienen realizando en biología molecular, biofísica molecular, genómica y proteómica[a] han mostrado además, que las funciones celulares, la comunicación intercelular, la respuesta inmune y la transducción de la información biológica están gobernadas por interacciones no covalentes que se establecen entre péptidos y proteínas [3-6]. Todo lo apuntado anteriormente implica un enorme potencial para el desarrollo de nuevos productos con valor terapéutico (hormonas, anticuerpos, vacunas,...) o industrial (enzimas, aditivos,...) [4-14] y, sin duda alguna, la aplicación de nuevas técnicas y metodologías analíticas para separar, identificar y caracterizar péptidos y proteínas, constituye una pieza clave para el descubrimiento, diseño, síntesis y control de estos nuevos productos [15-16].

Los péptidos y las proteínas consisten en cadenas de α-aminoácidos[b] (α-aa) unidos por enlaces amida entre el grupo amino de una unidad y el grupo carboxilo de otra [1-2,12-13]. En general, contienen un grupo amino libre (el extremo N-terminal) y un grupo carboxilo libre (el extremo C-terminal) (Figura 1.1). Denominamos péptidos a las secuencias más cortas, de entre 2 y 100 aminoácidos aproximadamente, aunque no existe una división clara entre ambas categorías.

[a] La proteómica se ocupa del estudio de todas las proteínas expresadas en un determinado organismo, célula o tejido. Trata de identificarlas, cuantificarlas, caracterizarlas estructuralmente, determinar su función, la interacción con otras proteínas y establecer la relación de estas proteínas con las enfermedades.

[b] En los α-aminoácidos los grupos carboxilo y amino están directamente unidos al mismo átomo de Carbono, que se denomina Carbono α.

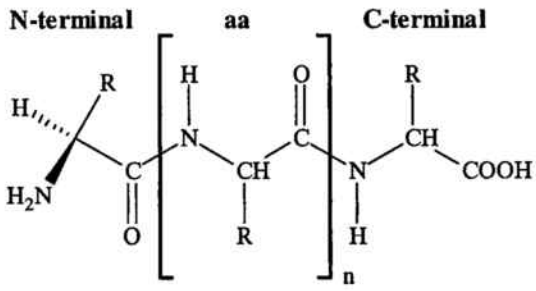
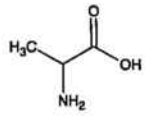


Figura 1.1. Representación esquemática de un péptido lineal con n aminoácidos (aa).

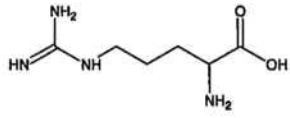
La mayoría de los aminoácidos presentes en la naturaleza se encuentran formando parte de péptidos y proteínas, aunque hay algunos que pueden desempeñar otras funciones, por lo que se puede diferenciar para su clasificación sistemática entre aminoácidos proteicos y no proteicos. Los 20 aminoácidos proteicos naturales aparecen en la Tabla 1.1. Con la excepción de la glicina, todos son quirales, debido a la presencia de al menos un carbono estereogénico, y pertenecen a la serie L. En algunas ocasiones, estos residuos aminoacídicos sufren modificaciones postranslacionales después de la biosíntesis en los ribosomas del correspondiente péptido o proteína, originándose estructuras hidroxiladas, carboxiladas, sulfatadas, fosforiladas o glicosiladas [1-2,12-13].

La estructura global de los péptidos y las proteínas es fundamental para explicar sus propiedades físico-químicas y biológicas y se organiza jerárquicamente en varios niveles de complejidad sucesiva. Al primer nivel estructural, que describe la secuencia aminoacídica, se le denomina estructura primaria. En la Tabla 1.2 aparece una clasificación de los aminoácidos proteicos naturales según el tipo de cadenas laterales que poseen.

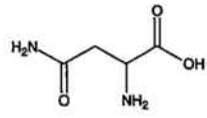
Tabla 1.1 Los 20 aminoácidos proteicos naturales.



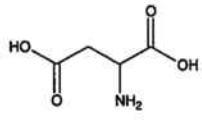
Alanina (Ala, **A**)



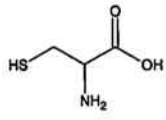
Arginina (Arg, **R**)



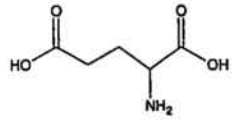
Asparagina (Asn, **N**)



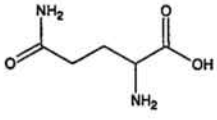
Ácido Aspártico (Asp, **D**)



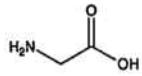
Cisteína (Cys, **C**)



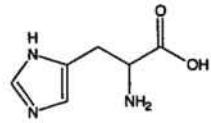
Ácido Glutámico(Glu, **E**)



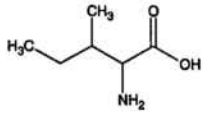
Glutamina (Gln, **Q**)



Glicina (Gly, **G**)



Histidina (His, **H**)



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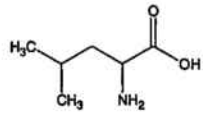

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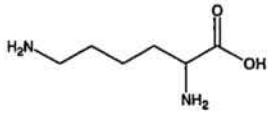

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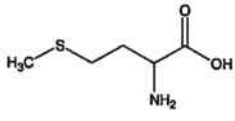

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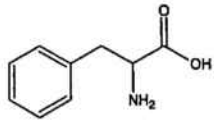

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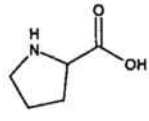
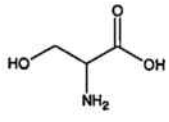


Prolina (Pro, **P**)



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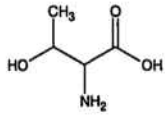
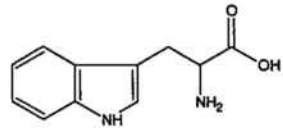
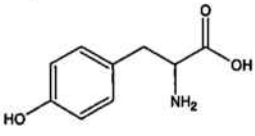


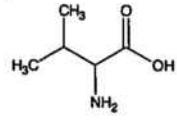
Treonina (Thr, **T**)



Triptófano (Trp, **W**)



Tirosina (Tyr, **Y**)



Valina (Val, **V**)

Nota: los códigos de tres y una letra para cada aa aparecen entre paréntesis al lado de su nombre químico

La estructura secundaria se debe al plegamiento de diferentes segmentos de cadena peptídica y origina estructuras regulares tales como las hélices α y las β. Las estructuras terciaria y cuaternaria sólo las presentan las proteínas. La terciaria describe cómo los elementos que componen la estructura secundaria de una cadena proteica, interaccionan para plegarse, generando la estructura nativa de la proteína. Algunas proteínas complejas llegan a presentar estructura cuaternaria, que se origina por la interacción entre las diferentes subunidades que las forman. Entre las interacciones más destacadas, que estabilizan los niveles estructuralmente más complejos, están las interacciones electrostáticas, los puentes de hidrógeno y los puentes disulfuro entre residuos de cisteína [1-2,12-13].

Tabla 1.2 Tipos de cadenas laterales y pK_a de los aa proteicos naturales [1-2].

Tipos de cadenas laterales	aa	pK_1	pK_2	pK_3
Alifáticas	Gly	2.4	9.8	
	Ala	2.4	9.9	
	Val	2.2	9.7	
	Leu	2.3	9.7	
	Ile	2.3	9.8	
Hidroxiladas	Ser	2.2	9.2	≅ 13
	Thr	2.1	9.1	≅ 13
	Tyr	2.2	9.1	10.1
Con átomos de azufre	Cys	1.9	10.8	8.3
	Met	2.1	9.3	
Con grupos ácidos o sus amidas	Asp	2.0	9.9	3.9
	Asn	2.1	8.8	
	Glu	2.1	9.5	4.1
	Gln	2.2	9.1	
Con grupos básicos	Arg	1.8	9	12.5
	Lys	2.2	9.2	10.8
	His	1.8	9.3	6.0
Con anillos aromáticos	His			
	Phe	2.2	9.2	
	Tyr			
	Trp	2.4	9.4	
Iminoácidos	Pro	2.0	10.6	

1.2. ANÁLISIS DE MEZCLAS PEPTÍDICAS COMPLEJAS

El análisis de mezclas complejas de péptidos y proteínas es un problema común a diversas áreas de la ciencia, para cuyo desarrollo resulta fundamental la combinación de técnicas de separación de alta resolución con métodos de detección cada vez más selectivos y sensibles, que permitan además la caracterización inequívoca de las sustancias presentes en las mezclas [15-16]. La Cromatografía de Líquidos (LC) [17-21] y la Electroforesis Capilar (CE) [22-27], acopladas o no a la Espectrometría de Masas (MS) [20-21,24-27] son, hoy en día, las técnicas de separación de alta resolución más ampliamente empleadas con esta finalidad.

1.2.1. Cromatografía de Líquidos (LC)

La Cromatografía de Líquidos (LC) ha sido la técnica tradicionalmente utilizada para la separación y purificación de mezclas peptídicas complejas, debido principalmente a su versatilidad, selectividad, reproducibilidad, poder de resolución y a la posibilidad de trabajar tanto a escala analítica como a escala preparativa [15-19]. La Espectrofotometría Ultravioleta (UV) ha sido el modo de detección más comúnmente empleado, trabajando a longitudes de onda comprendidas entre 210 nm y 230 nm, donde los enlaces peptídicos absorben intensamente, o a 280 nm si en la molécula hay triptófano (no oxidado) o tirosina. En los últimos años se ha generalizado el uso de las técnicas de ionización suaves en MS – la Ionización por Electrospray (ESI) [28] y la Ionización por Desorción con Láser Asistida por una Matriz (MALDI) [29-30] – para determinar la masa molecular de péptidos y proteínas. La Cromatografía de Líquidos acoplada a la Espectrometría de Masas con Ionización por Electrospray (LC-ESI-MS) [31] o la combinación de la LC con la Espectrometría de Masas con Ionización por Desorción con Láser Asistida por una Matriz y Analizador de Tiempo de Vuelo (MALDI-TOF) [32] permiten la

separación, identificación y caracterización de las sustancias presentes en una mezcla, cuando se pretende algo más que purificar un péptido o proteína concreto. Paralelamente, el gran desarrollo instrumental experimentado por la LC en los últimos 20 años (bombas, columnas, inyectores automáticos, detectores, interfases,...) [31-33], ha permitido su implantación como técnica de rutina en los laboratorios analíticos modernos.

Entre las diferentes modalidades de LC existentes, la Cromatografía de Líquidos de Exclusión por Tamaños (SEC), la Cromatografía de Líquidos de Intercambio Iónico (IEC) y la Cromatografía de Líquidos de Afinidad (ALC) son las más comúnmente empleadas para recuperar una proteína en su forma biológicamente activa [3,7-9,15-18]. La Cromatografía de Líquidos de Fase Inversa (RPLC) y la Cromatografía de Líquidos de Interacción Hidrofóbica (HIC) son preferentemente empleadas para la separación y purificación de péptidos, aunque a diferencia de la RPLC, la HIC permite realizar los análisis en condiciones no desnaturalizantes. La SEC es especialmente útil cuando se desea separar moléculas de muy diferente volumen o forma. Se emplea a menudo para estimar la masa molecular y la forma de las proteínas y para separarlas de sus agregados (dímeros, trímeros,...) [17-18,34]. En IEC, los péptidos o proteínas cargados, que quedan retenidos por interacciones electrostáticas sobre la resina de intercambio iónico, son eluidos mediante variaciones de pH o de fuerza iónica de la fase móvil [17-18,35]. En ALC se utilizan fases estacionarias muy selectivas aprovechando las propiedades específicas de un determinado analito o grupo de analitos. Fases estacionarias típicas contienen anticuerpos, lectinas, receptores, metales, proteína A o G,... y para la elución se utilizan fases móviles de pH ácido y fuerza iónica elevada [17,36]. En HIC se regula la hidrofobicidad de la fase móvil acuosa mediante variaciones en la concentración de sales inorgánicas, $(NH_4)_2SO_4$ o NaCl normalmente [17-18,35]. En presencia de una alta concentración salina, las proteínas son atraídas hacia la superficie hidrofóbica de la fase estacionaria, porque al estar menos solvatadas, sus

residuos aminoacídicos hidrofóbicos son más accesibles. Si se realiza en gradiente, las proteínas son eluidas a medida que desciende la concentración salina.

La RPLC presenta indiscutiblemente una serie de ventajas respecto a todas las anteriores modalidades, ya que ofrece mayor versatilidad y poder de resolución y las fases móviles empleadas pueden ser compatibles con la detección por MS [18,20-21,31]. Las separaciones de péptidos y proteínas mediante RPLC, se llevan a cabo empleando columnas de fases estacionarias hidrofóbicas (octilsilano, octadecilsilano, poliméricas,...) y normalmente fases móviles hidroorgánicas a pH ácido, con elución en modo isocrático o realizando un gradiente de la concentración del disolvente orgánico [18,20-21,31]. En general, al pH ácido de la fase móvil, el grupo carboxilo terminal y el resto de grupos ácidos de las cadenas laterales peptídicas, se encuentran protonados, mientras que los grupos amina, estarán cargados positivamente, por lo que la adición de un reactivo formador de par iónico puede disminuir las interacciones de las moléculas peptídicas con la fase estacionaria, reduciéndose los procesos de adsorción de dichos compuestos sobre ésta y provocando una mejora de la simetría y anchura de los picos cromatográficos [18]. El ácido trifluoroacético (TFA) es el agente de par iónico más comúnmente utilizado, por su acidez, gran solubilidad, transparencia al ultravioleta y adecuada volatilidad (punto de ebullición, 72.4 °C). También se suelen emplear el ácido heptafluorobutírico (HFBA), el ácido fosfórico, el ácido perclórico, el ácido clorhídrico, el ácido acético, el ácido fórmico y el acetato o el formiato de trietilamonio [15-18,37-39]. El disolvente orgánico más comúnmente empleado, es el acetonitrilo (MeCN), debido a su baja viscosidad, alta volatilidad y transparencia al ultravioleta a bajas longitudes de onda. También se utilizan el metanol, el etanol (mayoritariamente en aplicaciones preparativas), el isopropanol (para proteínas hidrofóbicas) y en menor medida el trifluoroetanol [15-18]. Otra de las ventajas de la RPLC, es que las fases móviles hidroorgánicas ácidas de fuerza iónica moderada que generalmente se emplean, son compatibles con el uso de interfases de ESI en

MS [31]. Del mismo modo, también es posible recolectar fracciones y analizarlas mediante MALDI-TOF [32], sin necesidad, en la mayoría de los casos, de realizar ningún pretratamiento de la muestra. La RPLC es la modalidad de LC que se ha empleado en este trabajo, por lo que en lo sucesivo al mencionar LC nos estaremos refiriendo a RPLC.

1.2.2. Electroforesis Capilar (CE)

La Electroforesis Capilar (CE) constituye actualmente una excelente alternativa para separar mezclas complejas de péptidos y proteínas [22-27], aunque la Electroforesis en geles (GE) y el Isoelectroenfoque (IEF) continúan siendo ampliamente utilizadas para tales efectos, sobre todo en el caso de las proteínas [15-16]. La CE, a diferencia de la GE y el IEF, proporciona mayores eficacias y mayor poder de resolución con tiempos de análisis más cortos, mayor versatilidad en la selección de los analitos o las metodologías de separación, menor consumo de muestras, reactivos y productos, automatización completa y posibilidad de acoplamiento en línea con la MS [40-43]. La gran versatilidad de la CE deriva parcialmente de sus numerosas y complementarias modalidades de operación [40-43]. Estas modalidades incluyen la Electroforesis Capilar de Zona (CZE), la Cromatografía Electrocinética Micelar (MEKC), la Electroforesis Capilar en Geles (CGE), el Isoelectroenfoque Capilar (CIEF), la Isotacoforesis Capilar (CITP), y la Electrocromatografía Capilar (CEC). En la Tabla 1.3 se muestra una breve descripción de los fundamentos de la separación en cada una de estas modalidades [40-43].

Los mecanismos en los que se basan las separaciones electroforéticas son diferentes a los que gobiernan en LC, lo que permite la complementariedad de ambas técnicas. Sin embargo, aún hoy en día no se ha logrado la introducción generalizada de la CE en los laboratorios de análisis químico del sector industrial

(farmacéutico, alimentario, cosmético, ...) o clínico, debido principalmente a la gran implantación de la Cromatografía de Gases (GC) y la LC, y a algunos inconvenientes, inherentes a la propia técnica, que trataremos en profundidad posteriormente [40-43]. Entre ellos destaca la imposibilidad de trasladar los métodos de separación a la escala preparativa, la poca reproducibilidad en los tiempos de migración observada en algunos casos [44-47], su relativamente baja sensibilidad [48-49]; y finalmente, el aún limitado desarrollo y reproducibilidad de las interfases y las metodologías empleadas en Electroforesis Capilar acoplada a la Espectrometría de Masas con Ionización por Electrospray (CE-ESI-MS) [50-51].

Tabla 1.3 Modalidades de CE.

Modo	Base de la separación
CZE	Diferencias entre las movilidades netas de analitos cargados
MEKC	Diferencias entre las movilidades netas de analitos neutros o cargados que interaccionan con micelas
CGE	Diferencias en el tamaño y la forma de los analitos cargados
CIEF	Diferencias entre los puntos isoeléctricos (pI) de analitos anfóteros en tampones discontinuos
CITP	Diferencias entre las movilidades netas de analitos cargados en tampones discontinuos
CEC	Diferencias entre las movilidades netas de los analitos neutros o cargados que se distribuyen entre la fase móvil y la fase estacionaria

La Electroforesis Capilar de Zona (CZE) es la más habitual de las modalidades empleadas en CE [40-43], y ha sido ampliamente utilizada para la separación de un variado número de analitos, entre ellos los péptidos y las proteínas [22-27] y es la modalidad electroforética que mayoritariamente se ha empleado en este trabajo. En general, está ampliamente extendido y aceptado el uso del término CE para referirse específicamente a la CZE, con lo que seguiremos esta nomenclatura en la mayoría de las explicaciones y publicaciones que se recopilan en esta memoria de tesis doctoral.

Tabla 1.4 Aditivos empleados en CE para la separación de péptidos y proteínas.

Aditivo	Ejemplo	Uso	[Ref.]
Surfactantes (aniónicos, catiónicos o neutros)	SDS, HTAB, BRIJ, TWEEN, CTAB	▪ Modificación del EOF y prevención de la adsorción ▪ Solubilización de solutos hidrofóbicos ▪ Formación de par iónico ▪ MEKC por encima de la CMC	[52-56]
Sustancias zwiteriónicas	MES, Tris, CHAPS, CHAPSO	▪ Modificación de la selectividad sin aumento de la conductividad.	[57]
Polímeros hidrofílicos lineales	Metil celulosa, poliacrilamida, PEG, PVA	▪ Reducción del EOF ▪ Minimización de la adsorción de la muestra si se emplean a baja concentración ▪ CGE a elevada concentración	[56,58-60]
Disolventes orgánicos	Metanol, acetonitrilo	▪ Modificación del EOF y la selectividad ▪ Solubilización de sustancias poco solubles en agua	[61-63]
Selectores quirales	Ciclodextrinas, antibióticos	▪ Separación de compuestos quirales ▪ Modificación de la selectividad ▪ Solubilización de solutos hidrofóbicos	[52,64]
Iones metálicos	K^+,Na^+,Cu^{2+},Li^+,Ca^{2+}	▪ Modificación de la selectividad	[65-66]
Agentes desnaturalizantes	Urea	▪ Solubilización de proteínas ▪ Modificación de la selectividad	[56,67]
Formadores de par iónico, agentes complejantes	TFA, HFBA, borato, ácido fítico	▪ Modificación de la selectividad ▪ Formación de par iónico o complejos	[68-69]
Aminas cuaternarias	putrescina, espermina, polybrene, PEI	▪ Modificación del EOF ▪ Formación de par iónico	[67,70-73]

SDS: *Sodium dodecyl sulfate, Lauryl sulfate*, HTAB: *n-hexadecyl-trimethylammonium bromide*, BRIJ: *Generic name of polyoxyethylene esters, p.e. Brij 35 = 23 Lauryl Ether* ($C_{12}E_{23}$), TWEEN: *Generic name of polyoxyethylenesorbitans , p.e. Tween 20 = Monolaurate*, CTAB: *Cetyltrimethylammonium Bromide*, MES: *2-[N-morpholino]ethanesulfonic acid*, TRIS: *Tris[hydroxymethyl]aminomethane*, CHAPS: *3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate*, CHAPSO: *3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonat*, PEG: *Polyethylene Glycol*, PEI: *Polyethyleneimine*, PVA: *Polyvinyl alcohol.*

En CE los analitos son separados en zonas discretas debido a las diferencias que se producen entre sus velocidades de migración, al aplicar una diferencia de potencial entre los extremos de un capilar de sílice fundida, o cualquier otro material, que contiene el electrolito de separación. El electrolito de separación consiste en una disolución tampón al pH adecuado, que puede contener un variado número de aditivos que modificarán sus propiedades con el objetivo de mejorar la selectividad,

reproducibilidad y sensibilidad de los análisis [40-43]. En la Tabla 1.4 se resumen los aditivos más ampliamente empleados en CE para las separaciones de péptidos y proteínas [22-27,52-73].

1.2.3. Optimización de los métodos de separación

La optimización de la separación de una mezcla mediante LC o CE, se realiza normalmente siguiendo largos y laboriosos procedimientos empíricos, que además generan un gran volumen de mezclas hidroorgánicas potencialmente contaminantes si la técnica empleada es la LC. Se pueden seguir varias estrategias para optimizar de forma rápida y sencilla las metodologías de separación empleadas en LC y CE [74-84]. Una de las más efectivas consiste en predecir la retención de los analitos empleando modelos que tengan en cuenta los mecanismos que gobiernan su comportamiento cromatográfico [76-80] o electroforético [81-84], respectivamente.

1.2.3.1. Optimización de la separación en LC

La retención de una sustancia en LC, es un proceso complejo que depende de las interacciones que se establecen entre el soluto, la fase móvil y la fase estacionaria [18,85]. La distribución de un soluto entre fase móvil y fase estacionaria depende de la diferencia de polaridad entre ambas. Así, aunque la estructura y composición de la fase estacionaria juegue un papel fundamental sobre la selectividad de las separaciones, si se emplea una columna cromatográfica concreta, la retención se puede estudiar teniendo únicamente en cuenta las propiedades de la fase móvil.

La composición y el pH de la fase móvil son dos factores fundamentales que influyen en las separaciones por LC [18,85]. El pH de la fase móvil determina

el grado de ionización de los solutos ionizables y el de las sustancias que se adicionan como formadores de par iónico, lo que puede provocar grandes variaciones en la selectividad cromatográfica [78-79,87-89]. Para optimizar el pH de la fase móvil, se pueden establecer relaciones entre éste y el factor de retención de los analitos [78-79,87-89], siendo muy recomendable medir el pH en la mezcla hidroorgánica utilizada, sobre todo si contiene un alto porcentaje de disolvente orgánico [90-92]. Para ello se han de emplear las disoluciones tampón de referencia adecuadas a cada una de las composiciones hidroorgánicas de fase móvil [90,92]. Una medida correcta del pH en medio hidroorgánico va a permitir la correcta interpretación de los resultados de retención cromatográfica y la determinación de los pK_a de los solutos en las mezclas hidroogánicas [93-94].

En general, en las separaciones de péptidos y proteínas se utilizan fases móviles de pH ácido y el TFA es el agente de par iónico más comúnmente utilizado, a concentraciones que varían entre un 0.05 % y un 0.1% (v/v) [17-20,78]. En estas condiciones el agente de par iónico se encuentra en exceso suficiente respecto a la concentración de los analitos y su proporción en la fase móvil no afecta significativamente la selectividad de las separaciones, aunque los péptidos a analizar difieran mucho en el número de grupos básicos [38,86].

Respecto a la proporción de disolvente orgánico, entre los parámetros que se han empleado con más éxito para describir la polaridad de las fases móviles destacan los parámetros solvatocrómicos de Kamlet y Taft, α y β, que evalúan la capacidad dadora [95] y aceptora [96] de enlaces por puente de hidrógeno, respectivamente, π^* que evalúa la dipolaridad/polarizabilidad del disolvente [97] y el parámetro E_T^N de Dimroth y Reichardt [98], que está relacionado con la polaridad y capacidad dadora de puentes de hidrógeno. Estos parámetros se utilizan en el Método de Comparación Sovatocrómico (*Linear Solvation Energy Relationships,* LSER), para modelizar la retención en LC [80,99-101]. El método LSER se basa en la ecuación multiparamétrica de Kamlet y Taft que relaciona una propiedad

determinada, en este caso el factor de retención del soluto, con los parámetros π^*, α y β de la fase móvil empleada para eluirlo, o bien con el parámetro E_T^N de la misma fase móvil [80,99-101].

1.2.3.2 Optimización de la separación en CE

Las separaciones en CE se basan en las diferentes velocidades de migración que experimentan los analitos cuando se someten a un campo eléctrico en capilares de diámetro interno pequeño (normalmente 50-75 μm). La velocidad de una especie cargada viene dada por la ecuación [40-43]:

$$v = m_e E$$

donde v es la velocidad del ión; E el campo eléctrico, que es función del voltaje aplicado y de la longitud del capilar; y m_e es la movilidad electroforética, que es constante para cada ion en un determinado medio, y se deduce a partir del balance entre la fuerza eléctrica y de fricción que experimenta la molécula:

$$m_e = \frac{q}{6\pi\eta r}$$

donde q es la carga del ión, η la viscosidad de la disolución y r el radio del ión. Así, la m_e de un determinado soluto es directamente proporcional a su carga e inversamente proporcional a su radio iónico, que en muchas ocasiones es directamente proporcional a su masa. Si los analitos son ionizables, su carga dependerá de su grado de ionización y resultará fundamental controlar el pH de la disolución para optimizar las separaciones [83-84,102-104]. En caso de analitos neutros o no ionizables se pueden utilizar para separarlos electrolitos de separación que contengan los aditivos adecuados (Tabla 1.4) [22-27,52-73] u otro modo de CE (Tabla 1.3) [40-43].

La presencia del Flujo Electroosmótico (EOF) también es fundamental en las separaciones por CE [40-43]. El EOF es el resultado del movimiento relativo del

electrolito de separación respecto a la superficie cargada de la pared interna del capilar. Cuando se emplean disoluciones acuosas, muchas superficies sólidas poseen un exceso de cargas negativas que pueden originarse por la propia ionización de los grupos ácidos de la superficie o por la adsorción de especies cargadas. En los capilares de sílice fundida ocurren probablemente ambos procesos, aunque el EOF está principalmente controlado por los numerosos grupos silanoles (SiOH) que pueden existir en forma aniónica (SiO^-) a partir de pH 2. Así, los contraiones del electrolito de separación se sitúan cerca de la superficie interna del capilar para contrarrestar esta carga negativa, forman una doble capa iónica y originan una diferencia de potencial muy próxima a la pared (potencial zeta (ξ)). Cuando el voltaje es aplicado a través del capilar en polaridad normal (ánodo=*inlet* y cátodo=*outlet*), los cationes que forman la doble capa difusa son atraídos hacia el cátodo. Como están solvatados, el efecto neto es el de arrastrar la disolución del interior del capilar hacia el cátodo, generando el EOF. La magnitud del EOF puede expresarse en términos de movilidad como:

$$m_{EOF} = \varepsilon\zeta/\eta$$

donde m_{EOF} es la "movilidad" del flujo electroosmótico, ξ es el potencial zeta y ε la constante dieléctrica del electrolito de separación. El potencial zeta y el EOF aumentan con el pH, al incrementarse la carga negativa de la superficie interna del capilar. ξ también depende de la fuerza iónica de la disolución: fuerzas iónicas mayores dan como resultado compresiones de la doble-capa, un potencial ξ menor y un menor EOF. Con electrolitos de separación con constantes dieléctricas mayores y viscosidades menores, el EOF es mayor. Lo mismo ocurre si el campo eléctrico aplicado es mayor. El efecto sobre el EOF de cada uno de los aditivos que aparecen en la Tabla 1.4 se debe estudiar en cada caso [40-43].

Para llevar a cabo la optimización de las separaciones por CE hay que tener en cuenta las contribuciones electroforética y electroosmótica sobre la migración de los analitos [40-43]. En presencia de EOF la movilidad neta de una especie es un

balance entre su movilidad electroforética y la debida al EOF. Debido a que a ambas les afecta el pH del electrolito de separación, éste será el principal parámetro a estudiar para optimizar las separaciones electroforéticas [83-84,102-104]. La evaluación del comportamiento electroforético es posible en intervalos amplios de pH, ya que los capilares empleados suelen ser más estables químicamente que las fases estacionarias empleadas en LC [40-43,18,85]. Por los mismos motivos que en LC, cuando se emplean electrolitos de separación que contienen un disolvente orgánico para modificar las condiciones de separación [105-111], también es recomendable medir el pH en las mezclas hidroorgánicas utilizadas [93-94,108-111]. Por otro lado, aunque la movilidad neta es lo que principalmente determina los tiempos de migración de los solutos en CE, la resolución entre picos adyacentes en una mezcla depende también de la obtención de picos estrechos. La eficacia en las separaciones electroforéticas se hace máxima al seleccionar una temperatura y un voltaje adecuados que no generen demasiado calor por efecto Joule, un volumen de inyección correcto que no sobrecargue el sistema y una longitud y diámetro interno de capilar adecuados [40-43]. Otros factores que influyen en la forma de los picos son la composición de la muestra, la difusión longitudinal de los analitos y las interacciones irreversibles del soluto con los grupos presentes en las paredes internas del capilar [40-43].

La adsorción en CE

De entre todos los parámetros que han de considerarse antes de comenzar la optimización de una separación electroforética con capilares de sílice fundida, destaca la necesidad de evitar la adsorción de los solutos sobre las paredes internas del capilar [40-43,46-47]. Este fenómeno es especialmente importante con péptidos de elevado peso molecular o proteínas, ya que se maximizan las interacciones entre los solutos cargados positivamente y los grupos silanoles de la superficie interna del

capilar. El resultado supone recuperaciones no cuantitativas de los analitos, poca reproducibilidad en el EOF y en los tiempos de migración, y ensanchamiento y asimetría de los picos, lo que hace disminuir la eficacia, la resolución y la sensibilidad.

Existen diferentes estrategias para reducir la adsorción irreversible de los analitos en la superficie interna de los capilares de sílice fundida [40-43,46-47].

- *Electrolitos de separación de elevada fuerza iónica.* Si se aumenta la fuerza iónica del electrolito de separación, se reduce la carga efectiva sobre la superficie interna del capilar y además el contraión positivo compite con el analito por unirse a ésta. Sin embargo, no son recomendables fuerzas iónicas demasiado elevadas, ya que si el calor de Joule que se genera no se disipa eficazmente, puede producirse un efecto negativo sobre la calidad y reproducibilidad de las separaciones.

- *Electrolitos de separación a pH extremo.* Trabajando a pH muy ácido (< 2-3), el número de grupos silanoles desprotonados en la superficie interna del capilar será muy pequeño, con lo que disminuirán las interacciones con las proteínas que se encuentran cargadas positivamente. A pH superior al punto isoeléctrico de la proteína, ésta estará cargada negativamente y será repelida por la pared interna del capilar. El problema de trabajar a valores de pH extremos con péptidos de elevado peso molecular y proteínas es que su estructura puede verse alterada a estos pH no fisiológicos.

- *Formación de par iónico.* La formación de pares iónicos entre los analitos y el electrolito de separación suele reducir la carga neta del analito, disminuyendo la interacción con las cargas negativas de la superficie interna del capilar.

- *Capilares recubiertos.* La forma más efectiva de prevenir la adsorción es recubriendo la superficie interna del capilar mediante recubrimientos dinámicos o permanentes [42,112-113]:

Recubrimientos dinámicos [42,112]

Para recubrir la superficie interna de un capilar de forma dinámica, se utilizan aditivos que interaccionan de forma reversible con los grupos silanoles. Según la naturaleza del aditivo empleado y el estado de ionización de los grupos silanoles, esta interacción reversible será más o menos intensa. La principal ventaja de este tipo de recubrimiento es la simplicidad de su preparación, ya que se añade la proporción del compuesto adecuado al electrolito de separación, de forma que el recubrimiento es constantemente renovado al interaccionar continuamente con la superficie del capilar. Su principal inconveniente es que la presencia de estos aditivos en el medio de separación puede provocar una disminución de la sensibilidad, si el compuesto añadido interfiere de alguna forma en la detección empleada, o bien, pueden interaccionar con los analitos, afectando a la selectividad, la eficacia y la resolución de la separación. En la Tabla 1.5 se resumen las características generales de estos recubrimientos dinámicos y algunos ejemplos de sus aplicaciones a las separaciones de péptidos y proteínas [52-56, 58-60,67,70-73].

Tabla 1.5 Recubrimientos en CE para la separación de péptidos y proteínas.

Tipo	Ejemplo	Comentarios	[Ref.]
DINÁMICOS			
Surfactantes	SDS, HTAB, BRIJ, TWEEN	▪ Efectividad limitada ▪ Pueden desnaturalizar irreversiblemente los analitos ▪ Incompatibilidad con detección por MS	[52-56]
Polímeros hidrofílicos	Alquilcelulosas, polivinilalcohol, dextranos, poliacrilamida, polybrene	▪ Efectividad elevada de los que implican interacciones electrostáticas (p.e. polybrene) ▪ Reproducibilidad elevada ▪ Incompatibilidad con detección por MS	[56,58-60,72-73]
Aminas cuaternarias	Putrescina, espermina, cadaverina	▪ Estabilidad limitada de sus disoluciones ▪ Reproducibilidad limitada ▪ Incompatibilidad con detección por MS	[67,70-71]
PERMANENTES			
Por adsorción			
Polímeros simples	Celulosa, PEO, PVA	▪ Estabilidad a largo plazo muy pobre ▪ Estabilidad entre pH 2 y 4 ▪ Son relativamente hidrofóbicos	[60,114-115]
Polímeros entrecruzados	PEI, polybrene, sulfato de dextrano	▪ Intervalos de pH de trabajo amplios ▪ Estabilidad elevada a largo plazo	[72,116-117]
Por enlace covalente			
Por silanización (Si-O-Si-R)	R= poliacrilamida, arilpentafluoro, proteína o aminoácido, ácidos sulfónicos, maltosa, PEG, polivinilirolidinona	▪ Gran versatilidad en el tipo de grupo funcional ▪ Relativamente sencillos de preparar ▪ Estabilidad del enlace Si-O-Si entre pH 4 y 7 ▪ Estabilidad a largo plazo limitada	[118-123]
Por enlace directo Si-C	Poliacrilamida	▪ El enlace directo evita el paso de silanización ▪ Estabilidad entre pH 2 y 10 ▪ Dificultad de preparación	[124-125]
Fases de LC	C8, C18	▪ Pueden favorecer la adsorción de las proteínas	[126]
Fases de GC	PEG	▪ Facilidad de hidrólisis	[123]

Ver pie de Tabla 1.4, PEO: *poly(ethyleneoxide)*

Recubrimientos permanentes [42,113]

Este tipo de recubrimientos se caracteriza por la modificación irreversible de los grupos silanoles de la superficie interna del capilar en una etapa previa a la separación, por lo que no se adicionan al electrolito de separación [42,113]. Se puede diferenciar entre los recubrimientos generados por la adsorción física del aditivo a la superficie interna del capilar y los que implican la modificación covalente de dicha superficie. Estos últimos requieren, en la mayoría de los casos, largos y laboriosos procesos de preparación, que generalmente implican la silanización y posterior derivatización de la superficie activada del capilar. En general, estos recubrimientos tienen una estabilidad más limitada y los intervalos de pH de trabajo suelen ser menos amplios que cuando se trabaja con los capilares sin recubrir. En la Tabla 1.5 se resumen las características generales de estos recubrimientos permanentes y algunos ejemplos de sus aplicaciones a las separaciones de péptidos y proteínas [60,72,114-126].

Mejora de la sensibilidad en CE

A pesar de las excelentes características de la CE, que incluyen la elevada selectividad y eficacia, el bajo consumo de muestra y reactivos, los tiempos de análisis cortos, la excelente sensibilidad por unidad de masa y las elevadas recuperaciones si se evita la adsorción de los analitos [40-43], la técnica tiene un inconveniente importante: la baja sensibilidad por unidad de concentración [48-49]. Esto es debido principalmente a los limitados volúmenes de muestra que es posible inyectar en los capilares de separación y, cuando se emplea detección UV, a los pequeños caminos ópticos que estos mismos proporcionan. Para solucionar este problema con los límites de detección, algunos investigadores han preferido emplear

las técnicas de preconcentración *off-line* [127-128]: recolección de fracciones en LC, extracción líquido-líquido, extracción en fase sólida (SPE), ultrafiltración,... que son laboriosas y difíciles de automatizar y presentan inconvenientes si hay que manipular pequeñas cantidades de muestras valiosas. Paralelamente, a lo largo de los años, se han ido proponiendo diferentes alternativas en línea para la mejora de la sensibilidad en CE [48-49]. Entre ellas podemos distinguir tres estrategias: las que se basan en modificaciones instrumentales, las que lo hacen en métodos electroforéticos y las basadas en el uso de fases estacionarias cromatográficas. Entre las primeras destaca el diseño de nuevos capilares (de mayor diámetro interno, rectangulares,...), celdas de detección UV (celda en forma Z, burbuja o de multireflexión, ...) y detectores (con iluminación y detección axial, de serie de diodos, electroquímicos, de fluorescencia, espectrómetros de masas...) [48-49]. De esta forma se pueden llegar a conseguir aumentos de la sensibilidad de alrededor de tres órdenes de magnitud. Entre los métodos electroforéticos destacan el *stacking* [129-130], la isotacoforesis [131], el isoelectroenfoque [132] y, recientemente, el *sweeping* [133], que permiten inyectar volúmenes de muestra de hasta 50 veces el tamaño del volumen habitual en CE, concentrando los analitos en pequeñas zonas de migración, lo que da como resultado una mejora de las eficacias de las separaciones y los límites de detección. Un efecto similar se puede conseguir inyectando electrocinéticamente la muestra, si los analitos están cargados adecuadamente al pH de la muestra [129]. Las mejoras conseguidas empleando estos métodos electroforéticos pueden variar entre un orden y los cinco órdenes de magnitud que se han llegado a observar con *sweeping* [133], dependiendo de los analitos, las muestras, las condiciones de separación y la metodología de preconcentración seleccionada [129-133]. Los limites de detección pueden ser aún mejorados, ya que, en la mayoría de los casos, los métodos electroforéticos son compatibles con las modificaciones instrumentales que hemos comentado anteriormente y con el uso de fases estacionarias cromatográficas que se presenta a continuación.

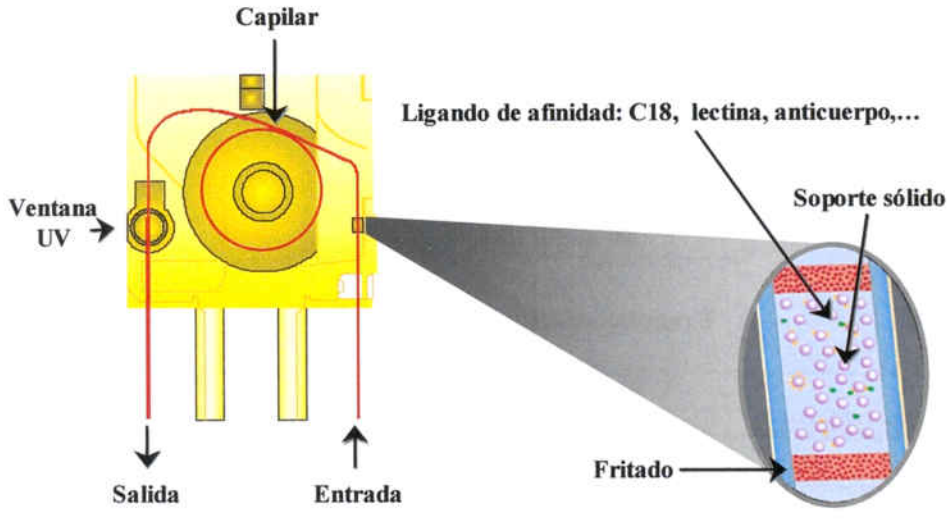


Figura 1.2 Cartucho de CE-UV con capilar modificado para realizar SPE-CE.

Los métodos basados en fases estacionarias cromatográficas para mejorar la sensibilidad en CE, se basan en la interacción reversible de los analitos con una fase estacionaria y pueden ser agrupados bajo la denominación de Extracción en Fase Sólida acoplada en línea a la CE (SPE-CE), aunque estas metodologías han recibido, a lo largo de los años, muy diversas denominaciones [48-49,134-139]. En SPE-CE, se inmoviliza un determinado ligando de afinidad sobre un soporte sólido situado cerca de la entrada del capilar de separación, tal y como se muestra en la Figura 1.2. Se han propuesto numerosos diseños y materiales para la construcción de los preconcentradores empleados en SPE-CE (Figura 1.3) [134-139]. Si se emplean fritados, es importante que su porosidad y tamaño sean los adecuados para evitar la formación de burbujas y el bloqueo de la migración de los analitos. Las fases estacionarias también han de cumplir este requisito, además de permitir máxima eficacia en la preconcentración. En general, si se emplea la superficie interna del capilar de separación como soporte para el ligando de afinidad, la superficie derivatizada suele ser menor que empleando partículas o una membrana porosa adecuada.

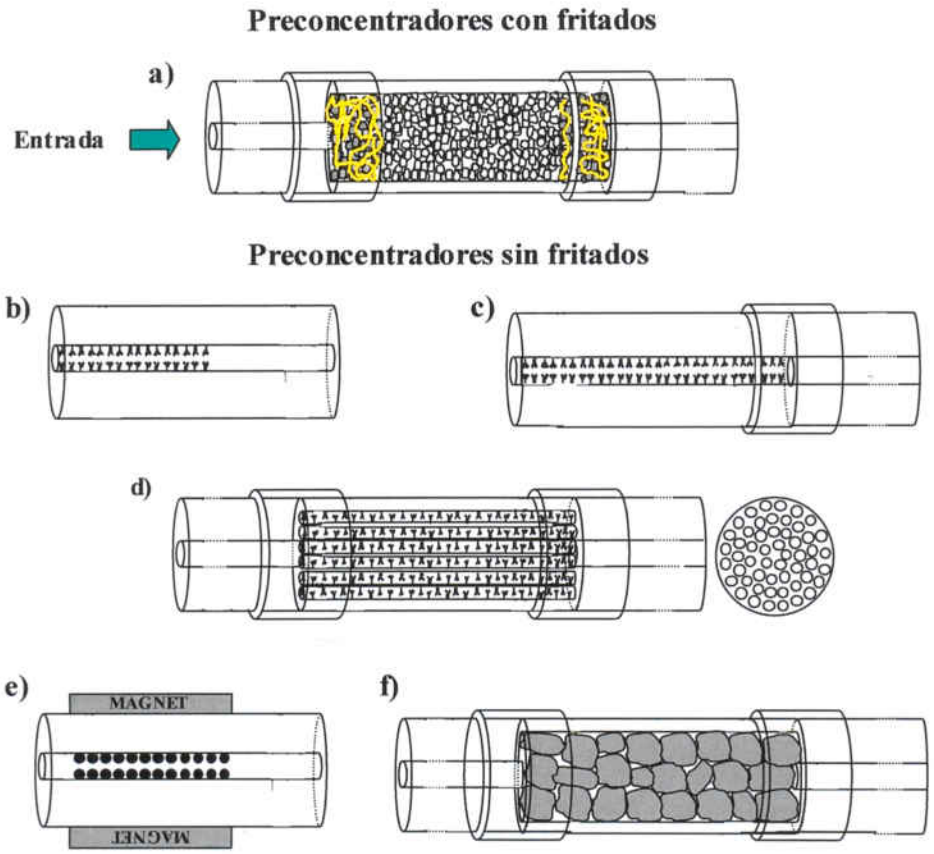


Figura 1.3 Tipos de preconcentradores empleados en SPE-CE. Con fritados. a) Doble fritado. El fritado de la entrada puede estar ausente. Sin fritados. b,c) Una porción del capilar de separación actúa de soporte sólido, d) Capilar en multicanal, e) Partículas magnéticas y f) Se retienen partículas de diámetro superior al del capilar de separación, o una membrana adecuada, o un soporte de afinidad monolítico o similar.

En la Figura 1.4 se describe esquemáticamente la metodología empleada en SPE-CE [134-139]. Después de acondicionar la fase estacionaria, se procede a introducir un relativamente elevado volumen de muestra. Las moléculas del analito interaccionan y quedan retenidas sobre el ligando de afinidad. Si estas interacciones son muy selectivas, el proceso de preconcentración permite a la vez la purificación

del analito deseado. Las moléculas de analito que no han interaccionado convenientemente, los contaminantes y las impurezas son eliminadas lavando adecuadamente. Las moléculas de analito retenidas son eluidas entonces con un pequeño volumen de la disolución adecuada, lo que da como resultado un aumento de la concentración.

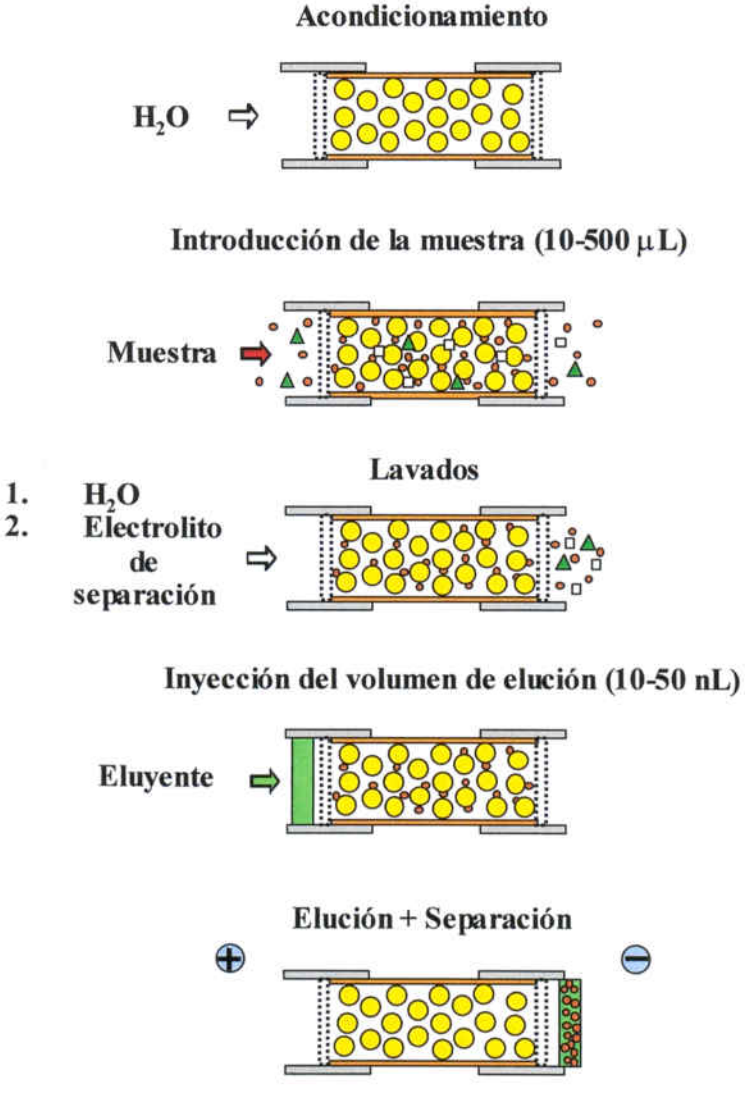


Figura 1.4 Representación esquemática de la metodología empleada en SPE-CE.

El factor de preconcentración conseguido depende en gran medida del ligando de afinidad y del analito y de la correcta y meticulosa optimización de las diferentes etapas del proceso de preconcentración y la posterior separación: acondicionamiento de la fase estacionaria, volumen de muestra introducido, de los lavados, de la elución, composición de la disolución de muestra, del eluyente, del electrolito de separación, ... [134-139.]

Desde los primeros trabajos de SPE-CE publicados por N. A. Guzmán *et al* a principios de los 90 empleando fases estacionarias de inmunoafinidad [140], han sido numerosas y variadas las aplicaciones desarrolladas empleándose todo tipo de fases estacionarias, comerciales o caseras [134-139]. En líneas generales se puede diferenciar entre las no selectivas (C8, C18, ...) y las selectivas (metales enlazados, lectinas, anticuerpos, ...). Las enzimas son ligandos de afinidad que reaccionan selectivamente con determinados substratos, pero que no permiten su preconcentración. En este caso concreto se habla de microreactores y no de preconcentradores. La Tabla 1.6 muestra una recopilación exhaustiva de las aplicaciones de la SPE-CE aparecidas hasta este momento [140-211]. Como podemos observar, se han analizado preferentemente péptidos y proteínas: mezclas de patrones, péptidos obtenidos en digestiones enzimáticas de proteínas, péptidos o proteínas en matrices biológicas, ... [141-190]. También se ha demostrado su aplicabilidad en el análisis de moléculas orgánicas de bajo peso molecular [140,191-201] y aniones inorgánicos [202-204]. En algunas aplicaciones se han llegado a alcanzar niveles de preconcentración de hasta cuatro órdenes de magnitud empleando anticuerpos [48-49,137-138]. También se ha combinado el *stacking* o la isotacoforesis con la SPE-CE para obtener mejoras en estos límites de detección [136,139,144]. Empleando anticuerpos o fragmentos de anticuerpos se ha demostrado también la extraordinaria utilidad de la SPE-CE para la captura selectiva y la separación de péptidos en matrices complejas, tales como tejidos o fluidos biológicos [138].

Tabla 1.6 Aplicaciones más destacables de la SPE-CE en el período 1991-2003.

Analitos	Tipo de fase estacionaria	CE	[Ref.]
BIOMOLÉCULAS			
Aminoácidos	RPLC	CE	[141]
Péptidos originados en digestiones enzimáticas			
Tripsinización de la β-caseína bovina	C18	CE	[142]
Tripsinización de la cadena β de la insulina	C18	CE	[143]
Tripsinización de la BSA y de las proteínas de la levadura	C18	CE (stacking)	[144]
Tripsinización de la BSA	C18	CE-ESI-MS	[145-148]
Tripsinización del dominio recombinante catalítico de la quinasa de la cadena pesada de la Miosina I	C18	CE-ESI-MS	[149]
	C18	tITP-ESI-MS	[147]
Proteólisis de la apomioglobina	C18	CE	[150]
Proteólisis de la bacteriorodopsina	C18	CE	[151]
Proteólisis de una lectina	C18	CE-ESI-MS	[152]
Tripsinización del citocromo C bovino	RPLC	CE	[141,143]
Tripsinización de la α-caseína y ribosoma de *S. Cerevisiae*	RPLC	CE-ESI-MS	[153]
Tripsinización de la proteína que une los ácidos grasos intestinales (IFABP)	Membrana SDB	tITP-ESI-MS	[154]
Péptidos patrones			
Péptidos sintéticos	C18	CE	[150,155]
Angiotensina II, bombesina, bradicinina, LHRH, α-MSH, Leu-encefalina, Met-encefalina, oxitocina, TRH	C18	CE	[155-156]
Angiotensina I, Leu-encefalina, péptido intestinal vasoactivo, [Glu]fibrinopéptido B	C18	CE	[157]
Fasciculina (toxina de serpiente)	C18	CE	[158]
Angiotensina II, gonadorelina	C18	TITP	[159]
Péptidos sintéticos	C18	CE-ESI-MS	[160]
Colecistoquinina$_{10-20}$, angiotensina I, Val$_4$,Ile$_7$-angiotensina III, sustancia P$_{3-11}$, sustancia P$_{1-9}$, Leu-encefalina. fibrinopéptido B	C18	CE-ESI-MS	[152]
Fibrinopéptido A, péptido relacionado con la fibronectina, α-endorfina, residuos de la cadena β de la insulina, Tyr-bradicinina	C18	CE-ESI-MS	[147]
	C18	tITP-ESI-MS	[147]

Analitos	Tipo de fase estacionaria	CE	[Ref.]
Oxitocina, angiotensina II	C18	CE-ESI-MS	[148]
Péptidos MHC clase I	C18	CE-ESI-MS	[148]
Angiotensina I, Leu-encefalina, péptido intestinal vasoactivo, [Glu]fibrinopéptido B, proteinasa K, deoxiribonucleasa I, ribonucleasa	Membrana SDB	CE	[157]
Angiotensina II, gonadorelina	Membrana SDB	tITP	[159]
Péptidos sintéticos	Membrana SDB	CE-ESI-MS tITP-ESI-MS	[161]
Péptidos sintéticos	Membrana SDB	tITP-ESI-MS	[162]
Péptidos MHC clase I	Membrana SDB	tITP-ESI-MS	[163-168]
Angiotensina II, bombesina, bradicinina, LHRH, α-MSH, Leu-encefalina, Met-encefalina, oxitocina, TRH	Membrana SDB	tITP-ESI-MS	[161,163,165, 168-169]
Angiotensina II, gonadorelina	Membrana SDB	tITP-ESI-MS	[170-171]
Péptidos sintéticos	Membrana polimérica	tITP-ESI-MS	[162,172]
Factores de crecimiento de la insulina	Membrana polimérica	tITP-ESI-MS	[172]
Bradicinina, des-R^9-bradicinina, dermorfina,dinorfina,endorfina	Membrana PSDB	tITP-ESI-MS	[173]
Fosfopéptidos, Met-encefalina, Leu-encefalina, GY, VYV	Fe(III)	CE-ESI-MS	[174]
Glicopéptidos	Concanavalina A	CE	[175]
Troponina humana cardiaca I	Anticuerpo monoclonal	CE	[176]
Ciclosporina A	Anticuerpo monoclonal	CE	[177]
Neurotensina, Met-encefalina	Mezcla de anticuerpos policlonales	CE	[48]
Citoquinas	Fragmentos Fab de anticuerpo policlonal	CE-LIF	[178]
Citoquinas recombinantes	Fragmentos Fab de anticuerpo monoclonal	CE-LIF	[179,180]
Hormona liberadora de gonadotropina (GnRH)	Fragmentos Fab de anticuerpo monoclonal	CE-ESI-MS	[181]
Proteínas			
Proteínasa K, deoxiribonucleasa I, ribonucleasa	C18	CE	[157]
Metalotioneína de conejo	RPLC	CE	[182-183]
Proteínas del humor acuoso	Membrana C8	tITP-CE-MS	[166,184]
Lisozima,mioglobina,anhidrasa carbónica, HSA	Membrana C2	tITP	[185]
Apomioglobina	Membrana SDB	tITP-ESI-MS	[169]
Proteínas Bence-Jones	Membrana SDB	tITP-ESI-MS	[166]
Proteínas Bence-Jones	Membrana polimérica	tITP-ESI-MS	[172]

Analitos	Tipo de fase estacionaria	CE	[Ref.]
Citocromo C, lisozima, ribonucleasa A, α-quimotripsinoógeno A	Fibras SPME	CE	[186]
Anhidrasa carbónica	Zn(II)	CE	[187]
IgE	Anticuerpo policlonal	CE	[188]
IgG	Anticuerpo monoclonal	CE	[189]
FITC-biotina	Anticuerpo policlonal	CE-LIF	[190]
	Otras		
Lipopolisacárido en extractos bacterianos	Membrana SDB	CE	[157]
Extracto de células de melanoma	Membrana PSDB	tITP-ESI-MS	[173]
MOLECULAS ORGÁNICAS DE BAJO PESO MOLECULAR			
Prometon, prometrina	C18	CE	[191]
Terbutalina y enantiómeros	C18	CE	[192]
Haloperidol	C18	CE	[193]
	C18	CE-ESI-MS	[160]
Terbutalina, bambuterol, efedrina, bromofeniramina	C18	CE (*stacking*)	[194]
Derivados tipo sulfonilurea	C18	CE	[195]
Verapamilo	C4	CE	[196]
Doxepina, propanolol	RPLC	CE	[197]
Haloperidol	Membrana C18	CE-ESI-MS	[166]
Haloperidol y análogos sintéticos	Membrana SDB	tITP	[169,198]
Haloperidol y análogos sintéticos	Membrana Polimérica	tITP-ESI-MS	[172]
3-fenilamino-1,2-propanodiol	Membrana polisulfonada	CE	[199]
Antidepresivos tricíclicos (amitriptilina, imipramina, nortriptilina, desipramina)	Fibras SPME	CE	[200]
Metanfetamina	Anticuerpo monoclonal	CE	[140]
Atrazina y Atrazina marcada con fluoresceína	Anticuerpo monoclonal	CE	[201]
ANIONES INORGÁNICOS			
NO_3^-, Br^-	Intercambio iónico	tITP	[202]
NO_3^-, Br^-, I^-, SCN^-, CrO_4^{2-}	Intercambio iónico	tITP	[203]
NO_3^-, Br^-, I^-, CrO_4^{2-}, MoO_4^{2-}	Intercambio iónico	tITP	[204]
REACTORES ENZIMÁTICOS			
β-caseína	Tripsina	CE-LIF	[205-207]
α_1-Glicoproteína ácida	Tripsina	CE	[207]
Cadena β de la insulina oxidada	Tripsina	CE-ES-MS	[208]
β-caseína	Pepsina	CE	[207]
Cadena β de la insulina oxidada	Carboxipeptidasa-Y	CE	[207]

Analitos	Tipo de fase estacionaria	CE	[Ref.]
Péptidos fosforilados	Fosfatasas	CE-ES-MS	[209]
p-nitrofenol	Fosfatasa alkalina	CE	[189]
Ácidos nucleicos	Ribonucleasa, hexoquinas, deaminasa	CE	[210]
Péptido derivatizado con fluoresceína	Proteasa	CE	[189]
Subunidad α de la 4-prolilhidroxilasa (digestión+derivatización en línea con FITC)	Proteasa / FITC complejado a un anticuerpo contra FITC	CE	[211]

Se han llegado a inmovilizar varios anticuerpos de moléculas diferentes en un mismo preconcentrador para capturarlas selectivamente en un mismo análisis [48]. Por otro lado, también se han realizado numerosos trabajos en los que se emplean microreactores en línea con la CE para realizar digestiones enzimáticas [205-211]. En uno de estos trabajos, se derivatizan también, en un segundo microreactor, los péptidos generados en la digestión enzimática de una proteína en un primer microreactor [211]. Las extraordinarias posibilidades de estas metodologías de preconcentración y microreacción aumentan si se emplea detección por MS, lo que permite la identificación y la caracterización de los analitos o productos de la reacción [181]. En esta línea, numerosos trabajos han demostrado su extraordinario potencial para la secuenciación de proteínas en matrices complejas diluidas, lo que la convierte en una excelente alternativa para la investigación en proteómica [212-213]. Recientemente, se ha presentado además un instrumento que permite realizar SPE-CE de varias muestras en paralelo, para las aplicaciones que requieren un gran número de análisis por unidad de tiempo [214]. Sin embargo, a pesar del extraordinario potencial de la SPE-CE y la SPE-CE-ESI-MS para la detección, separación y caracterización de cualquier tipo de analitos en matrices complejas diluidas, todavía existen hoy pocos estudios sistemáticos que determinen rigurosamente los parámetros que afectan a la preconcentración y separación. Este hecho, junto con su nula disponibilidad comercial, frena la aplicación generalizada de estas metodologías.

1.2.4. LC, CE y Espectrometría de Masas (MS).

En los últimos años la Ionización por Electrospray (ESI) y la Ionización por Desorción con Láser Asistida por una Matriz (MALDI), se han afirmado definitivamente como herramientas fundamentales en Espectrometría de Masas (MS) para la caracterización de biomoléculas altamente polares, termolábiles y de cualquier peso molecular [20-21,31,215-218]. Prueba de ello y de su importancia es el Premio Nobel de Química con el que fueron galardonados el pasado año 2002 J. Fenn y K. Tanaka por su contribución a la caracterización de biomacromoléculas mediante MS empleando ESI [28] y MALDI [29], respectivamente [219]. Hoy en día ambas técnicas resultan indispensables, en combinación con las técnicas de separación de alta resolución, para la separación, identificación y caracterización de mezclas complejas de todo tipo de moléculas, entre ellas los péptidos y proteínas [20-21,31,50-51,215-218].

1.2.4.1. Ionización por Electrospray (ESI).

En ESI se produce la ionización de los analitos a presión atmosférica, mediante la aplicación de una diferencia de potencial entre el extremo del capilar por donde fluye la muestra líquida (normalmente a flujos comprendidos entre 1 y 100 μL/min) y el contraelectrodo situado en la entrada del espectrómetro (Figura 1.5) [31]. Por efecto de este intenso gradiente eléctrico, la muestra emerge del capilar en forma de un aerosol de pequeñas gotas cargadas. A medida que estas gotas avanzan hacia el orificio de entrada del espectrómetro, su tamaño se va reduciendo, se van desolvatando, hasta que las fuerzas culombianas de repulsión entre los iones con múltiple carga generados en su interior, son capaces de vencer la tensión superficial, momento en que pasan a la fase gaseosa. En algunas ocasiones se suele favorecer la desolvatación de las gotas y la obtención de iones en fase gas empleando N_2 como

gas de secado, a alta presión y a temperatura moderada. En general, la nebulización se asiste mediante un flujo de gas nitrógeno, lo que además permite la introducción de flujos mayores de líquido [220]. Globalmente el proceso de ionización genera iones moleculares multicargados, lo que permite el análisis de moléculas de elevada masa molecular empleando analizadores con intervalos de m/z convencionales, siendo los cuadrupolos, las trampas iónicas y los analizadores de tiempo-de-vuelo (TOF) los más ampliamente empleados [31].

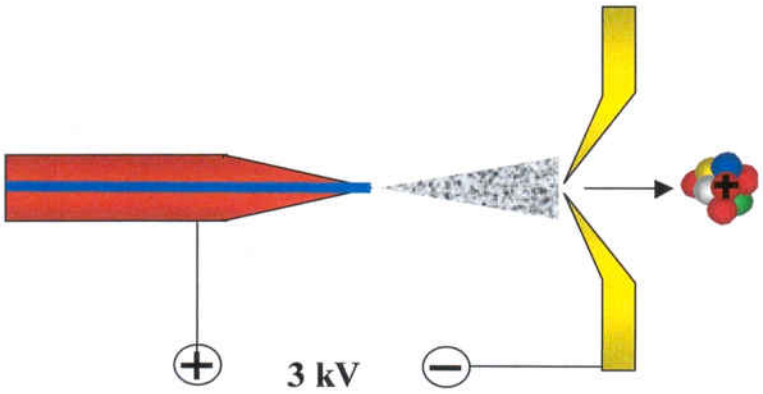


Figura 1.5 Representación esquemática de la Ionización por Electrospray (ESI).

La estructura y las propiedades físico-químicas de cualquier molécula determinan su capacidad intrínseca para ionizarse al pH de la fase móvil o del electrolito donde está disuelta, pero la eficacia de su ionización y, por lo tanto, la sensibilidad en la detección, están íntimamente relacionadas con las propiedades de esta disolución [31]. En ESI se suelen emplear disoluciones hidroorgánicas (MeCN-H_2O, MeOH-H_2O e iPrOH-H_2O) de tensión superficial y fuerza iónica moderadas, conteniendo tampones o aditivos preferentemente volátiles que aseguren la ionización de los analitos en modo positivo (TFA, ácido fórmico, ácido acético, ...) o negativo (trietilamina, hidrogenocarbonato amónico,...) [31,221-228]. A medida que

disminuye la volatilidad de la fase móvil disminuye la señal y la relación señal/ruido obtenida y, si además la disolución tiene una elevada fuerza iónica, se puede producir la obstrucción del orificio de entrada o la contaminación del espectrómetro de masas.

En la mayoría de las ocasiones, el análisis directo de una muestra compleja mediante ESI-MS no permite la identificación y la cuantificación de los analitos deseados, y es necesaria la separación previa de los componentes de la mezcla [31]. El acoplamiento en línea de la LC y la CE a la ESI-MS (LC-ESI-MS y CE-ESI-MS, respectivamente), se lleva a cabo utilizando interfases con el diseño adecuado en cada caso [31,42,50-51]. En cuanto a los analizadores, los cuadrupolos y las trampas iónicas son los de uso más generalizado [31,42,50-51,229-230]. Los actuales analizadores TOF, que proporcionan una frecuencia de barrido elevada (0.1 s), constituyen una de las mejores alternativas cuando se obtienen separaciones con picos de elevada eficacia, como en el caso de la CE-ESI-MS [231-232]. En un analizador TOF los iones son separados de acuerdo a sus tiempos de vuelo, que a su vez dependen de sus relaciones m/z: los iones de mayor m/z se desplazarán a lo largo del tubo de vuelo a menor velocidad y serán detectados después de los de menor m/z. Todos los iones que se generan durante la ionización llegan al detector, lo que permite una elevada sensibilidad. El detector es capaz de distinguir las diferentes masas, tanto mejor cuanto mayor es la trayectoria recorrida por los iones y cuanto menor es la dispersión de energías de los iones formados en la fuente. Así, si se dispone de un analizador TOF con reflectrón, éste permitirá reenfocar los iones de la misma masa sobre el detector. Ésta y otras modificaciones instrumentales, como la extracción retardada (DE), han permitido un aumento en la resolución y la exactitud del analizador TOF respecto a otros analizadores. Todo ello, junto con el uso de analizadores que permiten realizar Espectrometría de Masas en Tandem (MS-MS) en línea a la LC o la CE, proporciona herramientas de extraordinaria utilidad a la hora de separar mezclas complejas de péptidos y proteínas y caracterizar

exhaustivamente sus estructuras primarias (secuencias aminoacídicas, fosforilaciones, glicosilaciones, oxidaciones, ...) [31,233-235].

Cromatografía de Líquidos acoplada a la Espectrometría de Masas con Ionización por Electrospray (LC-ESI-MS)

En la actualidad la LC-ESI-MS es una técnica que se aplica de forma rutinaria en los laboratorios analíticos modernos. A ello ha contribuido sin duda el gran desarrollo instrumental que ha experimentado en los últimos años, lo que ha permitido el establecimiento de metodologías analíticas selectivas, sensibles y robustas. Muchas de las nuevas interfases de ESI permiten la ionización de los analitos en condiciones cada vez más extremas: flujos elevados, fases móviles de elevada fuerza iónica o con aditivos no volátiles, bajo contenido en disolvente orgánico, analitos difíciles de ionizar,... [31]. Sin embargo, sigue siendo recomendable operar a flujos bajos, con una fase móvil de composición adecuada, para obtener una elevada eficacia en la ionización de la muestra. Cuando se emplean columnas cromatográficas convencionales de 4.6 mm de d.i, es necesaria la división post-columna del flujo de fase móvil para adaptarse a los flujos óptimos de operación en ESI. De esta forma, parte de los analitos se eliminan, aunque alternativamente pueden ser detectados empleando otra técnica de detección y recolectados a la salida del divisor de flujo. En la actualidad, se ha extendido el uso de columnas capilares (25-500 μm i.d.) que permiten trabajar a flujos de fase móvil compatibles con la operación en ESI convencional (>1μL/min), o incluso a flujos muy inferiores (microESI o nanoESI, nL/min) evitando la pérdida de muestra ocasionada por la división del flujo [236-238].

En general, la RPLC es la modalidad más empleada en LC-ESI-MS, ya que como habíamos comentado, además de tener un mayor poder de resolución, el uso de fases móviles hidroorgánicas de fuerza iónica moderada permite la ionización

efectiva de los analitos y sensibilidades adecuadas, en modo positivo o negativo [221-228]. Los péptidos y las proteínas se han analizado empleando mayoritariamente fases móviles hidroorgánicas de acetonitrilo, metanol o mezclas de ambos, a pH ácido para asegurar su ionización positiva [222-224]. Habitualmente, se utiliza el TFA, un excelente formador de par iónico que proporciona una selectividad y resolución adecuadas, pero que puede suprimir de manera muy eficaz la carga de los iones positivos, disminuyendo la sensibilidad de los análisis. Una sencilla forma para mejorar la sensibilidad en algunos casos concretos, consiste en la adición post-columna de disolventes orgánicos que disminuyan la tensión superficial de la fase móvil [225-226] o disoluciones de ácidos débiles que desplacen el TFA, permitiendo la liberación de los cationes de la muestra [223]. También se ha propuesto la eliminación del reactivo de par-iónico mediante un procedimiento de extracción en línea inmediatamente anterior a la detección por MS [239] o la utilización de reactivos formadores de par-iónico más volátiles, como otros ácidos carboxílicos perfluorados [227-228]. Como alternativa al TFA, se suelen emplear los ácidos fórmico o acético, que no presentan problemas de supresión de señal, pero que pueden afectar negativamente a la selectividad y resolución en las separaciones.

Electroforesis Capilar acoplada a la Espectrometría de Masas con Ionización por Electrospray (CE-ESI-MS)

La CE-ESI-MS constituye actualmente una excelente alternativa a la LC-ESI-MS para el análisis de mezclas complejas de péptidos y proteínas [15-16,24-27], aunque las dificultades que plantea el acoplamiento continúan frenando el desarrollo de interfases y metodologías robustas y reproducibles [31,42,50-51,229-230], lo que unido a los problemas de reproducibilidad en los tiempos de migración

[44-45] y sensibilidad de la CE [48-49], dificultan su aplicación como técnica de rutina.

En la mayoría de los casos, las interfases desarrolladas para CE-ESI-MS son modificaciones de las interfases previamente diseñadas para LC-ESI-MS [31,42,50-51,229-230]. La dificultad radica en que para ello se ha de tener en cuenta que los flujos de electrolito de separación en los capilares de CE no exceden, en la mayoría de los casos, de los 100 nL/min y que en la interfase se debe cerrar la conexión eléctrica a la salida del capilar, que ya no está sumergido en un vial con electrolito de separación. Estos dos factores implican el diseño de interfases que proporcionando una conexión eléctrica adecuada, permitan operar a bajo flujo de electrolito de separación (microelectrospray o nanoelectrospray), o bien el uso de un líquido auxiliar que aumente el flujo que llega hasta la interfase para operar en ESI convencional. En ambos casos, se tendrá que considerar que la eficacia de la ionización dependerá en gran medida de la volatilidad del electrolito de separación o de la disolución resultante, y ésta estará seriamente comprometida si las disoluciones presentan fuerza iónica elevada o elevada concentración de aditivos no volátiles [221-228]. Este último es el principal motivo por el cual en la mayoría de las aplicaciones que se han descrito en CE-ESI-MS se emplea CZE, y solamente se han descrito algunas aplicaciones trabajando por ejemplo con MEKC o CGE [42,50-51].

Desde que las primeras interfases fueron introducidas por el grupo de Smith [240-241], se han propuesto y se siguen proponiendo numerosas modificaciones (Figura 1.6) [31,42,50-51,229-230]. El principal objetivo es obtener interfases robustas que permitan una gran versatilidad en la selección de las metodologías de separación y una elevada sensibilidad en la detección, a la vez que se mantiene la resolución de la separación electroforética. Entre las interfases empleadas podemos diferenciar básicamente entre las que utilizan un líquido auxiliar (*sheath-flow*) [240] y las que no lo utilizan (*sheathless*) [241].

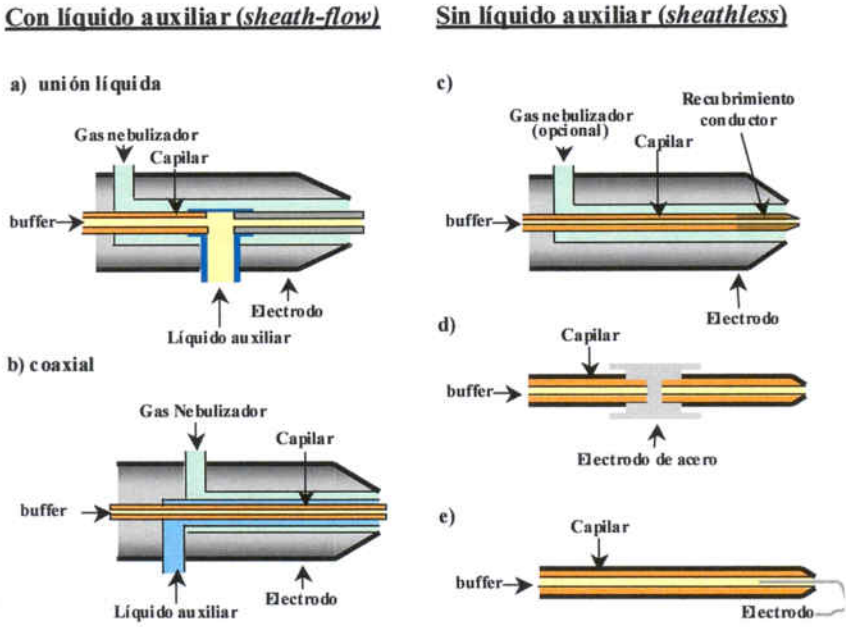


Figura 1.6 Representación esquemática de varias interfases de CE-ESI-MS

Interfases Sheath-flow

En este tipo de interfases la conexión eléctrica se consigue mediante un líquido auxiliar (*sheath-liquid*) que se mezcla con el electrolito de separación, bien a la salida del capilar de separación a través de un tubo coaxial o bien empleando una conexión adecuada inmediatamente antes de la ionización (Figura 1.6) [31,42,50-51,229-230,240]. En ambos casos, la dilución de los analitos provoca una disminución en la sensibilidad. El uso de conexiones para realizar la mezcla (Figura 1.6-a), implica generalmente un volumen muerto que puede tener efectos negativos sobre las separaciones [242-243]. Este volumen muerto es mucho menor cuando se utiliza un líquido auxiliar coaxial (Figura 1.6-b). Esta última interfase es además mucho más robusta y simple de preparar y manejar, por lo que ha sido ampliamente utilizada. La nebulización puede además asistirse, dependiendo del flujo de la

mezcla (normalmente de entre 1 y 10 μL/min), mediante gas N_2 también coaxial a la mezcla. Sin embargo, la presencia de este gas, genera un flujo laminar hidrodinámico positivo hacia la salida del capilar que puede afectar tanto a la eficacia de la nebulización, como a la separación y los volúmenes de inyección [244-245]. La composición del líquido auxiliar, ha de asegurar que la mezcla resultante con el electrolito de separación, sea fácilmente nebulizable y permitir la conexión eléctrica a la salida del capilar de separación. En general la composición del líquido auxiliar es uno de los parámetros que más afecta a la eficacia de la ionización y se debe optimizar para cada aplicación concreta [242,246-250]. Normalmente se trata de mezclas hidroorgánicas que contienen metanol, isopropanol o acetonitrilo y concentraciones moderadas de un ácido volátil (0.1-1% (v/v) HFor o HAc), si se trabaja en modo de ionización positivo. Tras la mezcla, la composición del electrolito de separación en ambos extremos del capilar no es idéntica, de manera que los contraiones del liquido auxiliar pueden difundirse hacia el interior del capilar de separación, generando un medio discontinuo que puede provocar modificaciones en los tiempos de migración de las especies y ensanchamientos de banda no deseados [247-248].

Interfases Sheathless

En este tipo de interfases la conexión eléctrica a la salida del capilar de separación se consigue mediante un contacto eléctrico directo entre un metal y el electrolito de separación (Figura 1.6) [31,42,50-51,229-230,241]. La sensibilidad es elevada porque no existe un líquido auxiliar que diluya los analitos. Sin embargo, a diferencia del caso anterior, se han de emplear electrolitos de separación de baja fuerza iónica, un cierto contenido en disolvente orgánico y no pueden utilizarse aditivos no volátiles, lo que limita considerablemente su versatilidad a la hora de desarrollar nuevas metodologías de separación [241]. En general se suele además

afilar el extremo de salida del capilar de separación para aumentar la eficacia de la ionización. En la Figura 1.6 aparecen algunos de los diseños descritos en la bibliografía. La opción más adecuada es emplear recubrimientos metálicos para hacer conductora la superficie externa de la salida del capilar (Figura 1.6-c), aunque éstos suelen degradarse rápidamente [50-51]. En el resto de alternativas, que emplean una conexión a la salida del capilar para acoplar una punta de nanoelectrospray o que utilizan electrodos, la resolución en las separaciones puede verse afectada (Figura 1.6-e). En este último caso también se pueden formar burbujas dentro del capilar debido a la electrolisis del agua. Estos y otros fenómenos electroquímicos pueden tomar especial relevancia cuando se emplea una interfase *sheathless* para trabajar en CE-ESI-MS [50-51].

1.2.4.2. Ionización por Desorción con Láser Asistida por una Matriz (MALDI)

En MALDI se produce la ionización de los analitos cuando se hace incidir sobre la muestra un láser de energía adecuada en el vacío elevado del espectrómetro de masas (Figura 1.7) [216-218]. Para que esto ocurra, se debe mezclar primero una disolución de la muestra con un gran exceso de una disolución de la matriz adecuada. Cuando el disolvente se evapora, las moléculas de ambas disoluciones cocristalizan adecuadamente para que la matriz, al absorber los fotones de la radiación láser, permita la ionización efectiva de la muestra. En los trabajos originales de K. Tanaka se utilizaba como matriz un polvo ultrafino de cobalto [29], pero F. Hillenkamp y M. Karas revolucionaron la técnica introduciendo el uso de ácidos orgánicos de bajo peso molecular [30]. El láser más empleado es el de N_2 a 337 nm, y se puede operar tanto en modo positivo como en negativo, dependiendo de la matriz empleada. Los iones que se generan tienen pocas cargas, incluso los de moléculas de masa molecular elevada, lo que da como resultado espectros de mayor simplicidad que los obtenidos en ESI-MS [216-218].

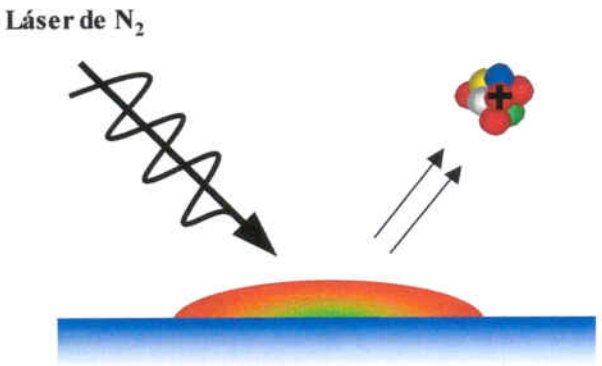


Figura 1.7 Representación esquemática de la Ionización por Desorción con Láser Asistida por una Matriz (MALDI).

Al igual que en ESI, el mecanismo por el que se produce la ionización no se entiende completamente y la selección de la matriz adecuada y la forma de preparar la mezcla se realiza muchas veces basándose en la propia experiencia. Las matrices más empleadas para péptidos son derivados del ácido cinámico (ácido α-ciano-4-hidroxicinámico) y del ácido dihidroxibenzoico (ácido 2,5-dihidroxibenzoico), mientras que los derivados del ácido sinapínico (ácido *trans*-3,5-dimetoxi-4-hidroxicinámico) son más utilizados para el análisis de proteínas [216-217]. La eficacia del proceso depende fundamentalmente del analito y de las matrices empleadas, así como de la composición de la disolución original del analito. Para facilitar la ionización, la muestra se disuelve normalmente en mezclas hidroorgánicas de MeCN con 0.1 % de TFA o sales de plata. Sin embargo, grandes cantidades de sal en las muestras pueden causar supresión de la señal y en ocasiones es conveniente desalarlas [251]. A diferencia de lo que ocurre al utilizar ESI, los iones no se generan de forma continua, sino en paquetes discretos y esto limita el tipo de analizadores que se emplean y su acoplamiento en línea con la LC o la CE. El analizador TOF es el más utilizado, ya que permite la medida de un espectro de

masas completo cada vez que se ioniza la muestra [216-218]. Recientemente la aparición de los equipos MALDI-Cuadrupolo-TOF y MALDI-TOF-TOF está permitiendo realizar experimentos MS-MS para caracterizar inequívocamente la estructura de los analitos [218]. Paralelamente se avanza continuamente en el diseño de instrumentos que automatizan la combinación *off-line* de la LC, la GE, el IEF o la CE con MALDI-TOF [32].

1.3. MÉTODOS DE OBTENCIÓN DE PÉPTIDOS Y PROTEÍNAS

Aislar cantidades considerables de péptidos o proteínas naturales, no es viable en la gran mayoría de los casos, ya que por un lado pueden encontrarse a muy bajas concentraciones en los fluidos biológicos o en tejidos de disponibilidad limitada, y por otro pueden estar contaminados con agentes tóxicos o infecciosos tales como los virus de la hepatitis o el SIDA [1-2,12-13]. En la actualidad, las técnicas modernas de síntesis química permiten la obtención de forma relativamente rápida y sencilla de una gran variedad de péptidos y proteínas de relativamente baja masa molecular [4,10-14]. Por otra parte, las técnicas biotecnológicas de ingeniería genética, se utilizan generalmente para la obtención de moléculas cuyo tamaño o complejidad estructural están fuera del alcance sintético [3,7-9,252-253]. Ambos procedimientos permiten introducir las modificaciones estructurales adecuadas en las moléculas naturales para obtener análogos con mejores propiedades y menores inconvenientes (actividad biológica, especificidad, conformación, solubilidad, estabilidad, resistencia a las enzimas digestivas o proteolíticas, rápida metabolización, baja toxicidad,...). Así por ejemplo, las hormonas peptídicas sintéticas: leuprolide, goserelina, buserelina, nafarelina o triptorelina, son más eficaces que la LHRH natural (Hormona Liberadora de la Hormona Luteinizante) [254] y la NESP (Nueva Proteína Estimuladora de la Eritropoyesis) presenta una actividad biológica similar a la de la EPO natural (Eritropoyetina), pero una mayor duración de su efecto [255-256]. Habitualmente, los péptidos naturales son extremadamente vulnerables a la acción de las enzimas digestivas y poco permeables a la membrana intestinal, lo que dificulta su administración oral [7]. En la actualidad, se trabaja activamente en el desarrollo de nuevos principios activos peptídicos y formulaciones que permitan producir fármacos peptídicos que no deban administrarse por vía intravenosa. Así, ya existen algunos productos en el mercado que se administran en forma de spray nasal (oxitocina) o gotas para los ojos

(eledoisina) [254] y varias compañías farmacéuticas tienen en avanzado estado de desarrollo nuevas formulaciones de insulina de administración oral o nasal, que prometen mejorar espectacularmente la calidad de vida de millones de diabéticos de todo el mundo [252-253].

1.3.1. Síntesis de Péptidos en Fase Sólida (SPPS)

En 1963, Bruce Merrifield introdujo la Síntesis de Péptidos en Fase Sólida (SPPS) [257], por la que fue galardonado con el Premio Nobel en el año 1984. En los últimos años, el desarrollo paralelo de técnicas de separación de alta resolución, ha contribuido enormemente a la evolución y optimización de las metodologías de SPPS [258-261]. Hoy en día, los procedimientos de SPPS son fácilmente automatizables, lo que está permitiendo acelerar el diseño y evaluación de nuevas estructuras con potenciales propiedades terapéuticas e industriales [262-266]. Sin embargo, aunque la SPPS comienza a aplicarse en mayor número de casos, los métodos clásicos de síntesis en disolución se siguen utilizando ampliamente en la producción a gran escala de péptidos moderadamente complejos, en el acoplamiento de segmentos peptídicos previamente preparados por SPPS y en algunos casos concretos que así lo exigen, como la síntesis de algún compuesto especial, que contenga por ejemplo aminoácidos no naturales [10-14].

En la primera etapa de una SPPS (Figura 1.8) el primer aminoácido de la secuencia peptídica, o un derivado de éste, se enlaza covalentemente a una resina polimérica insoluble convenientemente funcionalizada [10-14] . En la mayoría de los casos la cadena peptídica crece hacia el extremo N-terminal. Se incorporan sucesivamente los aminoácidos adecuados, convenientemente protegidos en el Nitrogeno α y en las cadenas laterales susceptibles de reaccionar con el aminoácido que se incorpora. Gran parte del éxito de este tipo de síntesis reside en la selección de una metodología de protección adecuada. La secuencia peptídica se genera a

través de sucesivos ciclos de desprotección/acoplamiento, en los que el exceso de reactivos solubles, que generalmente asegura rendimientos elevados, es fácilmente eliminable por filtración y lavado de la resina. En la última etapa, un tratamiento

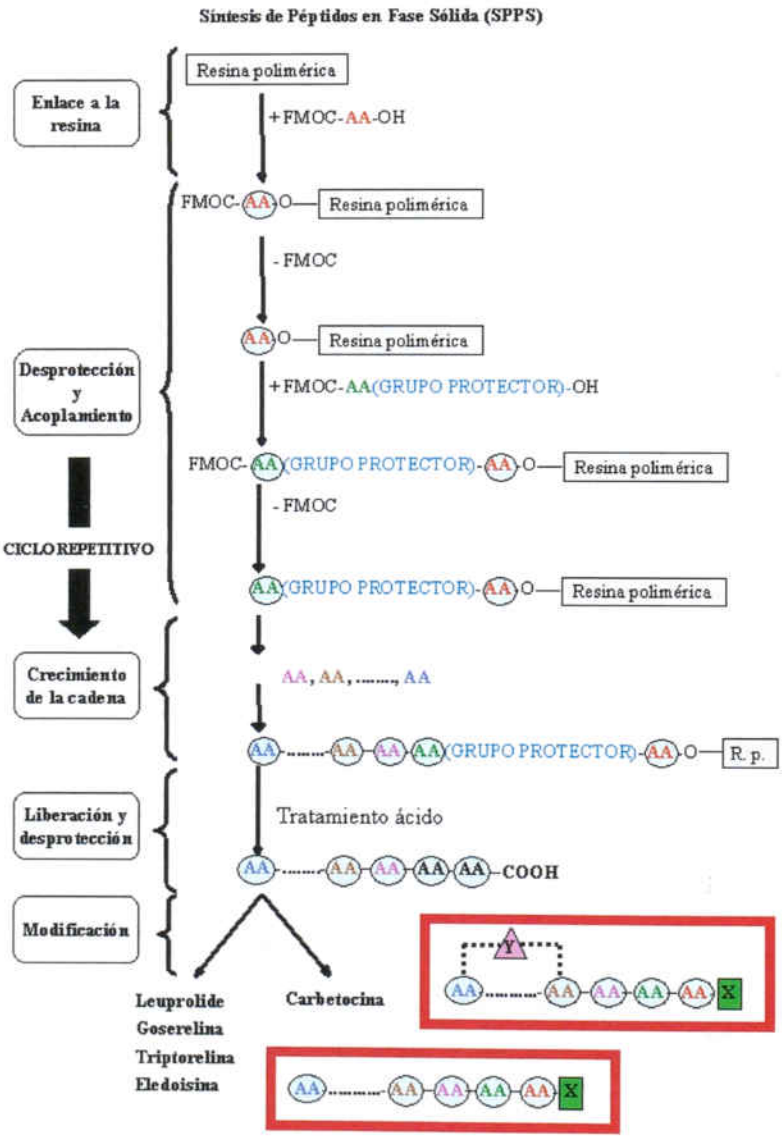


Figura 1.8 Representación esquemática del procedimiento de SPPS seguido para obtener alguno de los péptidos estudiados en este trabajo.

adecuado permitirá la liberación del péptido en crecimiento de la resina y, en general, su desprotección simultánea. Este péptido precursor puede sufrir tantas modificaciones sintéticas adicionales como sean necesarias hasta obtener el producto peptídico deseado.

En las numerosas alternativas de reacción que existen a la hora de diseñar un procedimiento de SPPS reside su enorme versatilidad y efectividad a la hora de obtener todo tipo de moléculas peptídicas con excelentes rendimientos, aunque una revisión y discusión exhaustiva de las estrategias y reactivos se escapan del objetivo de este trabajo [10-14]. La Figura 1.8 describe esquemáticamente a modo de ejemplo el proceso genérico de SPPS empleado para la síntesis de algunas de las hormonas peptídicas de bajo peso molecular estudiadas en esta tesis doctoral. En este caso se emplea el esquema de protección Fmoc/^{t}Bu (9-fluorenilmetoxicarbonilo/*tert*-butilo), es decir, el grupo Fmoc para la protección del Nitrógeno α de los aminoácidos entrantes y grupos protectores ^{t}Bu para las cadenas laterales. Un tratamiento con ácido TFA permite la liberación del precursor ácido y su desprotección simultánea. La síntesis finaliza con una serie de pasos adicionales en los que el precursor ácido experimenta las transformaciones adecuadas en solución hasta obtener el producto final.

Sin duda, parte del éxito de los procedimientos de SPPS ha estado íntimamente relacionada con la posibilidad de monitorizarlos, y posteriormente optimizarlos, empleando las técnicas analíticas modernas [10-14]. En los últimos años, el desarrollo tecnológico de LC, la CE y la MS y su uso generalizado para analizar los crudos peptídicos finales o los generados en etapas comprometidas de la síntesis [258-261,267-272], ha contribuido especialmente al desarrollo de metodologías de síntesis más rápidas y eficaces y a la obtención en condiciones óptimas de una extensa variedad de moléculas peptídicas con potenciales aplicaciones terapéuticas o industriales [262-266]. La LC-ESI-MS y la CE-ESI-MS pueden proporcionar valiosa información sobre la identidad y estructura de los

compuestos presentes en los crudos de síntesis, lo que puede ser muy útil para optimizar la etapa o etapas de reacción implicadas, mejorando los rendimientos al evitar la presencia de productos no deseados (enantiómeros o diastereoisómeros, productos de deleción, secuencias con aminoácidos adicionales, secuencias truncadas, subproductos de reacción, oligómeros, agregados, reactivos o disolventes no deseados, etc...) [258-261,267-272]. De la misma manera el uso de LC-ESI-MS y, en menor medida, CE-ESI-MS resulta hoy en día imprescindible para garantizar la pureza, homogeneidad, identidad y estructura del producto peptídico final [273-279], factores fundamentales para industrias sujetas a una estricta reglamentación como la industria farmacéutica [280-281].

1.3.2. Ingeniería genética

Para la obtención de polipéptidos complejos y proteínas se suelen emplear técnicas biotecnológicas de ingeniería genética [3,7-9]. Mediante técnicas de ADN recombinante, se puede transferir *in vitro* un determinado gen, cuya secuencia se puede haber modificado de alguna forma, a una célula que normalmente no lo presenta, para que la célula transformada exprese, o sea sintetice, el producto deseado. Para ello se han de seleccionar primero las células transformadas y posteriormente multiplicarlas para obtener células genéticamente idénticas. Todo el proceso es delicado y complejo [3,7-9] y la estructura y las propiedades fisicoquímicas y biológicas del producto final dependen del tipo y edad de las células huésped, del método de transferencia de la información genética, de las condiciones experimentales de cultivo celular, del método de expresión y de los procesos empleados para la recuperación del producto recombinante.

En la Figura 1.9 aparece a modo de ejemplo un esquema de un proceso genérico de clonación empleando un plásmido[b] como vector o vehículo de clonación [3,7-9]. En primer lugar el fragmento de ADN que codifica el gen se aísla cortándolo con la enzima de restricción adecuada. Seguidamente se corta el plásmido con la misma enzima de restricción y se le incorpora el fragmento de ADN, uniendo el conjunto mediante una enzima denominada ADN ligasa. El plásmido recombinante es incorporado a la célula procariota o eucariota huésped, en un proceso denominado transformación. Las células transformadas son separadas de las no transformadas mediante una selección, aprovechando que las células que contienen el plásmido vector son resistentes a algunos antibióticos. En este caso concreto (Figura 1.9), las células transformadas que contienen crecerían en presencia de ampicilina pero no en presencia de tetraciclina, ya que el fragmento que codifica la resistencia a la tetraciclina fue cortado al insertar el gen en el plásmido. De esta forma se puede llegar a seleccionar una única célula que contenga el plásmido con el gen humano deseado y que por división celular origine una colonia de células idénticas. Una vez finalizada la clonación de la célula de partida, se utilizan las colonias obtenidas para expresar el polipéptido o proteína deseado.

Células huésped típicas son la *Escherichia coli* (E. coli), *Saccharomyces cerevisiae*, líneas celulares de ovario de hámster chino (CHO) y células de riñón de crías de hámster (BHK). En general siempre que es posible, por su bajo coste económico y eficacia, se utilizan bacterias. En algunos casos es necesario utilizar directamente células eucariotas de animales o vegetales, debido a que las células procariotas carecen de la maquinaria biológica necesaria para expresar el polipéptido o proteína deseado o para introducir las modificaciones post-translacionales necesarias. Esto ocurre por ejemplo en la proteólisis parcial que experimenta la proinsulina en su conversión a insulina, o en la incorporación de azúcares que se produce para obtener las glicoproteínas [3,7-10].

[b] Los plásmidos son piezas de ADN circular presentes en casi todas las bacterias.

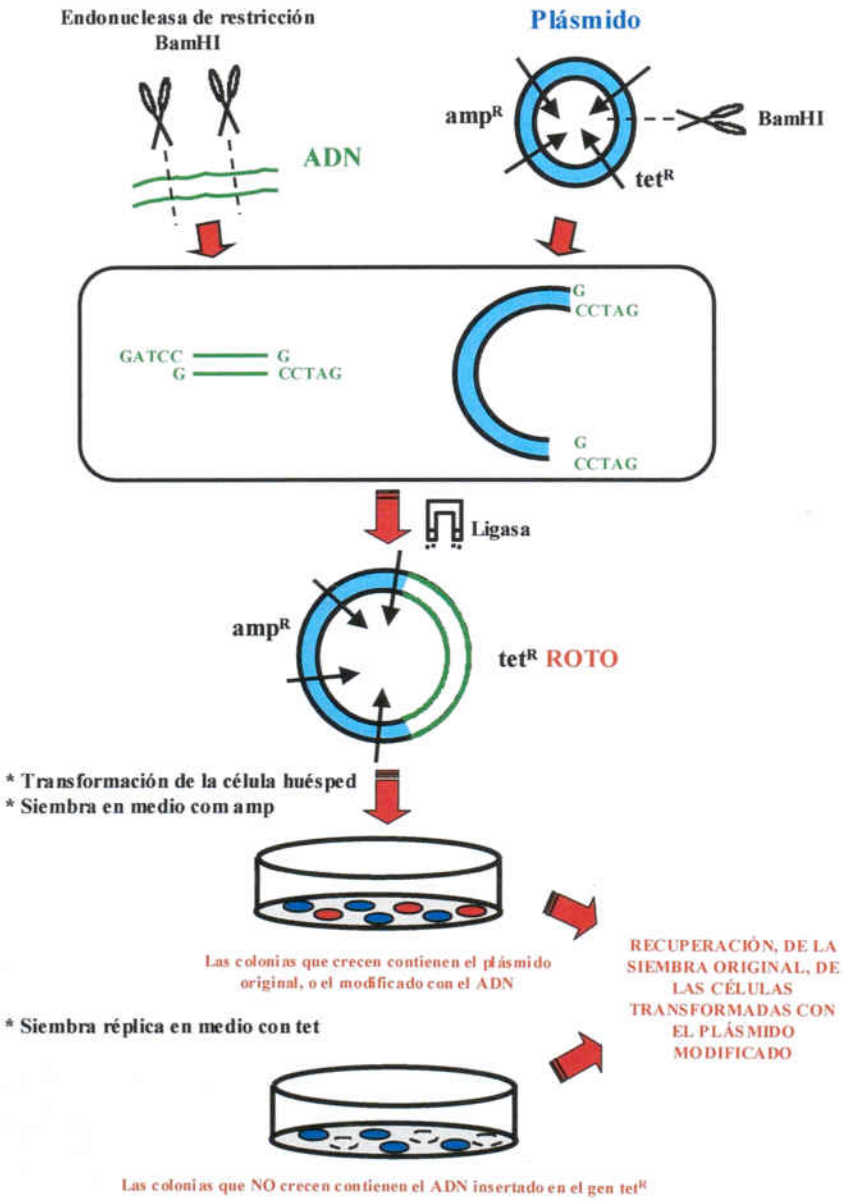


Figura 1.9 Esquema de la clonación de una célula empleando el plásmido pBR322 como vector de clonación (amp = ampicilina y tet = tetraciclina) [3,7-9].

Una vez el producto deseado ha sido fabricado por las células recombinantes, éste debe ser separado de las células y del resto de reactivos y productos presentes en el medio de reacción, y caracterizado convenientemente [7]. Normalmente las células bacterianas secretan las proteínas fabricadas al medio de reacción, de forma que se puede separar el producto recombinante de las células por centrifugación, extracción líquido-líquido, precipitación, filtración a contracorriente o a través de membranas. En cambio, en células de mamíferos, los polipéptidos o proteínas fabricados se encuentran en el interior de las células, de forma que se habrán de liberar primero provocando la rotura celular. A partir de aquí en ambos casos, se tendrá que purificar el producto recombinante para garantizar su homogeneidad, estabilidad y esterilidad, es decir, que no se encuentra contaminado con ácidos nucleicos, virus u otros agentes. Igual que ocurría con los productos obtenidos por síntesis química, la LC resulta fundamental para purificar grandes cantidades del producto recombinante. Concretamente se suelen emplear ALC [36], SEC [34], IEC [35], HIC [35], RPLC [17-21], o combinaciones de éstas [15-16]. Una vez purificado el producto, tradicionalmente se solía determinar la composición de aminoácidos, la secuencia de los últimos 15 aminoácidos del extremo amino o carboxi terminal y se caracterizaba empleando Electroforesis en Geles de Poliacrilamida con SDS (SDS-PAGE), IEF, o ambas combinadas en 2-D, empleando detección con azul de *Coomassie*, plata o *immunoblotting* [282-283]. Sin embargo, últimamente la LC, la CE y la MS (ESI-MS y MALDI-TOF) comienzan a ser ampliamente empleadas para separar y caracterizar los productos obtenidos por tecnologías de ADN recombinante [15-16,27,284-286]. Así, se está exigiendo que los péptidos de más de 20 aminoácidos se acompañen de un mapa peptídico en el que se separen o aíslen sus fragmentos obtenidos por digestión enzimática y se caractericen por MS [284]. También se suele obtener información sobre la estructura secundaria, terciaria y cuaternaria de la proteína utilizando técnicas como el dicroísmo circular (CD), la fluorescencia, la espectroscopia infrarroja, la difracción de rayos X o la RMN [15-

16]. Una vez purificada, identificada y caracterizada estructuralmente, la molécula recombinante es a menudo sometida a pruebas bioquímicas (enzimáticas, biológicas, inmunológicas, ...) para determinar su actividad y completar su caracterización [283].

1.4 HORMONAS PEPTÍDICAS

Las hormonas peptídicas naturales o sus análogos obtenidos artificialmente son en la actualidad las biomoléculas de más amplio uso farmacológico [1-3,7-14]. En la Tabla 1.7 aparecen algunas de las más empleadas en la actualidad. Entre éstas destacan algunas de las que se estudian en este trabajo, como los análogos de la LHRH, entre ellos el leuprolide y la goserelina, que se utilizan para combatir cierto tipo de tumores hormono-dependientes [254], y cuyas ventas a nivel mundial suponen alrededor de 1.5 billones de dólares [287]; y la EPO que se emplea en el tratamiento de diversos tipos de anemia [288-290], con ventas mundiales que generan alrededor de 2.5 billones de dólares, ingresos que superan los 2.3 billones de dólares que suponen el mercado de insulina humana a nivel mundial [287]. Leuprolide, EPO e insulina se encuentran entre los 50 productos farmacéuticos que han generado más ingresos a nivel mundial en los últimos años, y siguen despertando un enorme interés en todo el sector, quizá renovado últimamente debido a que algunas de las patentes que protegen a los análogos de la LHRH y a la EPO expiran en el año 2004 y esto permitirá la comercialización de nuevos productos genéricos [291].

1.4.1. Hormonas peptídicas de bajo peso molecular

En la Tabla 1.8 se muestran las secuencias detalladas de los péptidos de bajo peso molecular estudiados en este trabajo, destacando las diferencias con la secuencia de la molécula original cuando se trata de análogos estructurales. La citrulina y los tripéptidos se seleccionaron como una primera etapa para abordar posteriormente los estudios con las hormonas peptídicas que también se señalan en dicha tabla.

Tabla 1.7 Hormonas peptídicas con interés terapéutico más utilizadas en la actualidad [1-2,252].

Nombre	Molécula	Origen	Función
LH	Glicoproteína	Hipófisis anterior	Gonadotropina que regula el funcionamiento de las gónadas y la secreción de hormonas sexuales. Útil en el tratamiento de infertilidad.
FSH	Glicoproteína	Hipófisis anterior (Se obtiene de la orina de posmenopáusicas o se obtiene recombinante)	En mujeres, gonadotropina que estimula el desarrollo y maduración de los folículos, la ovulación. En combinación con la LH regula la síntesis de progesterona. Útil en el tratamiento de infertilidad. En hombres interviene en la espermatogénesis.
LHRH (GnRH)	Nonapéptido	Hipotálamo	
Gonadorelina, leuprolide, goserelina, triptorelina, buserelina, nafarelina, deslorelina, histrelina	Nona o decapéptidos	Análogos obtenidos por SPPS	Regulan los niveles de LH y FSH. Tratamiento de cáncer de próstata y mama, endometriosis, miomas uterinos, pubertad precoz y para inducir la ovulación en tratamientos de infertilidad en los que se emplean en combinación con gonadotropinas.
Oxitocina	Nonapéptido	Hipófisis posterior (obtenida de hipófisis de mamíferos)	Estimula las contracciones uterinas durante el parto y se emplean para estimular la bajada de la leche durante la lactancia. Los análogos dan repuestas mayores y de más larga duración que la oxitocina. La demoxitocina se administra vía oral en forma de pastillas.
Oxitocina (sint.), carbetocina, demoxitocina	Nonapéptidos	Análogos obtenidos por SPPS	
Vasopresina	Nonapéptido	Hipófisis posterior	Antidiurética. Vasoconstrictor. Regula la contracción de la musculatura lisa del intestino. Utilizadas en el diagnóstico y tratamiento de la diabetes insipidus, enuresis nocturna y para aumentar la concentración de factor VIII en pacientes hemofílicos.
Lipresina, desmopresina, argipresina, felipresina	Nonapéptidos	Análogos obtenidos por SPPS (La argipresina se puede obtener de hipófisis de todos los mamíferos. La Lipresina se puede obtener de hipófisis de cerdos	
Met-encefalina	Pentapéptido	Hipófisis anterior	Péptidos opiáceos. Funcionan como neurotransmisores o neurohormonas
Leu-encefalina	Pentapéptido	Hipófisis anterior	
Eledoisina	Undecapéptido	Glándulas salivares posteriores de ciertos pulpos.	Potente vasodilatador e incrementa la permeabilidad de los capilares. Se administra como gotas para los ojos en el tratamiento del síndrome de sequedad ocular y relacionados.
Eledoisina (sint.)	Undecapéptido	Análogos obtenidos por SPPS	

Nombre	Molécula	Origen	Función
Bradicinina	Nonapéptido	Se forma por acción de ciertas enzimas proteolíticas sobre una globulina plasmática	Vasodilatador, estimulación del músculo liso e incremento de la permeabilidad de los capilares.
Somatostatina	Tetradecapéptido cíclico	Hipotálamo	Inhiben la liberación de la hormona del crecimiento, la tirotropina, la corticotropina, el glucagón y la insulina y parece que regulan las secreciones gastro-duodenales.
Somatostatina (sint.), octreotide	Tetradecapéptido y octapéptido cíclicos	Análogos obtenidos por SPPS	
Calcitonina	Polipéptido 32 aa	Tiroides (de cerdo)	Regulación del calcio y fósforo y el metabolismo de los huesos. Tratamiento de osteoporosis, hipercalcemia, dolor óseo y similares.
Calcitonina humana (sint.), elcatonina y salcatonina.	Polipéptidos	Análogos obtenidos por SPPS (elcatonina y salcatonina son análogos de calcitonina de anguila y salmón respectivamente)	
Hormona del crecimiento humana	Proteína 191 aa	Hipófisis anterior	Estimulan el crecimiento de los tejidos y el anabolismo en general. Tratamiento del enanismo. Afecta al metabolismo de las grasas, los carbohidratos y los minerales.
Somatropina y Somatrem (metionil-somatropina)	Proteína 191 aa	Análogos recombinantes	
Gonadotropina coriónica	Glicoproteína	Placenta (se obtiene de la orina de mujeres embarazadas)	Actúa predominantemente como LH. Útil en el tratamiento de infertilidad en mujeres, y el criptorquidismo (fallo en el descenso de los testículos) e hipogonadismo en hombres.
Insulina	Proteína 51 aa	Páncreas (porcino o bovino)	Regula la concentración de glucosa en sangre, e interviene en la regulación del metabolismo de las proteínas y los lípidos. Tratamiento de diabetes mellitus dependiente de insulina.
Insulinas humanas sintéticas	Proteínas ≈ 51 aa	Proinsulina porcina sometida a reacciones enzimáticas o proinsulinas o insulinas recombinantes modificadas enzimática o sintéticamente.	
Eritropoyetina	Glicoproteína	Riñón	Regulan la eritropoyesis. Se usan ampliamente para el tratamiento de anemias y como alternativa a las transfusiones sanguíneas.
Eritropoyetina recombinante, NESP	Glicoproteína	Análogo recombinante	

Tabla 1.8 Hormonas peptídicas de bajo peso molecular [252].

Nombre propio internacional	Secuencia de aminoácidos
Diglicina	Gly-Gly
Ornitina[1] (Orn)	NH_2-CH_2-CH_2-CH_2-CH(NH_2)-COOH
Citrulina (Cit)	**NH_2-CO-**NH-CH_2-CH_2-CH_2-CH(NH_2)-COOH
Triglicina	Gly-Gly-Gly
N-(N-glicilglicil)-L-Valina	Gly-Gly-Val
N-(N-glicilglicil)-L-Isoleucina	Gly-Gly-Ile
N-(N-DL-alanil-DL-leucil)-Glicina	Ala-Leu-Gly
N-(N-glicilglicil)-L-Fenilalanina	Gly-Gly-Phe
N-(N-tirosilglicil)-Glicina	Tyr-Gly-Gly
Leucina-encefalina	Tyr-Gly-Gly-Phe----**Leu**
Metionina-encefalina	Tyr-Gly-Gly-Phe----**Met**
Angiotensina I[1]	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-**His-Leu**
Angiotensina II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Bradicinina	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
LHRH[1]	5-oxo-Pro-His-Trp-Ser-Tyr- ----------Gly---------- --Leu-Arg-- ------Pro----- ----Gly--- ---NH_2
Nafarelina[1]	5-oxo-Pro-His-Trp-Ser-Tyr- **-3-(2-naftil)D-Ala**--- --Leu-Arg-- ------Pro----- ----Gly--- ---NH_2
Triptorelina	5-oxo-Pro-His-Trp-Ser-Tyr- ---------**D-Trp**-------- --Leu-Arg-- ------Pro----- ----Gly--- ---NH_2
Goserelina	5-oxo-Pro-His-Trp-Ser-Tyr- -------**D-(tBu)Ser**---- --Leu-Arg-- ------Pro----- **--AzGly-** ---NH_2
Leuprolide	5-oxo-Pro-His-Trp-Ser-Tyr- ---------**D-Leu**-------- --Leu-Arg-- **--Pro-NHEt**
Buserelina	5-oxo-Pro-His-Trp-Ser-Tyr- -----**D-Ser (tBu)**----- --Leu-Arg-- **--Pro-NHEt**
Histrelina[1]	5-oxo-Pro-His-Trp-Ser-Tyr- **--D- (Nt-benzil)His**-- --Leu-Arg-- **--Pro-NHEt**
Eledoisina	5-oxo-Pro-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Oxitocina	S-S Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
Carbetocina	$COCH_2CH_2CH_2S$ Tyr(**OMe**)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

[1] No han sido analizadas en este trabajo.

La citrulina es un derivado de la ornitina, un aminoácido no proteico. Se utiliza frecuentemente en insuficiencias hepáticas como hepatoprotector e interviene como intermediario en la biosíntesis de la urea [1-2].

La Metionina-Encefalina y la Leucina-Encefalina son péptidos opiáceos endógenos que se forman en el sistema nervioso central. Actúan sobre los mismos receptores cerebrales específicos que otros agentes opiáceos, como la heroína y la morfina, produciendo un efecto analgésico que amortigua las sensaciones dolorosas. El desarrollo de nuevos agentes opiáceos que no produzcan depresión respiratoria, efectos sedantes, ni adicción, constituye en la actualidad un campo de investigación de elevado interés [1-2].

La angiotensina I, que no tiene actividad biológica conocida, es transformada en angiotensina II en los pulmones. La Angiotensina II es un potente vasoconstrictor que estimula intensamente la sed, libera vasopresina, corticotropina (que regula la liberación de glucocorticoides como el cortisol) y aldosterona (que regula la retención del sodio, y la eliminación del potasio y el agua en los riñones), además de ejercer su conocido efecto de elevación de la presión sanguínea del que deriva su nombre.

La bradicinina es un potente vasodilatador que interviene en la regulación de la presión sanguínea. Produce también una acción espasmogénica en varios tipos de músculos lisos, incluyendo el intestino, el útero y el sistema bronquial. Parece que también interviene en los procesos inflamatorios y alérgicos [252].

El resto de las hormonas peptídicas de bajo peso molecular estudiadas presentan un grupo amida en su extremo C-terminal. Esto ocurre en muchas hormonas peptídicas y es a menudo esencial para su actividad biológica, por lo que existe un gran interés en la síntesis química de péptidos amidados, ya que además son difíciles de obtener empleando técnicas biotecnológicas [10-14].

La eledoisina puede aislarse de forma natural de las glándulas salivares posteriores de cierta especie de pulpos [252,292]. Actualmente se obtiene

sintéticamente y debido, entre otras propiedades, a su acción vasodilatadora, se utiliza a nivel ocular como estimulante de la secreción lacrimal en casos de queratoconjuntivitis u otras condiciones relacionadas con la sequedad ocular. Un estudio reciente ha mostrado que la eledoisina presenta similar secuencia, hasta un 73 % de homología y actividad que los fragmentos Aβ 25-35 de la proteína β-amiloide y sus análogos, que juegan un papel fundamental en la aparición y el desarrollo del Alzheimer [292].

La oxitocina fue el primer péptido en sintetizarse para su uso farmacológico [1-2,252]. Es secretada por el hipotálamo y almacenada en la glándula pituitaria. Se emplea en medicina humana y veterinaria para provocar o controlar la contracción del útero durante el parto y la eyección de la leche materna durante la lactancia. Tiene también una ligera acción antidiurética. La carbetocina es un análogo estructural de la oxitocina más estable química y metabólicamente, lo que permite efectos farmacológicos más prolongados [252]. La respuesta que provoca es menos intensa, provocando un efecto vasoconstrictor menor y presentando además un efecto antidiurético casi nulo.

Buserelina, goserelina, leuprolide y triptorelina son análogos de la LHRH, también denominada GnRH (hormona liberadora de gonadotropinas) [252]. Tal y como indica su nombre, es una hormona que regula la secreción de LH (hormona luteinizante) y FSH (hormona estimulante del folículo), las que a su vez regulan la producción de hormonas sexuales. La LHRH tiene un papel fisiológico muy importante [1-2] y su descubrimiento supuso el desarrollo de nuevos campos de investigación para aprovechar su potencial uso terapéutico en el tratamiento de la infertilidad humana y otras enfermedades relacionadas con la secreción de hormonas sexuales, como son el cáncer de próstata, el de mama, el de ovario, la endometriosis, los fibroides uterinos y la pubertad precoz [252]. Al igual que la carbetocina, los análogos aquí considerados presentan una mayor resistencia a la degradación enzimática y a la eliminación biológica, junto con una mayor afinidad

por sus receptores lo que les hace presentar una mayor actividad biológica. En especial, en este caso, un mismo análogo puede actuar como agonista o antagonista, dependiendo de la dosis administrada [293-295]. Así, su administración a largo plazo primero estimula la secreción de LH y FSH, y más tarde puede provocar su inhibición, que es reversible cuando los niveles de LHRH vuelven a su estado normal.

1.4.1.1. Separación y caracterización de hormonas peptídicas de bajo peso molecular.

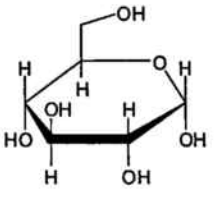
La separación y caracterización de mezclas complejas de péptidos de bajo peso molecular ofrece solución a la problemática que se plantea en muy diversos campos. Así, resultan de excepcional interés para determinar la homogeneidad de preparados farmacéuticos de base peptídica [273-279], separar y caracterizar los productos de síntesis en crudos de síntesis peptídicos [258-272], determinar nuevos marcadores en fluidos biológicos para el diagnóstico de una patología [176-181] o en alimentos para poner de manifiesto la adulteración de un determinado producto [24], determinar la huella peptídica de una proteína o proteínas, en una mezcla que previamente se ha digerido enzimáticamente [284],... Podríamos recopilar numerosas y variadas aplicaciones sobre separación y caracterización de péptidos empleando LC, LC-ESI-MS, CE, CE-ESI-MS y recientemente CEC y CEC-MS [15-16,24]. Sin embargo, es importante señalar, que sólo en algunas de estas aplicaciones se describen la separación y caracterización de crudos peptídicos de síntesis o mezclas de péptidos obtenidos por síntesis combinatoria [258-272] y la determinación de la pureza de preparados farmacéuticos peptídicos [272-279]. La importante repercusión a nivel industrial de este último tipo de aplicaciones, invitan a un desarrollo sistemático y riguroso de las metodologías analíticas empleadas, que permitan la selección rápida y sencilla de condiciones de separación y

caracterización óptimas, tal y como se pone de manifiesto en algunos de los trabajos recopilados en esta tesis doctoral.

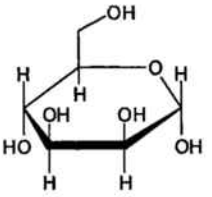
1.4.2. Hormonas peptídicas de elevado peso molecular. Glicoproteínas.

La glicosilación proteica es una de las modificaciones post-translacionales más comunes. Consiste en la unión covalente al núcleo peptídico, a través de enlaces glicosídicos, de cadenas de carbohidratos [1-2,296-297]. La adición de estos carbohidratos cambia el tamaño y la estructura de la proteínas, pudiendo afectar a sus propiedades fisicoquímicas (solubilidad, estabilidad, ...) y biológicas (actividad, comportamiento antigénico, destino metabólico, interacciones célula-célula, resistencia a la proteólisis, secreción, ...). Esta modificación es muy común en las proteínas de organismos superiores, aunque también puede estar presente en células y fluidos biológicos de microorganismos y virus. Las glicoproteínas actúan básicamente como enzimas, anticuerpos, hormonas, proteínas estructurales, mucinas de secreciones epiteliales, receptores y proteínas transportadoras a través de membranas [1-2,296].

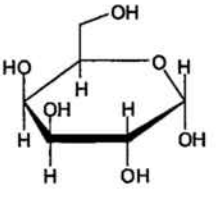
La cantidad de carbohidratos presente en las glicoproteínas está comprendida entre el 1 y el 85% del peso en seco de estas moléculas. Estos carbohidratos pueden variar en naturaleza y tamaño, desde monosacáridos a polisacáridos y puede haber desde una a cientos de unidades de carbohidratos unidas a una sola cadena polipeptídica. En la Figura 1.10 aparecen los monosacáridos más frecuentemente encontrados en las glicoproteínas [1,296]. El ácido siálico o ácido neuramínico puede presentarse con diferente tipo de substituyentes. El derivado más común es el ácido N-acetilneuramínico (NeuNAc), pero se han identificado alrededor de otros veinte derivados, algunos de los cuales se describen en la Figura 1.10 [296].



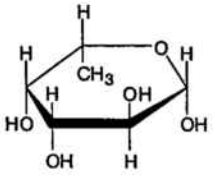
α-D-Glucosa (Glc)



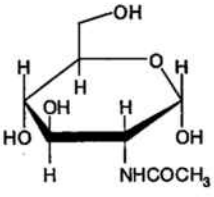
α-D-Manosa (Man)



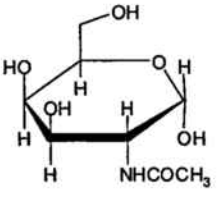
α-D-Galactosa (Gal)



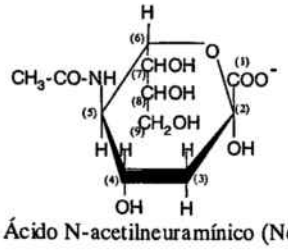
β-L-Fucopiranosa (Fuc)



α-D-Glucosamina (GlcNAc)



α-D-Galactosamina (GalNAc)



Ácido N-acetilneuramínico (NeuNAc)

Tipo de ácido siálico	C	Grupo
N-Acetilneuramínico	C(5)	CH_3CONH-
N-glicolilneuramínico	C(5)	$HOCH_2CONH$-
Derivados O-acetilados	C(9)	CH_3CO-
	C(7)	CH_3CO-
	C(4)	CH_3CO-

Figura 1.10 Estructura de los monosacáridos más frecuentemente encontrados en las glicoproteínas.

Las cadenas de azúcares pueden unirse al núcleo peptídico a través de dos tipos de enlaces covalentes: N-glicosídico u O-glicosídico (Figura 1.11) [1-2,296]. El primero se forma a través de un residuo de N-acetilglucosamina (GlcNAc), que se une a un grupo amida de una asparagina (Asn) de la cadena polipeptídica, originando una unión tipo asparagina (N-glicoproteína). En el caso de establecerse una unión tipo mucina, el extremo reductor de un residuo de N-acetilgalactosamina (GalNAc), se une con un grupo hidroxilo de un residuo de serina (Ser), de treonina (Thr), o en casos extraños a residuos de hidroxiprolina de la cadena polipeptídica

(O-glicoproteína). Las N,O-glicoproteínas presentan ambos tipos de enlace en sus moléculas. En la Figura 1.12 aparecen algunas de las estructuras que pueden presentar los N-oligosacáridos de una glicoproteína. En este caso se observan oligosacáridos con dos y tres ramificaciones, bi- y tri-antenarios.

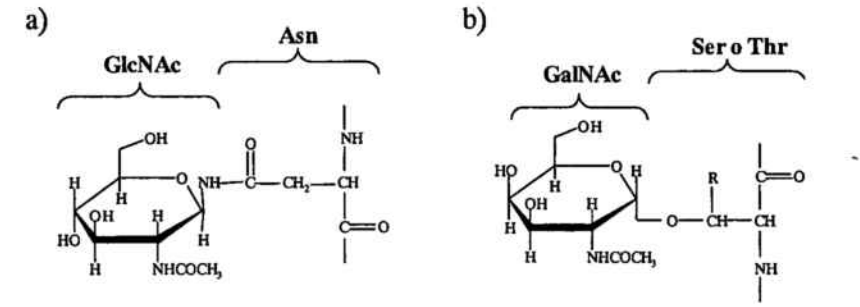


Figura 1.11 Enlaces glicosídicos. a) N-glicosídico y b) O-glicosídico.

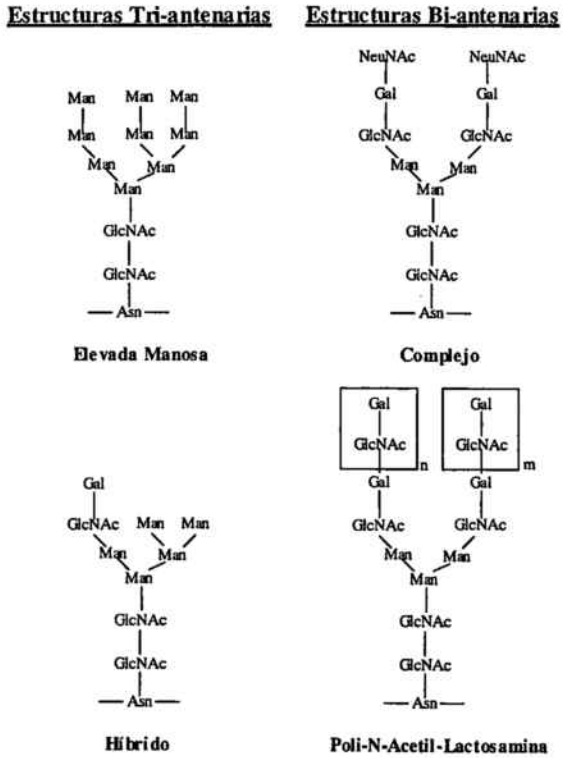


Figura 1.12 Principales estructuras de los N-oligosacáridos. En el tipo poli-N-acetillactosamina, los grupos m y n son estructuras repetitivas.

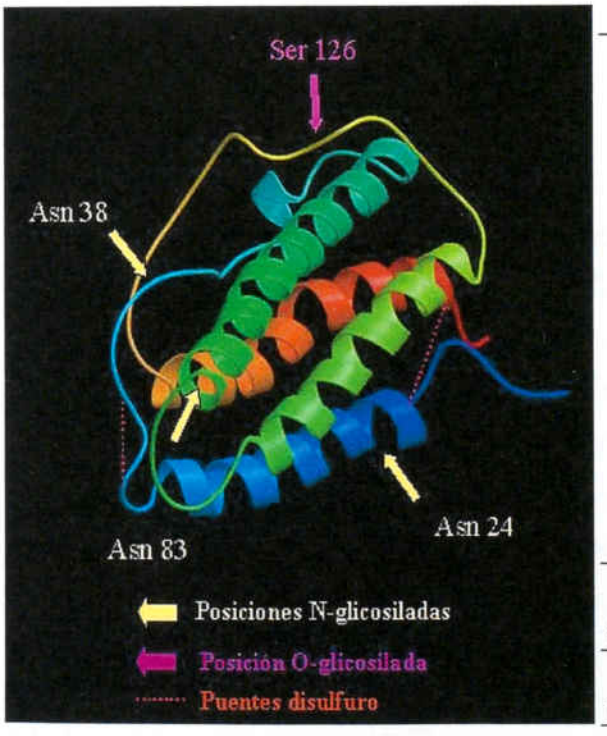
Al polimorfismo asociado con el tipo, el número y la posición de las cadenas de carbohidratos que poseen las glicoproteínas, se le denomina microheterogeneidad y a las diferentes especies que se generan se les denomina glicoformas [1-2,296]. Así, una glicoproteína cualquiera está compuesta por una mezcla de glicoformas que presentan el mismo núcleo peptídico, pero que difieren en el tipo, el número y la posición de las cadenas de carbohidratos. En general, las glicoproteínas naturales difieren de las glicoproteínas recombinantes en el número, la concentración y el tipo de glicoformas [3,7-9]. También pueden existir alteraciones en este perfil glicoproteico cuando el individuo se encuentra afectado por determinadas patologías. Del mismo modo, como el proceso de obtención de una proteína recombinante está afectado por numerosas variables, dos glicoproteínas recombinantes obtenidas mediante procedimientos diferentes pueden presentar diferente número, concentración y tipo de glicoformas. Entre los factores más importantes que afectan a la glicosilación proteica está el tipo de células y su estado fisiológico o patológico [7]. Quizá parte de la solución a estas problemáticas y a sus aplicaciones terapéuticas en humanos pasa por el avance de las investigaciones basadas en el uso de células madre procedentes de embriones humanos y que solamente están autorizadas en algunos países [299]. Otros factores que se han comprobado que pueden afectar a la mezcla de glicoformas obtenidas, son las condiciones de cultivo celular y los procesos de recuperación de las células huésped, lo que da una idea de la complejidad del problema [4,285-286,297].

1.4.2.1. Eritropoyetina (EPO)

La eritropoyetina (EPO) humana es una hormona glicoproteica formada por una cadena polipeptídica de 165 aminoácidos[c] con dos puentes disulfuro (entre la Cys7 y la Cys161, y entre la Cys29 y la Cys33) y cuya masa molecular es de alrededor de 30400 Da (18400 Da sin glicosilar) (Figura 1.13) [255,288]. Presenta un 30% de carbohidratos en su estructura, de los cuales 11% son ácidos siálicos [255,288]. Estos azúcares se distribuyen en cuatro cadenas de polisacáridos, mono-, bi- o tri-antenarias (tres de enlace N-glicosídico en Asn24, Asn38 y Asn83, y una de enlace O-glicosídico en Ser-126), Figura 1.13. Normalmente los residuos de ácido siálico se encuentran en los extremos de las cadenas N-glicosídicas [300-302].

La actividad *in vivo* de la EPO depende en gran medida de los residuos de ácido siálico, ya que previenen su rápida asimilación por los receptores hepáticos, que reconocen los residuos de galactosa expuestos. Por este motivo la EPO tratada con sialidasas para eliminar los residuos de ácido siálico, presenta una gran actividad biológica in vitro, pero una menor actividad in vivo. Por otro lado, la ramificación de las cadenas de carbohidratos y el tipo de enlace O-glicosídico, también afectan a la actividad biológica, siendo las cadenas que se enlazan mediante enlaces N-glicosídicos y que presentan configuraciones de sus carbohidratos tri-antenarias las que proporcionan mayor actividad biológica, por contener más residuos de ácido siálico del tipo NeuNAc [255-256].

[c]Secuencia aminoacídica de la EPO humana: Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Lys Ile Thr Thr Gly Cys Ala**30** Glu His**32** Cys Ser Leu Asn Glu Lys Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Lys Ser Ser Gln Pro**87** Trp**88** Glu Pro**90** Leu Gln Leu His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala AspThr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp Arg



Aminoácido	%
Ala	11.5
Arg	7.3
Asn	1.8
Asp	3.6
Cys	2.4
Glu	7.3
Gln	4.2
Gly	5.5
His	1.2
Ile	3.0
Leu	13.9
Lys	6.7
Met	0.6
Phe	2.4
Pro	4.8
Ser	6.1
Thr	6.7
Trp	1.8
Tyr	2.4
Val	6.7
Estructura	**Nº**
Hélice α	4
Hélice β	0
Carbohidratos	**30-40 %**
Ácido Siálico	11 %
Hexosa	11 %
N-acetilglucosamina	8 %

Figura 1.13 Estructura del núcleo aminoacídico de la EPO humana.

La EPO se produce principalmente en los riñones de seres adultos y en el hígado de fetos y recién nacidos y es transportada hasta las células de la médula ósea a través de la circulación sanguínea [288-290]. Es responsable de la proliferación, diferenciación y maduración de las células precursoras eritroides de la médula ósea, de forma que globalmente regula la producción de eritrocitos. Generalmente, la síntesis de EPO se estimula por la hipoxia -baja presión de oxígeno- causada por un volumen bajo de sangre, anemia, bajos niveles de hemoglobina, flujo sanguíneo pobre o desórdenes pulmonares. Como resultado se forman nuevos eritrocitos en los riñones, la capacidad portadora de oxígeno (VO_{2max}) aumenta y se eleva el nivel de hemoglobina (Hb), hematocrito (Hct) y receptores de transferrina solubles (sTfR) en la corriente sanguínea .

La EPO humana fue por primera vez purificada por Miyake et al. [303], a partir de orina procedente de pacientes afectados de anemia aplásica severa. Desde entonces, se han desarrollado varios métodos para purificar la EPO procedente de orina o suero humano [304-306]. Sin embargo, son procesos laboriosos y aunque este tipo de pacientes presentan elevados niveles de EPO comparados con un sujeto normal, se necesitan varios miles de litros de orina o suero para aislar unos pocos miligramos de hormona. Además, la EPO procedente de la orina humana corresponde a la fracción de la hormona que fue eliminada y sus carbohidratos presentan diferencias estructurales respecto a la que puede aislarse del suero, que por otra parte es más difícil de conseguir [307-309]. La EPO humana recombinante (rHuEPO) se encuentra disponible comercialmente desde 1988 [288] y es actualmente uno de los productos farmacéuticos, obtenidos por tecnología de ADN recombinante, más vendidos en todo el mundo [287,310]. Esto constituye un gran estimulo para la investigación en este área, pero también es el origen de conflictos entre las compañías biofarmaceuticas que participan o desean participar de tan lucrativo negocio [310].

En la Tabla 1.9 aparecen la mayoría de las rHuEPO comercializadas actualmente. Una misma rHuEPO puede presentar diferentes nombres comerciales si se ha concedido la licencia a diferentes compañías o en diferentes países para diversos usos clínicos. En la Tabla 1.9, también aparece la recientemente aprobada Nueva Proteína Estimuladora de la Eritropoyesis (NESP), un análogo hiperglicosilado que presenta algunas diferencias en su secuencia aminoacídica con la EPO humana nativa o los diferentes tipos de rHuEPO [255]. Su secuencia aminoacídica difiere en cinco posiciones de la de la EPO (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, y Pro90Thr (ver pie de la página 66), lo que permite la unión en las posiciones 30 y 88 de 2 cadenas adicionales de N-oligosacáridos. La NESP tiene un contenido total de carbohidratos del 50%, con mayor contenido medio de ácidos siálicos que la EPO nativa o las recombinantes y una masa molecular media

de 38,500 Da [255]. Este mayor contenido en ácidos siálicos, la hace tener una menor afinidad por el receptor y una vida media más larga en la circulación sanguínea (25.3 horas por alrededor de las 8.5 h de la EPO natural), lo que globalmente permite una administración menos frecuente del fármaco [256]. Se sigue explorando la posibilidad de conseguir este mismo efecto obteniendo otros análogos de la glicoproteína, modificados por ejemplo con polietilenglicol [311]. Otros grupos de investigación se centran en la obtención de peptidomiméticos de la EPO humana nativa, que permitan obtener fármacos efectivos de administración oral [312].

Tabla 1.9 Productos comerciales de rHuEPO disponibles o en últimas fases de experimentación .

Nombre genérico	Nombre propio	Nombre comercial	País	Compañía
rHuEPO	Epoyetina Alfa	Epogen	USA	Amgen
		Procrit	USA	Ortho
		Espo	Japón	Kirin, Sankyo
			China	Kirin
		Eprex	Europa, Australia, Canada	Ortho y sus afiliados
rHuEPO	Epoyetina Beta	Epogin	Japón	Chugai
		Recormon	Europa	Boehringer Mannheim
rHuEPO	Epoyetina Omega	Epomax	Europa del Este Ensayos clínicos en Europa, USA y Japón	Baxter
rHuEPO	Epoyetina Delta[a]	Dynepo	Ensayos clínicos (FIII)	Aventis
NESP[b]	Darbepoyetina Alfa	Aranesp	Europa, Japón, USA	Amgen

[a]Gene Activated – EPO (GA-EPO) : Una rHuEPO obtenida de forma recombinante activando el gen adecuado en células de tejido epitelial humano [310].
[b]NESP (Novel Erythropoiesis Stimulating Protein): Nueva Proteína Estimuladora de la Eritropoyesis.

La utilidad terapéutica de la rHuEPO y sus análogos, está bien establecida en muchas aplicaciones clínicas [256,288-289]. Se utiliza principalmente para el tratamiento de la anemia causada por fallos renales crónicos [288]. Además, se ha demostrado que es altamente efectiva en el tratamiento de otros tipos de anemia, asociadas con el cáncer, la quimioterapia, el SIDA, el nacimiento prematuro o la talasemia. También está indicada para reducir la transfusión de sangre alogénica y la recuperación rápida de eritrocitos en pacientes quirúrgicos. Las últimas aplicaciones clínicas estudiadas son la mejora de hematocritos y hemoglobina en pacientes que sufren artritis reumática o juvenil [289] y recientemente se ha demostrado que previene la muerte de células cerebrales en ratones, pudiendo convertirse en un eficaz agente para la prevención de enfermedades neurodegenerativas como el Alzheimer o el Parkinson [313]. Aunque la administración de rHuEPO se tolera normalmente bien, se han encontrado algunos efectos adversos (trombosis, hipertensión, empeoramiento del riego sanguíneo, desórdenes cardiovasculares) lo que sugiere precauciones en su uso, especialmente en sujetos sanos [256,288-289].

En los últimos años la EPO y sus análogos han sido popularmente conocidos por su uso generalizado para la mejora ilegal del rendimiento en deportes de resistencia (ciclismo, esquí, natación, atletismo, ...) (Figura 1.14) [314]. Los deportistas implicados la utilizan para mejorar su capacidad aeróbica, al incrementar la capacidad de transportar oxígeno al músculo. Su uso está oficialmente prohibido por el Comité Olímpico Internacional (COI) y otras importantes organizaciones deportivas nacionales e internacionales. En 1989, la comisión médica del COI introdujo una nueva categoría de doping, la de "Hormonas Peptídicas y Análogos", en la que se incluía la EPO, la gonadotropina humana coriónica (hCG), la hormona adrenocorticotrópica (ACTH), la hormona del crecimiento humana (hGH) y todos los compuestos relacionados con estas hormonas [314].

MARTES, 4 SEPTIEMBRE 1998 — DEPORTES — LA VANGUARDIA 37

CICLISMO: *53 Vuelta a España*

Züille implicó a todo el Once en el uso de EPO

"La veintena de corredores del equipo la consumía bajo el control del doctor Terrados", declaró a la justicia

Las seis drogas más utilizadas

ESTEROIDES. *Para deportistas de velocidad, de potencia y de resistencia (culturismo, natación).* Aumenta la potencia muscular. Masculinización en las mujeres, feminización en los hombres, daños en el corazón y en el hígado. Se detecta en la orina

EPO. *Deportistas de resistencia (maratón, ciclismo, triatlón, remo).* Produce glóbulos rojos. Anafilaxia, problemas cardíacos. En la orina y sangre, por vez primera en estos JJOO.

HORMONA DEL CRECIMIENTO. *Deportistas de velocidad y de potencia (culturismo, natación, ciclismo).* Aumenta la potencia muscular. Cigantismo indetectable

TESTOSTERONA. *Deportistas de potencia y de resistencia.* Aumenta la potencia muscular (culturismo, ciclismo, halterofilia). Masculinización en las mujeres, feminización en los hombres, daños en el corazón y en el hígado. En la orina

ANABOLIZANTES (Anabolizantes orales) y DHEA. (Dehidroepiandrosterona) *Deportistas de potencia y de resistencia (culturismo, natación, ciclismo).* Aumenta la potencia muscular. Masculinización en las mujeres, feminización en los hombres, daños en el corazón y en el hígado. En la orina

66 LA VANGUARDIA — MIÉRCOLES, 29 DICIEMBRE 1999

Nuevo escándalo de dopaje en Italia

Un famoso médico presuntamente dio EPO a un grupo de 22 deportistas de elite

LA CONFESIÓN

"Usaba EPO antes de cada carrera de importancia: dos inyecciones por semana entre tres y cuatro semanas antes de la competición y durante todo el transcurso de la misma"

MIÉRCOLES, 29 FEBRERO 1999

46 LA VANGUARDIA — DEPORTES

ESCÁNDALO EN EL ESQUÍ ▸▸ *La penosa vuelta a casa*

Muehlegg afronta dos años de sanción

El contraanálisis reafirma el positivo y sólo se puede apelar al Tribunal Arbitral del COI

MIÉRCOLES, 1 SEPTIEMBRE 1999 — LA VANGUARDIA 29

GOLF. Sergio García, "el Niño", se propone hacer a la chaca de 23 años Tiger" Woods

32 **Deportes**

FÚTBOL. El Espanyol negocia a Tamudo, que podría jugar en el Celtic Park 30

TENIS. Pete Sampras se retira del Open de Estados Unidos por una lesión 31

Antón y Fiz reivindican su inocencia

Los maratonianos se muestran indignados con los ataques de "Le Monde", que les acusa de doparse con EPO

12 LA VANGUARDIA Juegos olímpicos SÁBADO, 16 SEPTIEMBRE 2000

La eterna lucha contra el doping

14 LA VANGUARDIA lunes match 21 FEBRERO 2000

OLIMPISMO

El oro de Juanito ya no brilla

El COI retira la tercera medalla como campeón olímpico y excluye de los Juegos al esquiador hispanoalemán Johann Muehlegg

Figura 1.14 Resumen de algunas de las noticias más destacadas en relación al uso de rHuEPO y NESP en el deporte aparecidas en *La Vanguardia* entre 1998-2003.

1.4.2.2. Separación y caracterización de glicoproteínas. EPO.

La separación y caracterización de las diferentes glicoformas en las que se presenta la EPO nativa o sus análogos recombinantes es necesaria, entre otros aspectos, para controlar la calidad de los preparados farmacéuticos comerciales [285-286]. Por otro lado, la detección, separación y caracterización de estas glicoformas en fluidos biológicos humanos tiene una gran importancia farmacológica, clínica y en el control antidopaje [256,289-290,314]. Así, puede ser útil para observar diferencias en la glicosilación dependiendo del estado normal o patológico del paciente [256,289-290]; puede permitir realizar estudios farmacocinéticos en los que se tengan en cuenta sus diferentes glicoformas; y puede ayudar a demostrar el uso ilegal de la hormona o sus análogos recombinantes [314]. En general, cuando se analizan preparados farmacéuticos, se dispone de disoluciones de EPO suficientemente concentradas y la principal dificultad del análisis radica en la gran analogía que existe entre las diferentes glicoformas, lo que hace difícil su separación. En el análisis de EPO en fluidos biológicos, se encuentran varias dificultades adicionales: sus bajas concentraciones en suero y orina, 0.1-100 ng/ml ; la gran analogía entre la EPO nativa y las rHuEPO; y su corto tiempo de vida media [256,289-290,314].

Las técnicas electroforéticas constituyen hoy en día la elección más acertada para separar las glicoformas de cualquier glicoproteína [27,285-286]. Esto se debe, a que las diferencias estructurales entre las glicoformas (tipo y tamaño de los azúcares, especialmente el contenido en ácido siálico), a menudo implican diferencias en sus relaciones carga/masa, lo que se traduce en diferencias significativas en sus movilidades electroforéticas y puntos isoeléctricos. Aunque el IEF ha sido la técnica preferentemente empleada [285-286], la CE, por sus ventajosas características, y la MS comienzan a considerarse como una atractiva alternativa para el control de calidad de glicoproteínas recombinantes (hormonas,

anticuerpos, ...) o el establecimiento de nuevos tests de diagnóstico de patologías en los que se ve alterada la glicosilación proteica [285-286,315]. En los últimos años, la CE-ESI-MS y el análisis por MALDI-TOF de fracciones recolectadas por CE, han sido también empleadas para la caracterización de las glicoformas de varias glicoproteínas [316-320].

En la Tabla 1.10 se muestra una recopilación de las técnicas de análisis empleadas para la separación y caracterización de las glicoformas de EPO en los últimos años. Aunque la LC, en sus diferentes modalidades, ha sido ampliamente utilizada en los complejos protocolos de purificación de EPO, sus glicoformas son difíciles de separar empleando esta técnica [61,303-306,321-323]. El método tradicionalmente propuesto por la Farmacopea Europea para el análisis de disoluciones lo suficientemente concentradas es el IEF, al que en los últimos años también se le añadió una metodología que empleaba CIEF [334]. Esta última, fue substituida el pasado año 2002 por un método de CE que emplea un recubrimiento dinámico de putrescina y que permite resolver un mayor número de glicoformas [326-327]. Sin embargo, en la mayoría de las metodologías de CE que aparecen en la Tabla 1.10, aunque se utilicen capilares recubiertos, existen muchas dificultades para obtener separaciones reproducibles y las condiciones de trabajo son incompatibles con la detección en línea por MS [330]. A diferencia de las glicoformas de la EPO intacta, los glicopéptidos u oligosacáridos que se obtienen al digerirla enzimáticamente, sí que han sido ampliamente separados utilizando CE y LC y caracterizados por MS (Tabla 1.10) [340-345].

Tabla 1.10 Análisis de EPO.

Técnica	Condiciones de separación	Observaciones	[Ref.]
		EPO intacta	
LC	SEC, IEC, ALC, RPLC	▪ Purificación de la proteína a escala preparativa	[61,303-306,321-323]
LC-ESI-MS	RPLC, modo positivo	▪ No hay separación y mala señal. El espectro de masas no proporciona información definitiva.	[324]
MALDI-TOF	Modo positivo	▪ Sólo proporciona una M media	[301,324-325]
CE	Capilar sílice fundida. Buffer: 0.01 M tricina, 0.01 M NaCl, 0.01 M NaAc, 7 M urea, 2.5 mM putrescina, pH 5.5	▪ Máxima resolución. ▪ Incompatible con CE-ESI-MS ▪ Problemas de reproducibilidad ▪ Método de referencia de la Farmacopea Europea	[326-330]
	Capilar sílice fundida. Buffer: 200 mM fosfato de sodio, 1 mM cloruro de níquel hexahidratado, pH 4	▪ Menor resolución ▪ Incompatible con CE-ESI-MS.	[323]
	Capilar sílice fundida. Buffer: 100 mM acetato-fosfato, pH 4	▪ Menor resolución ▪ Incompatible con CE-ESI-MS.	[331]
	Capilar recubierto DB-1. Buffer: 10 mM acetato, 0.5% (p/v) Hidroxipropilmetilcelulosa, pH 5.7	▪ Menor resolución. ▪ Incompatible con CE-ESI-MS.	[330]
	Capilar recubierto C_8. Buffer: 30 mM fosfato, pH 7.	▪ Menor resolución. ▪ In compatible con CE-ESI-MS	[332]
	Capilar sílice fundida. Buffer: 100 mM acetato-fosfato, pH 4.	▪ Menor resolución ▪ Poco reproducible. ▪ Incompatible con CE-ESI-MS	[61]
	Capilar recubierto FEP. Buffer: 10 mM HEPPSO, contraión BTP, 0.2 % hidroximetiletilcelulosa	▪ Menor resolución ▪ Incompatible con CE-ESI-MS	[333]
CIEF	Capilar recubierto poliacrilamida. CIEF kits provistos por Beckman Coulter (3-10) y Pharmacia Biotech (2.5-5). Mezcla 1:10. Mezcla 1:50 con el gel de CIEF. 7 M Urea.	▪ Menor resolución. ▪ Incompatible con CE-ESI-MS ▪ Método de referencia de la Farmacopea Europea hasta el 2002	[328,334]
IEF	pI= 2.5-6.5	▪ Método de referencia de la Farmacopea Europea	[326-327,330,334]

Técnica	Condiciones de separación	Observaciones	[Ref.]
	Isoelectroenfoque + doble *immunoblotting*	▪ Método adoptado por el COI para la detección de EPO y análogos en orina ▪ Alta sensibilidad.	[307-308]
PAGE	SDS	▪ No consigue resolver las glicoformas	[307-309,335]
2D-GE	Isoelectroenfoque + SDS-PAGE	▪ Buena resolución (hasta 40 manchas)	[309,336]
Otras técnicas	RMN, Sedimentación, DC, Pruebas bioquímicas	▪ Información global de la glicoproteína	[334,337-339]
EPO digerida enzimáticamente			
LC	IEC, NPLC, RPLC	▪ Análisis de oligosacáridos	[300,301,309,335,340-344]
LC-ESI-MS	RPLC, modo positivo	▪ Análisis de glicopéptidos	[300,302]
	RPLC, modo positivo	▪ Análisis de la EPO parcialmente desglicosilada	[324]
	RPLC, modo positivo y negativo. MS/MS	▪ Análisis de oligosacáridos	[345]
ESI-MS	LC off-line. Modo positivo	▪ Análisis de glicopéptidos	[300,340,343]
FAB-MS	LC off-line. Modo positivo	▪ Análisis de glicopéptidos	[344]
MALDI-TOF	Modo positivo	▪ Análisis de la EPO parcialmente desglicosilada	[324]
	Modo positivo	▪ Análisis de glicopéptidos	[324,342]
	Modo negativo	▪ Análisis de oligosacáridos	[301]
CE	CZE en polaridad inversa	▪ Análisis de glicopéptidos	[340]
CE-ESI-MS	CZE en polaridad inversa	▪ Análisis de glicopéptidos	[341]
PAGE	SDS	▪ Análisis de las glicoformas parcialmente digeridas	[309]
RMN	^{1}H	▪ Análisis de oligosacáridos	[335]

DB-1: nombre comercial, FEP: *Fluorinated ethylene-propylene*, HEPPSO: *N-(hydroxyethyl)piperazin-N'-3-(2-hydroxy)propanesulfonic acid, BTP: 1,3-bis[tris(hydroxymethyl)-methylamino]peopane*

Además, no todas las metodologías que aparecen en la Tabla 1.10 son lo suficientemente sensibles para la detección de la EPO intacta en fluidos biológicos. Por ello, ante la imposibilidad de detectar EPO directamente, se empleaban en la mayoría de las ocasiones los métodos indirectos, que cuantificaban algún factor que se veía afectado por la variación en la concentración total de EPO [314,346]: masa o índice de hematocritos, los parámetros del metabolismo del hierro (receptores de

transferrina solubles (sTfR) o la relación sTfR/ferritina (sTfR/fr)) o los productos de degradación del fibrinógeno y la fibrina. Estos indicadores indirectos, han sido ampliamente criticados en los controles antidopaje, ya que exigen una muestra de sangre, dependen de cada individuo, de sus condiciones de entrenamiento, no han sido validados por estudios de población, ni se han establecido bien valores de referencia y son fácilmente manipulables empleando infusiones salinas [314]. El COI no se pronunció sobre este tema hasta Agosto del año 2000, cuando aprobó dos métodos para determinar de forma independiente el uso de rHuEPO [307-308,346]. Estos se pusieron en marcha durante los pasados Juegos Olímpicos de Sidney 2000, y para evitar falsos positivos, sólo se sancionó a los deportistas que no pasaron ambos tests. En el primero de los métodos [346], se determina semicuantitativamente mediante un inmunoensayo la concentración de EPO total en sangre así como otros cuatro factores que resultan afectados por un incremento en dicha concentración de EPO (reticulocitos (RetHct), receptor de transferrina soluble, hematocrito (Hct) y % macrocitos). En el otro método, se detecta directamente la presencia de EPO en orina y se separan sus diferentes glicoformas mediante IEF, porque su diferente contenido de ácido siálico conduce a diferencias en sus puntos isoeléctricos. La combinación de IEF seguido de doble *immunoblotting* y detección quimioluminiscente, permite obtener perfiles de migración electroforética de EPO nativa o recombinante en las muestras analizadas (Figura 1.15)[307-308]. El doble *immunoblotting* evita la interferencia del resto de proteínas presentes en la orina, antes de la detección quimioluminiscente de las diferentes manchas (Figura 1.15). Para cada glicoproteína se obtiene un perfil electroforético compuesto por varias manchas y cada mancha correspondería a un conjunto de glicoformas con puntos isoelectricos similares. Los perfiles electroforéticos difieren entre la EPO natural (Figura 1.15-a) y las recombinantes (Figura 1.15-b y c) y pueden ser identificados los individuos a los que ha sido administrada la hormona recombinante (Figura 1.15 e,f,g y h) . Aunque simple en concepto, todo el procedimiento resulta largo y

tedioso, además de ser únicamente semicuantitativo y de no confirmar la identidad por MS de las bandas separadas. Todo esto provoca contínuas protestas entre los deportistas de élite y estimula la investigación de posibles alternativas de análisis, entre las que destaca la posibilidad de utilizar la CE-ESI-MS, por las ventajas que esto representa. De cualquier manera, los métodos directos siempre tendrán el inconveniente de la rápida eliminación de la hormona, lo que hace difícil su detección directa en la orina si se suspende su uso varios días antes de la competición. Esto puede continuar haciendo indispensable el uso de los indicadores indirectos complementarios o los análisis periódicos fuera del período de competición [314,346,307-308].

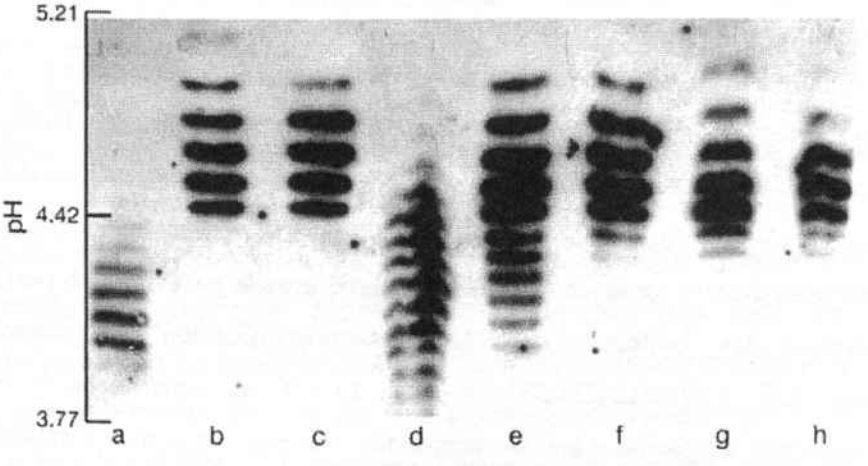


Figure 1.15 Análisis de diferentes tipos de EPO empleando el método de IEF seguido de doble immunoblotting propuesto por Lasne et al [307-308]. A) EPO humana natural (Sigma); b) rHuEPO (Neorecormon, Francia); c) rHuEPO- (Eprex, Francia); d) Orina control; e), f) Orina de dos pacientes tratados con rHuEPO Neorecormon EPO; g), h) Orina de dos ciclistas del Tour de Francia 1998 (muestras preconcentradas por ultrafiltración).

1.5 SEPARACIÓN Y CARACTERIZACIÓN DE PÉPTIDOS Y PROTEÍNAS. TENDENCIAS ACTUALES.

En los últimos años, tras la secuenciación del genoma humano, el gran reto científico consiste en descifrar el proteoma humano. La aparición de la investigación proteómica ha incrementado espectacularmente el interés por el desarrollo de tecnologías y metodologías analíticas que respondan a la necesidad de separar, cuantificar, identificar y caracterizar mezclas de péptidos y proteínas en matrices biológicas complejas (orgánulos celulares, células, tejidos, fluidos biológicos,...) [11-13,15-16,26,218,229-231,347-349]. La extrema complejidad del problema radica en que, el número de proteínas humanas es dos o tres órdenes de magnitud superior a los 40000 genes que las codifican, ya que, entre otras transformaciones, pueden experimentar numerosas modificaciones post-translacionales [350]. Además muchas de estas proteínas se encuentran a muy bajas concentraciones en las muestras analizadas y, a diferencia del genoma humano, no se dispone de un método tan eficiente para preconcentrarlas, como los basados en la Reacción en Cadena de la Polimerasa (PCR) [351]. En general, en Proteómica las muestras biológicas se tratan en una serie de etapas previas para obtener, purificar y preconcentrar las proteínas deseadas (inmunopurificación, fraccionamiento subcelular, LC,...) [26,218,233-235,347-349]. La GE es actualmente la técnica preferentemente empleada para la separación de estas mezclas complejas de proteínas. Las proteínas separadas son entonces identificadas y secuenciadas empleando MS y los resultados obtenidos interpretados utilizando bases de datos especializadas [218,233-235,347-349]. Para ello, o bien se analizan las proteínas separadas con MS-MS, o bien se analizan las mezclas peptídicas originadas en la digestión enzimática de la proteína deseadada. Cuando las mezclas son muy complejas, la Electroforesis en Geles bidimensional (2D-GE) es la técnica preferentemente empleada, aunque entre sus numerosas desventajas figuran su laboriosidad, las grandes cantidades de muestra necesarias y su limitada

sensibilidad. Por ello la tendencia actual en el estudio de las proteínas es el empleo de técnicas acopladas de separación y caracterización como la LC-ESI-MS y la CE-ESI-MS, que dan similares o mejores prestaciones y requieren tiempos de análisis mucho menores. La LC-ESI-MS y la LC-ESI-MS-MS también han sido muy empleadas para separar, identificar y caracterizar los péptidos generados tras la digestión enzimática de estas mezclas de proteínas [218,233-235,347-349]. Además, se han diseñado numerosas metodologías multidimensionales en las que se emplean secuencialmente dos modalidades de LC (LC-LC), la LC y la CE (LC-CE) o LC-LC y CE (LC-LC-CE), algunas de forma off-line y otras de forma totalmente automatizada, que permiten la preconcentración y la purificación en línea de las muestras [352-355]. Sin embargo, en todos los casos parece todavía utópico pensar en consumir pequeñas cantidades de muestra y poder realizar varios análisis en paralelo en espacios muy cortos de tiempo. Tendrán que pasar años hasta que las separaciones en formato de microchip permitan detectar, separar y caracterizar péptidos y proteínas que se encuentren a baja concentración en matrices complejas, aunque continuamente se avanza en la integración y en la mejora de los límites de detección empleando estos microdispositivos [356-360]. Recientemente, la utilización del concepto de preconcentración en línea mediante ligandos immovilizados en los capilares de separación [359], está aplicándose para la mejora de los límites de detección en las separaciones en microchips, ya se han acoplado con éxito a la ESI-MS [358] y existen algunos ejemplos de microchips que integran secuencialmente varias etapas del análisis [360].

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Capítulo 2. Separación de péptidos y hormonas peptídicas de bajo peso molecular mediante LC y CE

En los artículos que componen este capítulo, se describen modelos que explican el comportamiento electroforético y cromatográfico de las sustancias ionizables en función del pH de la fase móvil cromatográfica o del electrolito empleado en la separación electroforética, los pK_a de los analitos y los coeficientes de actividad implicados. Las ecuaciones más sencillas corresponden a los casos en los que los pK_a de la sustancia son lo suficientemente diferentes como para que sus equilibrios de disociación puedan considerarse independientemente dentro de un intervalo de pH, llegando incluso a poder utilizarse expresiones lineales. En el resto de casos, se pueden obtener las expresiones concretas, a partir de los modelos generales que se han establecido, para explicar el comportamiento electroforético y cromatográfico de especies polipróticas. Las ecuaciones deducidas permiten de manera simultánea la determinación de los valores de pK_a de las sustancias estudiadas y la predicción, a partir de un reducido número de datos experimentales, del pH al que se obtiene la resolución óptima de estas sustancias cuando forman parte de una misma mezcla. Para determinar rigurosamente los valores de pK_a en las mezclas hidroorgánicas empleadas en LC o CE, además de tener en cuenta los coeficientes de actividad, se realizan medidas correctas del pH en las mezclas consideradas. En este caso, se utiliza una escala de referencia de pH previamente establecida en nuestro grupo de investigación.

Los modelos establecidos permiten explicar el comportamiento cromatográfico y electroforético de series de péptidos y hormonas peptídicas de diferente peso molecular, determinar sus valores de pK_a y el pH óptimo para su separación cromatográfica y electroforética. Se aplican los modelos establecidos a dos series de péptidos y hormonas peptídicas y se estudia el efecto de la presencia del disolvente orgánico sobre los pK_a. Los valores de pK_a obtenidos mediante las diferentes técnicas son comparados entre si y con los datos bibliográficos. Una vez seleccionado el pH óptimo para su separación mediante LC o CE, se estudia la diferente selectividad de las separaciones.

Las investigaciones realizadas han conducido a la publicación de los siguientes artículos, que se reproducen a continuación en su formato original:

- ***Electrophoretic Behavior of Peptides in Capillary Electrophoresis. Influence of Ionic Strength and pH in Aqueous-Organic Media***
  V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa
  J. Chromatogr. A, 921 (2001) 69-79.

- ***pK_a Values of Peptides in Aqueous and Aqueous-Organic Media. Prediction of Chromatographic and Electrophoretic Behaviour***
  V. Sanz-Nebot, I. Toro, F. Benavente, J. Barbosa
  J. Chromatogr. A, 942 (2002) 145-156.

- ***Migration Behavior of Therapeutic Peptide Hormones: Prediction of Optimal Separation by Capillary Electrophoresis***
  V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa
  Electrophoresis, 22 (2001) 4333-4340.

- ***Evaluation of Chromatographic versus Electrophoretic Behaviour of a Series of Therapeutical Peptide Hormones***
  V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa
  J. Chromatogr. A, 985 (2003) 411-423.

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Journal of Chromatography A, 921 (2001) 69–79

JOURNAL OF CHROMATOGRAPHY A

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Electrophoretic behavior of peptides in capillary electrophoresis Influence of ionic strength and pH in aqueous–organic media

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Abstract

Through correct pH, pK_a and activity coefficients values, a model describing the effect of pH on electrophoretic mobility of substances has been applied to a series of peptides in water and in acetonitrile–water mixtures. The derived equations permit prediction of the optimum pH for the electrophoretic separation from only a few experimental values and they also permit determination of pK_a values of analytes in the aqueous–organic media employed. Furthermore, the electrophoretic resolution between pairs of substances can be predicted, in order to evaluate electrophoretic separations of the studied peptides. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Dissociation constants; Buffer composition; Migration prediction; Resolution; Peptides

1. Introduction

The recent increased use of peptides in biomedical therapy is a result of their large range of activity and specificity, usually with low toxicity and rapid metabolization [1]. For the near future, an increasing activity in design and synthesis of new peptide-based drugs is expected, as a result of combined advances in proteomic research and biotechnology [2]. Thus, separation and analysis of peptides and peptide hormones has become increasingly important for an ever-widening range of research disciplines. Development of highly efficient and selective separation methods is necessary before overcoming the unambiguous characterization of complex peptide mixtures generated during solid-phase peptide synthesis (SPPS) or enzymatic digestion of noncharacterized proteins [3,4]. Capillary electrophoresis (CE) is regarded as one of the first choices for rapid and efficient separation of a wide variety of peptide substances [5–7].

The use of nonaqueous solvents in general and aqueous–organic mixtures in particular extends the application range of CE. There is enhancement of electrophoretic separation versatility, because it is possible to work in media with a wide variety of dielectric constants, polarities, densities, viscosities and acid–base properties [8–10]. Other advantages of nonaqueous CE (NACE), are improved solubility of nonsoluble analytes and low operating currents when a high voltage is applied [11]. As a result, less Joule heat is produced in NACE conditions than in buffered aqueous systems, allowing much higher electric field strengths than those currently used in CE [12]. Furthermore, in most cases it is possible to use the same additives as in aqueous CE, e.g., salts, chiral selectors or electroosmotic flow modifiers [13]. Various aqueous–organic mixtures have been

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00730-0

used as separation media in NACE [12–14]. Acetonitrile (MeCN) and its mixtures with water are widely used, due to the excellent characteristics of the pure solvent. MeCN is a very weak base and a very weak acid and therefore a good differentiating solvent for both acids and bases. Furthermore, low viscosity and good UV transparency make it ideal for NACE [11,15].

Although a desired separation can be obtained by trial and error, this may take many attempts with subsequent loss of time and final substance yield, and a particular problem could arise when only small amounts of sample are available. The total number of attempts to achieve an optimum separation can be minimized by using a limited number of experimental data to establish accurate quantitative relationships capable of predicting electrophoretic migration of compounds under different conditions [16,17]. In the electrophoretic separation of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual solute [18,19]. Thus, for prediction of electrophoretic behavior of ionizable substances, relationships between electrophoretic mobility, dissociation constants and pH can be very helpful [20,21]. However, the inclusion of pH as an optimization parameter in aqueous–organic mixtures raises several problems. Thermodynamically valid pH and pK_a values must be used to interpret ionic equilibria in NACE media. Moreover, activity coefficients can be neglected in water for dilute solutions (<0.01 *M*) because of the high permittivity of the medium, but they must be considered in aqueous–organic mixtures. The measurement of pH in a mixed solvent is based upon the operational definition of pH [22,23], in which pH is estimated by:

$$pH_X = pH_S + \frac{(E_S - E_X)}{k_g} \quad (1)$$

Where the unknown pH of solution *X*, pH_X, is related to the pH of a standard reference solution, pH_S, and the e.m.f. values of the potentiometric cell containing the standard, E_S, and the unknown solution, E_X. k_g must be used for practical measurements, usually carried out in cells with glass electrodes, and corresponds to the practical slope of the *E* versus pH function [24,25]. pH measurements in MeCN–water mixtures can be performed in a manner similar to that in water [24,26], taking into account the pH values previously assigned to primary standard buffer solutions in these media [27], according to the NIST multiprimary standard scale. Furthermore, in compliance with IUPAC rules [28,29], the activity coefficients of the species in aqueous–organic mixtures can be calculated from the ionic strength through the classical Debye–Hückel equation [22,23].

In the work described here, through correct pH, pK_a and activity coefficient values, a model describing the effect of pH on electrophoretic mobility of substances was applied to a series of peptides in water and in MeCN–water mixtures with 10% (w/w) of MeCN, in order to establish separation methodologies for peptide mixtures using CE, but on the basis of just a few experimental data. The suggested model in aqueous–organic medium, uses the pH value in MeCN–water mixtures instead of pH value in water and takes into account the effect of activity coefficients. The equations proposed have two possible applications: they permit prediction of electrophoretic migration as a function of pH of the separation medium using a minimum number of measurements, and they permit determination of the pK_a values of the analytes in the aqueous–organic media used. From pH, pK_a, activity coefficients and electrophoretic mobilities, the resolution between pairs of substances was predicted and the optimum conditions for the separation of the studied peptides were established.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetonitrile, phosphoric acid (85%), sodium hydroxide, hydrochloric acid (25%), formic acid (98%), acetic acid (glacial), acetone and potassium hydrogenphathalate were supplied by Merck (Darmstadt, Germany). Tris [Tris(hydroxymethyl)aminomethane] was purchased from J.T. Baker (Deventer, Holland). Water with a conductivity lower than 0.05 mS cm^{-1}

was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). All peptides used in this study were purchased from Sigma (St. Louis, MO, USA): D-2-amino-5-ureidovaleric acid (citrulline), *N*-(*N*-glycyl)glycine (Gly–Gly), *N*-(*N*-glycylglycyl)glycine (Gly–Gly–Gly), *N*-(*N*-L-tyrosylglycyl)glycine (Tyr–Gly–Gly), *N*-(*N*-glycylglycyl)-L-valine (Gly–Gly–Val), *N*-(*N*-glycylglycyl)-L-isoleucine (Gly–Gly–Ile), *N*-(*N*-DL-alanyl-DL-leucyl)-glycine (Ala–Leu–Gly) and *N*-(*N*-glycylglycyl)-L-phenylalanine (Gly–Gly–Phe). In the case of DL-Ala–DL-Leu–Gly, it was possible to separate two diastereoisomer mixtures from the commercial product, and they are referred in the text as I and II, respectively.

2.2. Electrolyte solutions

A buffer consisting of 50 m*M* acetic acid–50 m*M* formic acid, was prepared to cover the acidic pH range (2.5–4.5 approximately), adjusting to the appropriate pH with 1 *M* NaOH. For the determination of the electrophoretic mobility of the fully protonated peptides a 20 m*M* phosphate buffer was used [pH 2.1 and pH 2.2 in water and in MeCN–water (10:90, w/w), respectively]. A 50 m*M* Tris solution was the operating buffer in the basic pH range (7–9.5 approximately), adjusting to the appropriate pH with 1 *M* HCl. For the determination of the electrophoretic mobility of the fully deprotonated peptides 50 m*M* Tris buffer was used. When working in MeCN–water (10:90, w/w), the background solvent was prepared by mixing water and MeCN in the appropriate amounts to obtain a stock solvent solution. All the buffers and solutions used in MeCN–water (10:90, w/w), medium were prepared using this stock solvent. 150 μ*M* peptide solutions were separately prepared in water, containing acetone at 3% (v/v) as the electroosmotic flow (EOF) marker [18,19]. A mixture containing all the peptides at 150 μ*M* was prepared. Samples and running electrolytes were passed through a 0.45-μm nylon filter (MSI).

2.3. Instrumental parameters

A Beckman P/ACE system 5000 (Beckman Instruments, Fullerton, CA, USA) was used in all the electrophoretic experiments. A 57-cm×75-μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was inserted in a capillary cartridge, thermostated to 25°C (±0.1°C). Samples were injected hydrodynamically at 0.5 p.s.i. for 4 s (1 p.s.i=6894.76 Pa). Experiments were conducted under normal polarity, applying a voltage of 25 kV during electrophoretic separations. The detection window was placed at 50 cm from the inlet of the capillary. A photodiode array detector was used. All data were recorded and analyzed by a computer program supplied by Beckman (P/ACE Station 1.0 with Golden System interface). pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

2.4. Capillary treatment

All capillary rinses were performed at high pressure (20 p.s.i.). New capillaries were activated by flushing for 20 min with aqueous 1 *M* NaOH, followed by 15 min with water and 30 min with working buffer. Between days or after a change of buffer, the capillary was conditioned by rinsing successively for 5 min with water, 5 min with aqueous 0.1 *M* NaOH, 15 min with water and 30 min with buffer. After the last flush with buffer, to complete capillary activation and conditioning, a voltage of 25 kV was applied for 15 min. It was empirically demonstrated that this final step accelerate capillary equilibration. Between runs, the capillary is successively rinsed for 2 min with 0.1 *M* aqueous NaOH, 3 min with water and 5 min with buffer, in order to reequilibrate it and thereby minimize hysteresis effects. The capillary was stored overnight filled with working buffer electrolyte.

2.5. Procedures

Individual solutions of peptides were first injected at each pH until the electrophoretic mobility was constant. m_e values were calculated as the difference between the apparent mobility of each peptide and the mobility of acetone used as neutral marker [30]. Each electrophoretic mobility was obtained as the average of three replicates.

All the peptides studied have only two relevant ionisable functional groups in terminal positions, which means that the acid–base chemistry involves two equilibria. The pK_a value in the acidic range can be associated with carboxylic acid function and the pK_a value in the basic range can be assigned to the protonated amino group dissociation [31,32]. In all cases the two pK_a values are different enough to consider these two equilibria independently [31,32]. In order to study the influence of pH on the electrophoretic behavior of substances, we can relate pH, pK_a and electrophoretic mobility. The established relationships are based on the principle that a solute has its maximum electrophoretic mobility when it is fully ionized, has no electrophoretic mobility in its neutral form, and has an intermediate mobility in the pH region surrounding its pK_a [21,30,33,34]. Thus, the electrophoretic mobility, m_i, of a compound coexisting in the form of various species, is a function of the mobility and the molar fraction, X_i, of these species. In the case of the studied peptide substances, the first dissociation constant is related to the concentration of protonated $(H_2Z)^+$ and undissociated (HZ) species [30–32]. Thus, effective mobility can be expressed as follows:

$$m_e = X_{H_2Z^+} m_{H_2Z^+} + X_{HZ} m_{HZ} \quad (2)$$

where the term corresponding to the HZ species is considered nil, because the HZ species has no charge and migrates with the electroosmotic flow. Assuming that the mobility of the fully protonated species is $m_{H_2Z^+} = m_a$ and replacing the molar fraction by its expression for ampholytes: $X_{H_2Z^+} = a_{H^+}/(a_{H^+} + K_1 y)$ where y is the activity coefficient. The electrophoretic mobility, m_e, can therefore be obtained:

$$m_e = \frac{a_{H^+} m_a}{a_{H^+} + K_1 y} \quad (3)$$

The activity coefficient, y, is obtained, according to IUPAC rules [21,30,35], from the Debye–Hückel equation, $\log y = -z_i^2 A\sqrt{I}/(I + a_o B\sqrt{I})$, where A and B are the Debye–Hückel constants, a_o is the ion size parameter in the solvent mixture and I is the ionic strength. Values of A and $a_o B$ parameters at 25°C, at different percentages of MeCN in water were reported in previous works [36,37]. Eq. (3) can be written in a linearized form as:

$$\frac{1}{m_e} = \frac{1}{m_a} + \frac{K_1 y}{m_a a_{H^+}} \quad (4)$$

Similar expressions to Eqs. (3) and (4) can be deduced in basic media [30], this permits determination of the second dissociation constant. Thus, considering that the electrophoretic mobility is maximum for Z^- species, the expression for mobility in the pH region surrounding the pK_2 is $m_e = m_b X_b$, where m_b is the electrophoretic mobility of the fully deprotonated species, Z^-, whose sign is opposite to that of the mobility of the fully protonated form, H_2Z^+. Replacing X_b by its expression for ampholytes, the following equation for mobility in basic media is obtained:

$$m_e = \frac{K_2 m_b}{K_2 + a_{H^+} y} \quad (5)$$

This equation can be written in a linearized form as:

$$\frac{1}{m_e} = \frac{1}{m_b} + \frac{a_{H^+} y}{K_2 m_b} \quad (6)$$

In order to calculate dissociation constants for each peptide, data pairs of m_e–pH values in water and in MeCN–water (10:90), were fitted to Eqs. (3)–(6), using a least-squares regression program. Using this procedure, m_a and m_b values were also determined.

Eqs. (3)–(6) allow us to determine the dissociation constants of analytes when several data pairs of m_e–pH are available. In addition, the influence of pH on the electrophoretic mobility of substances can be predicted when the pK_a of compounds are known, allowing prediction of optimum pH for separation. Likewise, using Eqs. (4) and (6), only two experimental measurements of m_e–pH pair for each compound, varying their migration in the pH range considered, are enough for describing and predicting their migration behavior and therefore, optimizing their electrophoretic separation by predicting resolution in a fixed medium.

3. Results and discussion

Experimental electrophoretic mobility for all the studied peptides were determined as an average of

three replicates, over the pH ranges considered. The electrophoretic migration behavior of these peptides are described by Eqs. (3)–(6), which at a given pH, relate electrophoretic mobilities of the fully protonated or deprotonated species, m_a or m_b, respectively, and the dissociations constants, K_1 or K_2, taking into account the activity coefficients effect. The electrophoretic mobilities for all the studied peptides obtained in water and in MeCN–water (10:90, w/w) were plotted against the buffer pH in Fig. 1A,B,

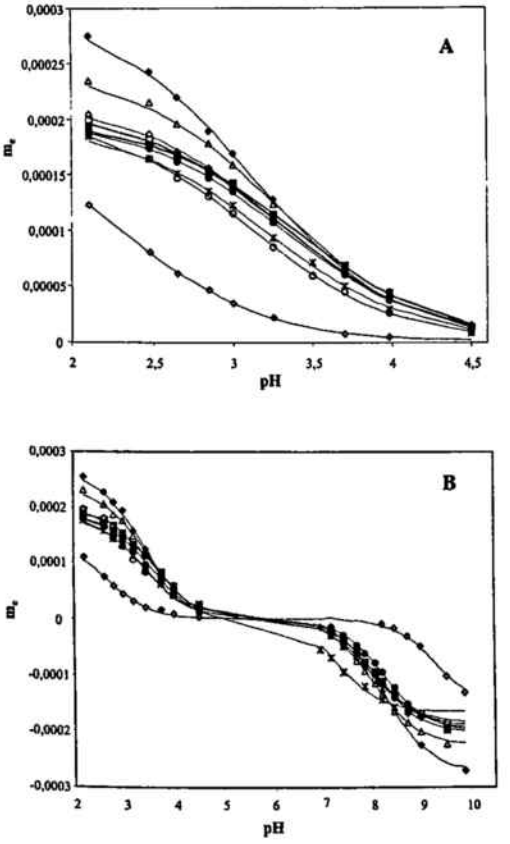

Fig. 1. Plots of experimental (points) and predicted (lines) electrophoretic mobilities, m_e, versus pH for Gly–Gly (♦), Gly–Gly–Gly (△), Gly–Gly–Val (◇), Gly–Gly–Ile (□), Ala–Leu–Gly (I) (■), Ala–Leu–Gly (II) (●), Gly–Gly–Phe (○), Tyr–Gly–Gly (*), citrulline (◆). (A) In water; (B) in MeCN–water (10:90, w/w).

respectively. Symbols stand for the experimental data and solid lines indicate the best nonlinear regression fits for each peptide, using Eqs. (3) and (5).

On the other hand, Fig. 2 plots $1/m_e$ versus y/a_{H^+} and ya_{H^+}, for each peptide in water and in MeCN–water (10:90, w/w) in acidic (Fig. 2A,B) and basic conditions (Fig. 2C). In accordance with Eqs. (4) and (6) good linearities are observed. Fig. 2a,b permits m_a and pK_1 determination while m_b and pK_2 can be obtained from linear correlations corresponding to basic medium. The activity coefficients were calculated from the Debye–Hückel equation, although the higher buffer ionic strengths are 6×10^{-2} M, slightly higher than the traditionally accepted upper limits of the simple Debye–Hückel correlation. In MeCN–water (10:90, w/w), $A=0.5404$ and $a_0B=1.5206$ [36,37]. Citrulline presents much higher migration times than the other peptides studied, due to its low pK_1 and high pK_2 values. Thus, adjustments corresponding to citrulline are shown off-scale in Fig. 2, in order to facilitate reading of the plots. Negative slopes of lines in Fig. 2c are due to negative values of mobilities presented by peptides when the pH studied is higher than their pK_a.

Models described by Eqs. (3)–(6) established using data pairs pH–m_e and ionic strengths over the whole pH range in water and MeCN–water mixtures, allow pK_a determinations. pK_a values determined for the studied peptides using nonlinear regression method, Eqs. (3) and (5), and the linear model, Eqs. (4) and (6), are shown in Table 1, with their respective standard deviations. As Table 1 shows, results obtained using both methodologies are similar. Only slight differences were observed in the case of citrulline due to its extreme pK_a values. Final pK_a values in Table 1 were calculated averaging the results obtained from both methods. Thus, both methodologies have demonstrated suitability for pK_a determinations using CE.

There are only a few pK_a values of peptides reported in the literature. Table 1 summarizes the pK_a values previously determined using liquid chromatography (LC) [32] and potentiometric techniques [31]. As shown in Table 1, the values obtained in water are similar to bibliographic data. In MeCN–water mixtures, pK_a values established by CE are

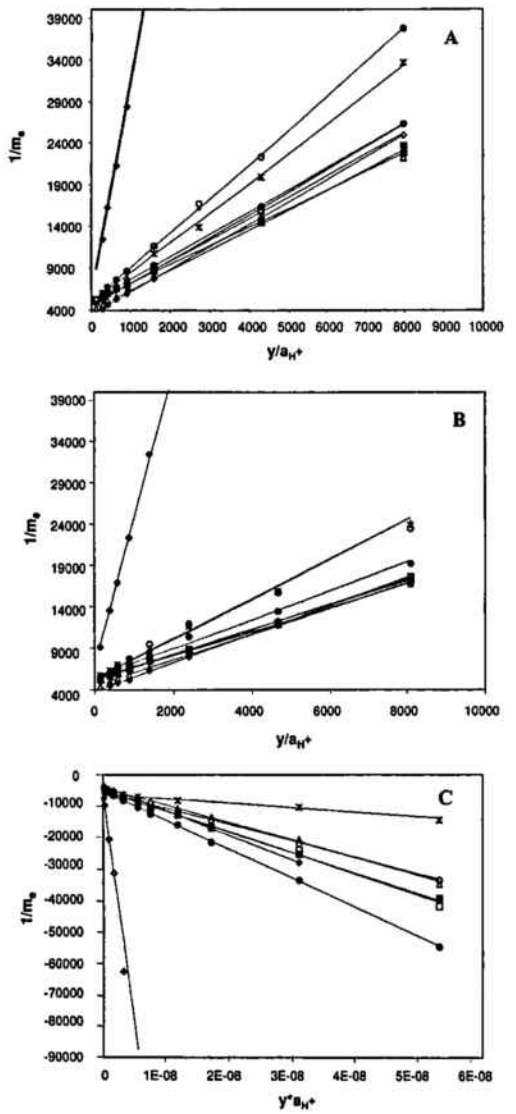

Fig. 2. Plots of experimental (points) and predicted (lines) $1/m_e$ values versus (A) y/a_H^+ in water, (B) y/a_H^+ in MeCN–water (10:90, w/w) and (C) ya_H^+ in MeCN–water (10:90, w/w) for Gly–Gly (♦), Gly–Gly–Gly (△), Gly–Gly–Val (◇), Gly–Gly–Ile (□), Ala–Leu–Gly (I) (■), Ala–Leu–Gly (II) (●), Gly–Gly–Phe (○), Tyr–Gly–Gly (*), citrulline (◆).

practically identical to the values previously determined by potentiometry. In contrast, the values previously obtained by LC methodologies at 5.5% (w/w) of MeCN are slightly higher than the ones determined by CE and potentiometric techniques. This fact was explained in a previous work [38], taking into account that the retention mechanism in LC includes interactions other than those of a hydrophobic nature. In general, as can be seen in Table 1, the acidic dissociation constants for deprotonation of carboxylic acid and protonated amino groups decrease as the solvent becomes enriched in the organic component. These variations of pK_a values are lower than those expected because of the preferential solvation of water in MeCN–water mixtures [39] and have been interpreted in previous works [40]. Finally, notice that the pK_a of both Ala–Leu–Gly diastereoisomer mixtures can be differentiated, as previously observed with LC [32]. This makes capillary electrophoresis specially attractive for pK_a determinations of diastereoisomers, because they cannot be differentiated by potentiometry [31].

The usefulness of Eqs. (3)–(6) is two-fold. They can be effectively used to calculate the pK_a values of the substances, and they permit us to obtain accurate values of their electrophoretic mobilities at any pH of the medium. Thus, Eqs. (3)–(6) allow prediction of the optimum pH for electrophoretic separations from a minimum number of experimental m_e–pH pairs. Fig. 3 shows plotted experimental versus predicted values of m_e in water, using Eq. (2), for all the studied peptides over the whole pH range assayed. All data pairs are aligned with a slope value of 1.000 and correlation coefficients of 0.999. Similar results were obtained for all the studied peptides in MeCN–water (10:90, w/w). Thus, studied models permit accurate migration prediction over the whole pH range studied.

Once the usefulness of the models for predicting electrophoretic mobilities has been demonstrated, it is possible to consider the combined calculated curves for all the peptides (Figs. 1 and 2) in order to predict the optimum pH to achieve the best separation of their mixtures. Based on these predicted curves, m_e can be determined at any pH by performing a few experimental measurements. Consequently, it is possible to establish the pH range where the

Table 1
pK_a values for the studied peptides in water and aqueous–organic mixtures

Peptide	pK_a	MeCN (%, w/w)								
		0%				5.5%	10%			
		Linear method	Non-linear method	Average value (CE)	Ref. [32]	Ref. LC [31]	Linear method	Non-linear method	Average value (CE)	Ref. [32] Potentiometry
Gly–Gly	1	3.10 (0.02)	3.08 (0.04)	3.09 (0.03)	3.14	3.35 (0.06)	3.25 (0.02)	3.33 (0.02)	3.29 (0.02)	3.33
	2				8.04		8.35 (0.04)	8.32 (0.02)	8.33 (0.03)	8.30
Gly–Gly–Ile	1	3.26 (0.02)	3.26 (0.07)	3.26 (0.04)		3.59 (0.02)	3.52 (0.02)	3.53 (0.02)	3.52 (0.02)	3.55
	2				8.09		8.10 (0.07)	8.11 (0.01)	8.10 (0.04)	8.09
Gly–Gly–Gly	1	3.21 (0.02)	3.22 (0.02)	3.21 (0.02)	3.30	3.57 (0.06)	3.46 (0.03)	3.41 (0.02)	3.43 (0.02)	3.46
	2				7.96		8.11 (0.06)	8.08 (0.01)	8.09 (0.03)	8.09
Tyr–Gly–Gly	1	3.14 (0.04)	3.18 (0.02)	3.16 (0.03)		3.54 (0.05)	3.35 (0.02)	3.36 (0.02)	3.35 (0.02)	3.46
	2						7.36 (0.05)	7.41 (0.04)	7.38 (0.04)	7.45
Gly–Gly–Val	1	3.20 (0.02)	3.23 (0.02)	3.21 (0.02)		3.59 (0.02)	3.53 (0.02)	3.50 (0.02)	3.51 (0.02)	3.54
	2				8.12		8.09 (0.05)	8.15 (0.02)	8.12 (0.03)	8.08
Ala–Leu–Gly (I)	1	3.34 (0.02)	3.34 (0.01)	3.34 (0.01)		3.71 (0.04)	3.56 (0.01)	3.56 (0.03)	3.56 (0.02)	
	2						8.08 (0.02)	8.13 (0.02)	8.10 (0.02)	
										3.62
										8.12
Ala–Leu–Gly (II)	1	3.26 (0.02)	3.27 (0.01)	3.26 (0.01)		3.61 (0.05)	3.49 (0.01)	3.46 (0.03)	3.47 (0.02)	
	2						8.23 (0.05)	8.30 (0.02)	8.26 (0.03)	
Gly–Gly–Phe	1	3.08 (0.01)	3.06 (0.01)	3.07 (0.01)		3.30 (0.02)	3.33 (0.04)	3.33 (0.05)	3.33 (0.04)	3.33
	2				8.04		7.98 (0.02)	8.01 (0.02)	7.99 (0.02)	8.02
Citrulline	1	2.39 (0.08)	2.30 (0.04)	2.34 (0.06)	2.4	2.37 (0.11)	2.58 (0.07)	2.60 (0.06)	2.59 (0.06)	2.39
	2				9.69		9.43 (0.02)	9.44 (0.03)	9.43 (0.02)	9.58

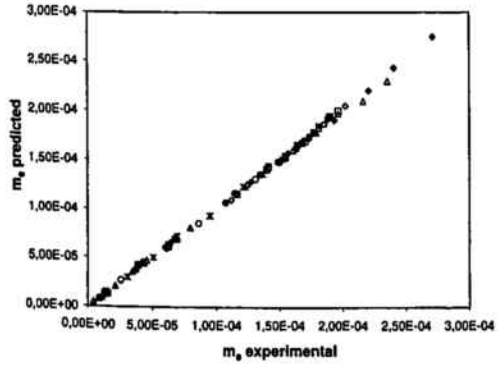

Fig. 3. Experimental versus predicted with non-linear model m_e for the studied peptides in water.

mobility values of all the studied peptides differ most and hence the pH where the separation of a mixture of all of them is optimum. According to Figs. 1 and 2, optimum separations are expected between pH 2.5 and 2.6, in water and in MeCN–water (10:90, w/w), because at these pH their electrophoretic mobility values show the greatest difference. From Figs. 1 and 2 their migration orders at any pH could be also deduced.

In order to predict pH conditions for optimum CE separations, Eqs. (4) and (6) can be especially useful. Using these equations, only two experimental measurements of migration times for each compound, varying their electrophoretic mobility in the pH range considered, are enough for characterizing and predicting their migration behavior and, therefore, optimizing their electrophoretic separation and resolution in a fixed composition medium.

The best way to evaluate separation between

critically adjacent peak pairs when a mixture of all the peptides is injected, is by predicting their resolution (R_s) according to the following equation [19,20,41]:

$$R_s = \frac{N^{1/2}}{4} \cdot \frac{(m_1 - m_2)}{(m_{avg} + m_{EOF})} \quad (7)$$

where m_i is the predicted electrophoretic mobility of solutes (linear model values in this case), m_{avg} is the average of m_i values, m_{EOF} is mobility of the electroosmotic flow, which can be evaluated from the study of m_{EOF} versus pH (data not shown) and N the number of theoretical plates. This equation only finds application when analyzing peaks with similar N value, which means that they are close enough to show similar efficiency. In our case, where all the critical pairs elute in a very narrow time window, the minimum N value of 4×10^4 in water and 3×10^4 in MeCN–water (10:90, w/w) were chosen. The N number is different because of the lower efficiencies observed in the analysis performed in MeCN–water media. Overestimations of the resolutions are thus avoided.

Changes in migration order of studied peptides over the studied pH range (see curves in Figs. 1 and 2), recommend performing resolution calculations considering peaks labelled from 1 to 9, according to their migrating order at each pH. Thus, minimum resolution between all analytes in the mixture is calculated. Fig. 4 shows plotted predicted resolution of adjacent peaks versus pH. Resolution between peaks number 8 and 9 is higher than the limits of the current representation in the whole range of studied pH. Solid lines correspond to predicted resolution, while some experimental values of resolution, calculated with Eq. (8), are represented by points:

$$R_s = 2 \times \frac{(t_2 - t_1)}{(w_1 + w_2)} \quad (8)$$

where w_i is the base width and t_i the migration time. The agreement between experimental and predicted

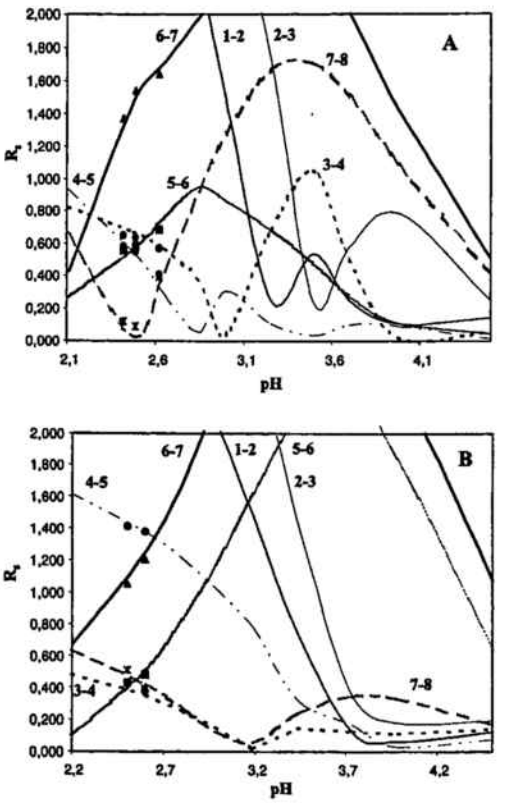

Fig. 4. Plots of experimental (points) and predicted (lines) minimum resolutions for a mixture of the studied peptides versus pH, in (A) water and (B) in MeCN–water.

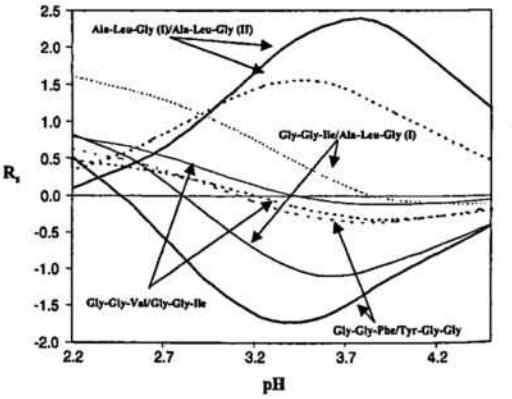

Fig. 5. Plots of predicted resolutions for some pairs of peptides versus pH, in water (solid lines) and in MeCN–water (10:90, w/w) (dashed lines).

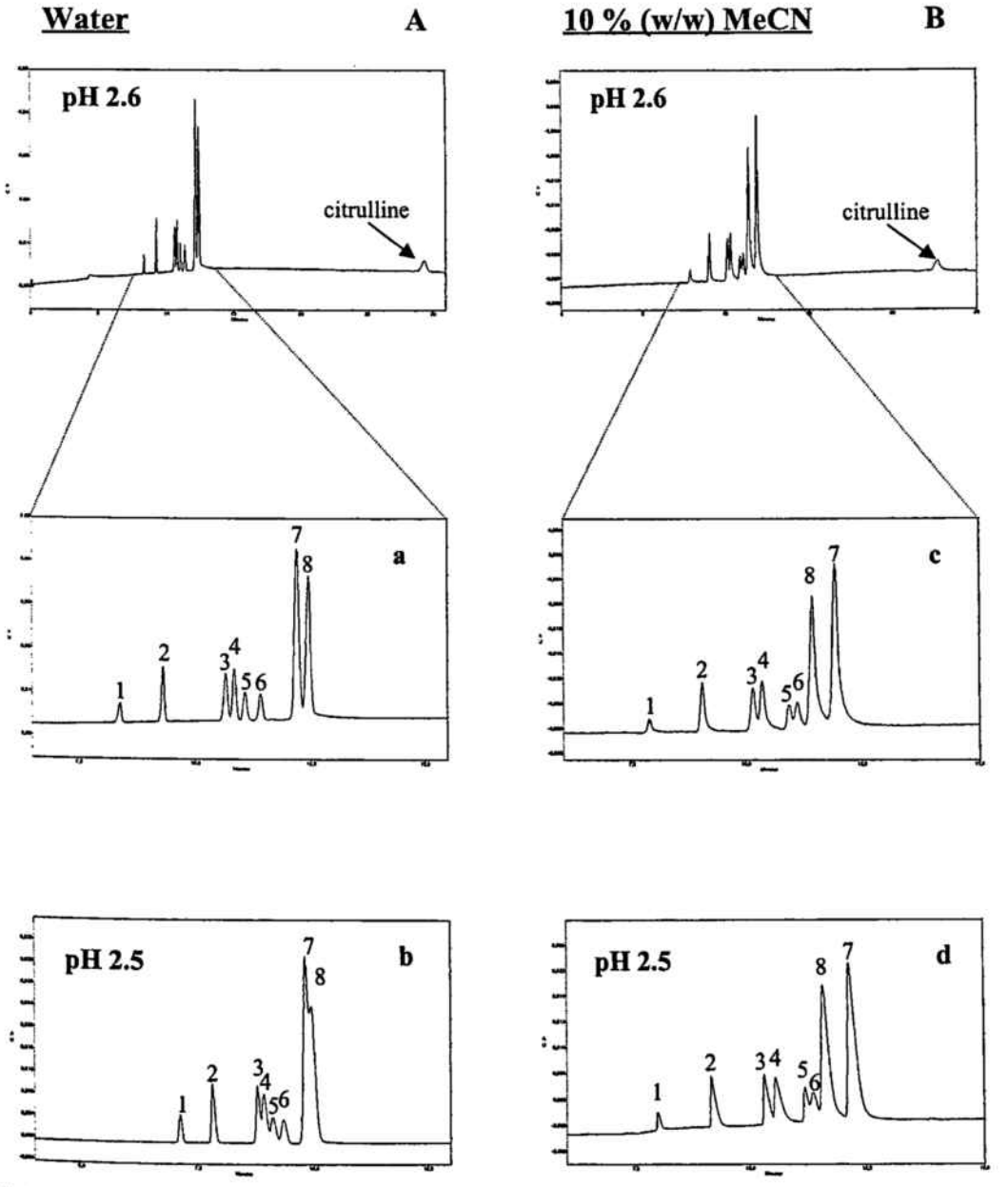

Fig. 6. Electropherograms at 25 kV of a mixture of the studied peptides in 50 m*M* formic acid–50 m*M* acetic acid buffer in (A) water and in (B) MeCN–water (10:90, w/w). Peaks: (1) Gly–Gly, (2) Gly–Gly–Gly, (3) Gly–Gly–Val, (4) Gly–Gly–Ile, (5) Ala–Leu–Gly (I), (6) Ala–Leu–Gly (II), (7) Tyr–Gly–Gly, (8) Gly–Gly–Phe, (9) citrulline.

resolution values confirms the accuracy of the studied models. At pH around 3.5 in water (Fig. 4A), maximum resolutions are predicted for all the studied pairs, except for peaks 4 and 5 with a value close to zero. In the case of Fig. 4B the presence of acetonitrile has made resolution between 4 and 5 slightly higher but resolution between 3 and 4 has dramatically decreased. According to Fig. 4 optimum separation of all peptides present in the mixture are achieved in the pH range between 2.5 and 2.7 in the aqueous and aqueous–organic media studied.

However, for a better understanding of parameters affecting resolution, resolution between adjacent peaks can be studied in the same pH range, without considering changes in migration order. In Fig. 5 predicted resolution in water (solid lines) and MeCN–water (10:90, w/w) (dashed lines) is plotted versus pH for some critical pairs: Gly–Gly–Val/Gly–Gly–Ile, Gly–Gly–Ile/Ala–Leu–Gly (I), Ala–Leu–Gly (I)/Ala–Leu–Gly (II) and Tyr–Gly–Gly/Gly–Gly–Phe. Negative resolution values indicate a change in migration order between analyzed peaks. When this occurs resolution with the next adjacent peak, when analyzing a mixture, must be considered. This change in migration order is affected by MeCN content and pH. In general, MeCN addition results in a loss of resolution. But in some cases, Gly–Gly–Ile/Ala–Leu–Gly (I), a gain of resolution can be observed in MeCN–water media. In both media the mixtures of Ala–Leu–Gly diastereoisomers can be baseline resolved at pH around 3.7, due to their pK_a differences. This indicates the great importance of pH optimization when structurally related ionisable compounds must be separated [13,42]. Critical influence of pH on separations is confirmed by comparison of the electropherograms of the mixture at pH 2.5 and 2.6 in water in Fig. 6a,b, respectively. Influence of acetonitrile on analysis time, resolution and efficiency is verified by comparison with the electropherograms of the mixture in MeCN–water (10:90, w/w) shown in Fig. 6c,d. Analysis time is decreased in the presence of MeCN, while efficiencies and resolutions are higher in water. Fig. 6c,d also show how MeCN presence affects the migration order of Tyr–Gly–Gly and Gly–Gly–Phe.

In conclusion, we have shown the suitability of the studied models for predicting electrophoretic behavior of peptides in capillary electrophoresis from a limited number of experimental data. Thus, resolution between solutes in a complex mixture can be easily predicted, making simple and rapid pH selection to conduct optimum electrophoretic separations. In a parallel way, dissociation constants can be evaluated. Calculated dissociation constants agree with the ones determined by potentiometry.

Acknowledgements

Financial support for this project by DGICYT (Project PB98-1174) is gratefully acknowledged. F.B. also thanks the Generalitat de Catalunya for an F. I. Grant.

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JOURNAL OF CHROMATOGRAPHY A

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pK_a values of peptides in aqueous and aqueous–organic media Prediction of chromatographic and electrophoretic behaviour

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Received 14 June 2001; received in revised form 10 October 2001; accepted 10 October 2001

Abstract

In the present work, models describing the effect of the pH on the chromatographic and electrophoretic behaviour for polyprotic peptides were compared. The proposed models can be simultaneously used for determination of dissociation constants and selection of the optimum pH for the separation of peptides, in water and acetonitrile–water mixtures widely used in liquid chromatography and in capillary electrophoresis. The models use the pH value measured in the acetonitrile–water mixture instead of the pH value in water and take into account the effect of the activity coefficients. They permit the determination of the acidity constants in the aqueous and hydro–organic mobile phase from chromatographic retention and electrophoretic migration measurements, respectively. The values obtained by both proposed techniques agree with the potentiometric values previously determined. The suitability of the proposed models for predicting chromatographic and electrophoretic behaviour of compounds studied from a limited number of experimental data was also compared. The separation between solutes by both techniques in a complex mixture can be easily predicted, making simple and rapid pH selection to achieve optimum separation.

Keywords: Dissociation constants; Mobile phase composition; Relation prediction; Buffer composition; pH effects; Optimization; Peptides

1. Introduction

The use of synthetic peptides in pharmaceutical research and in human and veterinary medicine is widespread. In this way, peptide and protein separations have been extensively explored, especially when high efficiency and resolution are desirable [1–4]. Novel applications in this field are continuously described, whereas the major drawbacks of the technique are tried to be solved on its way to maturity [4–8]. In the near future, the combined advances of biotechnology and proteomic research must lead to an increasing activity in the discovery and synthesis of new biologically active peptides [9], which is necessary for the development of new separation and characterization technologies. At present, liquid chromatography (LC) and capillary electrophoresis (CE) are orthogonal techniques for peptide and protein separation and identification [1–6], improvement of hyphenated techniques [10,11] must increase their value also in other fields.

The development of rapid, efficient and selective separation methods requires optimization of separation conditions. Although a desired peptide sepa-

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01398-X

ration may be obtained by trial and error, this may take many attempts with subsequent loss of time and final peptide yield, and could be a particular problem when only limited amounts of sample are available. We can minimize the total number of attempts by using experimental data to select the best conditions and by making use of accurate quantitative relationships able to predict elution of peptides under different separation conditions.

The optimization of chromatographic and electrophoretic separation of ionogenic solutes is a task that has been actively researched [12–15]. Due to the specific acid–base characteristics of ionogenic solutes, the two most useful optimization parameters are the pH and the organic modifier concentration.

The use of non-aqueous solvents in general, and the binary water–organic solvent system in particular, extends the range of aqueous CE applications. This provides a more versatile electrophoretic separation, since it is possible to work in a medium with different dielectric constants, polarities, densities, viscosities and acid–base properties [5,16,17]. Other advantages of non-aqueous CE are improved solubility of analytes with low solubility in pure aqueous buffer solutions, and low operating currents when voltage is applied [18]. As a result, less Joule heat is produced under non-aqueous CE conditions than in buffered aqueous systems, allowing much higher electrical field strengths than those currently used in CE [19].

The inclusion of the pH as an additional optimization parameter raises several problems [20]. The pH of the mobile phase is usually taken to be the same as that of the aqueous fraction. However, the pK_a values of the acids used to prepare the buffers, change with the solvent composition [21,22] so does the pH of the buffer [23,24]. Sometimes the pH is measured after mixing the buffer with the organic modifier [25]. But even in this instance, the potentiometric system is usually calibrated with aqueous standards, and the measured pH is not the true pH of the mobile phase. Additionally, the proposed models do not consider the effect of the activity coefficients. This effect can be neglected in water, which has a high dielectric constant, but it may be considerable in acetonitrile–water mixtures [21,22].

pH measurements in acetonitrile–water, the most widely used mobile phase, can be performed in a manner similar to that in water [26–28], taking into account the pH values previously assigned to primary standard buffer solutions in acetonitrile–water mixtures [23,24] according to the National Institute of Standards and Technology (NIST) multiprimary standard scale [26]. Also, in compliance with IUPAC rules [28,29], the activity coefficients of the species in acetonitrile–water mixtures can be calculated from the ionic strength through the classical Debye–Hückel equation [29].

Several semi-empirical approaches have been proposed to relate chromatographic retention or electrophoretic mobility with structural parameters of proteins and peptides, but their application is limited by the assumptions made in their development [30–33]. Furthermore, there is no general rule to a priori select the appropriate relationship for each type of compound [31]. Likewise, in the separation of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual solute [15]. Accurate quantitative relationships between chromatographic retention and electrophoretic mobility, respectively and pH, can be very useful [12,34,35].

In this way, modelling the chromatographic and electrophoretic behaviour is a key tool to predict separations, avoiding long and tedious separation optimizations performed by trial and error procedures. Furthermore, these retention or migration models can be used to perform physico–chemical and conformational characterization of biomolecules like acidity constants [31,36–38].

The activity of many biological molecules depends on the presence of charged groups. Consequently, the dissociation constant can be a key parameter for understanding and quantifying chemical phenomena or biological activity. Thus, the passage of many drugs into cells and across other membranes is a function of pH in the internal environment and the pK_a of the drug [35].

In this work models describing the effect of pH on retention in LC and CE were compared. The suggested models use the pH value in the acetonitrile–water mixture used as mobile phase, instead of pH value in water and take into account the effect of the activity coefficients. The model has been tested for a series of peptides in acetonitrile–water (7:93, v/v) for LC and in water and acetonitrile–water

(12.5:87.5, v/v) mixtures for CE. The usefulness of the proposed equations is twofold. They permit the determination of the acidity constants in water and in the hydro–organic mobile phases, and also can be used, to establish a general model that relates the elution behaviour of the solute with the significant mobile phase properties: composition, pH and ionic strength [37,39]. The advantages of using LC and CE to determine accurate thermodynamic pK_a values of compounds are numerous: the use of these techniques requires small amounts of sample at low solute concentration and the procedure does not require solute measurement or titrant concentration, like potentiometric techniques, but only retention or migration times. Calculations are independent of solutes impurities, since impurities can be separated from the solutes of interest [40,41]. Moreover, CE permits pK_a determination in aqueous solution without difficulties [41] which is not the case for LC, in which the retention could be unsuitable without the addition of an organic modifier [14].

2. Experimental

2.1. Chemical and reagents

Water with a conductivity lower than 0.05 μS/cm was obtained using a Milli-Q water purification system (Millipore, Molsheim, France); acetonitrile (Merck, Darmstadt, Germany) was LC grade. Trifluoroacetic acid (TFA), phosphoric acid (85%), sodium hydroxide, hydrochloric acid (25%), formic acid (98%), acetic acid (glacial), acetone, potassium bromide and potassium hydrogen phthalate were supplied by Merck. Tris [tris(hydroxymethyl)-aminomethane] was purchased from J.T. Baker (Deventer, The Netherlands). All reagents were analytical grade. The peptides used in this study were purchased from Sigma (St. Louis, MO, USA) and are: citrulline, Gly–Gly, Gly–Gly–Gly, Tyr–Gly–Gly, Gly–Gly–Val, Gly–Gly–Ile, Ala–Leu–Gly and Gly–Gly–Phe. In the case of Ala–Leu–Gly, it was possible to separate two diastereoisomer mixtures named Ala–Leu–Gly (1) and Ala–Leu–Gly (2). Citrulline, Gly–Gly and Gly–Gly–Gly were kept at room temperature and the remainder were stored in a freezer at 0°C when not in use.

2.1.1. Chromatographic analysis

Mobile phases of 7% (v/v) of acetonitrile containing 0.05% TFA were prepared varying the pH of the mobile phase from 2 to 6.5 with 1 *M* NaOH. Stock solutions of the peptides were prepared by dissolving approximately 10 mg of each peptide and diluting to 5 ml; working solutions were prepared by 10-fold dilution of the stock solution. The mixtures of the peptides studied was prepared by 100-fold dilution of the stock solution in the mobile phase.

2.1.2. Electrophoretic analysis

A buffer containing 50 m*M* acetic acid–50 m*M* formic acid was prepared to cover the acidic pH range (2.5–4.5 approximately), adjusting to the appropriate pH with 1 *M* NaOH. For the determination of the electrophoretic mobility of the fully protonated peptides a 20 m*M* phosphate buffer was used [pH 2.11 and pH 2.20 in water and in 12.5% (v/v) MeCN, respectively]. A 50 m*M* Tris solution was the operating buffer in the basic pH range (7–9.5 approximately), adjusting to the appropriate pH with 1 *M* HCl. When working in the 12.5% (v/v) MeCN medium, the background solvent was prepared by mixing water and MeCN in the appropriate amounts to obtain a stock solvent solution. All the buffers and solutions used in the 12.5% (v/v) MeCN medium study were prepared using this stock solvent. 150 μ*M* peptide solutions were separately prepared in water, containing acetone at 3% (v/v) as the electroosmotic flow (EOF) marker [15]. A mixture containing all the peptides at 150 μ*M* was prepared.

All the eluents and mobile phases used for the chromatographic and electrophoretic methods were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication. The samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Instrumental

2.2.1. Chromatographic apparatus

The chromatographic equipment consisted of an ISCO Model 2350 (Lincoln, NE, USA) pump with a 10-μl injection valve and a variable-wavelength V^4 absorbance detector (ISCO) operating at 214 nm. The chromatographic system was controlled by

ChemResearch Chromatographic Data Management System Controller software (ISCO) running on a personal computer. A Merck LiChrospher 100 RP-18 (5 μm) column 250×4 mm I.D. was used at room temperature.

2.2.2. Electrophoretic apparatus

A Beckman P/ACE system 5000 (Beckman Instruments, Fullerton, CA, USA) was used in all the electrophoretic experiments. A 57 cm×75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was inserted in a capillary cartridge, thermostated to 25°C (±0.1°C). Samples were injected hydrodynamically at 0.5 p.s.i. for 4 s (1 p.s.i.=6894.76 Pa). Experiments were conducted under normal polarity, applying a voltage of 25 kV during electrophoretic separations. The detection window was placed at 50 cm from the inlet of the capillary. A photodiode array detector allows monitoring studied peptides at 195 nm. All data were recorded and analyzed by a computer program supplied by Beckman (P/ACE Station 1.0 with Golden System interface).

2.2.3. Capillary treatment

All capillary rinses were performed at high pressure (20 p.s.i.). New capillaries were activated flushing them during 20 min with aqueous 1 *M* NaOH, followed by 15 min with water and 30 min with working buffer. Between days or after a change of buffer, capillary was conditioned rinsing successively for 5 min with water, 5 min with aqueous 0.1 *M* NaOH, 15 min with water and 30 min with buffer. Both activation and conditioning methods, include, after the last flush with buffer, 15 min of 25 kV application. It was empirically demonstrated that this final step accelerates capillary equilibration. Between runs, the capillary is successively rinsed with 2 min of aqueous 0.1 *M* NaOH, 3 min of water and 5 min of buffer, in order to reequilibrate it and thereby minimize hysteresis effects. The capillary was stored overnight filled with working buffer electrolyte.

2.2.4. pH measurements

The electromotive force (e.m.f.) values used to evaluate the pH of the mobile phase were measured with a potentiometer (±0.1 mV) Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 ROSS combination pH electrode (Orion Research, Boston, MA, USA). All solutions were thermostated externally at 25±0.1°C. The electrodes were stabilized in the appropriate acetonitrile–water mixtures before the e.m.f. measurements, which were performed in triplicate to ensure potentiometric system stability.

To calibrate the potentiometric system in hydro–organic media, solutions of reference standards in these media were used. The pH value of these reference solutions, pH_S was previously assigned [23,24], in accordance with IUPAC rules [28,29] and on the basis of the NIST multiprimary standard scale [26].

2.3. Experimental procedures

2.3.1. Chromatographic procedure

In order to study the influence of the eluent pH on the chromatographic separation, the mobile phase was adjusted to different pH values, from 2 to 6.5, with sodium hydroxide at 7% (v/v) of MeCN. This percentage was chosen because it corresponds to the optimum composition of the mobile phase for the separation of the studied peptides. The mobile phase optimization was performed previously [42] using the solvatochromic parameter E_T^N as solvent descriptor and taking into account that log k values and E_T^N solvent parameter correlate linearly [42].

Retention factors were calculated from $k=(t_R-t_0)/t_0$, where t_0 is the retention time of the potassium bromide (hold-up time) which is established for each mobile phase composition and pH studied, and t_R is the retention time of peptides. The flow-rate of the mobile phase was maintained at 1 ml/min. Each retention time was obtained as the average of three replicates and the corresponding relative standard deviations are lower than 2%.

2.3.2. Electrophoretic procedure

In order to study the influence of the eluent pH on the electrophoretic separation, the mobile phase was adjusted to different pH values from 2 to 9.5 in water and in aqueous–MeCN (87.5:12.5, v/v) mixtures. Individual solutions of peptides were injected at each pH until the electrophoretic mobility was constant. m_e values were calculated as the difference between the apparent mobility of each peptide and the

mobility of acetone used as neutral marker [15]. Each electrophoretic mobility was obtained as the average of three replicates. Relative standard deviations lower than 2% for the m_e values were obtained.

2.3.3. pH measurement procedure

The pH was measured in the mixed mobile phase, where the chromatographic and electrophoretic separation takes place, taking into account the reference pH values of primary standard buffer solutions, pH_S, for the standardization of potentiometric sensors in acetonitrile–water mixtures. The knowledge of pH_S values allows one to perform pH measurements in a mixed solvent as easily as in water taking into account the operational definition of pH [21,28]:

$$pH_X = pH_S + \frac{E_S - E_X}{k_g} \quad (1)$$

where E_X and E_S denote the e.m.f. measurements on the sample solution at unknown pH_X and on the standard primary reference solution at known pH_S, respectively, and $k_g = (\ln 10)RT/F$. In this study we used potassium hydrogenphthalate (0.05 mol/kg) or phosphate (0.03043 mol/kg disodium hydrogenphosphate, 0.008695 mol/kg potassium dihydrogenphosphate) as primary standard buffer reference solution in the acetonitrile–water mixtures studied [27].

The molar activity coefficient, y, was calculated using the classical Debye–Hückel expression:

$$\log y = \frac{-AI^{1/2}}{1 + a_0 BI^{1/2}} \quad (2)$$

where A and B are the Debye–Hückel constants and a_0 is the ion size parameter in the solvent mixture [29,40]. The ionic strength, I, of the mobile phases used can be easily calculated over the entire range of pH explored [37].

3. Results and discussion

The retention factor values, k, in MeCN–water (7:93, v/v) mixtures, for the series of peptides studied at every mobile phase pH considered, are shown in Fig. 1 [42]. The peptides studied here usually have two relevant functional groups. pK_a values in the acid range can be associated with

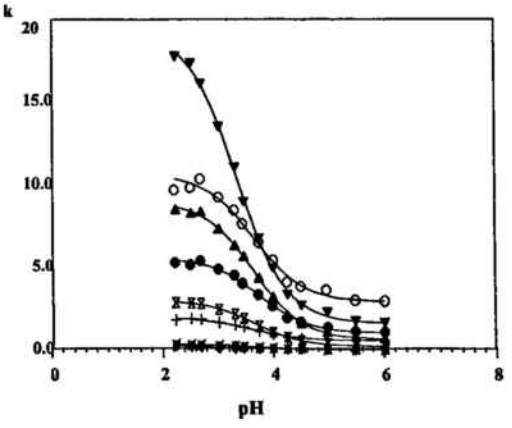

Fig. 1. Plots of retention factors, k, versus pH for Gly–Gly–Phe (▼), Ala–Leu–Gly (2) (○), Gly–Gly–Ile (▲), Ala–Leu–Gly (1) (●), Gly–Gly–Val (×), Tyr–Gly–Gly (+), Gly–Gly–Gly (x), Gly–Gly (■), citrulline (♦) in MeCN–water (7:93, v/v). Symbols stand for the experimental data and solid lines indicate the best non-linear regression fits.

carboxylic acid function and pK_a values in the basic range can be assigned to the protonated amino groups dissociation [43]. Thus, peptides can be considered as typical zwitterion forming compounds. However, the applied octadecylsilica (ODS) stationary phase may only be used in the pH range 2–7, so it was no possible to study the retention of peptides as typical ampholytes, because correlation between k values and the pH of the mobile phase cannot be obtained over the entire range of pH. Thus, from a chromatographic point of view, with the widely used ODS stationary bonded phase, only the protolytic equilibria corresponding to pK_a values in the acid range are relevant. In this way, the pK_a value, corresponding to the dissociation of the terminal carboxylic group has been considered. The retention of peptides is high at low pH values, Fig. 1, where the peptide exists as a single charged cation. When pH increases, the k value decreases and levels off at isoelectric point pH and stays constant, Fig. 1.

Although the effect of solute ionization on retention is known, the theoretical interpretation of this phenomenon is hampered by the lack of a rigorous treatment of protolytic equilibria in hydro–organic mixtures. In doing this, pK_a values, the pH, the ionic

strength, and the mean ionic activity coefficient, must be determined at each mobile phase composition studied after mixing the aqueous solutions with the organic modifier.

An equation which describes retention factor as a function of pH of the mobile phase, considering the activity coefficient, and accounts for every peptide equilibria that influences the retention in ODS columns, can be derived [35] taking into account that the ionization of peptides in the mobile phase takes place according to the following equilibrium:

$$H_2Z^+ \Leftrightarrow HZ + H^+ \quad K_{a1}$$

where H_2Z^+ is the protonated form and HZ the zwitterionic form of peptides.

Equations that relate the retention of a compound in LC columns with the pH of the eluent, consider that the observed retention factor, k, is a weighted average of the k of the ionic and neutral forms of the solute [20,35,37], according to the molar fractions of these forms in the mobile phase. The overall observed, k, for peptides can be given as:

$$k = x_{H_2Z^+} k_{H_2Z^+} + x_{HZ} k_{HZ} \tag{3}$$

where $k_{H_2Z^+}$ and k_{HZ} are the retention factors and $x_{H_2Z^+}$ and x_{HZ} the molar fraction of the protonated and zwitterionic forms of peptides, respectively, that can be written as:

$$x^+_{H_2Z} = \frac{a_{H^+}}{a_{H^+} + K_{a1} y} \tag{4}$$

$$x_{HZ} = \frac{K_{a1} y}{a_{H^+} + K_{a1} y} \tag{5}$$

By replacing Eqs. (4) and (5) in Eq. (3):

$$k = \frac{k^+_{H_2Z} a_{H^+} + k_{HZ} K_{a1} y}{a_{H^+} + K_{a1} y} \tag{6}$$

or

$$k = \frac{k_{H_2Z^+} \frac{a_{H^+}}{K_{a1} y} + k_{HZ}}{\frac{a_{H^+}}{K_{a1} y} + 1} \tag{7}$$

The pK_a values of the substances studied were determined from the experimental k values, Fig. 1, the pH data and the calculated y values, by a non-linear least-squares fit of the data to Eq. (7) and are shown in Table 1. The activity coefficient, y, is obtained according to IUPAC rules [28,29] from the Debye–Hückel equation. In Fig. 1, symbols stand for the experimental data and solid lines indicate the best non-linear regression fits for each peptide, using Eq. (7). The agreement between both values is good, with correlation coefficients of 0.999.

In order to study the influence of pH on the electrophoretic behaviour of substances, we can relate pH, pK_a and electrophoretic mobility. The established relationships are based on the principle that a solute has its maximum electrophoretic mobility when it is fully ionized, has no electrophoretic mobility in its neutral form, and has an intermediate mobility in the pH region surrounding its pK_a [15,38,41]. Thus, the electrophoretic mobility, m_e, of a substance is a function of the mobility and the molar fraction, x_i, of its species. In the case of the studied peptide substances, the first dissociation constant is related to the concentration of protonated $(H_2Z)^+$ and undissociated (HZ) species [37,44]. Thus, effective mobility can be expressed as follows:

$$m_e = x_{H_2Z^+} m_{H_2Z^+} + x_{HZ} m_{HZ} \tag{8}$$

where the term corresponding to the HZ species is considered nil, because the HZ species has no charge and migrates with the electroosmotic flow. Assuming that the mobility of the fully protonated species is $m_{H_2Z^+} = m_a$ and replacing the molar fraction by its expression for ampholytes, the electrophoretic mobility, m_e, can therefore be obtained:

$$m_e = \frac{a_{H^+} m_a}{a_{H^+} + K_{a1} y} \tag{9}$$

Expressions similar to Eq. (9) can be deduced in basic media [44], that permit electrophoretic determination of the second dissociation constant of the studied peptides. Thus, considering that the electrophoretic mobility is maximum for Z^- species, the expression for mobility in the pH region surrounding the pK_{a2} is $m_e = x_{Z^-} m_b$, where $m_{Z^-} = m_b$ is the electrophoretic mobility of the fully deprotonated species, Z^-, whose sign is opposite to that of the mobility of the fully protonated form, H_2Z^+. Replac-

Table 1
pK_a values determined from potentiometric, chromatographic and electrophoretic method for the peptides studied in water and hydro–organic mixtures

Peptide	pK_a	0% MeCN		7% MeCN		12.5% MeCN	
		CE method	Bibliography	LC method	Potentiometric method	CE method	Potentiometric method
Gly–Gly	1	3.08 (0.04)	3.14	3.35 (0.06)	3.18 (0.01)	3.33 (0.02)	3.33
	2		8.04			8.32 (0.02)	8.30
Gly–Gly–Ile	1	3.26 (0.07)		3.59 (0.02)	3.54 (0.03)	3.53 (0.02)	3.55
	2		8.09			8.11 (0.01)	8.09
Gly–Gly–Gly	1	3.22 (0.02)	3.30	3.57 (0.06)	3.50 (0.02)	3.41 (0.02)	3.46
	2		7.96			8.08 (0.01)	8.09
Tyr–Gly–Gly	1	3.18 (0.02)		3.54 (0.05)	3.45 (0.01)	3.36 (0.02)	3.46
	2					7.41 (0.04)	7.45
Gly–Gly–Val	1	3.23 (0.02)		3.59 (0.02)	3.47 (0.02)	3.50 (0.02)	3.54
	2		8.12			8.15 (0.02)	8.08
Ala–Leu–Gly 1	1	3.34 (0.01)		3.71 (0.04)	3.57 (0.02)	3.56 (0.03)	3.62
	2					8.13 (0.02)	8.12
Ala–Leu–Gly 2	1	3.27 (0.01)		3.61 (0.05)	3.57 (0.02)	3.46 (0.03)	3.62
	2					8.30 (0.02)	8.12
Gly–Gly–Phe	1	3.06 (0.01)		3.30 (0.02)	3.21 (0.01)	3.33 (0.05)	3.33
	2		8.04			8.01 (0.02)	8.02
Citrulline	1	2.30 (0.04)	2.40	2.37 (0.11)	2.57 (0.01)	2.60 (0.06)	2.39
	2		9.69			9.44 (0.03)	9.58

ing x_{Z^-} by its expression for ampholytes, the following equation for mobility in basic media is obtained:

$$m_e = \frac{K_{a2} m_b}{K_{a2} + a_{H^+} y} \quad (10)$$

The electrophoretic migration behaviour of these peptides can be described by Eqs. (9) and (10), which at a given pH, relate electrophoretic mobilities to the corresponding mobilities of the fully protonated or deprotonated species, m_a or m_b, respectively, and the dissociation constants, pK_{a1} or pK_{a2}, taking into account the activity coefficients effect. The electrophoretic mobilities for all the studied peptides obtained in water and in MeCN–water (12.5:87.5, v/v) mixtures were plotted against the buffer pH in Fig. 2A and B, respectively. Symbols stand for the experimental data and solid lines indicate the best non-linear regression fits for each peptide, using Eqs. (9) and (10). The agreement between both values is good, with correlation coefficients of 0.999. From this regression analysis, dissociation constants for each peptide were calculated in addition to m_a and m_b values.

In this way, models described by Eqs. (7), (9) and (10), that were established using data pairs pH–k or pH–m_e, respectively, and ionic strength over the whole pH range in water and MeCN–water mixtures, allow pK_a determinations. pK_a values determined for the studied peptides using the non-linear regression method, Eqs. (7), (9) and (10), are shown in Table 1, with their respective standard deviations. Moreover, Table 1 summarizes the pK_a values previously determined by potentiometric techniques [43].

In general, the values obtained by LC methodologies at 7% (v/v) MeCN are slightly higher than the ones determined by potentiometric techniques. This fact can be explained taking into account the non-

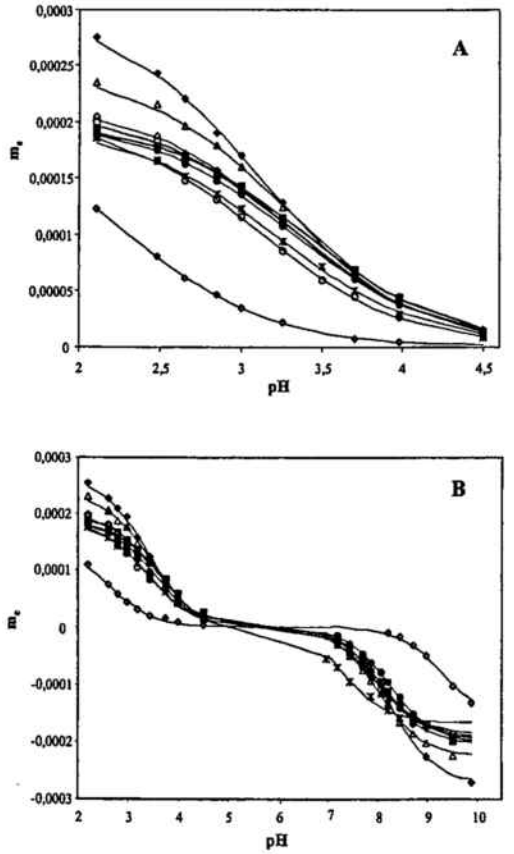

Fig. 2. Plots of electrophoretic mobilities, m_e, versus pH for Gly–Gly (♦), Gly–Gly–Gly (△), Gly–Gly–Val (◇), Gly–Gly–Ile (□), Ala–Leu–Gly (1) (■), Ala–Leu–Gly (2) (●), Gly–Gly–Phe (○), Tyr–Gly–Gly (✱) and citrulline (◆). Symbols stand for the experimental data and solid lines indicate the best non-linear regression fits.

hydrophobic interactions of the compound studied with the residual silanol groups that remain intact after chemical modification of the silica surface in the used column. These interactions increase the retention factor of peptides and, therefore, the obtained pK_a values are higher than potentiometric ones.

In contrast, values obtained in water by CE are similar to potentiometric values. Only slight differences in some peptides can be observed. These differences could also be explained by the presence of residual silanols groups in the inner surface of the capillary. The specific interaction between positively charged peptides and the fused-silica capillary wall diminishes the electrophoretic mobility of peptides, resulting in slightly lower pK_a values than the potentiometric ones. Citrulline has an exceptional behaviour due to its stronger acid character.

pK_{a2} values determined by CE in 12.5% MeCN media, are very similar to potentiometric ones. In basic media, amino group of peptides becomes deprotonated diminishing interactions with the capillary wall. Differences between electrophoretic and potentiometric values are positive as well as negative, therefore, they are probably reflective of the random error of the procedure, not of specific interactions.

Finally, the slightly differences observed between pK values obtained from LC, CE and potentiometric methods, demonstrate the suitability of these techniques for pK_a determination. In general, as can be seen in Table 1, the acid dissociation constants for deprotonation of carboxylic acid and protonated amino groups decrease as the solvent become enriched in the organic component. These variations of pK_a values are lower than expected because of the preferential solvation of water in MeCN–water mixtures [45,46] and have been interpreted in previous works.

Moreover, note that pK_a of both Ala–Leu–Gly diastereoisomers can be differentiated by LC and CE. This makes both techniques specially attractive for pK_a determinations of diastereoisomers, because potentiometry is not able to differentiate between them [43].

On the other hand, Eq. (7) can be written in a linearized form:

$$\boxed{k\left(\frac{a_{H^+}}{K_{a1}y}+1\right)} = k_{H_2Z^+}\boxed{\frac{a_{H^+}}{K_{a1}y}} + k_{HZ} \qquad (11)$$

When pK_a values of substance are known, plots of the terms in the boxes can be used in order to optimize the pH of the mobile phase. Fig. 3 shows

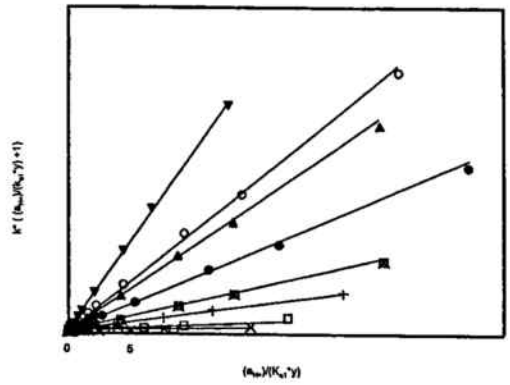

Fig. 3. Plots of experimental (points) and predicted with linear models (lines) $k[(a_{H+})/(K_{a1}y)+1]$ versus $(a_{H+})/(K_{a1}y)$ in MeCN–water (7:93, v/v). Symbols: (▼) Gly–Gly–Phe, (○) Ala–Leu–Gly (2), (▲) Gly–Gly–Ile, (●) Ala–Leu–Gly (1), (×) Gly–Gly–Val, (+) Tyr–Gly–Gly, (□) Gly–Gly–Gly, (x) Gly–Gly and (♦) citrulline.

these plots for the peptides studied. From these it is also possible to obtain k values of the species of the peptides from the intercept and slope. In accordance with Eq. (11), all present good linearities. Once the linearity has been verified only two experimental measurements of k for each compound considered at two different pH values are enough for predicting k values at any pH of the mobile phase, and then, for optimizing the chromatographic separation.

In the same way, Eqs. (9) and 10 obtained by the electrophoretic method, also can be written in a linearized form, respectively:

$$\boxed{\frac{1}{m_e}} = \frac{1}{m_a} + \frac{1}{m_a} \cdot \boxed{\frac{K_{a1}y}{a_{H^+}}} \tag{12}$$

and

$$\boxed{\frac{1}{m_e}} = \frac{1}{m_b} + \boxed{\frac{a_{H^+}y}{K_{a2}}} \cdot \frac{1}{m_b} \tag{13}$$

When pK_a values of substance are known, plots of the boxed terms can be used, in order to optimize the pH of the electrolyte solutions. Fig. 4 shows these

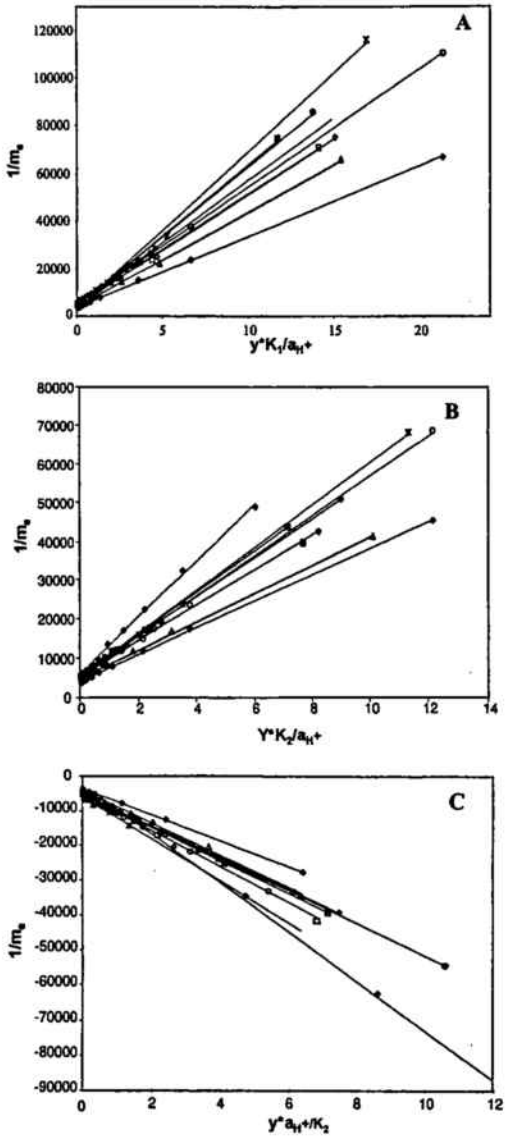

Fig. 4. Plots of experimental (points) and predicted with linear models (lines)$1/m_e$ values versus (A) $K_{a1}y/a_H^+$ in water, (B) $K_{a1}y/a_H^+$ in MeCN–water (12.5:87.5, v/v) and (C) $K_{a1}y/a_H^+$ in MeCN–water (12.5:87.5, v/v) for Gly–Gly (♦), Gly–Gly–Gly (△), Gly–Gly–Val (◇), Gly–Gly–Ile (□), Ala–Leu–Gly 1 (■), Ala–Leu–Gly 2 (●), Gly–Gly–Phe (○), Tyr–Gly–Gly (⋇), citrulline (◆).

plots for each peptide in water and in 12.5% (v/v) MeCN in acidic (Fig. 4A and B) and basic conditions (Fig. 4C). In accordance with Eqs. (12) and (13), all present good linearities. As in the LC analysis, once the linearity has been verified, only two experimental measurements of m_e–pH for each compound are sufficient for predicting their electrophoretic behaviour and for optimizing their separation.

The k and m_e predicted values have been then used to calculate selectivity and resolution for solute adjacent pairs in the usual way [37,38]. In Figs. 5 and 6 the lines indicate theoretical values obtained from two measurements by compound using Eqs. (11), (12) and (13). The points of Fig. 5 are α values obtained from experimental data. Thus it appears clear that two measurements of retention by compound are sufficient to predict accurately the optimum pH of the mobile phase if pK_a values are known. From Figs. 5 and 6, optimal separation conditions can be obtained Fig. 5 indicates that good chromatographic separation can be obtained for the peptides considered in the series of peptides studied

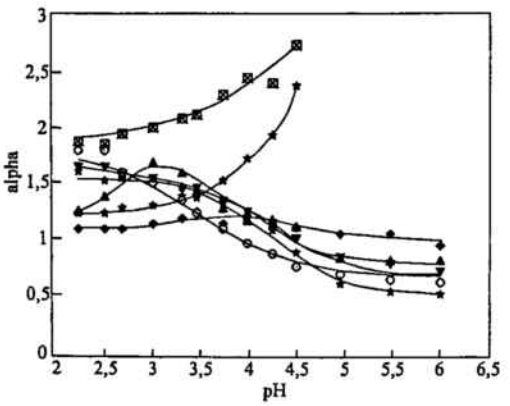

Fig. 5. Plots of experimental (points) and predicted with non-linear LC model (lines) selectivities for a mixture of the studied peptides versus pH, in MeCN–water (7:93, v/v). (⊠) Gly–Gly–Gly/Gly–Gly, (♦) Gly–Gly–Val/Tyr–Gly–Gly, (★) Ala–Leu–Gly 1/Gly–Gly–Val, (▼) Gly–Gly–Ile/Ala–Leu–Gly 1, (★) Ala–Leu–Gly 2/Ala–Leu–Gly 1 and (O) Gly–Gly–Phe/Ala–Leu–Gly 2.

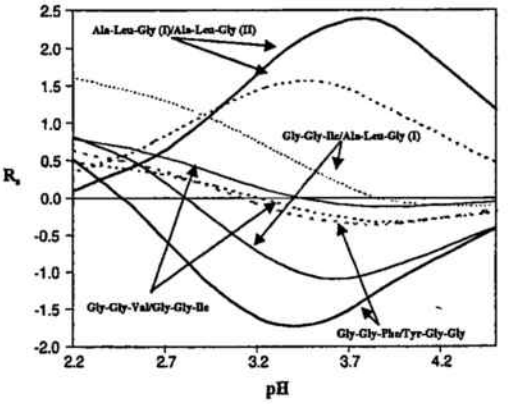

Fig. 6. Plots of predicted resolutions for some pairs of peptides versus pH, in water (solid lines) and in MeCN–water (12.5:87.5, v/v) (dashed lines).

in acetonitrile–water (7:93, v/v) mixtures [41] and at a pH value of the hydro–organic mixtures between 2.5 and 3. Fig. 7A shows a chromatogram of the separation of the nine substances studied at pH 2.8

According to Fig. 6 optimum electrophoretic separation of all peptides presents in the mixture are achieved in the pH range between 2.5 and 2.7 in the aqueous and aqueous–organic media studied. In general, in electrophoretic separations MeCN addition results in a loss of resolution. Fig. 7B and C show electropherograms of the separation of the nine substances studied at pH 2.6 in water and in acetonitrile–water (12.5:87.5, v/v) mixtures.

The analysis time obtained for two methodologies is similar and around 25 min. Likewise, the optimum pH for the best separation of the series of peptide compounds studied is similar (pH 2.8 in the chromatographic separation and pH 2.6 in the electrophoretic separation) by both techniques. The selectivities differ, as was expected, because the retention behaviour is based on different mechanisms and in consequence the elution order for the studied compounds is not the same. In this way, citrulline was the first substance eluted by LC and the last substance eluted by CE.

In conclusion, the suitability of the chromato-

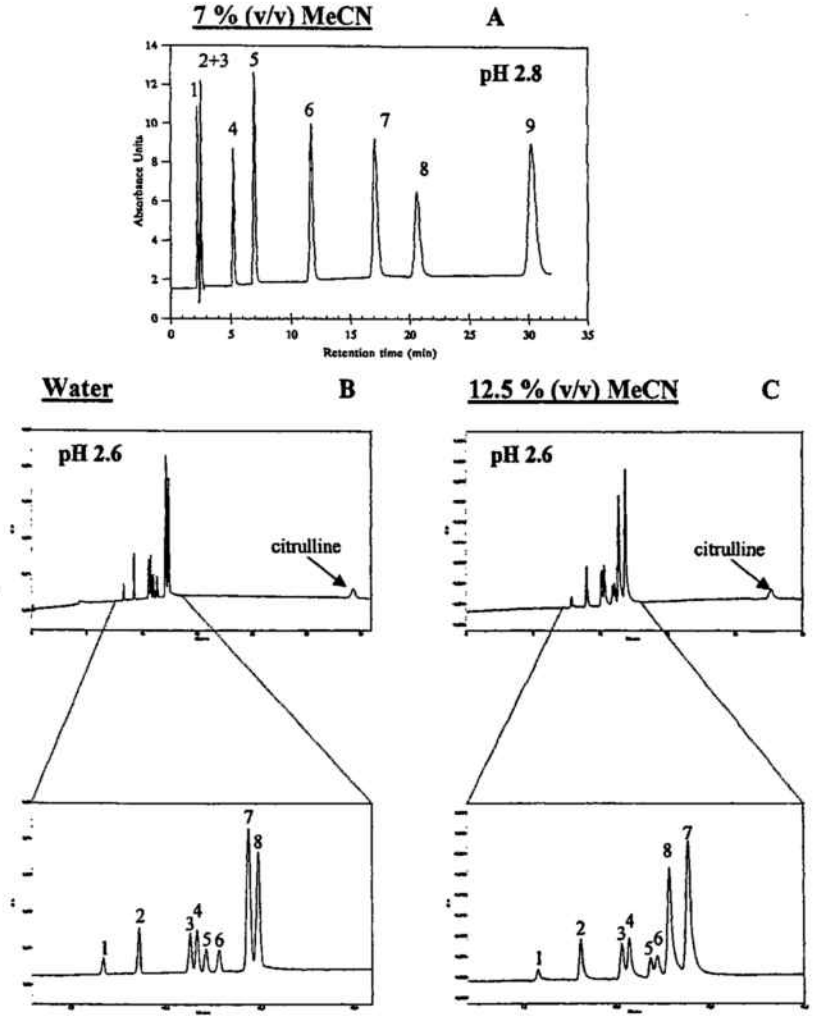

Fig. 7. Chromatogram of a mixture of the studied peptides with a mobile phase of 0.05% TFA, (A) MeCN–water (7:93, v/v). Electropherograms at 25 kV of a mixture of the studied peptides in 50 m*M* formic acid–50 m*M* acetic acid buffer (B), water (C) MeCN–water (12.5:87.5, v/v). (1) Citrulline, (2) Gly–Gly, (3) Gly–Gly–Gly, (4) Gly–Gly–Val, (5) Tyr–Gly–Gly, (6) Ala–Leu–Gly 1, (7) Gly–Gly–Ile, (8) Ala–Leu–Gly 2 and (9) Gly–Gly–Phe.

graphic and electrophoretic studied models to the determination of dissociation constants and to predict chromatographic and electrophoretic behaviour of peptides from a limited number of experimental data has been shown. In a parallel way, separation between solutes in a complex mixture can be easily

predicted, making a simple and rapid pH selection to achieve optimum separations.

Acknowledgements

Financial support of this project by DGICYT (Project PB98-1174) is gratefully acknowledged.

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Electrophoresis 2001, *22*, 4333–4340

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Migration behavior of therapeutic peptide hormones: Prediction of optimal separation by capillary electrophoresis

A general equation that relates electrophoretic mobility of polyprotic peptide substances and pH of the running electrolytes is established, taking into account the species in solution and the activity coefficients. Modelling electrophoretic mobility as a function of pH can be simultaneously used for determination of ionization constants and selection of the optimum pH for separation of mixtures of the modelled compounds. The proposed relationships allow an important reduction of the experimental data needed for development of new separation methods. The accuracy of the proposed equations is verified by modelling the migration behavior of a heterogeneous series of polyprotic amphoteric peptide hormones. By calculating the values of predicted resolutions, selection of the optimum pH to perform separation of their mixtures becomes a rapid and simple process.

Keywords: Peptide hormones / Capillary electrophoresis / Electrophoretic migration prediction / Ionization constants / Resolution

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1 Introduction

Capillary electrophoresis (CE) has demonstrated its versatility in the analysis of biomolecules, and has become a major analytical tool in biotechnological research [1, 2]. Peptide and protein separations have been extensively explored, especially when high efficiency and resolution are desirable [3–6]. Novel applications in this field are continuously described, and many attempts have been made to solve the main drawbacks of the technique in its way to maturity [1, 6–9]. In the near future, the combined advances of biotechnology and proteomic research are expected to boost the discovery and synthesis of new biologically active peptides [10], which will necessitate the development of new separation and characterization technologies. At present, CE is the premier choice for peptide and protein separation and identification [1–6], and improvement of the emerging hyphenated techniques [11, 12] may also increase its value also in other nonexploited fields.

The development of rapid, efficient and selective separation methods requires optimization of separation conditions. Separation procedures in CE are still being developed in time consuming and nonsystematic procedures. Moreover, in most cases, the final experimental conditions are not optimum. Fast optimization of the separation conditions is essential in the purification and characterization of newly synthesized products. Modelling electrophoretic behavior can be used to predict electrophoretic separations [13–19], avoiding long and tedious separation optimizations performed by trial and error. Furthermore, these migration models can be used to perform physicochemical and conformational characterization of biomolecules [13, 17–26].

Several semi-empirical approaches have been proposed to relate electrophoretic mobility to structural parameters of proteins and peptides, but their application is limited by the assumptions made in their development [27–30]. Thus, correct values of ionization constants must be known in order to calculate the charge of the molecules, although only a few of them are reported [28, 31]. Furthermore, there is no general rule for *a priori* selection of the appropriate relationship for each type of compound [28]. On the other hand, in the electrophoretic separation of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual solute [32, 33]. Accurate quantitative relationships between electrophoretic mobilities and pH, can be very useful [20–22, 34]. These models allow determination of ionization constants [17–26] and selection of the optimum pH for the separation of complex mixtures of the modelled compounds [13, 14, 16–19], by resolution calculations. Several authors have also used combinations of these two approaches to model migration behavior and simultaneously determine ionization constants and other physicochemical parameters of ionizable compounds [28, 35].

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Table 1. Structures of the studied peptide hormones (polyprotic formulation)[a)]

Peptide	Structure	Ionizable groups		Formula	
		Acidic (A)	Basic (B)	$H_nAB_z^z$	H_nX^z
Oxytocin	**Cys**-**Tyr**-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2[b)]	1 phenol (-Tyr-)	1 amino group (Cys-)	$HABH_2^{2+}$	H_3X^{2+}
Eledoisin	Pyr-Pro-Ser-**Lys**-**Asp**-Ala-Phe-Ile-Gly-Leu-Met-NH_2	1 carboxylic acid (-Asp-)	1 amino group (-Lys-)	$HABH^+$	H_2X^+
Bradykinin	**Arg**-Pro-Pro-Gly-Phe-Ser-Pro-Phe-**Arg**	1 carboxylic acid (-Arg)	1 amino group (Arg-) 2 guanidine groups (Arg-/-Arg)	$HABH_3^{3+}$	H_4X^{3+}
Met-enkephalin	**Tyr**-Gly-Gly-Phe-**Met**	1 carboxylic acid (-Met) 1 phenol (Tyr-)	1 amino group (Tyr-)	H_2ABH^+	H_3X^+
Leu-enkephalin	**Tyr**-Gly-Gly-Phe-**Leu**	1 carboxylic acid (-Leu) 1 phenol (Tyr-)	1 amino group (Tyr-)	H_2ABH^+	H_3X^+
Triptorelin	Pyr-Pro-**His**-Trp-Ser-**Tyr**-D-Trp-Leu-**Arg**-Pro-Gly-NH_2[b)]	1 phenol (-Tyr-)	1 imidazole group (-His-) 1 guanidine group (Arg)	$HABH_3^{3+}$	H_4X^{3+}
Buserelin	Pyr-Pro-**His**-Trp-Ser-**Tyr**-D-Ser (^{t}Bu)-Leu-**Arg**-Pro-NHC_2H_5[b)]	1 phenol (-Tyr-)	1 imidazol group (-His-) 1 guanidine group (Arg)	$HABH_3^{3+}$	H_4X^{3+}

a) Ionizable groups are marked with bold letters.
b) One extra basic group is considered in addition to marked groups. Explanation is given in the text.

In this work, general equations relating electrophoretic mobility, pH, pK_a and activity coefficients are presented for polyprotic peptide substances. Validation of the studied model is performed for a series of polyprotic amphoteric peptide hormones with therapeutic interest. Selectivity and resolution can be easily predicted [13, 14], hence optimum separation conditions for the mixture are systematically selected, on the basis of a few experimental data. Simultaneously, pK_a is determined by taking into account activity corrections in the model development [19, 23, 24].

2 Materials and methods

2.1 Chemical and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetonitrile, phosphoric acid (85%), sodium hydroxide, hydrogen chloride acid (25%), formic acid (98%), acetic acid (glacial), diethylmalonic acid, boric acid and acetone were supplied by Merck (Darmstadt, Germany). Tris was purchased from J. T. Baker (Deventer, Holland). Water with a conductivity lower than 0.05 μScm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Oxytocin, eledoisin, bradykinin, Met-enkephalin, Leu-enkephalin, triptorelin and buserelin were purchased from Sigma (St. Louis, MO, USA). Structures are shown in Table 1.

2.2 Electrolyte solutions and samples

Buffers covering the pH range 2–12 were prepared at the following concentrations and adjusted to the appropriate pH values using 1 M NaOH or 1 M HCl:20 mM H_3PO_4 (pH 2), 50 mM acetic acid:50 mM formic acid (pH 2.5–5), 20 mM diethylmalonic acid (pH 5.5–6.5), 50 mM Tris (pH 7–9), 50 mM H_3BO_3 (pH 9.5–10.5) and 10 mM H_3PO_4 (pH 11–12). Peptide hormones solutions (250 ppm) were separately prepared in water, containing 3% v/v acetone as the electroosmotic flow (EOF) marker. A mixture containing all the peptides at 250 ppm was prepared. Samples and running electrolytes were passed through a 0.45 µm nylon filter (MSI).

2.3 Instrumental parameters

A Beckman P/ACE system 5000 (Beckman Instruments, Fullerton, CA, USA) was used in the electrophoretic experiments. A 57 cm × 75 µm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was inserted in a capillary cartridge and thermostated to 25°C (± 0.1°C). Samples were injected hydrodynamically at 0.5 psi for 3 s (1 psi = 6894.76 Pa). Experiments were conducted under normal polarity, applying a voltage of 25 kV during electrophoretic separations. The detection window was placed at 50 cm from the inlet of the capillary. A photodiode array detector was used. The electropherograms shown correspond to detection at 195 nm. All data were recorded and

analyzed by a computer program supplied by Beckman (P/ACE Station 1.0 with Golden System interface). pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

2.4 Capillary treatment

All capillary rinses were performed at high pressure (20 psi). New capillaries were activated by flushing them for 20 min with aqueous 1 M NaOH, followed by 15 min with water and 30 min with working buffer. Between days or after a change of buffer, the capillary was conditioned by rinsing successively for 5 min with water, 5 min with aqueous 0.1 M NaOH, 10 min with water and 20 min with buffer. Both activation and conditioning methods include, after the last flush with buffer, 15 min of 25 kV application. It was empirically demonstrated that this final step accelerates capillary equilibration. Between runs capillary is successively rinsed 2 min with aqueous 0.1 M NaOH, 2 min with water and 2 min with buffer, in order to reequilibrate it and thereby minimize hysteresis effects. Capillary was stored overnight filled with working buffer electrolyte.

2.5 Procedures

In order to study the influence of pH upon migration behavior of ionizable substances, relationships between pH, pK_a and electrophoretic mobilities can be adequately established [20–22]. Polyprotic compounds undergo successive ionization equilibria when the pH of the running electrolyte is gradually changed. The dissociation equilibria for a generic fully protonated polyprotic species H_nX^z, are:

$$\begin{aligned} &H_nX^z \Leftrightarrow H_{n-1}X^{z-1} + H^+ && K_1 \\ &\vdots \\ &H_{n-(i-1)}X^{z-(i-1)} \Leftrightarrow H_{n-i}X^{z-i} + H^+ && K_i \\ &\vdots \\ &H_{n-(n-1)}X^{z-(n-1)} \Leftrightarrow X^{z-n} + H^+ && K_n \end{aligned} \quad (1)$$

where n is the total number of ionogenic groups, z the maximum positive charge, given by the protonated basic groups, and K_i is the dissociation equilibrium constant of the i^{th} dissociation step, that is given by the expression:

$$K_i = \frac{\left[H_{n-i}X^{z-i}\right]y^{z-i}a_{H^+}}{\left[H_{n-(i-1)}X^{z-(i-1)}\right]y^{z-(i-1)}} \quad (2)$$

where a_{H^+} is the activity of protons. The effect of ionic strength (I) upon dissociation constants has been taken into account, considering the activity coefficients of the solutes, y. The activity coefficients are obtained, according to IUPAC rules [36], from the Debye-Hückel equation, $\log y = -z^2A\sqrt{I}/\left(1 + a_0B\sqrt{I}\right)$, where A and B are the Debye-Hückel constants, a_o is the ion size parameter in the solvent mixture and I is the ionic strength. Deviations for other ions than univalent, due to ion association, have not been considered. Apparent dissociation constants can also be defined as

$$K_i' = \frac{y^{z-(i-1)}}{y^{z-i}}K_i \quad (3)$$

The effective electrophoretic mobility, m_e, of a polyprotic compound, H_nX^z, coexisting in the form of various species at a given pH, is a function of the mobility and the molar fraction, x_i, of the individual species [19–24]:

$$m_e = \sum_{i=0}^{n} x_{H_{n-i}X^{z-i}} m_{H_{n-i}X^{z-i}} \quad (4)$$

Replacing the molar fraction by its expression,

$$m_e = \sum_{i=0}^{n} \frac{\left[H_{n-i}X^{z-i}\right]}{\sum_{i=0}^{n}\left[H_{n-i}X^{z-i}\right]} m_{H_{n-i}X^{z-i}} \quad (5)$$

Eq. (5) can be rewritten as a function of dissociation constants, dividing numerator and denominator by an appropriate concentration $[H_{n-r}X^{z-r}]$, where $0<r<n$, that is, $r = 0$ for the fully protonated form, $r = n$ for the fully deprotonated species and $r = z$ if we refer to the zwitterionic form:

$$m_e \frac{\sum_{i=0}^{r-1} \frac{a_{H^+}^{i-r}}{\prod_{j=i+1}^{r} K_j'} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} \frac{a_{H^+}^{i-r}}{\prod_{j=i+1}^{r} K_j'} + 1 + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}}} \quad (6)$$

Furthermore, considering that and that $a_{H^+}^n = 10^{-npH}$ and that $K_i' = 10^{-pK_i'}$, Eq. (6) can be rearranged more conveniently as a function of pH to give:

$$m_e = \frac{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j'\right]} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j'\right]} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j'\right]} + 1 + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j'\right]}} \quad (7)$$

Equation (5) can be divided by the concentration of any species involved in the equilibria at the pH range studied, resulting in different equations. In any case, the solution of the model is always unique. However, when the zwitterionic form is present in the pH range considered, it is advantageous to take $r = z$ because the resulting zwitterionic species $H_{n-z}X$, has no net charge, and hence its mobility is assumed to be nil [20–24]. In this way, further simplifications regarding Eqs. (6) and (7) can be made.

Equations (6) and (7) are general expressions that can predict electrophoretic mobilities of ionogenic substances as a function of pH when several data pairs of m_e-pH are available. In addition, the model simultaneously allows determination of dissociation constants by considering activity corrections. To obtain these data pairs, individual solutions of peptide hormones were first injected at each pH until the electrophoretic mobility was constant. Buffers indicated in Section 2.2 were run in sequence from low to high pH. The m_e values were calculated as the difference between the apparent mobility of each peptide, m_{app}, and the mobility of acetone used as neutral marker, m_{EOF} [32, 33]: $m_e = m_{app} - m_{EOF} = L_C L_D / V(1/t_{app} - 1/t_{EOF})$, where L_D is the distance from the injection point to the detector, L_C is the capillary length, and t_{app} and t_{EOF} are the migration time of the peptide hormone and the neutral marker, respectively. Each electrophoretic mobility was obtained as the average of three replicates. In order to calculate the pK_i' of the studied substance, data pairs of m_e-pH where fitted to Eq. (7) using nonlinear regression analysis. Thus, pK_i values can be easily determined by using Eq. 3 and the Debye-Hückel expression

$$pK_i = pK_i' + \log\frac{y^{z-(i-1)}}{y^{z-i}} \tag{8}$$

Peptides are polyprotic amphoteric compounds with a variable number of acidic (*C*-terminus, tyrosine, cysteine, aspartic and glutamic acid) and basic groups (*N*-terminus, histidine, lysine, arginine). In this work, Eq. (7) is applied to study the electrophoretic behavior of some peptide hormones. The general formula and ionizable groups present in each peptide structure [37] are shown in Table 1. The generic formula that represents fully protonated amphoteric compounds H_nX^z can also be written in the more usual form as $H_aABH_z^z$, where *a* is the number of acidic groups and *z* is the number of basic groups. Therefore, the total number of ionizable groups *n*, $n = a+z$, is easily obtained and the formula H_nX^z derived. Thus, particular equations can be obtained from general Eq. (7) in each case, taking into account the relevant dissociation constants over the pH range studied. As an example, eledoisin is a diprotic compound, H_2X^+, with an acidic and a basic group, $HABH^+$. Its electrophoretic mobility can be expressed according to Eq. (4) as:

$$m_e = x_{H_2X^+} m_{H_2X^+} + x_{HX} m_{HX} + x_{X^-} m_{X^-} \tag{9}$$

where the term corresponding to the uncharged zwitterionic species (HX) can be considered nil. Equation (9) can be transfromed to Eq. (10) by substituting the molar fractions by their expressions and taking into account the ionization constants (Eq. 2) and their relation with apparent ionization constants (Eq. 3),

$$m_e = \frac{\dfrac{a_{H^+}}{K_1'} m_{H_2X^+} + \dfrac{K_2'}{a_{H^+}} m_{X^-}}{\dfrac{a_{H^+}}{K_1'} + 1 + \dfrac{K_2'}{a_{H^+}}} \tag{10}$$

Equation (10) is the particular case of the general Eq. (6) for $n = 2$ and $z = 1$. Therefore, it can be rearranged as

$$m_e = \frac{a_{H^+}^2 m_{H_2X^+} + K_1' K_2' m_{X^-}}{a_{H^+}^2 + K_1' a_{H^+} + K_1' K_2'} \tag{11}$$

Equation (11) was previously applied for modelling the migration behavior of quinolones, that are also amphoteric compounds [23, 24], and is also given as a function of pH and pK_i' in Table 2. In a previous work [19], a series

Table 2. Electrophoretic models for the studied peptide hormones

Peptide	H_nX^z	Equation (7)
Eledoisin	H_2X^+	$m_e = \dfrac{10^{-2pH} m_{H_2X^+} + 10^{-(pK_1' + pK_2')} m_{X^-}}{10^{-2pH} + 10^{-(pK_1' + pH)} + 10^{-(pK_1' + pK_2')}}$
Oxytocin	H_3X^{2+}	$m_e = \dfrac{10^{(pK_1' + pK_2' - 2pH)} m_{H_4X^{2+}} + 10^{(pK_2' - pH)} m_{H_3X^+} + 10^{(pH - pK_3')} m_{HX^-}}{10^{(pK_1' + pK_2' - 2pH)} + 10^{(pK_2' - pH)} + 10^{(pH - pK_3')}}$
Bradykinin	H_4X^{3+}	$m_e = \dfrac{10^{(pK_1' - pH)} m_{H_4X^{3+}} + m_{H_3X^{2+}} + 10^{(pH - pK_2')} m_{H_2X^+}}{10^{(pK_1' - pH)} + 1 + 10^{(pH - pK_2')}}$
Met-enkephalin Leu-enkephalin	H_3X^+	$m_e = \dfrac{10^{(pK_1' - pH)} m_{H_3X^+} + 10^{(pH - pK_2')} m_{HX^-} + 10^{(2pH - pK_2' - pK_3')} m_{X^{2-}}}{10^{(pK_1' - pH)} + 1 + 10^{(pH - pK_2')} + 10^{(2pH - pK_2' - pK_3')}}$
Triptorelin Buserelin	H_4X^{3+}	$m_e = \dfrac{10^{(pK_1' + pK_2' + pK_3' - 3pH)} m_{H_3X^{3+}} + 10^{(pK_2' + pK_3' - 2pH)} m_{H_4X^{2+}} + 10^{(pK_3' - pH)} m_{H_3X^+}}{10^{(pK_1' + pK_2' + pK_3' - 3pH)} + 10^{(pK_2' + pK_3' - 2pH)} + 10^{(pK_3' - pH)} + 1}$

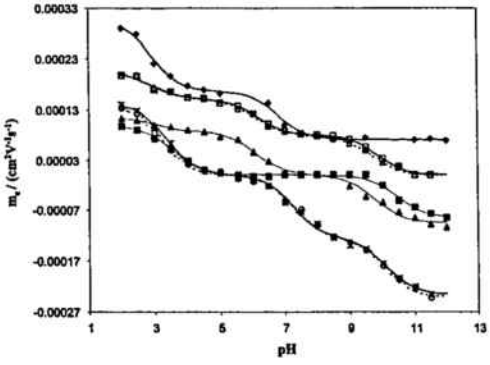

Figure 1. Experimental (points) and predicted (lines) electrophoretic mobility *vs.* pH of the running electrolyte for (◆) bradykinin, (△) triptorelin, (□) buserelin, (○) Met-enkephalin, (✱) Leu-enkephalin, (▲) oxytocin and (■) eledoisin.

of diprotic peptides of the type $HABH^+$ was studied. In this case, both acid-base equilibria were considered separately, permitting further simplifications and linearization of Eq. (11).

3 Results and discussion

The electrophoretic migration behavior of the studied peptide hormones can be described as a function of pH and pK'_a by Eq. (7). Particular formulations of Eq. (7) are deduced, taking into account the number and type of ionogenic groups present in each molecule (Table 2). The existence of the zwitterionic species is considered in all the cases, excepting for bradykinin, which never exists as these species over the pH range studied. Further simplifications developing Eq. (7) in each case can be performed considering only the relevant dissociation constants in this pH interval. Thus, the dissociation equilibrium due to the presence of the guanidine group is never determined, because the guanidine pK_a is usually higher than 12. Experimental pairs m_e-pH for each peptide have been fitted to the corresponding equation shown in Table 2. Good correlations have been observed between experimental and predicted values. pK_a have also been estimated from this relationships.

Once validity of Eq. (7) is verified, only a few experimental data pairs m_e-pH are necessary to predict the m_e values of the studied substances at any buffer pH. The predicted electrophoretic mobilities for all the studied peptide hormones are plotted against buffer pH in Fig. 1. The symbols stand for the experimental data and solid lines indicate the predicted m_e values obtained using only a few experimental data pairs. Selection of only a few experimental data means a great saving in analysis time. Smith *et al.* [13] have shown that for a weak acid, four evenly-spaced experimental points for each ionizable group are enough to accurately predict its migration behavior as a function of pH.

Special attention must be paid to oxytocin, triptorelin and buserelin. In oxytocin only two ionizable groups are theoretically present and in triptorelin and buserelin there are three. However, the plot of mobility *vs.* pH for these substances shows an unexpected increase at strong acidic pH value as can be seen in Fig. 1. The simplest interpretation for this abnormal behavior is the presence of an additional very acidic proton binding site common to the three hormones, as was observed by Castagnola *et al.* [35] in water-trifluoroethanol buffers. An alternative explanation for this abnormal mobility shift at low pH could be due to a temperature gradient that results in higher mobilities than expected. Thus, Eq. (7) in the case of oxytocin, triptorelin and buserelin was deduced considering an additional basic group (see Table 2). Using these equations, the concordance between experimental and predicted curves is excellent.

Equation (7) can be used to determine apparent pK_a values (pK'_a) of the studied peptide hormones [19, 23, 24]. Thus, pK_a values are calculated using Eq. (8), taking into account the activity coefficients of the species involved in each acid-base equilibrium. Apparent pK'_a and pK_a are summarized in Table 3 together with the few values

Table 3. pK_a values of the studied peptide hormones

Peptide		pK'_a	pK_a	Micro-titration (36)	CE (37)	(38)
Eledoisin	1	3.55 (0.05)	3.48			
	2	10.46 (0.06)	10.54			
Oxytocin	1	2.98 (0.17)	2.98			
	2	6.11 (0.09)	6.04			
	3	9.76 (0.08)	9.84			
Bradykinin	1	2.86 (0.06)	2.68			
	2	6.80 (0.07)	6.66			
Triptorelin	1	3.01 (0.15)	2.80			
	2	6.14 (0.09)	5.93			
	3	9.71 (0.06)	9.63			
Buserelin	1	2.84 (0.13)	2.66			
	2	6.12 (0.07)	5.92			
	3	9.83 (0.05)	9.76			
Met-enkephalin	1	3.22 (0.06)	3.17	3.45	3.52	3.20
	2	7.26 (0.06)	7.30	7.36		7.70
	3	10.05 (0.09)	10.30	10.36		10.30
Leu-enkephalin	1	3.36 (0.06)	3.31	3.69	3.69	3.20
	2	7.25 (0.06)	7.29	7.40		7.70
	3	10.09 (0.09)	10.34	10.34		10.30

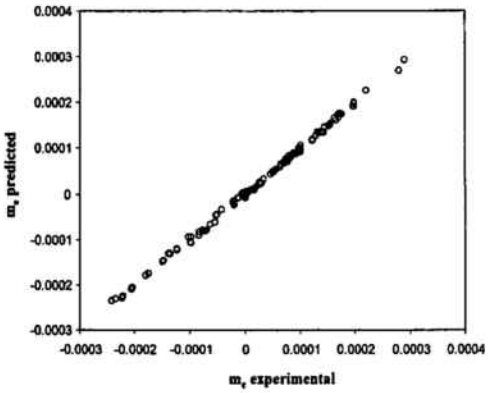

Figure 2. Experimental *vs.* predicted electrophoretic mobility for the studied peptides.

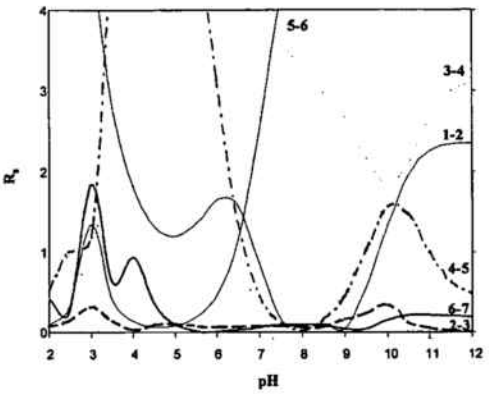

Figure 3. Predicted resolution (R_s) between adjacent peaks *vs.* pH of the running electrolyte.

reported in the literature [38–40]. As can be observed, the larger the charges on the reaction partners, the larger will be the influence of the Debye-Hückel correction on the activity coefficients, and hence the bigger the differences between both values. The pK_a values obtained for Met-enkephalin and Leu-enkephalin are similar to the ones reported by Castagnola *et al.* [38, 39], and also to the average values given by Shields [40]. The pK_a values of the structurally-related pairs triptorelin/buserelin and Met-enkephalin/Leu-enkephalin are very similar, and the electrophoretic separation of these mixtures would be possible only when differences due to different size are predominant, wich occurs when they are fully charged or uncharged, at extreme pH values. The usefulness of the equations in Table 2 is twofold because they simultaneously permit pK_a and mobility calculations of a solute as a function of pH. The correlation between predicted and experimental electrophoretic mobility for all the studied peptide hormones over the studied pH range is shown in Fig. 2. All data pairs are aligned with a slope value close to one and the correlation coefficient is greater than 0.99, showing good concordance between predicted and experimental m_e values.

The combined calculated curves for all the peptide hormones over the pH range studied are shown in Fig. 1. These curves can be used to perform a systematic and rapid selection of their optimum separation conditions, by selecting the appropiate pH to obtain the greatest differences between the electrophoretic mobilities of adjacent peaks. However, it is preferable to predict other parameters that quantitatively describe the extent of separation of the analytes present in the mixture [13, 14, 16–19]. In this way, predicting resolution (R_s) between adjacent peaks is the best way to evaluate separation between critically adjacent peaks, because efficiency and selectivity are simultaneously taken into account.

$$R_s = \underbrace{\frac{N^{1/2}}{4}}_{\text{efficiency}} \underbrace{\frac{(m_1 - m_2)}{(m_{avg} + m_{EOF})}}_{\text{selectivity}} \quad (12)$$

where m_i is the predicted electrophoretic mobility of the solutes obtained by equations of Table 2, m_{avg} is the average of m_i values, m_{EOF} is the mobility of electroosmotic flow, that can be evaluated from the study of m_{EOF} *vs.* pH (data not shown) and N is the number of theoretical plates. Similar N values for all the peaks present in the mixture are desirable when using Eq. (12) to perform accurate resolution estimations. In the peptide hormone mixture, where the peaks show typical N values of 15 000, this value was selected to predict R_s. Although higher N values would lead to greater resolution, the ability to predict optimum pH would not be altered [13]. Fixing efficiency term, resolution is governed by selectivity, and this can be misleading when having a large value of m_e at low pH because diffusion becomes important [14].

Resolution between adjacent peaks is then calculated, considering the changes in migration orders that can be observed in Fig. 1 [19]. Although resolution between the worst-resolved peak pair is the criterion for separation optimization [13, 14], plotting predicted resolution between adjacent peaks over the pH range studied for all the solute pairs gives more information about the separation of the overall mixture [17–19] (Fig. 3), and permits selection of various pH ranges when resolution is not fully achieved, as in our case, at a single pH. Thus, according to variation of predicted resolution *vs.* pH (Fig. 3), a mixture of the studied peptide hormones can-

Table 4. Experimental and predicted resolution values between adjacent peaks

pH	2.61			2.85			10.00	
	R_s exp.	R_s pred.		R_s exp.	R_s pred.		R_s exp.	R_s pred.
Bradykinin/triptorelin	6.01	6.09	Bradykinin/triptorelin	5.21	5.22	Bradykinin/buserelin	1.26	1.34
Triptorelin/buserelin	0	0.21	Triptorelin/buserelin	0	0.24	Buserelin/triptorelin	0.42	0.34
Buserelin/Leu-enkephalin	7.32	7.30	Buserelin/Leu-enkephalin	8.57	8.18	Triptorelin/eledoisin	1.79	1.84
Leu-enkephalin/Met-enkephalin	1.22	1.23	Leu-enkephalin/oxytocin	0.93	0.99	Eledoisin/oxytocin	1.32	1.40
Met-enkephalin/oxytocin	0.31	0.42	Oxytocin/Met-enkephalin	1.17	1.08	Oxytocin/Leu-enkephalin	5.47	5.25
Oxytocin-eledoisin	2.01	2.14	Met-enkephalin/eledosin	2.05	1.91	Leu-enkephalin/Met-enkephalin	0	0.15

not be fully resolved at only one pH because only partial separations for the pairs triptorelin/buserelin and Met-enkephalin/Leu-enkephalin can be obtained, and not at the same pH. Thus, two pH ranges must be selected to obtain the best separations in each case. One very narrow pH around 2.85 and another wider around pH 10.0, where the pairs Met-enkephalin/Leu-enkephalin and triptorelin/buserelin are respectively separated.

In order to check the accuracy of these predictions, a solution of all the studied peptide hormones was injected at pH 2.60, 2.85 and 10.00, where the last ones are included in the optimum pH ranges previously predicted. Experimental resolution is then calculated with: $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ (17–19), where t_1, t_2 and w_1, w_2 are, respectively, the migration time and the base width of peaks 1 and 2. Table 4 summarizes experimental and predicted values of resolution. Good concordance between both sets of resolution values was observed in all the cases that confirmed the accuracy of migration behavior prediction with the equations given in Table 2, and suitability of Eq. (12) to estimate resolution. The experimental electropherograms at pH 2.85 and 10 are shown in Figs. 4b and c, respectively. Figure 4b shows that at pH 2.85 all the pairs are separated, except triptorelin and buserelin, which comigrate. pH selection in the acidic region is very critical as can be seen comparing separation selectivities and order of migrations in Figs. 4a and b, where the pH differs only in 0.25 units. On the other hand, at pH 10.00 triptorelin and buserelin are only partially resolved, while the Met-enkephalin/Leu-enkephalin pair comigrates. Therefore, other variables of the buffer composition must be investigated in order to improve resolution of the studied mixtures.

4 Concluding remarks

In conclusion, in CE separations the pH has been shown to be the first variable of buffer composition to optimize in a mixture of ionizable compounds. A general equation was presented to simultaneously predict electrophoretic

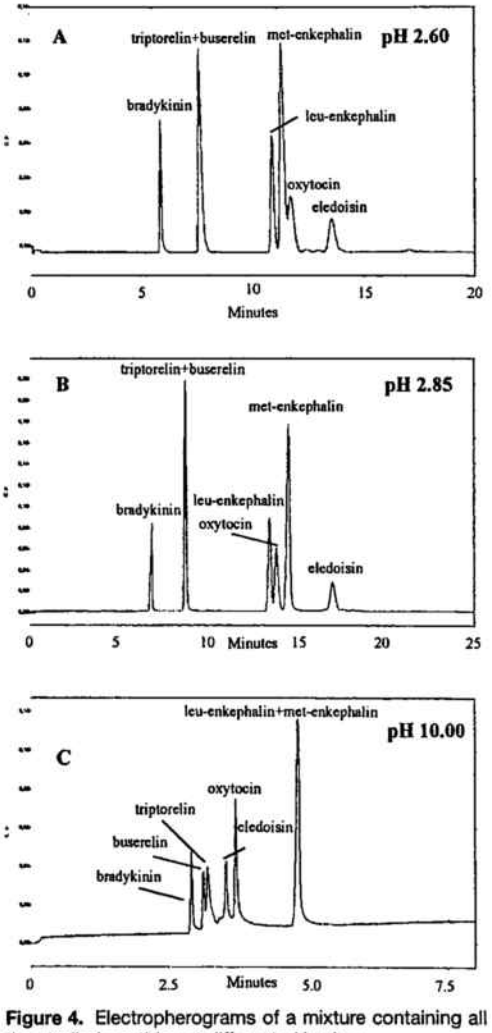

Figure 4. Electropherograms of a mixture containing all the studied peptides at different pH values.

mobility of polyprotic compounds and perform apparent pK'_a determinations from a limited number of experimental data. pK_a values can be calculated taking into account the Debye-Hückel correction on the activity coefficients for the charged species involved in the studied equilibrium. Predicted mobilities for any solute can be used to calculate resolution between them when they coexist in the same mixture. Accuracy of migration behavior predictions and pK_a determinations have been demonstrated for a series of polyprotic amphoteric peptide hormones and for other kinds of compounds [19, 23, 24]. These relationships have provided a simple, rapid and systematic approach for selecting optimum pH conditions to perform CE separations.

Financial support of this project by DGICYT (Project PB98–1174) is gratefully acknowledged. Fernando Benavente also thanks the Generalitat de Catalunya for an F. I. Grant.

Received March 6, 2001

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Evaluation of chromatographic versus electrophoretic behaviour of a series of therapeutical peptide hormones

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Abstract

In this work, models describing the effect of pH on chromatographic and electrophoretic behaviour for a series of polyprotic therapeutic peptide hormones were compared, taking into account the species in solution and the activity coefficients. The usefulness of the proposed equations is twofold, they permit the determination of the acidity constants in water and in the hydroorganic mobile phases used in liquid chromatography (LC) and capillary electrophoresis (CE) and can also be used for the selection of the optimum pH for the separation of mixtures of the modelled compounds. The proposed relationships allow an important reduction of the experimental data needed for the development of new separation methods. The accuracy of the proposed equations is verified by modelling the chromatographic and electrophoretic behaviour of a series of polyprotic therapeutic peptide hormones. By calculating the values of predicted resolutions, selection of the optimum pH to perform LC or CE separations of their mixtures becomes a rapid and simple process.

Keywords: Optimization; Retention prediction; Migration prediction; Ionization constants; Peptides; Hormones

1. Introduction

The recent increased use of native and synthetic peptide hormones and proteomimetics in biomedical therapy is a result of their large range of activity and specificity, usually with low toxicity and rapid metabolization [1]. For the near future, proteomic research is expected to play a major role in discovery of new peptide-based drugs, as new disease biomarkers, molecular targets for therapy and end points for therapeutic efficacy and toxicity are being described [2]. Thus, separation and analysis of peptides and peptide hormones has become increasingly important for an ever-widening range of research disciplines. Development of highly efficient and selective separation methods is necessary before overcoming the determination and characterization of peptide hormones in complex matrices. At present, liquid chromatography (LC) and capillary electrophoresis (CE) are considered orthogonal and the most useful techniques for rapid and efficient separation of a wide variety of peptides and proteins [3–5]. Furthermore, their coupling with mass spectrometry has become a powerful tool for the systematic separation, determination and characterisation of peptides and proteins in complex matrices [5–7].

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01902-7

The development of rapid, efficient and selective separation methodologies requires a previous optimization of the separation conditions. Although a desired peptide separation may be obtained by trial and error, this may take many attempts with subsequent loss of time and final peptide yield, and could be a particular problem when only limited amounts of sample are available. Modelling chromatographic and electrophoretic behaviour of substances can be used to predict separations using a few experimental data [8–18]. Furthermore, migration models can be used to perform physicochemical and conformational characterization of biomolecules [12,16–25].

Several semiempirical approaches have been proposed to relate chromatographic retention or electrophoretic mobility with structural parameters of peptides and proteins [26–29]. Nevertheless, their application is limited by the assumptions made in their development and there is no general rule to a priori select the appropriate relationship for each type of compound [27]. Likewise, in the separation of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual solute [8,10,30,31]. Accurate quantitative relationships between chromatographic retention or electrophoretic mobilities and pH can be very useful [8,20,32]. These models allow determination of ionization constants [16–25] and simultaneous selection of the optimum pH for the separation of complex mixtures of the modelled compounds, by resolution calculations [12,13,15–18,27,33].

In this work, general equations relating chromatographic retention or electrophoretic mobility with pH, pK_a and activity coefficients are presented for polyprotic peptide substances. Validation of the studied model is performed for a series of polyprotic amphoteric peptide hormones with therapeutic interest. The usefulness of the proposed equations is twofold. They permit the determination of the acidity constants and can also be used to establish a general model that relates the elution behaviour of the solute with pH and ionic strength. Selectivity and resolution can be easily predicted, and hence optimum separation conditions for the mixture are systematically selected on the basis of a few experimental data.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetonitrile, trifluoroacetic acid (TFA), potassium bromide, potassium hydrogenphthalate, phosphoric acid (85%), sodium hydroxide, hydrogen chloride (25%), formic acid (98%), acetic acid (glacial), diethylmalonic acid, boric acid and acetone were supplied by Merck (Darmstadt, Germany). Tris [Tris(hydroxymethyl)aminomethane] was purchased from J.T. Baker (Deventer, The Netherlands). Water with a conductivity lower than 0.05 mS cm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). The selected peptide hormones studied were: oxytocin, bradykinin, Leu-enkephalin, Met-enkephalin, triptorelin, buserelin and eledoisin. The peptide hormones were purchased from Sigma (St. Louis, MO, USA), except buserelin which was purchased from Hoechst Ibérica (Barcelona, Spain) and triptorelin which was purchased from Lasa (Barcelona, Spain). They were stored in a freezer at −4 °C when not in use. The structures of the selected substances are shown in Table 1.

2.1.1. LC analysis

Stock solutions of peptide hormones were prepared by dissolving ~5 mg of each substance in 5 ml of water; working solutions were prepared by 10-fold dilution of the stock solution. The mixture of the studied peptide hormones was prepared daily by 100-fold dilution of the stock solution. The solvent used as mobile phase was MeCN–water (35:65), 0.1% (v/v) TFA. This percentage of organic modifier in the mobile phase was previously optimized using linear solvation energy relationships [34]. All the eluents and mobile phases were passed through a 0.22 μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication. The samples were passed through a 0.45 μm nylon filter.

2.1.2. CE analysis

Electrolyte solutions covering the pH range 2–12 were prepared at the following concentrations and adjusted with 1 *M* NaOH or 1 *M* HCl: 20 m*M*

Table 1
Structures of the studied peptide hormones. Polyprotic formulation*

Peptide	Structure	Ionisable groups		Formula	
		Acidic (A)	Basic (B)	$H_nAB_z^-$	H_nX^z
Oxytocin	**Cys–Tyr–Ile–Gln–Asn–Cys**–Pro–Leu–Gly–NH_2	1 Phenol (–Tyr–)	1 Amino group (Cys–)	$HABH^+$	H_2X^+
Eledoisin	Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2	1 Carboxylic acid (–Asp–)	1 Amino group (–Lys–)	$HABH^+$	H_2X^+
Bradykinin	**Arg**–Pro–Pro–Gly–Phe–Ser–Pro–Phe–**Arg**	1 Carboxylic acid (–Arg)	1 Amino group (Arg–) 2 Guanidine groups (Arg–/–Arg)	$HABH_3^{2+}$	H_4X^{3+}
Met-enkephalin	**Tyr**–Gly–Gly–Phe–Met	1 Carboxylic acid (–Met) 1 Phenol (Tyr–)	1 Amino group (Tyr–)	H_2ABH^+	H_3X^+
Leu-enkephalin	**Tyr**–Gly–Gly–Phe–Leu	1 Carboxylic acid (–Leu) 1 Phenol (Tyr–)	1 Amino group (Tyr–)	H_2ABH^+	H_3X^+
Triptorelin	Pyr–Pro–**His**–Trp–Ser–Tyr–D–Trp–Leu–**Arg**–Pro–Gly–NH_2	1 Phenol (–Tyr–)	1 Imidazol group (–His–) 1 Guanidine group (Arg)	$HABH_2^{2+}$	H_3X^{2+}
Buserelin	Pyr–Pro–**His**–Trp–Ser–Tyr–D–Ser (^{t}Bu)–Leu–**Arg**–Pro–NHC_2H_5	1 phenol (–Tyr–)	1 Imidazol group (–His–) 1 Guanidine group (Arg)	$HABH_2^{2+}$	H_3X^{2+}

* Ionizable groups are marked with bold letters.

H_3PO_4 (pH 2), 50 m*M* acetic acid–50 m*M* formic acid (pH 2.5–5), 20 m*M* diethylmalonic acid (pH 5.5–6.5), 50 m*M* Tris (pH 7–9), 50 m*M* H_3BO_3 (pH 9.5–10.5) and 10 m*M* H_3PO_4 (pH 11–12). Solutions (250 ppm) of each peptide hormone were prepared in water, containing acetone at 3% (v/v) as the electroosmotic flow (EOF) marker. A mixture containing all the peptides at 250 ppm was prepared. Samples and running electrolytes were passed through a 0.45 μm nylon filter (MSI).

2.2. Instruments

The chromatographic equipment consisted of an ISCO Model 2350 (Lincoln, NE, USA) pump with an injection valve with a 10 μl sample loop and a variable wavelength V^4 absorbance detector (ISCO) operating at 214 nm. The chromatographic system was controlled by ChemResearch Chromatographic Data Management System Controller Software (ISCO) running on a Peceman AT Supermicro personal computer. A Merck LiChrospher 100 RP-18 (5 mm) column 250×4 mm I.D. was used at room temperature. The electrophoretic system consisted of a Beckman P/ACE system 5000 (Beckman Instruments, Fullerton, CA, USA). A 57 cm×75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was inserted in a capillary cartridge, thermostated to 25 °C (±0.1 °C). Samples were injected hydrodynamically at 0.5 p.s.i. for 3 s (1 p.s.i=6894.76 Pa). Experiments were conducted under normal polarity, applying a voltage of 25 kV during electrophoretic separations. The detection window was placed at 50 cm from the inlet of the capillary. A photodiode array detector was used. Electropherograms shown correspond to detection at 195 nm. All data were recorded and analyzed by a computer program supplied by Beckman (P/ACE Station 1.0 with Golden System interface). pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

2.3. Experimental procedures

2.3.1. Chromatographic procedure

In order to study the influence of the eluent pH on the chromatographic separation, the mobile phase was adjusted to different pH values, from 2 to 7, with sodium hydroxide using the percentage of acetonitrile previously optimized [33–37]. The LiChrospher C_{18} column was equilibrated at 1 ml/min with new mobile phase conditions for 30 min. Retention factors (k) were calculated from $k = (t_R - t_0)/t_0$, where t_0 is the retention time of the potassium bromide (hold-up time) which is established for each mobile phase composition and pH studied, and t_R is the retention time of each substance. The t_R of the solutes were determined from three different in-

jections of their individual solutions at every mobile phase considered. pH measurements in the mixed mobile phase, where the chromatographic separation takes place, were made taking into account the reference pH values of primary standard buffer solutions, pH_S, for the standardization of potentiometric sensors in MeCN–water mixtures. These were assigned in previous works [38,39], in accordance with IUPAC rules [40,41] and on the basis of multiprimary standard scale, according to the US National Institute of Standards and Technology (NIST) [42].

2.3.2. Electrophoretic procedure

New capillaries were activated by flushing them at 20 p.s.i. during 20 min with 1 *M* aqueous NaOH, followed by 15 min with water and 30 min with working buffer. Between days or after a change of buffer, capillary was conditioned by rinsing successively for 5 min with water, 5 min with 0.1 *M* aqueous NaOH, 10 min with water and 20 min with buffer. Both activation and conditioning methods, include, after the last flush with buffer, 15 min of 25 kV application. It was empirically demonstrated that this final step accelerates capillary equilibration. Between runs, the capillary is successively rinsed with 2 min 0.1 *M* aqueous NaOH, 2 min of water and 2 min of buffer, in order to reequilibrate it and thereby minimize hysteresis effects. Capillary was stored overnight filled with working buffer electrolyte. Electrolyte solutions indicated in the previous section were run in sequence from low to high pH. Individual solutions of each peptide hormone were injected at each pH until the electrophoretic mobility (m_e) was constant. m_e values were calculated as the difference between the apparent mobility of each peptide and the mobility of acetone used as neutral marker [30,31]. Each electrophoretic mobility was obtained as the average of three replicates.

2.4. Data analysis

In order to study the influence of pH upon chromatographic and electrophoretic behaviour of ionisable substances, relationships between pH, pK_a and retention factors or electrophoretic mobilities can be adequately established [33–37]. Polyprotic compounds undergo successive ionisation equilibria when the pH of the solvent is gradually changed. The dissociation equilibria for a generic fully protonated polyprotic species H_nX^z, are:

$$\begin{aligned} &H_nX^z \Leftrightarrow H_{n-1}X^{z-1} + H^+ && K_1 \\ &\vdots \\ &H_{n-(i-1)}X^{z-(i-1)} \Leftrightarrow H_{n-i}X^{z-i} + H^+ && K_i \\ &\vdots \\ &H_{n-(n-1)}X^{z-(n-1)} \Leftrightarrow X^{z-n} + H^+ && K_n \end{aligned} \quad (1)$$

where n is the total number of ionogenic groups, z the maximum net charge, given by the protonated basic groups, and K_i is the dissociation equilibrium constant of the i^{th} dissociation step, that is given by the expression:

$$K_i = \frac{[H_{n-i}X^{z-i}]y^{z-i}a_{H^+}}{[H_{n-(i-1)}X^{z-(i-1)}]y^{z-(i-1)}} \quad (2)$$

In Eq. (2), the effect of ionic strength (I) upon dissociation constants has been taken into account, considering the activity coefficients of the solutes, y. The activity coefficients are obtained, according to IUPAC rules [41], from the Debye–Hückel equation, $\log y = -z^2 A\sqrt{I}/(1 + a_0 B\sqrt{I})$, where A and B are the Debye–Hückel constants, a_0 is the ion size parameter in the solvent mixture and I is the ionic strength. In another way, apparent dissociation constants can be defined as,

$$K_i' = \frac{y^{z-(i-1)}}{y^{z-i}} \cdot K_i \quad (3)$$

The retention factor, k, of a polyprotic compound, H_nX^z, coexisting in the form of various species at a given pH, is a weighted average of the retention factors, k_i, of the individual species [43], according to the molar fraction, x_i, of these species in the mobile phase. Thus the overall observed k values for the peptide hormones can be given as:

$$k = \sum_{i=0}^{n} x_{H_{n-i}X^{z-i}} k_{H_{n-i}X^{z-i}} \quad (4)$$

In the same way, the effective electrophoretic mobility, m_e, is a function of the mobility and the molar fraction, x_i, of the individual species [18–23]:

$$m_e = \sum_{i=0}^{n} x_{H_{n-i}X^{z-i}} m_{H_{n-i}X^{z-i}} \quad (5)$$

Replacing the molar fraction by its expression the following equations can be obtained:

$$k=\sum_{i=0}^{n}\frac{\left[H_{n-i}X^{z-i}\right]}{\sum_{i=0}^{n}\left[H_{n-i}X^{z-i}\right]}\cdot k_{H_{n-i}X^{z-i}} \quad (6)$$

$$m_e=\sum_{i=0}^{n}\frac{\left[H_{n-i}X^{z-i}\right]}{\sum_{i=0}^{n}\left[H_{n-i}X^{z-i}\right]}\cdot m_{H_{n-i}X^{z-i}} \quad (7)$$

Eqs. (6) and (7) can be rewritten as a function of dissociation constants, dividing numerator and denominator by an appropriate concentration $[H_{n-r}X^{z-r}]$. Thus, the overall observed k and m_e values for the compounds considered can be given as:

$$k=\frac{\sum_{i=0}^{r-1}\frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r}K_j'}k_{H_{n-i}X^{z-i}}+k_{H_{n-r}X^{z-r}}+\sum_{i=r+1}^{n}\frac{\prod_{j=r+1}^{i}K_j'}{a_{H^+}^{i-r}}k_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1}\frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r}K_j'}+1+\sum_{i=r+1}^{n}\frac{\prod_{j=r+1}^{i}K_j'}{a_{H^+}^{i-r}}} \quad (8)$$

$$m_e=\frac{\sum_{i=0}^{r-1}\frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r}K_j'}m_{H_{n-i}X^{z-i}}+m_{H_{n-r}X^{z-r}}+\sum_{i=r+1}^{n}\frac{\prod_{j=r+1}^{i}K_j'}{a_{H^+}^{i-r}}m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1}\frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r}K_j'}+1+\sum_{i=r+1}^{n}\frac{\prod_{j=r+1}^{i}K_j'}{a_{H^+}^{i-r}}} \quad (9)$$

Furthermore, considering that $a_{H^+}^{n}=10^{-npH}$ and that $K_i'=10^{-pK_i'}$, Eqs. (8) and (9) can be rearranged more conveniently as a function of pH to give

where r can take any value higher than zero and less or equal to the maximum net charge z. Eqs. (10) and (11) are general expressions that can predict retention factors and electrophoretic mobilities of ionogenic substances as a function of pH when several data pairs of k–pH or m_e–pH are available. Particular equations can be obtained from general equations for each peptide hormone, taking into account the relevant dissociation constants over the pH range studied.

In the case of chromatographic analysis with ODS columns, only pK associated to the C-terminal carboxylic group of Met-enkephalin, Leu-enkephalin and bradykinin and the pK associated to the imidazol group of triptorelin and buserelin are within the pH range studied. Oxytocin has functional groups that are not within this pH range. The equations deduced in each particular case are summarized in Table 2.

In the case of electrophoretic data, further simplifications to Eq. (11) are made when in the pH range studied the r value can be taken as z, because the species considered is the zwitterionic form $H_{n-z}X$ that has no net charge, and hence its mobility is assumed to be nil [18–23]. Moreover, in the pH range studied, the dissociation equilibrium due to the presence of the guanidine group is never determined, because the guanidine pK_a is usually higher than 12. The corresponding equations are shown in Table 2.

3. Results and discussion

The retention factor values, k, for the series of peptide hormones considered at different pH values of the mobile phase are shown in Fig. 1. Relative standard deviation lower than 5% for the k values

$$k=\frac{\sum_{i=0}^{r-1}10^{\left[-(r-i)pH+\sum_{j=i+1}^{r}pK_j'\right]}k_{H_{n-i}X^{z-i}}+k_{H_{n-r}X^{z-r}}+\sum_{i=r+1}^{n}10^{\left[(i-r)pH-\sum_{j=r+1}^{i}pK_j'\right]}k_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1}10^{\left[-(r-i)pH+\sum_{j=i+1}^{r}pK_j'\right]}+1+\sum_{i=r+1}^{n}10^{\left[(i-r)pH-\sum_{j=r+1}^{i}pK_j'\right]}} \quad (10)$$

$$m_e=\frac{\sum_{i=0}^{r-1}10^{\left[-(r-i)pH+\sum_{j=i+1}^{r}pK_j'\right]}m_{H_{n-i}X^{z-i}}+m_{H_{n-r}X^{z-r}}+\sum_{i=r+1}^{n}10^{\left[(i-r)pH-\sum_{j=r+1}^{i}pK_j'\right]}m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1}10^{\left[-(r-i)pH+\sum_{j=i+1}^{r}pK_j'\right]}+1+\sum_{i=r+1}^{n}10^{\left[(i-r)pH-\sum_{j=r+1}^{i}pK_j'\right]}} \quad (11)$$

Table 2
Electrophoretic and chromatographic models for the studied peptide hormones

Peptide	H_nX^z	
		Chromatographic models (Eq. (10))
Bradykinin	H_4X^{3+}	$k=\frac{10^{(pK_1'-pH)}k_{H_4X^{3+}}+k_{H_3X^{2+}}}{10^{(pK_1'-pH)}+1}$
Met-enkephalin Leu-enkephalin	H_3X^{+}	$k=\frac{10^{(pK_1'-pH)}k_{H_3X^{+}}+k_{H_2X}}{10^{(pK_1'-pH)}+1}$
Triptorelin Buserelin	H_3X^{2+}	$k=\frac{10^{(pK_1'-pH)}k_{H_3X^{2+}}+k_{H_2X^{+}}}{10^{(pK_1'-pH)}+1}$
		Electrophoretic models (Eq. (11))
Eledoisin	H_2X^{+}	$m_e=\frac{10^{-2pH}m_{H_2X^{+}}+10^{-(pK_1'+pK_2')}m_{X^-}}{10^{-2pH}+10^{-(pK_1'+pH)}+10^{-(pK_1+pK_2')}}$
Oxytocin*	H_3X^{2+}	$m_e=\frac{10^{(pK_1'+pK_2'-2pH)}m_{H_3X^{2+}}+10^{(pK_2'-pH)}m_{H_2X^{+}}+10^{(pH-pK_3')}m_{X^-}}{10^{(pK_1'+pK_2'-2pH)}+10^{(pK_2'-pH)}+1+10^{(pH-pK_3')}}$
Bradykinin	H_4X^{3+}	$m_e=\frac{10^{(pK_1'-pH)}m_{H_4X^{3+}}+m_{H_3X^{2+}}+10^{(pH-pK_2')}m_{H_2X^{+}}}{10^{(pK_1'-pH)}+1+10^{(pH-pK_2')}}$
Met-enkephalin Leu-enkephalin	H_3X^{+}	$m_e=\frac{10^{(pK_1'-pH)}m_{H_3X^{+}}+10^{(pH-pK_2')}m_{HX^-}+10^{(2pH-pK_2'-pK_3')}m_{X^{2-}}}{10^{(pK_1'-pH)}+1+10^{(pH-pK_2')}+10^{(2pH-pK_2'-pK_3')}}$
Triptorelin* Buserelin*	H_4X^{3+}	$m_e=\frac{10^{(pK_1'+pK_2'+pK_3'-3pH)}m_{H_4X^{3+}}+10^{(pK_2'+pK_3'-2pH)}m_{H_3X^{2-}}+10^{(pK_3'-pH)}m_{H_2X^{+}}}{10^{(pK_1'+pK_2'+pK_3'-3pH)}+10^{(pK_2'+pK_3'-2pH)}+10^{(pK_3'-pH)}+1}$

* One extra basic group is considered in addition to marked groups on Table 1. Explanation is given in the text.

were obtained. The percentage of acetonitrile in the hydro–organic mixture used as mobile phase, 35% (v/v) acetonitrile, was optimized using the solvatochromic parameter E_T^N as solvent descriptor [44] and taking into account that log k values and E_T^N solvent parameters correlate linearly [34,37]. The octadecylsilica (ODS) stationary phase used may only be used in the pH range 2–7, so it was not possible to study the retention of peptide hormones as typical ampholytes, because correlation between k values and the pH of the mobile phase cannot be obtained over the entire range of pH. Thus, from a chromatographic point of view, with the widely used ODS stationary bonded phase, only the protolytic equilibria corresponding to pK_a values associated to carboxylic C-terminal groups and to imidazol groups of histidine residues are relevant, Table 1.

The retention of Met-enkephalin, Leu-enkephalin and bradykinin is high at low pH values (Fig. 1), where the compound exists as a charged cation and forms an ionic pair with TFA anions; when pH increases, the k value decreases, levels off at the isoelectric point pH and stays constant; this decrease in the chromatographic retention could be explained due to the equilibrium between the double charged zwitterionic and neutral forms being displaced to the first one. In the case of oxytocin, no variation of chromatographic retention with pH was observed because in the pH range studied, oxytocin was not involved in acid–base equilibria. k values of tri-

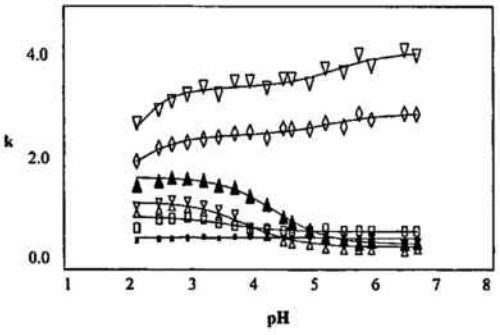

Fig. 1. Experimental (points) and predicted (lines) retention factor, k, vs. pH of the mobile phase for the peptide hormones: ▽, buserelin; ◊, triptorelin; ▲, Leu-enkephalin; ⧖, Met-enkephalin; □, bradykinin; ■, oxytocin. Experimental conditions: MeCN–water (35:65, v/v), 0.1% TFA and adjusting pH values up to 7 with sodium hydroxide.

ptorelin and buserelin slightly increase due to the low dissociation of their protonated basic imidazol groups when pH increases.

Although the effects of solute ionization on retention are known, the theoretical interpretation of this phenomenon is hampered by the lack of a rigorous treatment of protolytic equilibria in hydro-organic mixtures. Here, a general equation that

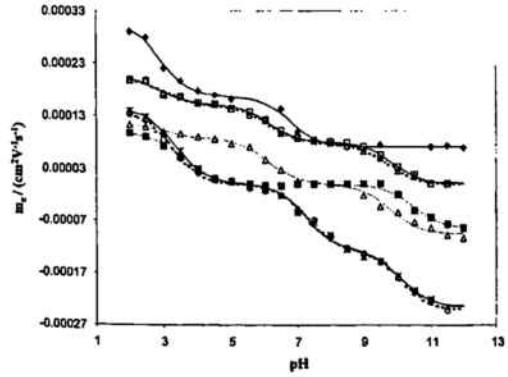

Fig. 2. Experimental (points) and predicted (lines) electrophoretic mobility vs. pH of the running electrolyte for bradykinin (♦), triptorelin (△), buserelin (□), Met-enkephalin (○), Leu-enkephalin (*), oxytocin (▲) and eledoisin (■).

describes the chromatographic retention as a function of pH of the mobile phase and of activity coefficients, has been deduced (Eq. (10)). Particular formulations of this equation have been derived, taking into account the number and type of ionogenic groups present in each molecule and involved in the pH range studied (Table 2). Experimental pairs k–pH for each peptide hormone have been fitted to the corresponding equation and good correlations have been observed between experimental and predicted values ($r>0.99$) as it is shown in Fig. 1.

In the same way, the m_e values for the series of peptide hormones considered have been plotted against pH of the electrolyte solutions as can be seen in Fig. 2. The electrophoretic migration behaviour of the studied peptide hormones can be described as a function of pH and pK_a' by general Eq. (11). Particular formulations of Eq. (11) are deduced, taking into account the number and type of ionogenic groups present in each molecule and involved in the pH range studied (Table 2). The existence of the zwitterionic species, with net charge zero, is considered in all cases, except for bradykinin, which never exists as these species over the pH range studied.

In contrast to LC experiments using ODS columns, CE experiments using bare fused-silica capillaries permit to study the electrophoretic migration behaviour of the peptide hormones in a wide pH range between 2 and 12. Therefore, electrophoretic equations summarized in Table 2 are more complex than relations obtained to explain their chromatographic behaviour in the pH range 2–7. Eq. (11) for oxytocin, triptorelin and buserelin, was deduced considering an additional basic group. The plot of mobility vs. pH for these substances shows an unexpected increase at strong acidic pH value (Fig. 2), that has been interpreted as an additional very acidic proton binding site common to the three hormones, as was observed by Castagnola et al. [45] in water–trifluoroethanol buffers.

Experimental pairs m_e–pH for each peptide have been fitted to the corresponding equation shown in Table 2. Good correlations have been observed between experimental and predicted values ($r>0.99$) as shown in Fig. 2. Considering the additional basic group for oxytocin, triptorelin and buserelin the concordance between experimental and predicted curves was also excellent.

Once the validity of Eqs. (10) and (11) is verified, only a few experimental data pairs m_e–pH and k–pH are necessary to predict the m_e and k values of the studied substances at any buffer pH. The predicted chromatographic retentions and electrophoretic mobilities for all the studied peptide hormones are plotted against buffer pH in Figs. 1 and 2, respectively. Symbols stand for the experimental data and solid lines indicate the predicted k and m_e values obtained using only a few experimental data pairs. Selection of only a few experimental data means a great saving in analysis time.

Figs. 1 and 2 can be used to perform a systematic and rapid selection of the optimum separation conditions, by selecting the appropriate pH to obtain the greatest differences between retention factors or electrophoretic mobilities of adjacent peaks. However, it is preferable to predict other parameters that quantitatively describe the extent of separation of the analytes present in the mixtures. In this way, predicting resolution (R_s) between adjacent peaks is the best way to evaluate separation between critically adjacent peaks, because efficiency and selectivity are simultaneously taken into account. In the case of chromatographic separations predicted resolution can be calculated from the expression:

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k_2}{1 + k_2}\right) \quad (12)$$

efficiency selectivity

where N is the number of theoretical plates, α the predicted selectivity ($\alpha = k_i/k_j$) and k the predicted retention factors calculated using equations in Table 2. In a similar way, predicted resolution can be expressed as follows for the electrophoretic separations:

$$R_s = \frac{N^{1/2}}{4} \cdot \frac{(m_1 - m_2)}{(m_{avg} + m_{EOF})} \quad (13)$$

efficiency selectivity

where m_i is the predicted electrophoretic mobility of the solutes obtained by equations of Table 2, m_{avg} is the average of m_i values, m_{EOF} is the mobility of electroosmotic flow, that can be evaluated from the study of m_{EOF} vs. pH (data not shown) and N the number of theoretical plates. Similar N values for all the peaks present in the mixture are desirable to perform accurate resolution estimations with Eqs. (12) and (13). Here, average typical values of 5000 and 15 000 theoretical plates have been selected for chromatographic and electrophoretic resolution calculations, respectively.

The k and m_e predicted values have then been used to calculate resolution for solute adjacent pairs, considering the changes in migration orders that can be observed in Figs. 1 and 2 [18]. Plotting predicted resolution over the pH range studied gives more information about the separation of the overall mixture (Figs. 3 and 4), and permits selection of a suitable pH in order to achieve the best separation, or the selection of various pH ranges when resolution is not fully achieved at a single pH. In Fig. 3, points stand for experimental resolution and solid lines indicate predicted chromatographic resolution values obtained using Eq. (12) and retention factors estimated using equations in Table 2. Good concordance between both sets of resolution values has been observed in all cases confirming the accuracy of chromatographic behaviour prediction with equations in Table 2, and suitability of Eqs. (12) and (13) to estimate resolution. Thus, according to variation of predicted resolution vs. pH (Fig. 3) and Fig. 1, a good chromatographic separation in a reasonable retention time can be obtained in a pH range between 3 and 3.5. Fig. 5 shows the chromatogram of the

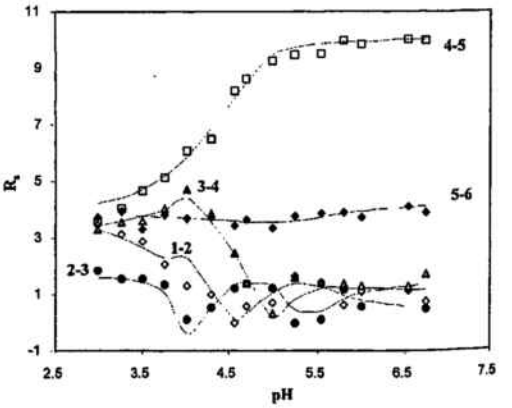

Fig. 3. Chromatographic resolution (R_s) between adjacent peaks vs. pH of mobile phase. Solid lines indicate predicted R_s values and points stand for experimental R_s data.

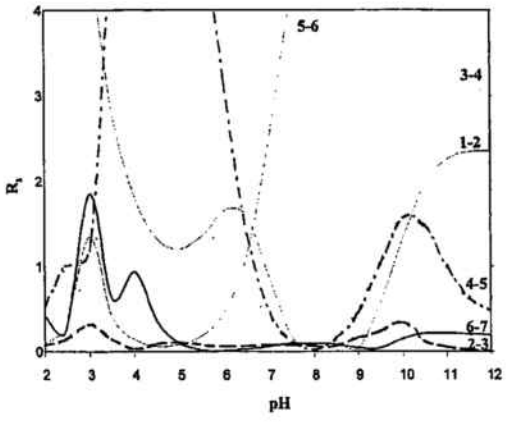

Fig. 4. Predicted electrophoretic resolution (R_s) between adjacent peaks vs. pH of the running electrolyte.

overall peptide mixture at pH 3.3 (35% (v/v) MeCN, 0.1% TFA).

On the other hand, this peptide mixture cannot be fully resolved by CE at only one pH, because only partial separations for the pairs triptorelin/buserelin and Met-enkephalin/Leu-enkephalin can be obtained, and not at the same pH (Figs. 2 and 4). Thus, two pH ranges must be selected to obtain the best separations in each case, one very narrow around pH 2.85 and another wider around pH 10.00. The experimental electropherograms at pH 2.85 and 10 are shown in Fig. 6a and b, respectively. At pH 2.85 all the pairs are separated, excepting triptorelin and buserelin that comigrate. At pH 10.00 triptorelin and buserelin are partially resolved, while the pair Met-enkephalin and Leu-enkephalin comigrates. Table 3 summarizes experimental and predicted values (Eq. (13)) of the electrophoretic resolution at pH 2.60, 2.85 and 10 and it shows a good concordance between both sets of values.

The separations obtained by both methodologies show different selectivities, even around the same pH values (Figs. 5 and 6a). In general, this orthogonality between the partitioning mechanism in LC and the charge-to-mass ratio in CE separations can be used to obtain combined separations with enhanced selectivities [46].

Various authors [20,21,47,48] have remarked on the advantages of the LC and CE methodologies for evaluating the ionization constants of substances. Small quantities of compounds are required, poor water-solubility is not a serious drawback and the purity of the substance is not a critical factor if

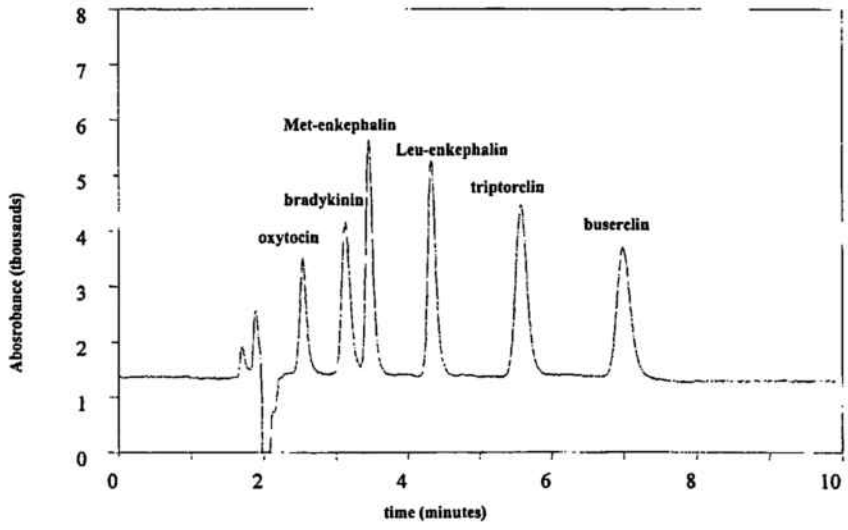

Fig. 5. Chromatogram of peptide hormones at pH 3.3 (35% MeCN, 0.1% TFA).

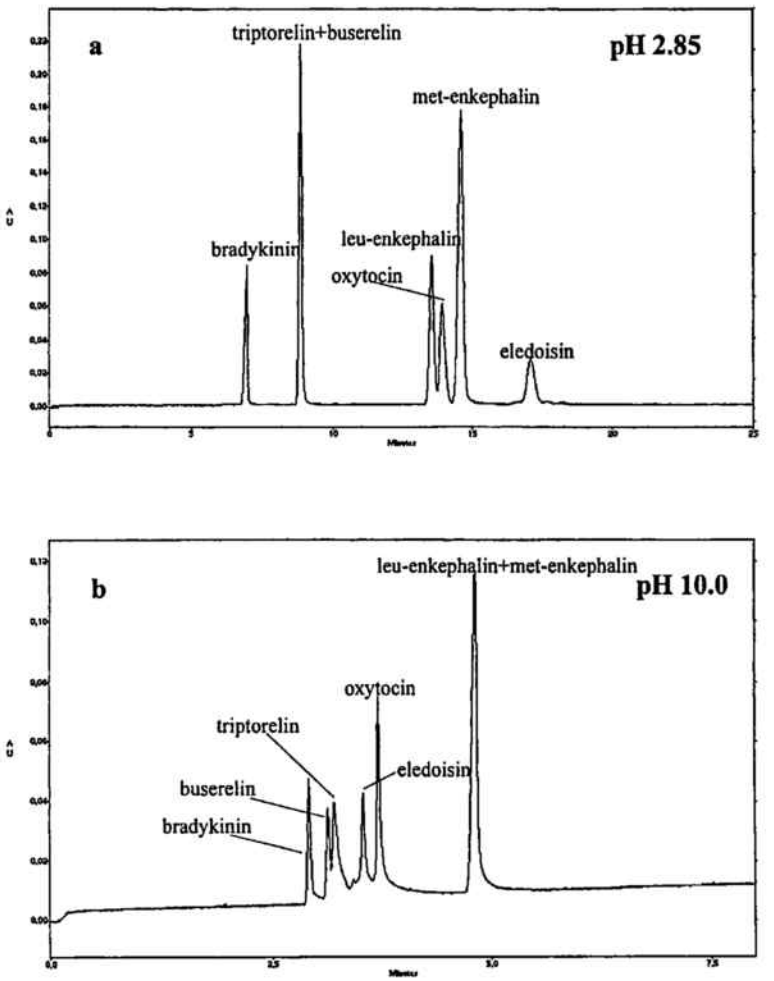

Fig. 6. Electropherograms of a mixture containing all the studied peptides at different pH values.

impurities can be separated from the target analyte on the columns. pK_a values of the studied substances can be calculated from the measured k or m_e values and the corresponding pH using Eqs. (10) and (11) [18,22,23], taking into account the activity coefficients of the species involved in each acid–base equilibrium (Eq. (3)). pK_a values are summarised in Table 4 together with the few values reported in the literature [49–51]. The values obtained by LC methodology are higher than the CE values because they have been determined in acetonitrile–water mixtures with an acetonitrile percentage of 35% (v/v). They cannot be compared with literature data because hydro–organic media values are not available. On the other hand, CE has proved suitable to perform pK_a determinations in aqueous solutions, which is not the case for LC, where retention could be unsuitable without the addition of an organic modi-

Table 3
Experimental and predicted resolution values between adjacent peaks

	pH 2.61		pH 2.85		pH 10.00	
	R_s exp.	R_s pred.	R_s exp.	R_s pred.	R_s exp.	R_s pred.
Bradykinin/triptorelin	6.01	6.09	5.21	5.22	1.26	1.34
Triptorelin/buserelin	0	0.21	0	0.24		
Buserelin/Leu-enkephalin	7.32	7.30	8.57	8.18		
Leu-enkephalin/Met-enkephalin	1.22	1.23				
Met-enkephalin/oxytocin	0.31	0.42				
Oxytocin–eledoisin	2.01	2.14				
Leu-enkephalin/oxytocin			0.93	0.99		
Oxytocin/Met-enkephalin			1.17	1.08		
Met-enkephalin/eledoisin			2.05	1.91		
Buserelin/triptorelin					0.42	0.34
Triptorelin/eledoisin					1.79	1.84
Eledoisin/oxytocin					1.32	1.40
Oxytocin/Leu-enkephalin					5.47	5.25
Leu-enkephalin/Met-enkephalin					0	0.15

Table 4
pK_a values of the studied peptide hormones

Peptide		35% (v/v) MeCN, LC (34)	0% (v/v) MeCN, CE (23)	0% (v/v) MeCN, Microtitration (49)	0% (v/v) MeCN, CE (50)	0% (v/v) MeCN, (51)
Eledoisin	1		3.48			
	2		10.54			
Oxytocin	1		2.98			
	2		6.04			
	3		9.84			
Bradykinin	1	3.21	2.68			
	2		6.66			
Triptorelin	1	4.71	2.80			
	2		5.93			
	3		9.63			
Buserelin	1	5.11	2.66			
	2		5.92			
	3		9.76			
Met-enkephalin	1	3.96	3.17	3.45	3.52	3.20
	2		7.30	7.36		7.70
	3		10.30	10.36		10.30
Leu-enkephalin	1	4.38	3.31	3.69	3.69	3.20
	2		7.29	7.40		7.70
	3		10.34	10.34		10.30

fier. The electrophoretic aqueous pK_a values obtained for Met-enkephalin and Leu-enkephalin are similar to the ones reported by Castagnola et al. [49,50], and also to the average values given by Shields [51]. The pK_a values obtained by CE for the structurally-related pairs triptorelin/buserelin and Met-enkephalin/Leu-enkephalin are very similar. However, the values obtained by LC show great differences, suggesting that elution behaviour of these closely related pairs is influenced by a certain kind of non-hydrophobic, hydrogen bonding or ionic interaction with the stationary phase. Thus, they will be easily resolved by LC, although the pK_a values obtained by this methodology would be less rigorous than the electrophoretic ones. Likewise, the electrophoretic separation of these mixtures would be only possible when differences due to their size are predominant, which in this case occurs when they are fully charged, at very acidic pH values. Several experiments are currently being developed in our laboratory using new LC stationary phases and coated capillaries to avoid undesired analyte–stationary phase or analyte–capillary wall interactions that negatively affect pK_a determinations. Furthermore, these investigations may be used to obtain stationary phases or coated capillaries with improved separation selectivities.

In conclusion, the suitability of the chromatographic and electrophoretic models studied to determine dissociation constants and to predict chromatographic and electrophoretic behaviour of peptide hormones from a limited number of experimental data has been shown. In a parallel way, separation between solutes in a complex mixture can be easily predicted, making a simple and rapid selection to achieve optimum separations.

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Capítulo 3. Separación y caracterización de crudos de síntesis de hormonas peptídicas de bajo peso molecular mediante LC-ESI-MS y CE

En este capítulo se describe la separación y la caracterización de las sustancias presentes en crudos de síntesis de hormonas peptídicas de amplio uso terapéutico (Leuprolide, Goserelina, Triptorelina, Carbetocina y Eledoisina) empleando LC, CE y LC-ESI-MS. Estos crudos de síntesis se generan durante la síntesis del péptido en Fase Sólida (SPPS), y son mezclas complejas donde el péptido sintetizado está acompañado de un gran número de sustancias, de origen peptídico (diastereoisómeros, productos de deleción, secuencias truncadas,...) o no peptídico (disolventes, reactivos, contaminantes,...). El método de comparación solvatocrómico (LSER), que relaciona linealmente el logaritmo del factor de capacidad y el parámetro de polaridad de Reichardt (E_T^N) de la fase móvil, se utiliza para explicar el comportamiento cromatográfico de las sustancias presentes en cada una de las mezclas complejas. Empleando estas relaciones lineales, se pueden predecir la selectividad o la resolución entre picos correspondientes a analitos adyacentes, a partir de un reducido número de datos experimentales. De esta manera se puede seleccionar de forma sencilla la proporción adecuada de acetonitrilo en la fase móvil para posteriormente purificar a escala preparativa el péptido sintetizado o para identificar cada uno de los analitos presentes en la mezcla mediante LC-ESI-MS.

Antes de proceder a la separación y caracterización de los crudos de síntesis por LC-ESI-MS, se optimizan los parámetros del detector, para obtener la máxima sensibilidad y la menor fragmentación. Se discute el efecto de la composición de la fase móvil sobre la sensibilidad en LC-ESI-MS. En algunos casos se realizan adiciones post-columna de disolventes adecuados para conseguir mejoras en la sensibilidad. En las condiciones de separación y detección óptimas, la obtención de las masas moleculares de las sustancias presentes en cada uno de los crudos de reacción, permite su identificación, lo que proporciona la información necesaria para una posterior reoptimización de las síntesis.

Sin embargo, en la mayoría de los casos, cuando se tiene una mezcla compleja, una única técnica de separación no es suficiente para resolver todas las sustancias que la forman. Esto es lo que ocurre en el caso de emplear LC o CE para la separación y caracterización de los crudos de síntesis de leuprolide. En estos casos es conveniente investigar la combinación de diferentes técnicas de separación para conseguir resolver el mayor número de sustancias posible en la mezcla. Así, en el último artículo de este capítulo se utiliza una combinación secuencial de la LC y la CE para separar las sustancias presentes en el crudo de síntesis del leuprolide. Las fracciones recolectadas por LC y el crudo de síntesis fortificado con éstas mismas, son analizados por CE. Los picos electroforéticos son identificados de acuerdo a la información previamente obtenida por LC-ESI-MS. Una vez identificados los picos electroforéticos, se emplean varios modelos semiempíricos que relacionan la movilidad electroforética de los péptidos con su estructura, para corroborar las asignaciones estructurales previamente realizadas en base únicamente a la masa molecular de las especies detectadas mediante LC-ESI-MS. De esta forma, se pone de manifiesto la complementariedad de estas técnicas de separación y la utilidad de los modelos semiempíricos que explican la migración de péptidos por CE, para obtener información estructural de las sustancias.

Las investigaciones realizadas se recogen en las siguientes publicaciones, que aparecen a continuación en su formato original:

- ***Separation and Characterization of Multicomponent Peptide Mixtures by Liquid Chromatography-Electrospray Ionization Mass Spectrometry. Application to Crude Products of the Synthesis of Leuprolide***
  V. Sanz-Nebot, F. Benavente, J. Barbosa
  J. Chromatogr. A, 870 (2001) 315-334.

- ***Liquid Chromatography-Electrospray Mass Spectrometry of Multicomponent Peptide Mixtures. Characterization of a Mixture from the Synthesis of the Hormone Goserelin***
  V. Sanz-Nebot, F. Benavente, A. Castillo, J. Barbosa
  J. Chromatogr. A, 889 (2000) 119-133.

- ***Optimization of HPLC Conditions for the Separation of Complex Crude Mixtures Produced in the Synthesis of Therapeutic Peptide Hormones***
  V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa
  Chromatographia, 53 (2001) S167-S173.

- ***Separation and Characterisation of Complex Crude Mixtures Produced in the Synthesis of Therapeutic Peptide Hormones by Liquid Chromatography Coupled to Electrospray Mass Spectrometry (LC-ES-MS)***
  V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa
  J. Chromatogr. A, (2003) enviado.

- ***Liquid Chromatography-Mass Spectrometry and Capillary Electrophoresis Combined Approach for Separation and Characterization of Multicomponent Peptide Mixtures. Application to Crude Products of Leuprolide Synthesis***
  V. Sanz-Nebot, F. Benavente, J. Barbosa
  J. Chromatography A, 950 (2002) 99-111.

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Journal of Chromatography A, 870 (2000) 315–334

JOURNAL OF CHROMATOGRAPHY A

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Separation and characterization of multicomponent peptide mixtures by liquid chromatography–electrospray ionization mass spectrometry
Application to crude products of the synthesis of leuprolide

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Abstract

Leuprolide is a synthetic structural analogue of luteinizing hormone-releasing hormone used for the treatment of a large number of diseases related with the regulation of sexual hormones. Solid-phase peptide synthesis is used to obtain leuprolide peptidic hormone, but this synthetic procedure results in complex mixtures that need separation and characterization. Here, liquid chromatography coupled with mass spectrometry using electrospray ionization, (LC–ES-MS), was used for the separation and characterization of multicomponent peptide mixtures of crudes of synthesis of leuprolide. To optimize the LC separation process, the method of linear solvation energy relationships was applied and the powerful coupling LC–ES-MS permitted rapid and reliable characterization of the reaction product.

Keywords: Peptides; Hormones; Leuprolide

1. Introduction

The use of peptides in biomedical therapy has increased in the last few years, because they have a large range of activity and specificity, usually with a low toxicity and a rapid metabolization [1,2].

Leuprolide {CAS: [53714-56-0] (leuprolide or leuprorelin) and [74381-53-6] (leuprorelin acetate)} is a synthetic structural analogue of luteinizing hormone-releasing hormone (LHRH), which regulates the production of sexual hormones. Leuprolide presents a higher affinity for the LHRH receptors and a higher resistance to enzymatic degradation than the natural hormone, which increases its biological potential. It can thus be used for the treatment of a large number of diseases related with the regulation of sexual hormones, like: masculine and feminine infertility, uterine myomas and prostatic and mammalian tumors (Fig. 1) [3,4].

The interest in peptidic hormone analogues has led to a parallel development of new strategies within the solid-phase peptide synthesis (SPPS) method. Since Merrifield [5] first introduced this procedure in 1962, it has been used in the synthesis of peptides and small proteins, with new modifications and a large number of improvements. However, this procedure results in complex crudes of reaction, in which the target peptide is mixed with undesired side products, for example incomplete side chains, products resulting from amino acidic insertions and

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)01088-2

Leuprolide

(5-oxo-L-prolyl-L-histidyl-L-tryptophanyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-1-L-arginyl-L-prolyl-ethylamide)

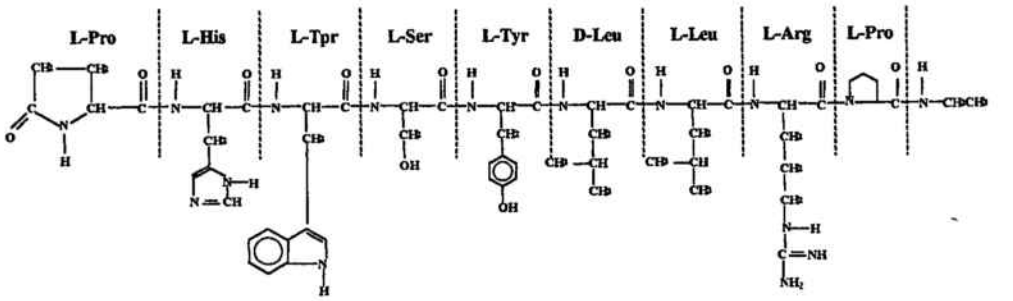


Fig. 1. Structure of leuprolide.

parallel reactions of the side chains of some amino acidic residues, *tert.*-butylated products and racemics [6]. So, the purification of the target peptide and the characterization of the impurities of the crude of synthesis must be solved before the peptidic analogue can be commercialized, in order to obtain a product that fulfils the legal requirements [7]. Therefore, laboratory preparative scale purification of these peptide mixtures needs to be considered. Taking the complexity of crudes of synthesis into account, the preparation of significant amounts of purified peptides requires firstly assessment of specifically analytical scale conditions. Also, purification and characterization allow optimization of the synthetic process, to make it economically beneficial.

The two most commonly used techniques to purify and characterize new synthesis products, liquid chromatography (LC) and capillary electrophoresis (CE) [8–11], lack the mass specificity necessary to allow unambiguous characterization of such complex products. LC and/or CE coupled with mass spectrometry (MS) have proved to be highly efficient for fast and reliable analysis and characterization of crude synthetic products [12–15]. Moreover, in order to achieve a fast optimization of the separation process, the method of linear solvation energy relationships (LSERs) can predict the chromatographic retention [16–19]. In this study, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^N scale of solvent polarity and the chromatographic retention measured by the capacity factor, k'. Also characterization of the side products associated with the target peptide leuprolide has been successfully carried out by coupling LC to MS using an electrospray ionization interface (LC–ES-MS). Although new ionization techniques have been developed, electrospray ionisation is the most used in the case of peptides [20–24], due to its sensitivity and its ability to analyze large, thermally labile biomolecules and to provide accurate molecular mass measurements using mass analyzers with limited range such as quadrupoles (m/z 2500).

The powerful coupling LC–ES-MS has become an important aid for rapid and reliable identification of the target peptide and has furnished comprehensive information on other reaction products in the multicomponent peptide mixtures of crude of synthesis of leuprolide peptidic hormone.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm, acetonitrile (MeCN) (Merck, Darmstadt, Germany) and dioxane (Merck) were of LC grade. Trifluoroacetic acid (TFA), potassium hydrogenphthalate (dried at 110°C before use) were all analytical grade obtained from Merck. The crudes of synthesis of leuprolide (5-oxo–Pro–His–Trp–Ser–Tyr–D-Leu–

Leu–Arg–Pro–NHEt) used in this study was supplied by Lipotec (Barcelona, Spain). Leuprolide crudes were dissolved using the mobile phase as solvent, at concentrations of 1 and 3 mg/ml for LC–UV analysis and LC–ES-MS analysis, respectively, and were stored in a freezer at 0°C when not in use. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by bubbling helium. The samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Apparatus

For the LC–UV experiments an ISCO (Lincoln, NE, USA) Model 2350 chromatographic pump with a Valco injection valve with a 20-μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 220 nm were used. The chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller Software (ISCO) running on a personal computer. A 5 μm Kromasil C_8 column (250×4.6 mm I.D.) (BC Aplicaciones Analíticas, Barcelona, Spain) was used at room temperature.

The electromotive force (e.m.f.) values used to calculate the pH of the mobile phase, as in previous works [25], were measured with a potentiometer Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA) with a precision of ±0.1 mV. The potentiometric system was calibrated using a standard reference solution of potassium hydrogenphthalate 0.05 mol/kg [17] whose reference pH values in the acetonitrile–water mixtures studied were known [26–28].

LC–ES-MS experiments were performed using two Phoenix 20 syringe pumps (CE Instruments, Milan, Italy) with a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 100-μl sample loop, coupled to a VG Platform II quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with a nebulizer-assisted electrospray source. The high-flow nebulizer was operated in a standard mode with N_2 as both nebulizing (15–20 l/h) and drying (400 l/h) gas. Separation was performed at ambient temperature on a 5 μm Kromasil C_8 column (250×4.6 mm I.D.) with 1 ml/min flow-rate. 0.3 ml/min of dioxane were added post-column to improve sensitivity. The total flow was split to allow an effective flow 60 μl/min into the source of the spectrometer. Instrument control and data analysis were performed using Masslynx application software from Micromass. The mass data represent the average of three separate measurements.

2.3. Procedures

2.3.1. LC–UV procedure

For the optimization of the mobile phase composition, the solution used as mobile phase was made of different acetonitrile–water mixtures containing 0.1% (v/v) TFA (pH 1.9–2.0) [16], at several concentrations of acetonitrile from 25% to 35% (v/v). The flow-rate of mobile phase was 1 ml/min. Capacity factors were calculated from $k'=(t_R-t_0)/t_0$, where t_0 is the hold-up time, established for every mobile phase composition using 0.01% (w/v) bromide solution in water [29], and t_R is the retention time of the target peptide and the associated products. The retention times and the capacity factors of the solutes were determined from three injections of 1 mg/ml solution of leuprolide crude at each mobile phase composition considered and monitoring the signal at 220 nm. The pH was measured in the mixed mobile phase in which the chromatographic separation took place [30].

2.3.2. LC–MS procedure

2.3.2.1. Optimization of the source and analyzer parameters

The source and analyzer parameters were optimized using electrospray ionization of 0.1 mg/ml crude solution in MeCN–water (31:69), 0.1% (v/v) TFA, introduced directly into the ES source. Parameters were optimized in order to obtain the best signal, stability of the measurement and the highest sensitivity of the target peptide leuprolide. Optimum conditions were as follows: flow-rate, 60 μl/min; capillary voltage 4000 V; counter electrode voltage, 250 V; sample cone voltage, 70 V; source temperature, 90°C; ion energy, 3 V. Fragmentation was also studied under these working conditions. At lower sample cone voltages ES yields simple mass spectra

with negligible fragmentation, allowing correct identification of the molecular identities. For this reason, spectra were obtained using extraction voltages of 40 V, as far as possible. At percentages of acetonitrile of 25 to 29%, extraction voltages of 70 V were used owing to the analyte signal reduction observed at lower acetonitrile compositions, probably due to the higher surface tension of the solution, which results in an inefficient spray process [31].

2.3.2.2. Identification of side products

For the identification of the side products of the crude of synthesis, 3 mg/ml solution of leuprolide crude was injected into the LC–ES-MS system, using acetonitrile–water containing 0.1% (v/v) TFA (28:72 and 33:67, v/v) as mobile phase. The pH measured in these mixtures ranged between 1.9 and 2. ES spectra of target peptide leuprolide and associated side products were obtained at the optimum conditions of the mass spectrometer and working at full scan mode (m/z 400–1500).

3. Results and discussion

3.1. LC–UV

The synthesis crude of leuprolide was first examined by analytical LC with UV detection. The resulting chromatograms contain a major peak corresponding to the target peptide leuprolide, as well as a number of peaks of unidentified peptidic substances (I1, I2, I3, I4, I5 and I6) (Fig. 2). To optimize the composition of mobile phase, the LSER method, which is based on the Kamlet–Taft multiparameter scale, was used in the same way as in previous works [16–19]. The LSER approach applied to chromatographic processes [32–35] correlates retention parameters of solutes with characteristic properties of solutes and both stationary and mobile phases. This correlation can be expressed as follows in a system with a fixed pair of solute and stationary phase [16,17]:

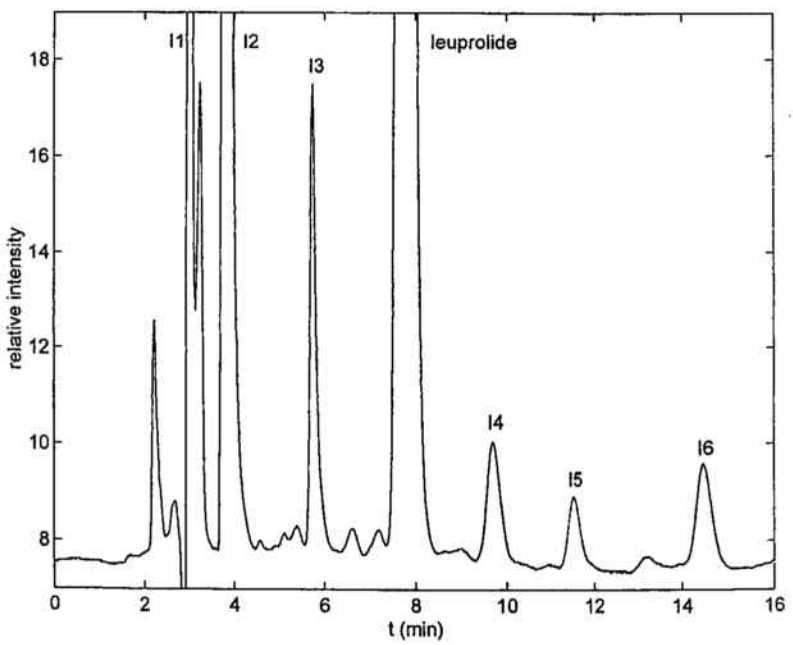


Fig. 2. UV chromatogram of a solution containing 1 mg/ml of a crude of synthesis of leuprolide. Mobile phase: MeCN–water (31:69), 0.1% (v/v) TFA.

$$\log k' = (\log k')_0 + s\pi^* + a\alpha + b\beta \quad (1)$$

where the coefficients $(\log k')_0$, s, a and b depend on the solute and the stationary phase properties and π^*, α and β are the solvatochromic parameters used to evaluate solvent dipolarity/polarizability [36], hydrogen-bond acidity [37] and hydrogen-bond basicity [38], respectively. Taking into account that the values of β are constant over most of the acetonitrile composition range [39,40] and the parameters E_T^N [41], π^* and α correlate according to $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [42], Eq. (1) can be reduced to Eq. (2) as follows:

$$\log k' = c + eE_T^N \quad (2)$$

This relation has been applied in previous studies for predicting the retention of analytes with very different structural characteristics [16–19]. In this work, k' values of leuprolide and associated side products, were obtained using acetonitrile–water systems as mobile phases, with acetonitrile percentages from 25% to 35% (v/v) and are shown in Table 1. The values of log k' were plotted versus the E_T^N values of the mixtures studied in Fig. 3, where a good linearity, greater than 0.99, was observed. This linearity allows prediction of the elution behavior of peptide derivatives and hence optimization of eluent composition for best separation from only two experimental measurements of k' values [16–19].

In order to examine the accuracy of retention prediction by Eq. (2), the selectivity for adjacent solute pairs calculated in the usual way, $\alpha = k_i'/k_j'$, was employed. Theoretical selectivity values were obtained using predicted k', calculated through Eq. (2) from only two experimental points, corresponding to the lowest and the highest acetonitrile percentages of the interval studied. Fig. 4 plots experimental α values as points, calculated using experimental k' values given in Table 1, and theoretical α values as solid lines vs. the acetonitrile percentage, showing a good concordance of both selectivity values. For our particular case, the composition interval between 28% and 32% of acetonitrile provided the best ratio selectivity/analysis time: working under 28% of acetonitrile the analysis times were too large, and over 32% of acetonitrile selectivity between impurity 3 and leuprolide was poor.

Moreover, a good resolution between all the analytes of interest in the leuprolide crude was our main goal. Fig. 5 shows variation of resolution (R_s) for the solute pairs versus percentage of acetonitrile in the mobile phase. Solid lines indicate theoretical resolution values obtained using Eq. (3):

$$R_s(\text{theo}) = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{1 + k_2'}\right) \quad (3)$$

where N is the number of theoretical plates [$N = 16(t_R/w)^2$], α the theoretical selectivity and k' the capacity factors calculated using Eq. (2). The points indicate experimental resolution values obtained from the typical relation: $R_s(\text{exp}) = (t_{R2} - t_{R1})/(w_2 - w_1)$.

Only resolution between solutes whose separation was critical was considered. The concordance of the theoretical and experimental R_s values in Fig. 5 confirms accurate estimations of resolution via Eq.

Table 1
Capacity factor values of the leuprolide and the associated products, and the E_T^N parameter values, at the percentages of acetonitrile assayed in the mobile phase

% (v/v) MeCN	E_T^N	k'(I1)	k'(I2)	k'(I3)	k'(leuprolide)	k'(I4)	k'(I5)	k'(I6)
25	0.877	0.360	0.878	2.21	14.84	22.70	28.61	33.33
26	0.873	0.339	0.816	2.04	10.57	15.85	19.74	23.74
27	0.869	0.337	0.778	1.93	7.60	11.10	13.88	17.03
28	0.865	0.330	0.750	1.84	5.87	8.42	10.48	13.12
29	0.862	0.318	0.711	1.72	4.357	6.10	7.59	9.64
30	0.859	0.310	0.665	1.61	3.067	4.24	5.30	6.91
31	0.855	0.300	0.631	1.51	2.332	3.24	4.03	5.33
32	0.852	0.295	0.616	1.44	1.860	2.55	3.16	4.24
33	0.849	0.299	0.599	1.38	1.492	2.02	2.52	3.39
34	0.847	0.282	0.572	-	1.161	1.56	1.94	2.65
35	0.844	0.281	0.562	-	0.920	1.23	1.54	2.09

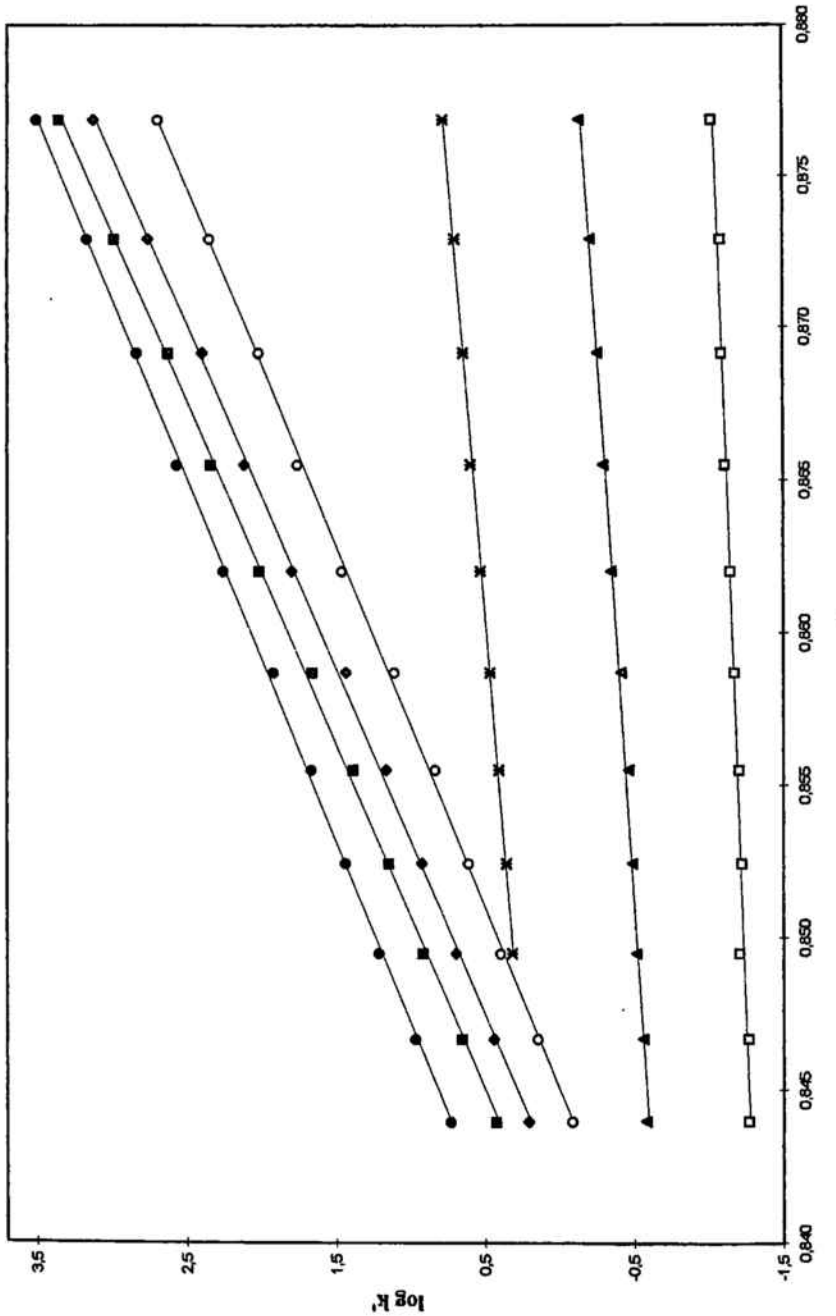


Fig. 3. Plots of log k' of leuprolide and impurities versus E_T^N parameters of the mobile phase: impurity I1 (□), impurity I2 (▲), impurity I3 (*), leuprolide (○), impurity I4 (◆), impurity I5 (■) and impurity I6 (●).

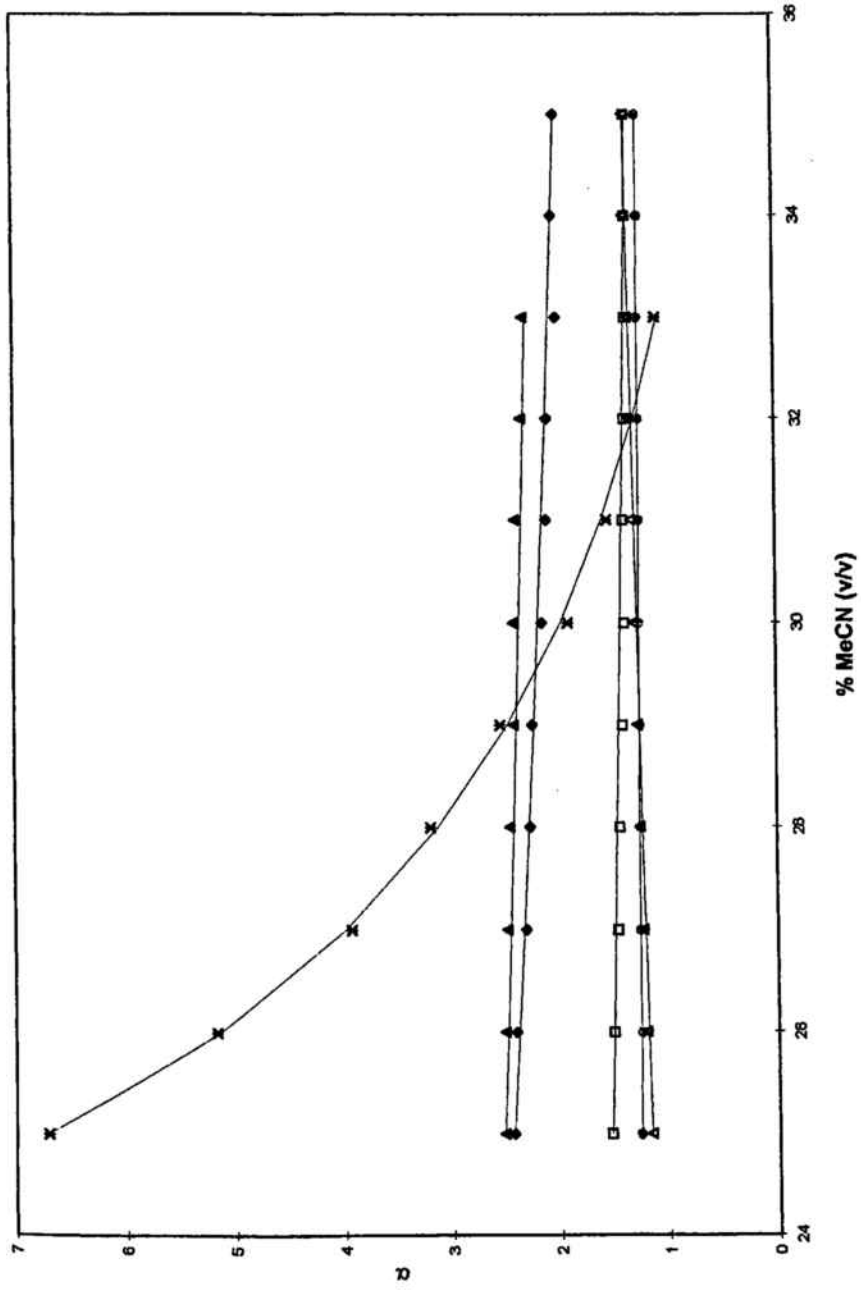


Fig. 4. Plots of selectivity between adjacent pairs of solutes versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of selectivity and points indicate experimental values of selectivity: impurity I1/impurity I2 (♦), impurity I2/impurity I3 (▲), impurity I3/leuprolide/impurity I4 (□), impurity I4/impurity I5 (●) and impurity I5/impurity I6 (△).

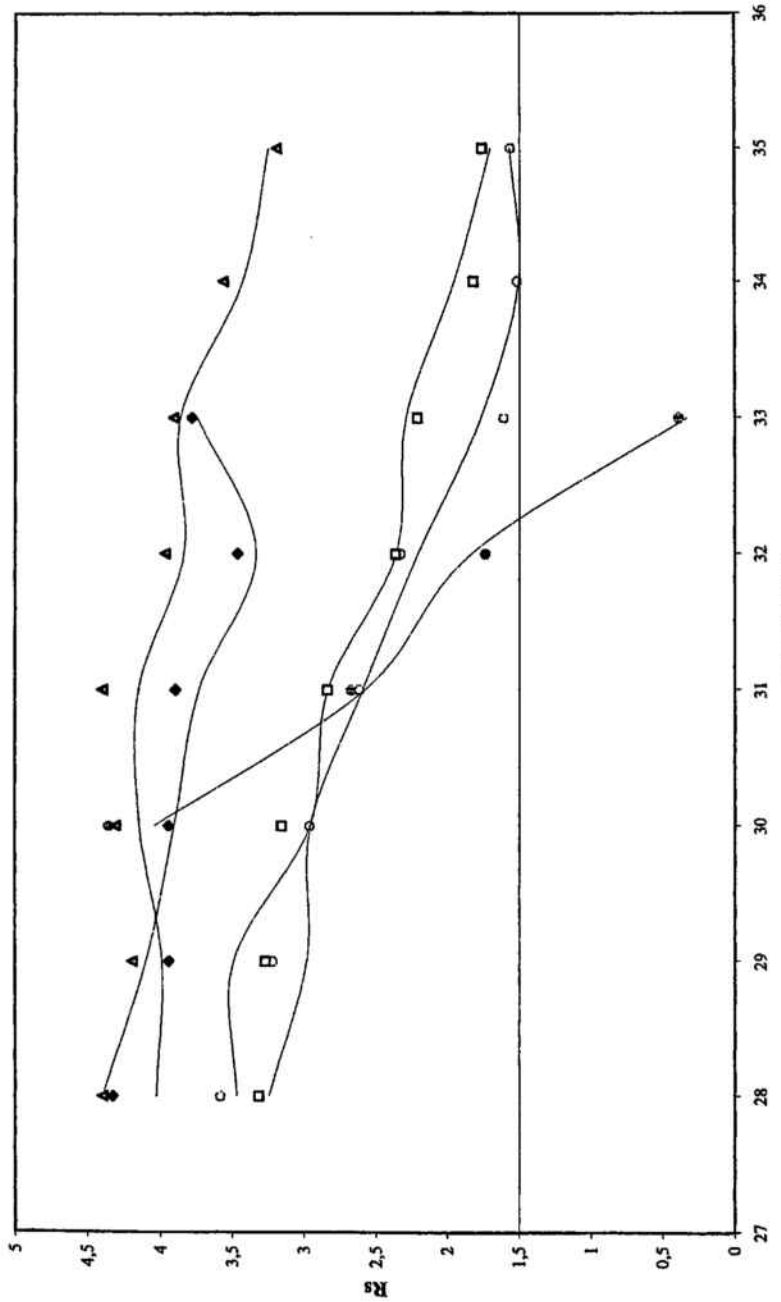


Fig. 5. Plots of resolution between adjacent pairs of solutes versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of resolution from Eq. (3) and points represent experimental values of resolution: impurity 12/impurity 13 (♦), impurity 13/leuprolide (●), leuprolide/impurity 14 (○), impurity 14/impurity 15 (□) and impurity 15/impurity 16 (▲).

(2) from only two experimental measurements. From Figs. 3, 4 and 5 we conclude that the optimum separation between the target peptide and the side products present in the crude could be achieved at percentages of acetonitrile in the mobile phase between 28 and 32% (v/v). The chromatogram of the crude of synthesis of leuprolide using UV detection obtained from injection of 1 mg/ml solution at optimal conditions is shown in Fig. 2. On the other hand, the mobile phase recommended for the purification of leuprolide using preparative chromatography is 31% (v/v) acetonitrile in water, 0.1% (v/v) TFA; this eluent provides high resolutions ($R_s \cong 3$) and suitable analysis times.

3.2. LC–MS

LC–MS was performed under the conditions described in the experimental section. In order to improve the analytical signal obtained, post-column additions of several solvents (acetonitrile, propionitrile, ethanol, methanol, dioxane and tetrahydrofuran) were made [31,43]. The addition of 0.3 ml/min of dioxane provided a signal enhancement (Fig. 6), whereas no net improvement was observed with the other solvents.

In order to study the synthetic subproducts that eluted near to leuprolide, a mobile phase with an acetonitrile percentage of 28% (v/v) was chosen (Fig. 7a). On the other hand, working with richer acetonitrile mobile phases (Fig. 7b), additional impurities (I7 to I15) were observed. These side products did not interfere in the leuprolide purification because of their different retention times, but their characterization could be very useful for the later synthetic process optimization. Comparing the resulting total ion current (TIC) chromatogram of Fig. 7a with the UV chromatogram of Fig. 2, very different intensities for some impurities as I1, I2 and I3 can be observed. They could be non-peptidic substances involved in the synthesis such as solvents and auxiliary reagents, with chromophor groups, which show good UV detection but they can not be observed using mass spectrometry. This is in good agreement with the different slopes observed in the plot log k' vs. E_T^N for these compounds with respect to the peptidic impurities (Fig. 3). On the other hand, the peaks A–E in Fig. 7a and Fig. 7b, are side products detected by mass spectrometry but not assigned in the UV chromatogram.

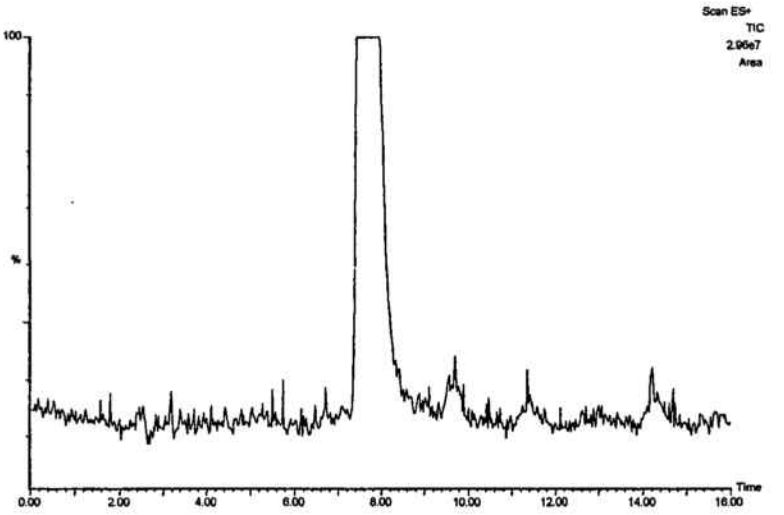
All the spectra associated with the chromatographic peaks observed in the TICs are shown in Figs. 8 and 9. The mass-to-charge ratios observed, the respective charged states and the molecular masses measured for each substance are summarized in Table 2, as well as the assignment of each ion on each peptide. The molecular masses were calculated from both the singly and doubly charged present in the spectra [44]. It is important to consider the method of the peptide synthesis and the reagents added in each step of the synthesis procedure in order to identify the target peptide and most associated impurities [45]. Leuprolide was prepared by Lipotec applying a linear SPPS method previously developed and optimized, which consists in coupling successively each amino acid of the sequence until it has been completed. After the last coupling, peptide was cleaved from the resin and deprotected by acidolytic treatment, and amidated to obtain the final product leuprolide, Fig. 1. In order to avoid consecutive additions [46] in a coupling step, protection of the α-amino group was carried out using the group 9-fluorenylmethoxycarbonyl (Fmoc). In the same way, reactivity of side chains was avoided by protection with 2,2,5,7,8-pentamethylcroman-6-sulfonyl (Pmc) to block the α-amino group of proline, *tert.*-butyl groups (^{t}Bu) for the hydroxyls of the side chains of tyrosine and serine and triphenylmethyl group (Trt) to block the tertiary amino group of the imidazole ring of tryptophan [6].

3.2.1. Target peptide

The electrospray spectra of the target peptide leuprolide, at retention time $t_R = 13.14$ min (28% MeCN) and 5.82 min (33% MeCN), are shown in Fig. 8e and Fig. 9a, respectively. The two charge states observed are: m/z 1210.0 $[L+H]^+$ and m/z 605.8 $[L+2H]^{2+}$. These ions are associated with the molecular mass 1209.3 which is in good agreement with the expected molecular mass 1209.4. No significant fragmentation is observed at a cone sample voltage of 40 V, but using 70 V the fragmentation present does not difficult this unambiguous assignment of the molecular peak with one and two charges.

The spectra of the side products identified in the

a) Without post-column addition



b) With post-column addition of 0.3 ml/min of dioxane

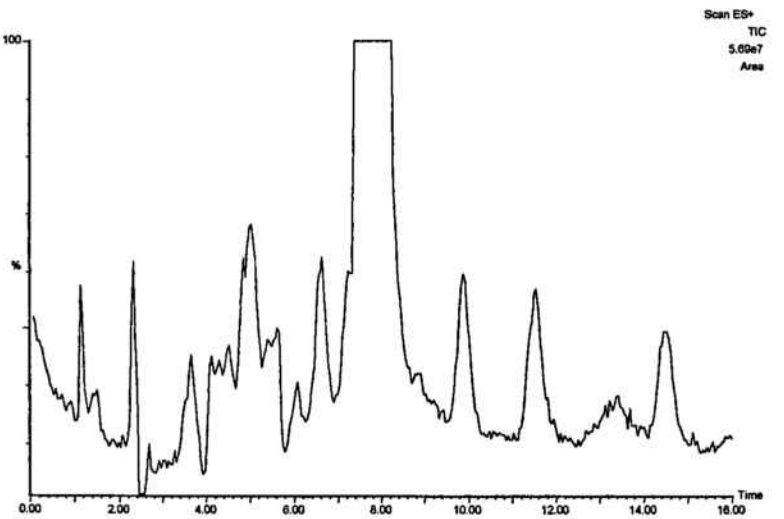


Fig. 6. Total ion current (TIC) chromatogram of a 3 mg/ml solution of leuprolide crude, with a mobile phase of MeCN–water (31:69), 0.1% (v/v) TFA. (a) Without post-column addition, (b) with post-column addition of 0.3 ml/min of dioxane. Time scales in min.

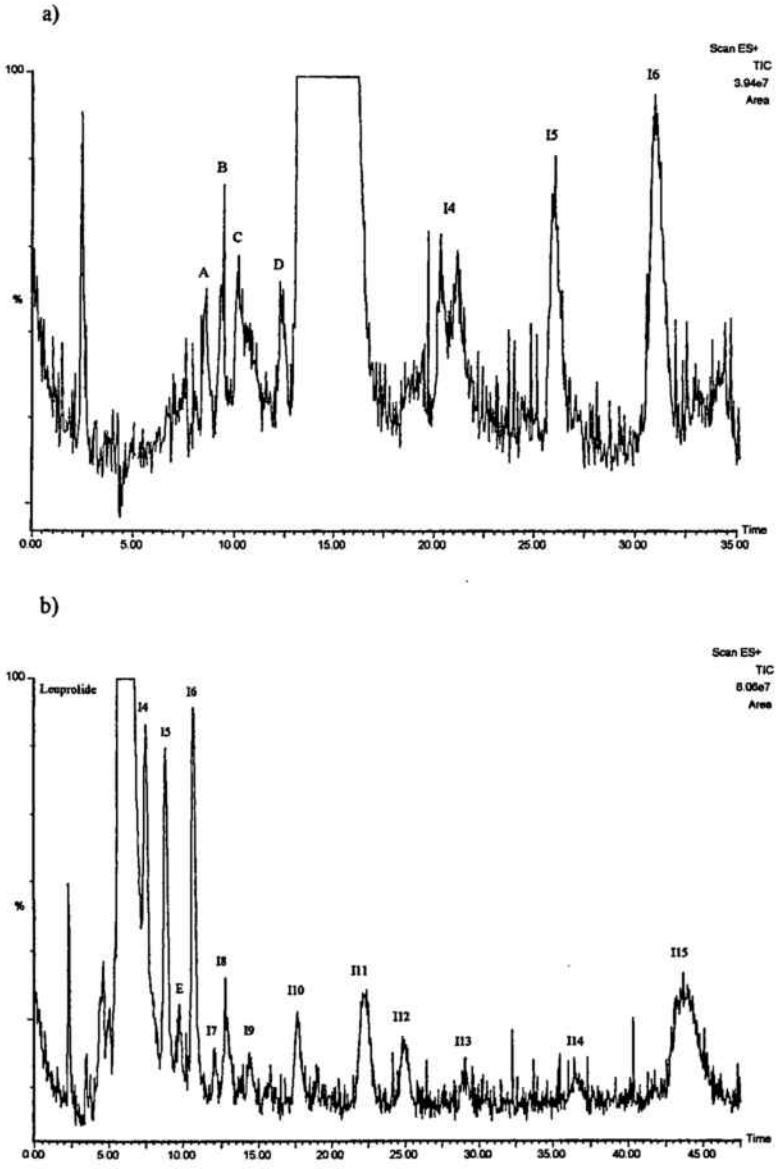


Fig. 7. (a) Total ion current (TIC) chromatogram of a 3 mg/ml solution of leuprolide crude, with a mobile phase of MeCN–water (28:72), 0.1% (v/v) TFA. (b) TIC chromatogram of a 3 mg/ml solution of leuprolide crude, with a mobile phase of MeCN–water (33:67), 0.1% (v/v) TFA. Time scales in min.

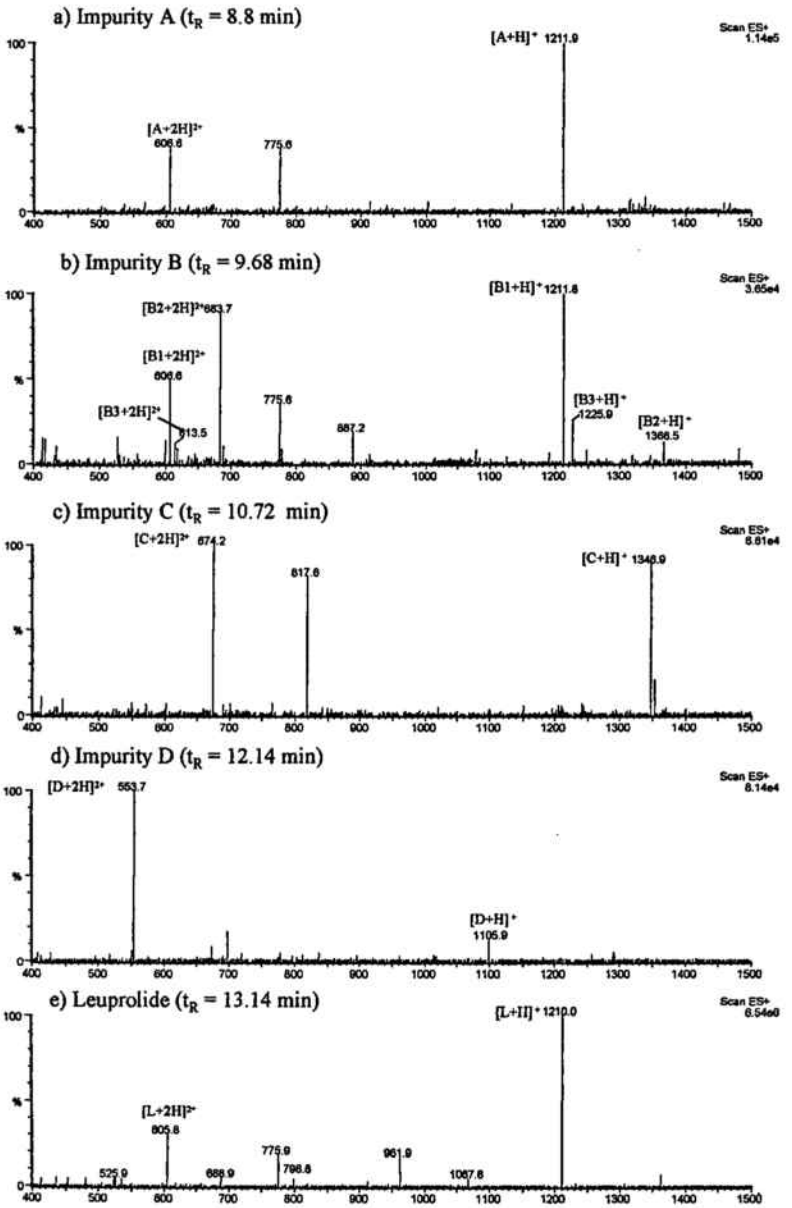


Fig. 8. Electrospray mass spectra associated with the TIC peaks at the retention time indicated in each case. Sample cone voltage: 70 V, mobile phase: MeCN–water (28:72), 0.1% (v/v) TFA. *x*-Axes: *m/z*.

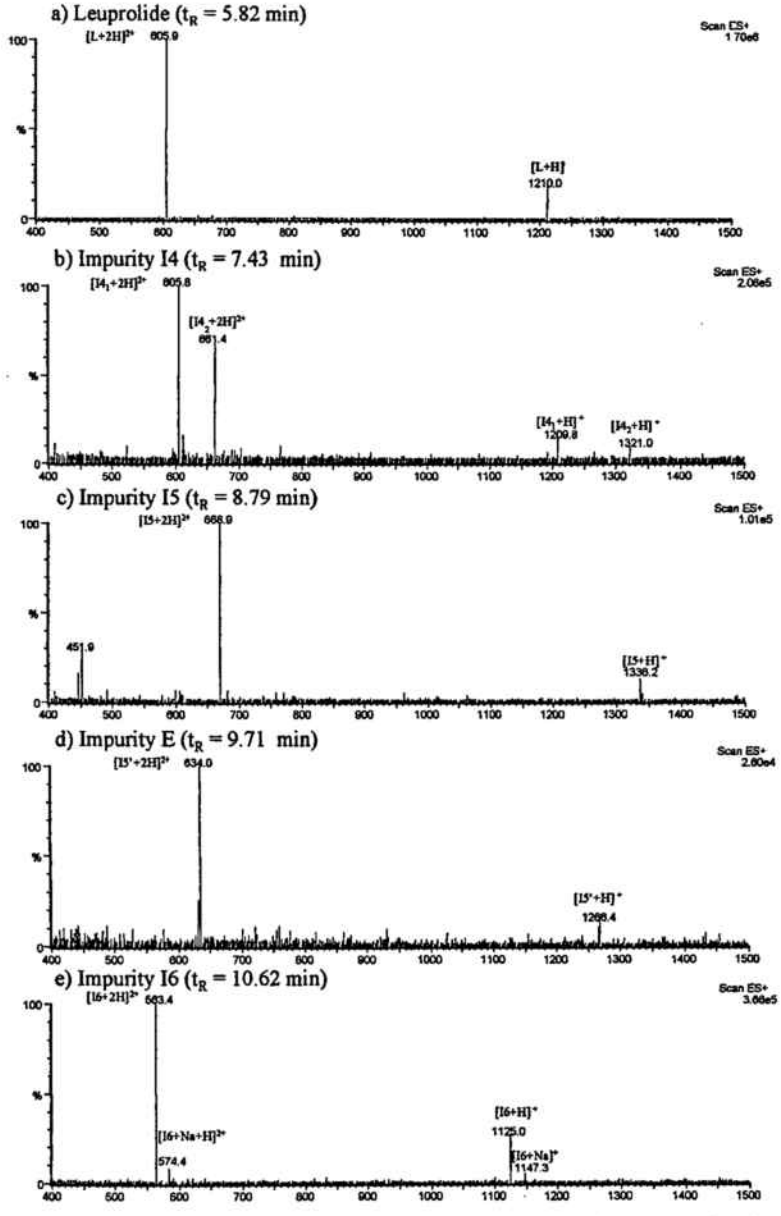


Fig. 9. Electrospray mass spectra associated with the TIC peaks at the retention time indicated in each case. Sample cone voltage: 40 V, mobile phase: MeCN–water (33:67), 0.1% (v/v) TFA. *x*-Axes: *m/z*.

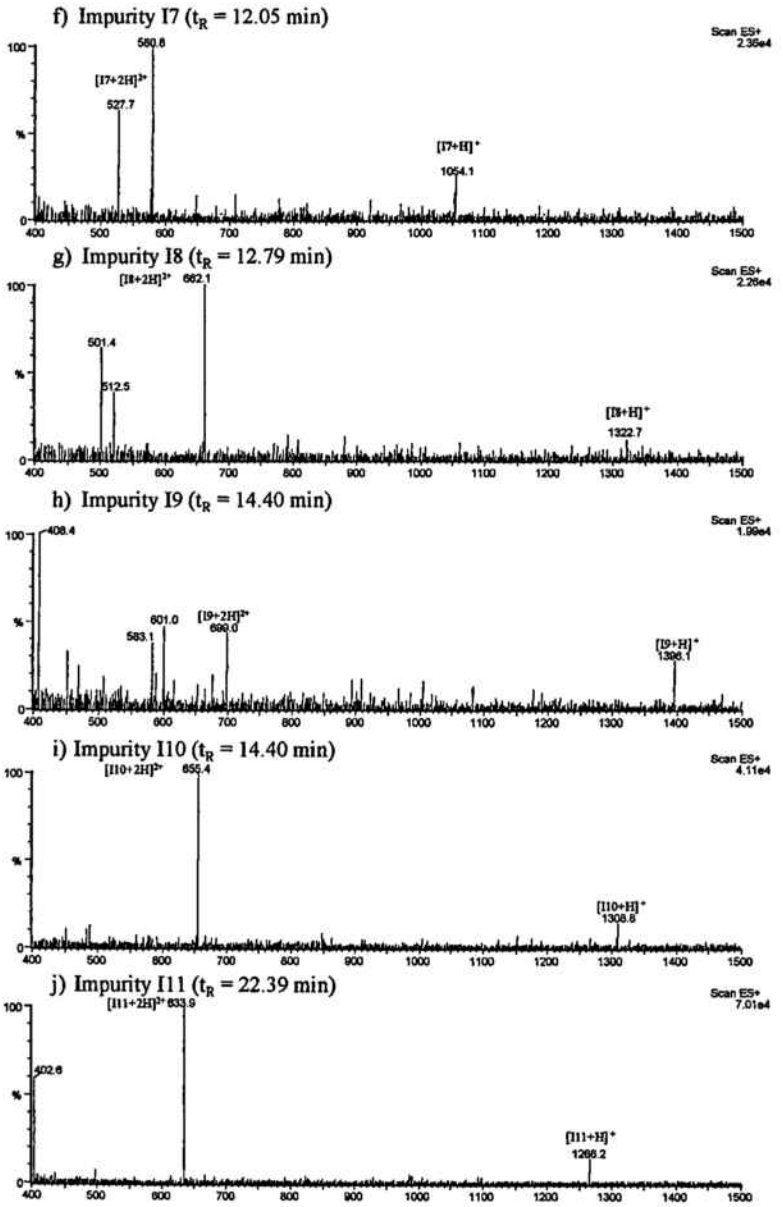


Fig. 9 (*continued*).

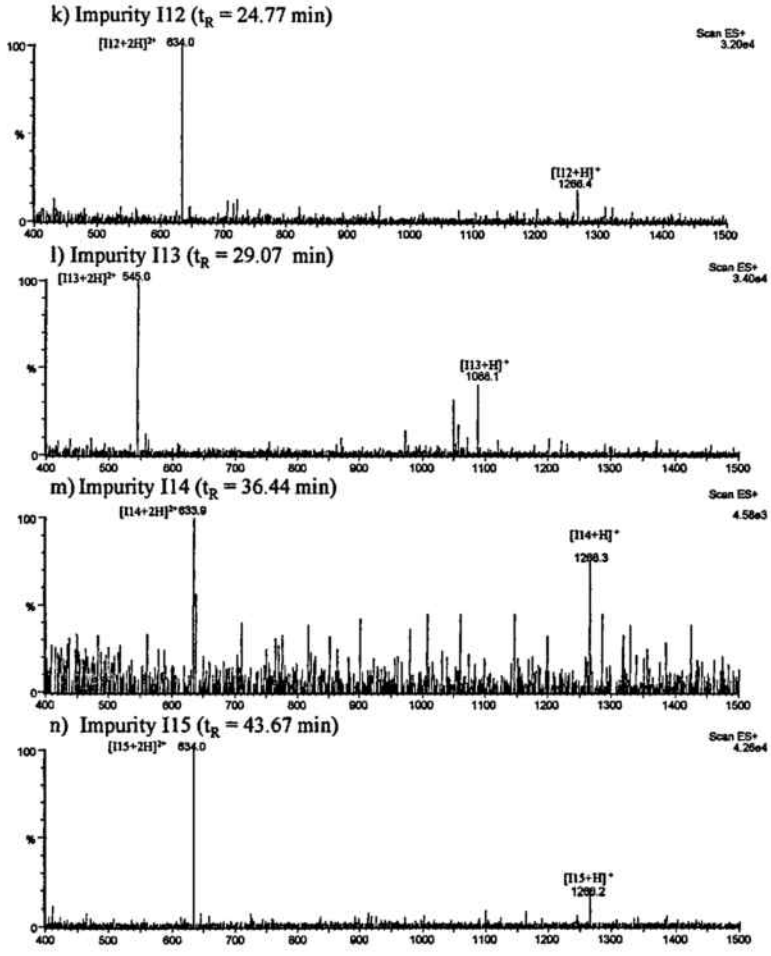


Fig. 9 (*continued*).

synthesis crude are shown in Figs. 8 and 9 and their identification is based on the experimental mass differences between the expected molecular mass of leuprolide, 1209.4, and the observed molecular mass of the side product [47].

3.2.2. Amino acidic insertions

There are some impurities in the crude of leuprolide, attributed to the insertion of an additional amino acid in the sequence while coupling step was carried out. The major reason for this occurrence in solid-phase peptide chemistry is the use of an excess of equivalents in the coupling step to assure the maximum coupling efficiency, taking into account the low coupling rates of some amino acids [46]. The spectrum corresponding to the chromatographic peak I9 (t_R = 14.4) (Fig. 9h) presents a double-charge state distribution: the m/z 1396.1 $[I9+H]^+$ and the m/z 699.0 $[I9+2H]^{2+}$, that yield the molecular mass 1395.5, 186.1 higher than the leuprolide molecular mass. This difference agrees with the insertion of an additional tryptophan in the sequence of leuprolide:

Table 2
Measured relative molecular masses (M_r) and proposed sequences associated with the TIC peaks in Fig. 7

Impurity	t_R (min)		Observed m/z[a]	Measured M_r	ΔM	Proposed sequence
	28% ACN	33% ACN				
			$[M+2H]^{2+}$ $[M+H]^{+}$			
A	8.80	–	606.6 1211.9	1211.1	+1.7	Leuprolide with a reduced tryptophan
B1	9.68	–	606.6 1211.8	1211.1	+1.7	Leuprolide with a reduced tryptophan
B2	9.68	–	683.7 1366.5	1365.5	+156.1	Additional arginine insertion
B3	9.68	–	613.5 1225.9	1225.0	+15.6	Leuprolide with an oxidized tryptophan
C	10.73	–	674.2 1346.9	1346.1	+136.7	Additional histidine insertion
Leuprolide	13.14	5.82	605.8 1210.0	1209.3	0	Leuprolide
$I4_1$	–	7.43	605.8 1209.8	1209.2	−0.2	Leuprolide, racemic
$I4_2$	–	7.43	661.4 1321.0	1320.4	+111.0	Fmoc–Hys–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–CO–$NHCH_2CH_3$
I5	–	8.79	668.9 1336.2	1335.5	+126.1	Formation of a substituted guanidine
E	–	9.71	634.0 1266.4	1265.7	+56.3	*tert.*-Butylated leuprolide
I6	–	10.62	563.4 1125.0 $[M+H+Na]^{2+}$ $[M+Na]^{+}$ 574.3 1147.2	1224.4	−85.0	Lack of a serine
I7	–	12.05	527.8 1054.0	1053.3	−156.1	Lack of an arginine
I8	–	12.79	662.1 1322.7	1321.9	+112.5	Doubly *tert.*-butylated leuprolide
I9	–	14.4	699.0 1396.1	1395.5	+186.1	Additional tryptophan insertion
I11	–	22.39	633.9 1266.2	1265.5	+56.1	*tert.*-Butylated leuprolide
I12	–	24.77	634.0 1266.4	1265.7	+56.3	*tert.*-Butylated leuprolide
I14	–	36.44	633.9 1266.3	1265.5	+56.1	*tert.*-Butylated leuprolide
I15	–	43.67	634.0 1266.2	1265.6	+56.2	*tert.*-Butylated leuprolide

Not identified: D, I10, I13

[a] Observed m/z represent the mean of three LC–ES-MS analyses.

5-oxo-Pro-His-(Trp)-Trp-Ser-Tyr-D-Leu-L-Leu

-Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity I9

The charge-to-mass ratios presents in the spectrum of peak B (t_R=9.68) (Fig. 8b): m/z 1366.5 $[B2+H]^+$ and m/z 683.7 $[B2+2H]^+$, show that one of the impurities that coelutes in this peak has a molecular mass of 1365.5 (difference of mass respect to leuprolide $\Delta M=+156.1$) attributed to the presence of an additional arginine in the sequence of leuprolide:

5-oxo-Pro-His-Trp-Ser-Tyr-D-Leu-L-Leu-(Arg)-

Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity B2

In the case of the chromatographic peak C (t_R= 10.73) (Fig. 8c) the charge-to-mass ratios: m/z 1346.9 $[C+H]^+$ and m/z 674.2 $[C+2H]^+$ are present, yielding a molecular mass of 1346.1 (leuprolide $\Delta M=+136.7$) that implicates the presence of an additional histidine in the leuprolide sequence:

5-oxo-Pro-(His)-His-Trp-Ser-Tyr-D-Leu-L-Leu

-Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity C

3.2.3. Incomplete deprotection: protected peptides, deletion peptides and truncated sequences

3.2.3.1. Protected peptides

The presence of impurities with a molecular mass that exceeds 56 the molecular mass of leuprolide is frequent in this peptidic mixture. This has been attributed to the presence in the sequence of a *tert.*-butylated amino acid, as a result of a failed deprotection of the side chains blocked with the *tert.*-butyl group during the chain elongation [6,46]. The spectra associated with the chromatographic peaks E (t_R= 9.71), I11 (t_R=22.39), I12 (t_R=24.77), I14 (t_R= 36.44) and I15 (t_R=43.67) show similar profiles as can be seen in Fig. 9d, j, k, m and n, respectively: they contain two charge state ratios corresponding to about the values of: m/z 1266 $[Ii+H]^+$ and m/z 634 $[Ii+2H]^{2+}$, yielding measured molecular masses between 1265.5 and 1265.7, that differ from the leuprolide mass by approximately +56. There are two possible *tert.*-butylation places: the hydroxyl groups of the side chains of serine and tyrosine, respectively, but also the indole ring of the side chain of tryptophan could undergo *tert.*-butylation. We also found in the mixture studied the impurity corresponding to the doubly terbutylated leuprolide: the spectrum of the chromatographic peak I8 (t_R= 12.79) (Fig. 9g) shows a charge state distribution with the m/z 1322.7 $[I8+H]^+$ and the m/z 662.1 $[I8+2H]^{2+}$; these ions are associated with the molecular mass 1321.9, and the difference of 112.5 with the molecular mass of leuprolide indicates the presence of two *tert.*-butyl groups in the molecule.

3.2.3.2. Truncated sequences

Problems during the deprotection process of the g-fluorenylmethoxycarbonyl (Fmoc) blocking groups could originate incomplete protected sequences [6,46].The spectrum of the peak I4 (t_R=7.43) (Fig. 9b) shows the coelution of two substances. The charge states m/z 1321.0 $[I4_2+H]^+$ and m/z 661.5 $[I4_2+2H]^{2+}$, correspond to the minor substance, yielding the molecular mass of 1320.4 (difference of mass with leuprolide, $\Delta M=+111.0$). This mass is attributed to this sequence:

Fmoc–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro

–NH–CH$_2$CH$_3$ impurity I4$_2$

3.2.3.3. Deletion peptides

Imperfections in the removal of blocking groups can also lead to the formation of chains from which one of the amino acid residues is absent. Such materials were designated "failure sequences" or "deletion sequences" [47]. The spectrum of peak I6 (t_R=10.62) (Fig. 9e) shows four charge states, corresponding to: m/z 1125.0 $[I6+H]^+$, m/z 563.4 $[I6+2H]^{2+}$ and m/z 1147.2 $[I6+Na]^+$, m/z 574.3 $[I6+Na+H]^{2+}$, yielding a molecular mass of 1124.4 ($\Delta M=-85.0$), which implies the loss of a serine residue:

5-oxo-Pro-His-Trp-( )-Tyr-D-Leu-L-Leu-Arg

-Pro-CO-NHCH$_2$CH$_3$ Impurity I6

In the case of peak I7 ($t_R = 12.05$)) (Fig. 9f) the spectrum shows two charge states: m/z 1054.0 $[I7+H]^+$, m/z 527.8 $[I7+2H]^{2+}$, that means that the impurity has a molecular mass of 1053.3 (difference of mass compared with leuprolide $\Delta M = -156.1$), associated to a side product with an arginine deletion in the leuprolide sequence:

5-oxo-Pro-His-Trp-Ser-Tyr-D-Leu-L-Leu-

Pro-CO-NHCH$_2$CH$_3$ Impurity I7

3.2.4. Side chain reactivity

The side chain of tryptophan is quite reactive in acidic conditions, and it can undergo oxidations, dimerizations or reductions [48]. In the case of chromatographic peak B ($t_R = 9.68$) (Fig. 8b) one of the products which coelute presents the charge states m/z 1225.9 $[B3+H]^+$ and m/z 613.5 $[B3+2H]^{2+}$, corresponding to a molecular mass of 1225.0, attributed to the oxidation of the indole ring of tryptophan, because the difference of mass compared with leuprolide is 15.6. But the reaction conditions change throughout the synthesis process, so the spectrum associated with peak A ($t_R = 8.8$) (Fig. 8a) shows two charge states, m/z 1211.9 $[A+H]^+$ and m/z 606.6 $[A+2H]^{2+}$, associated with the molecular mass 1211.1 ($\Delta M = +1.7$) that could be tentatively considered as an impurity with the same sequence of leuprolide but with the indole ring of tryptophan reduced. This reduction could be attributed to the presence of triisopropylsilane during the cleavage of the peptide–resin bond [49]. In the same way, one of the impurities that coelutes in chromatographic peak B (Fig. 8b) shows the same charge distribution (m/z 1211.8 $[B1+H]^+$ and m/z 606.6 $[B1+2H]^{2+}$) and it has been associated to a racemic of impurity A.

Histidine also shows reactivity in its side chain. The spectrum associated with peak I5 ($t_R = 8.79$) (Fig. 9c), shows the charge-to-mass ratios m/z 1336.2 $[I5+H]^+$ and m/z 668.9 $[I5+2H]^{2+}$. These ions are associated to the molecular mass 1335.5 ($\Delta M = +126.2$), attributed to the formation of a substituted guanidine by the reaction of the imidazol ring of the histidine side chain when a carbodiimide is added in the coupling step (Fig. 10) [46].

3.2.5. Racemization products

Among the undesired reactions that accompany various operations of peptide synthesis, racemization is the most general cause for concern, occurring in several steps of the synthesis process, for example during the activation and coupling or during the deprotection step and cleavage from the resin, influencing a lot of factors difficult to control [50]. High concentration of reactives are usually used in the coupling steps to secure high coupling rates, minimizing in this way the racemization; the use of additives like 4-dimethylaminopyridine (4-DMAP) or 1-hydroxybenzotriazole (HOBt) with the same purpose is also extended [51]. A racemic of leuprolide has been identified in I4 ($t_R = 7.43$) (Fig. 9b), which shows the charge states: m/z 1209.8 $[I4_1+H]^+$ and m/z 605.8 $[I4_1+2H]^{2+}$, yielding a measured molecular mass of 1209.2, that differs by 0.2 with respect to the expected mass of leuprolide.

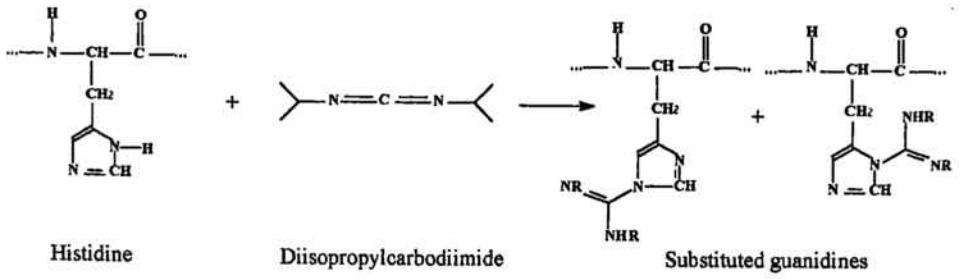


Fig. 10. Reaction of formation of a substituted guanidine.

4. Conclusions

The LC–ES-MS system has furnished fast and reliable information on the various products present in the crude of synthesis of leuprolide. The procedure followed for the synthesis of leuprolide appears to have resulted in partially deprotected peptide species, modified sequence peptide species due to the insertion of some amino acid, deletion peptide sequences, racemic peptides and modified species due to the reactivity of the side chains. So, the knowledge of the identities of the impurities present in the crude of synthesis allows one to optimize the synthesis procedure, improving the steps implicated, and fulfil the requirements for leuprolide commercialization as a therapeutic product.

Acknowledgements

Financial support of this project by DGICYT (Project PB95-0966) is gratefully acknowledged. The authors thank Lipotec, S.A. for the supply of the crude of leuprolide and F.B. also thanks the Generalitat de Catalunya for an F.I. Grant.

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Journal of Chromatography A, 889 (2000) 119–133

JOURNAL OF CHROMATOGRAPHY A

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Liquid chromatography–electrospray mass spectrometry of multicomponent peptide mixtures Characterization of a mixture from the synthesis of the hormone goserelin

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Abstract

In order to separate and characterize the target peptide and the side-product peptide compounds of a synthesis mixture of the peptide hormone goserelin, liquid chromatography coupled to high-flow electrospray ionization mass spectrometry (LC–ES-MS) has been used. Goserelin is an important drug with recognized therapeutical application for palliative treatment of prostatic and breast carcinomas. Stepwise solid-phase peptide synthesis commonly results in unwanted side-products associated with incomplete peptide chains. Consequently, this procedure requires extensive purification and characterization of the final synthesis mixture. The method of linear solvation energy relationships has been applied to optimize the proportion of organic modifier of the mobile phase used in the established LC method. On the other hand, ES-MS has allowed rapid and reliable identification of the target peptide and the other impurities present in the goserelin synthesis products.

Keywords: Linear solvation energy relationships; Pharmaceutical analysis; Peptides; Goserelin; Hormones

1. Introduction

Nowadays, the importance of peptides is evident in many fields. These biomolecules play an important role as hormones and neurotransmitters in the design of new drugs, in clinical diagnosis, etc., [1,2]. Discovery of new, biologically active peptides over the past few decades boosted the need for simplified and rapid methods for synthesizing them. Addressing these needs, in 1962 Merrifield introduced solid-phase peptide synthesis (SPPS) that, indeed, caused a revolution in the entire peptide field, and its influence spread to other areas. Goserelin is among several peptide hormones that were synthesized using this procedure.

Goserelin is a parenteral synthetic analog of luteinizing hormone-releasing hormone (LHRH) or gonadotropin-releasing hormone (GnRH). It is used for the palliative treatment of advanced prostatic carcinoma and for the management of endometriosis. Substitution of two amino acids normally found in GnRH leads to sustained activity that aids in hormonal control of prostate and breast carcinomas [3].

Peptide hormones are manufactured increasingly for various purposes. Those synthesized for therapeutic uses or preclinical investigations must be rigorously tested for their purity. Because peptides synthesized by the SSPS method may contain closely

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00394-0

related peptide impurities that result from incomplete or side reactions, purification must be accomplished before the peptidic analog can be commercialized [4,5].

Liquid chromatography (LC) is the most versatile method for separation and purification of polypeptides [6–10]. Although a desired peptide separation may be obtained by trial and error, this may take many attempts with subsequent loss of time. The ability to predict the elution profiles of peptides on the basis of accurate quantitative relationships would greatly enhance the value of LC. In previous studies, the linear solvation energy relationship (LSER), based on the Kamlet–Taft multiparameter scale and on the E_T^N scale of polarity, was used to predict the retention of series of peptides [11], quinolones [12], diuretics [13] and anabolic steroids [14] in LC. This approach can be used to develop an LC methodology to separate target peptides from other, similar impurity peptide components, on the analytical scale and also on the preparative scale without lack of resolution.

The LSER formalism applied to chromatographic processes, and when a system with a fixed pair of solute and stationary phase is considered, can be expressed as follows [15–18]:

$$\log k = (\log k)_s + s\pi^*_m + a\alpha_m + b\beta_m \quad (1)$$

The independent term and the coefficients in Eq. (1) depend on solute and stationary phase parameters; the solvatochromic π^*_m parameter evaluates solvent dipolarity/polarizability [19]; and the solvatochromic parameters α_m and β_m evaluate solvent hydrogen bond acidity [20] and solvent hydrogen bond basicity [21] of the mobile phase, respectively. Taking into account that β_m values for acetonitrile (MeCN)–water mixtures, used here as mobile phases, are nearly constant [22,23] and that the observed correlation between the normalized Dimroth and Reichardt polarity parameter, E_T^N, [24] and π^* and α parameters, $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [25], Eq. (1) can be reduced to the single solvent parameter-dependent expression:

$$\log k = C + eE_T^N \quad (2)$$

The linear correlation between the chromatographic retention, represented by the logarithm of the retention factor, and the E_T^N provides a useful tool for predicting retention due to the good linearity obtained [11–14] and because a suitable prediction of retention for a specific solute in a fixed stationary phase can be achieved from only two experimental measurements of k at two different mobile phase compositions. Because of its accuracy and simplicity, we judged it to be the best available as descriptor of retention as a function of percentage of organic solvent in the mobile phase.

Commercialization of therapeutic peptides not only requires purification but also characterization of the side-products present in the mixture of synthesis. Furthermore, this characterization allows one to improve the process of synthesis by suitable modification of those steps in which potentially by-products are produced.

LC coupled with mass spectrometry (MS) has proved to be a highly valuable technique for detailed structural characterization and purity evaluation of peptide mixtures and crude synthetic products [26–30]. Electrospray ionization (ES), a relatively gentle technique generally leads to the formation of multiply charged analyte ions, permits the analysis of very large, intact biomolecules and has become one of the most successful interfaces between LC and MS [31].

In this study, a mixture from the synthesis of goserelin has been examined. Firstly, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^N parameter of solvent polarity and the retention data. The synthesis mixture was then analyzed by LC–ES-MS in the chromatographic conditions optimized with this LSER methodology. The molecular masses of various side products within the mixture were determined, and, on the basis of these molecular masses, their sequence has been proposed.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm, acetonitrile (Merck, Darmstadt, Germany) and dioxane (Merck) were of LC grade. Trifluoroacetic acid

(TFA), potassium bromide and potassium hydrogenphthalate (dried at 110°C before use) were all analytical grade obtained from Merck. The mixtures from the synthesis of goserelin [Pyr–His–Trp–Ser–Tyr–D-(^{t}Bu)Ser–Leu–Arg–Pro–AzGly–NH_2] used in this study were supplied by Lipotec (Barcelona, Spain). Goserelin mixtures were dissolved in the mobile phase at concentrations of 1 and 3 mg/ml, and were stored in a freezer at 0°C when not in use. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by bubbling helium through the solution. The samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Apparatus

For the LC–UV experiments, an ISCO (Lincoln, NE, USA) Model 2350 chromatographic pump, a Valco injection valve with a 20-μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 220 nm were used. The chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller Software (ISCO) running on a personal computer. A 5 μm Kromasil C_8 column (250×4.6 mm I.D.) (BC Aplicaciones Analíticas, Barcelona, Spain) was used at room temperature.

The electromotive force (e.m.f.) values used to calculate the pH of the mobile phase were measured with a Model 2002 potentiometer (±0.1 mV) (Crison Instruments, Barcelona, Spain) using an Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA). The potentiometric system was calibrated with a standard reference solution of 0.05 mol/kg potassium hydrogenphthalate [18], whose reference pH values in the acetonitrile–water mixtures studied were previously assigned [32].

LC–ES-MS experiments were performed using two Phoenix 20 syringe pumps (CE Instruments, Milan, Italy) with a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 100-μl sample loop, coupled to a VG Platform II single quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with a nebulizer-assisted electrospray source. The high-flow nebulizer was operated in a standard mode with N_2 as nebulizing (15–20 l/h) and drying (400 l/h) gas. Separation was performed at ambient temperature on a 5 μm Kromasil C_8 column (250×4.6 mm I.D.) with 1 ml/min flow-rate. Dioxane (0.3 ml/min) was added post-column to improve sensitivity. The total flow was split to allow an effective flow of 60 μl/min into the source of the spectrometer. Instrument control and data analysis were performed using Masslynx application software from Micromass.

Mass calibration of the mass spectrometer was made using NaI. After calibration, a standard of goserelin was injected seven times to test the accuracy and precision of the mass measurements. The molecular mass obtained for goserelin was 1269.2, which is in good agreement with the expected 1269.4, and the relative standard deviation was 0.01%.

2.3. Procedures

2.3.1. LC–UV procedure

For the optimization of the mobile phase composition, different acetonitrile–water mixtures containing 0.1% (v/v) TFA were used. The acetonitrile percentage of these mixtures ranged from 24% to 30% (v/v). The flow-rate of mobile phase was 1 ml/min. Retention factors were calculated from $k = (t_R - t_0)/t_0$, where t_0 is the hold-up time, established for every mobile phase composition by means of a potassium bromide solution 0.01% (w/v) in water. The calculated average t_0 in this composition range was 2.20 min. The retention times and the retention factors of the solutes were determined from three injections of 1 mg/ml solution of goserelin crude at each mobile phase composition considered and monitoring the signal at 220 nm. The pH was measured in the mixed mobile phase in which the chromatographic separation took place, and it ranged from 1.9 to 2.0.

2.3.2. LC–MS procedure

2.3.2.1. Optimization of the source and analyzer parameters

The source and analyzer parameters were optimized, using electrospray ionization of a 0.1 mg/ml mixture of synthesis solution in MeCN–water (27:73), 0.1% (v/v) TFA, introduced directly into the ES source. Parameters were optimized in order to

obtain the best signal stability and the highest sensitivity of the target peptide, goserelin. Optimum conditions were as follows: flow-rate, 60 μl/min; capillary voltage 4000 V; counter electrode voltage, 250 V; sample cone voltage, 90 V; source temperature, 100°C; ion energy, 3 V. Fragmentation was negligible under this working conditions, which allowed correct identification of the molecular identities.

In order to improve the analytical signal obtained, post-column additions of several solvents at different flow-rates were made. The best results were obtained using dioxane at 0.3 ml/min.

2.3.2.2. Identification of side-products

For the identification of the side-products of the crude of synthesis, a 3 mg/ml solution of goserelin synthesis mixture was injected into the LC–ES-MS system, using MeCN–water (27:73, v/v) containing 0.1% (v/v) TFA as mobile phase (pH 1.94) with 0.3 ml/min of dioxane post-column addition. ES spectra of target peptide goserelin and associated side products were obtained in the positive ion mode at the optimum conditions of the mass spectrometer and working at full scan mode (m/z 400–1500).

3. Results and discussion

3.1. LC–UV

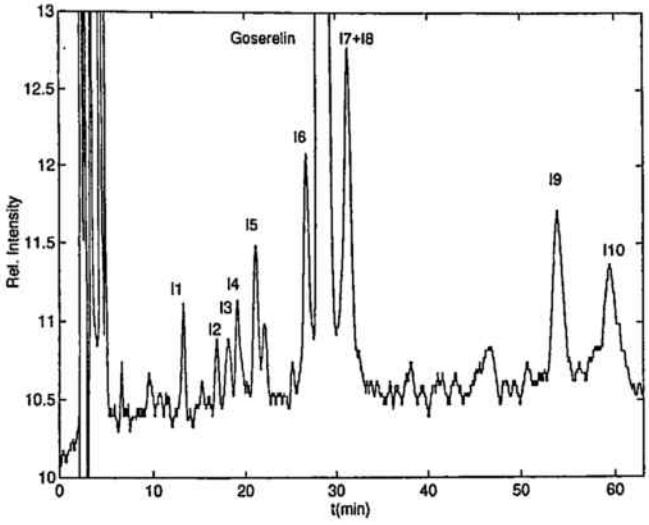
The synthetic product was first examined by analytical LC. The retention factor values, k, were obtained for the target peptide and for all the impurities at different percentages of acetonitrile from 24 to 30% (v/v) (Table 1). Owing to the large retention times for some of the substances studied, it has not been possible to obtain their retention factors in the whole acetonitrile–water range of mixtures. Selected UV chromatograms with mobile phases of MeCN–water (25:75) and (30:70) mixture, 0.1% TFA, are shown in Fig. 1. As it can be seen, chromatograms contain a major peak associated with the target peptide goserelin, as well as a number of minor peaks corresponding to unidentified peptide sequences, I1 to I13.

Chromatographic retention has been correlated with properties of the hydro–organic mixtures used as mobile phases; that is, the solvatochromic Reichardt's E_T^N parameter. Plots of log k of the substances studied here versus E_T^N values of acetonitrile–water systems are shown in Fig. 2. As can be observed and according to Eq. (2), log k and E_T^N correlate linearly ($r>0.99$) over the whole experimental range of acetonitrile content studied, which provides a good tool for predicting chromatographic retention of peptide derivatives. The use of Eq. (2) involves an important reduction of experimental retention data for the optimization of separation of solutes. Once the linearity of plots log k vs. E_T^N values has been verified, only two experimental measurements of retention factors for each compound considered at two different mobile phase compositions are sufficient to predict their retention behavior and hence for optimize their chromatographic separation and resolution in a fixed stationary phase.

Table 1
Logarithms of the retention factor values of the goserelin and the associated products, and the E_T^N parameter values, at the percentages of acetonitrile assayed in the mobile phase

MeCN (%, v/v)	E_T^N	Impurity I1	Impurity I2	Impurity I3	Impurity I4	Impurity I5	Impurity I6	Goserelin	Impurity I7	Impurity I8	Impurity I9	Impurity I10	Impurity I11	Impurity I12
24	0.881	0.8008	0.9160	0.9793	1.009	1.055	1.167	1.195	1.257	1.257	1.524	1.572	–	–
25	0.877	0.6943	0.8157	0.8520	0.8773	0.9298	1.041	1.068	1.115	1.115	1.365	1.409	–	–
26	0.873	0.5497	0.6449	0.6987	0.7286	0.7736	0.8784	0.9051	0.9324	0.9690	1.178	1.206	–	–
27	0.869	0.4339	0.5131	0.5808	0.6094	0.6523	0.7527	0.7791	0.7791	0.8448	1.031	1.052	1.309	1.387
28	0.865	0.3180	0.3826	0.4599	0.4853	0.5235	0.6534	0.6534	0.6534	0.7238	0.8955	0.8955	1.141	1.236
29	0.862	0.2068	0.2413	0.3385	0.3680	0.4035	0.5274	0.5274	0.5274	0.5925	0.7329	0.7329	0.9680	1.099
30	0.859	0.1348	0.1348	0.2667	0.2667	0.3040	0.4133	0.4133	0.4133	0.4780	0.5883	0.5883	0.7935	0.9527

a) Mobile phase: 25% MeCN : 75% H_2O, 0.1% TFA



b) Mobile phase: 30% MeCN : 70% H_2O, 0.1% TFA

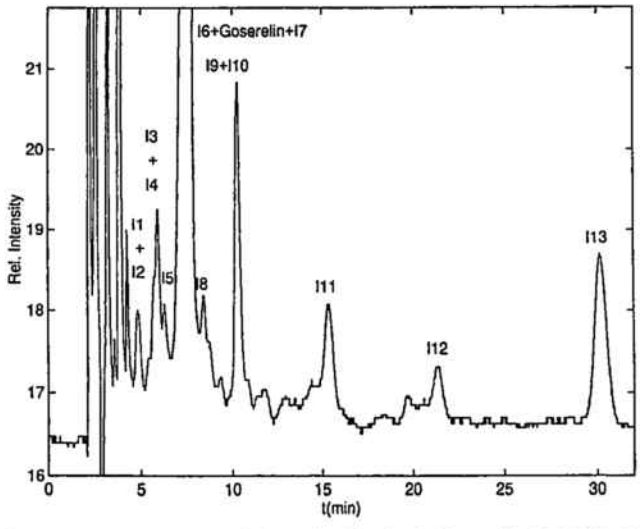


Fig. 1. UV chromatogram of a solution containing 1 mg/ml of a crude of synthesis of goserelin. (a) Mobile phase: MeCN–water (25:75), 0.1% (v/v) TFA. (b) Mobile phase: MeCN–water (30:70), 0.1% (v/v) TFA.

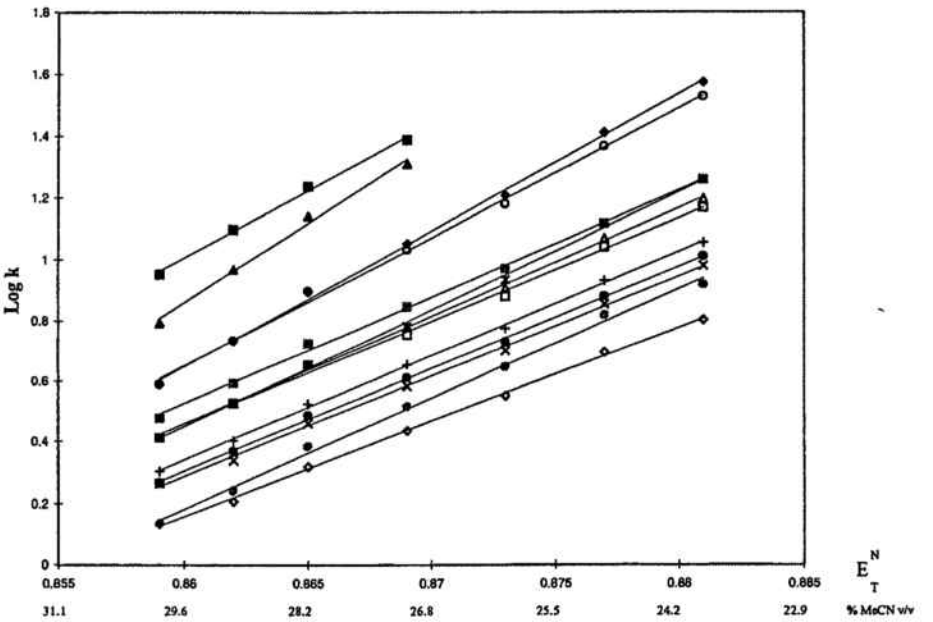


Fig. 2. Plots of log k of goserelin and impurities versus E_T^N parameters of the mobile phase: impurity I1 (◇), impurity I2 (⊗), impurity I3 (×), impurity I4 (●), impurity I5 (+), impurity I6 (□), goserelin (△), impurity I7 (✱), impurity I8 (⊠), impurity I9 (○), impurity I10 (♦), impurity I11 (▲) and impurity I12 (■).

In terms of fundamental chromatographic parameters, the resolution, R_s, between two adjacent peaks is given by:

$$R_s = \underbrace{\frac{1}{4}\cdot\sqrt{N_2}}_{\text{efficiency}} \cdot \underbrace{\left(\frac{\alpha-1}{\alpha}\right)}_{\text{selectivity}} \cdot \underbrace{\left(\frac{k_2}{1+k_2}\right)}_{\text{retention}} \quad (3)$$

Although the selectivity term is generally regarded as the most important in LC, to optimize resolution full attention must be given to all three terms in the fundamental resolution Eq. (3). Fig. 3 shows variations of R_s for solute pairs with the percentage of acetonitrile in the mobile phase. Only resolution between solutes whose separation was difficult was considered. Solid lines indicate resolution values obtained from two retention measurements using Eqs. (2) and (3), and points represent experimental resolution values obtained from the usual relation:

$$R_s = 2\cdot\frac{(t_{R2}-t_{R1})}{w_2+w_1} \quad (4)$$

where w_2 and w_1 are peak widths.

The concordance of the two sets of R_s values in Fig. 3 confirms accurate estimations of retention predictions and resolution via Eqs. (2) and (3) from only two experimental measurements per compound.

From Figs. 2 and 3, it can be concluded that good chromatographic separation between the target peptide and the impurities present in the crude can be achieved at percentages of acetonitrile in the mobile phase of 26–27% (v/v). On the other hand, the best resolution between goserelin and the adjacent impurities is achieved when the acetonitrile content in the mobile phase is 24%. Thus, the mobile phase recommended for the purification of Goserelin if preparative chromatography is used is acetonitrile–water (24:76), 0.1% TFA. For reliable identification of the target peptide and associate side-products by MS detection, an acetonitrile percentage of 27% (v/v) is to be preferred, owing to the improvement of the analytical response at higher acetonitrile percentages. This mobile phase composition provides a

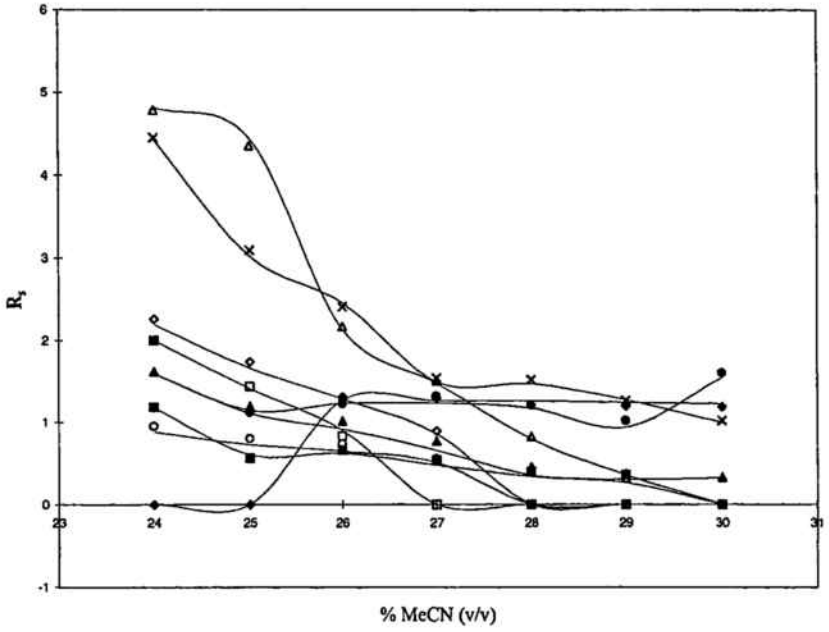


Fig. 3. Plots of resolution between adjacent pairs of solutes versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of resolution and points represent experimental values of resolution: impurity I1/impurity I2 (△), impurity I2/impurity I3 (●), impurity I3/impurity I4 (■), impurity I4/impurity I5 (▲), impurity I5/impurity I6 (×), impurity I6/goserelin (○), goserelin/impurity I7 (□), impurity I7/impurity I8 (♦) and impurity I9/impurity I10 (◇).

good separation among almost all the substances present in the synthesis mixture, allowing their characterization by MS without confusing overlaps.

3.2. LC–ES-MS

3.2.1. Characterization of the mixture of synthesis

LC–ES-MS was performed under the conditions described in the Experimental section. The effect of solvent composition on the detection of peptides in a complex mixture is critical. It has been observed that the use of high water percentages under ES conditions weakens the ES response because water does not allow an efficient droplet charging, and is less volatile and more difficult to spray than organic solvents [33,34]. The choice of an acetonitrile percentage of at least 27% (v/v) and the post-addition of a 20% (v/v) of dioxane provided a signal enhancement and allowed characterization of side-products. The total ion chromatogram of the crude of synthesis is given in Fig. 4. Besides the impurities assigned in Fig. 1 using UV detection, I1 to I13, other peaks detectable only by MS (A to F) appear in Fig. 4.

Figs. 5–7 show the spectra of the target peptide goserelin and also the spectra associated with some of the chromatographic peaks I1–I13 and A–F observed in the total ion current (TIC). The mass-to-charge ratios observed, the respective charged forms, and the average molecular masses calculated for each substance as well as the proposed sequences for each ion are summarized in Table 2. It can be observed in Table 2 that some chromatographic peaks contain several co-eluting impurities. The identification of these co-eluting impurities has been made from the data obtained at other acetonitrile percentages. In the same way, the impurity I13 has been identified from the ES data obtained at 29% (v/v) MeCN composition.

The method used to synthesize Goserelin provides

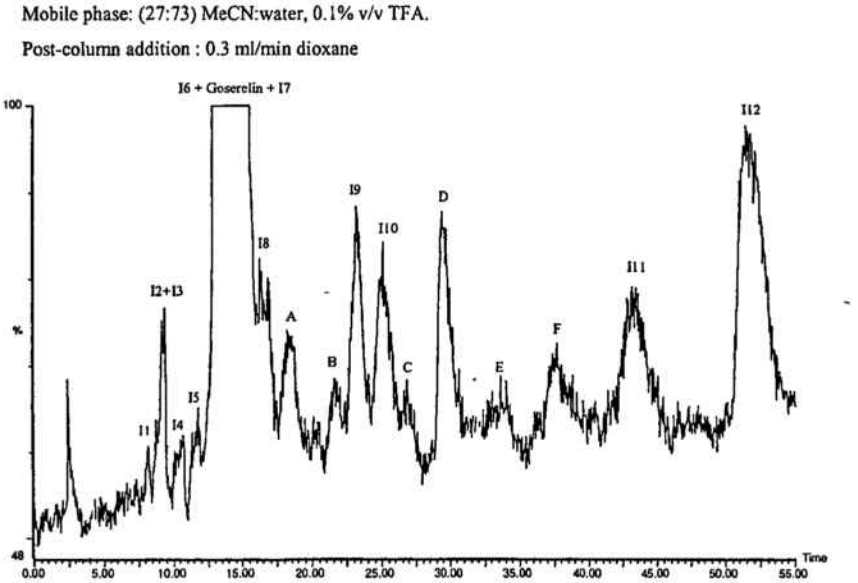


Fig. 4. Total ion current (TIC) chromatogram of the synthesis crude, with a mobile phase of MeCN–water (27:73), 0.1% (v/v) TFA, at optimal experimental conditions. Time scale in min.

useful information about the identity of the side products. Goserelin was prepared by a SPPS method, with the following protection scheme: 9-fluorenylmethoxycarbonyl (Fmoc) group to protect the α-amino group, 2-Cl-Trt (2-chloro-triphenylmethyl) group to protect the hydroxyl group of tyrosine side chain and Mmt (*p*-methoxytriphenylmethyl) group to protect the NH group in the imidazole ring of the histidine. After the last coupling, the peptide was cleaved from the resin and deprotected by acidolytic treatment, and finally amidated with the group azylglycinamida to obtain the final product.

3.2.2. Target peptide

The electrospray spectrum of the target peptide, eluting at $t_R = 13.27$ min, is shown in Fig. 5. The ion at m/z 1270.1, corresponding to $[M+H]^+$, and that at m/z 635.5 corresponding to $[M+2H]^{2+}$, associated with a molecular mass of 1269.1 are in good agreement with the calculated molecular mass of the goserelin, 1269.4.

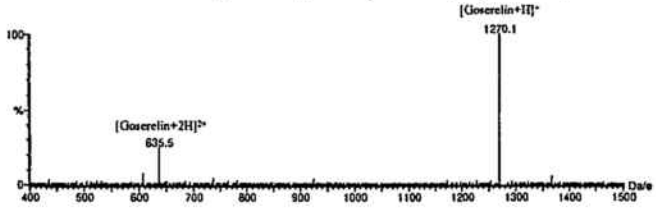
3.2.3. Impurities

The present identification of the side products [35,36] is solely based on the observed mass differences between the mass of goserelin, 1269.4, and the mass of the side product [37].

3.2.3.1. Racemization products

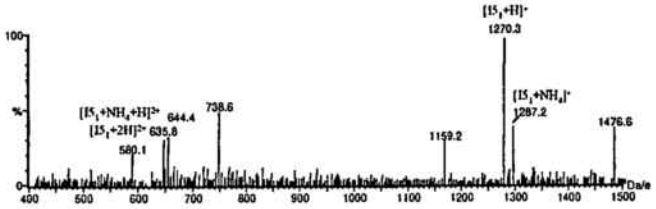
Racemization or isomerization of amino acids during the SPPS has been observed frequently [38], mainly in the activation and coupling steps. The use of a high concentration of reactives to secure high coupling rates and of some additives normally allows minimization of this undesired reaction. A racemate of goserelin has been identified in the chromatographic peak I5, the spectrum of which contains ions $m/z = 1270.3$ and $m/z = 635.8$, associated with a molecular mass of 1269.5 ($\Delta M = -0.1$).The $[M+NH_4]^+$ and $[M+NH_4+H]^{2+}$ adduct ions are also present in the spectrum. The remainder of the m/z ratios present in the spectrum will be assigned to other impurities afterwards.

a) TARGET PEPTIDE Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2



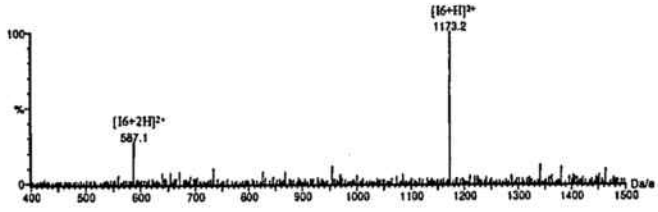
b) RACEMATE

• I5

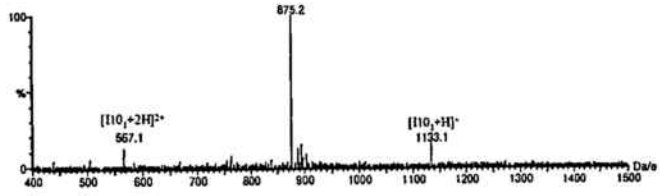


c) DELETION SEQUENCES

• I6 Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-□-AzGly-NH_2



•I10: I10$_1$ Pyr-□-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2



•I5$_3$ □-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2

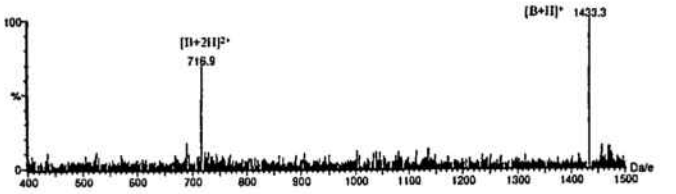
•I8$_1$ Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-□-NH_2

•E$_2$ Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-NH-NH_2

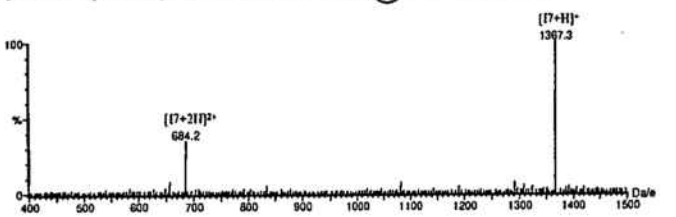
Fig. 5. Electrospray mass spectra associated with the chromatographic peaks indicated and structures associated with: (a) goserelin, (b) racemate of goserelin and (c) deletion sequences. Da/e=m/z.

a) AMINO ACIDIC INSERTIONS

•**A, B** Pyr-His-Trp-Ser-(Tyr)-Tyr-D-Ser(ᵗBu)-Leu-Arg-Pro-AzGly-NH_2



•**I7** Pyr-His-Trp-Ser-Tyr-D-Ser(ᵗBu)-Leu-Arg-(Pro)-Pro-AzGly-NH_2



•**I2** Pyr-His-Trp-Ser-Tyr-D-Ser(ᵗBu)-Leu-(Arg)-Arg-Pro-AzGly-NH_2

•**$I4_2$** Pyr-(His)-His-Trp-Ser-Tyr-D-Ser(ᵗBu)-Leu-Arg-Pro-AzGly-NH_2

•**E_1, F_1** Pyr-His-Trp-Ser-Tyr-D-Ser(ᵗBu)-(Leu)-Leu-Arg-Pro-AzGly-NH_2

•**I12** Pyr-His-Trp-[ ]-Tyr-D-Ser(ᵗBu)-Leu-Arg-(Pro)-Pro-AzGly-NH_2

b) DERIVATIVES OF THE GOSERELIN PRECURSOR

•**I9** Pyr-His-Trp-Ser-Tyr-D-Ser(ᵗBu)-Leu-Arg-Pro-CO-$N(CH_3)_2$

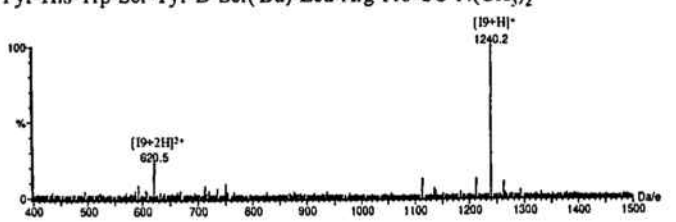


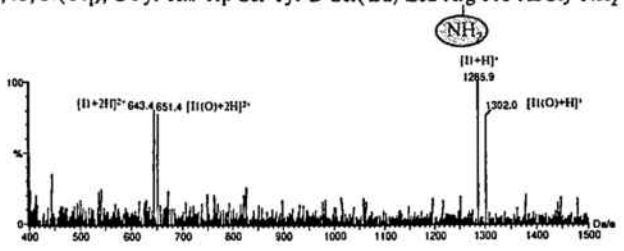
Fig. 6. Electrospray mass spectra associated with the chromatographic peaks indicated and structures associated with: (a) products of amino acidic insertions and (b) derivative of the goserelin precursor. Da/e = m/z.

3.2.3.2. Deletion sequences

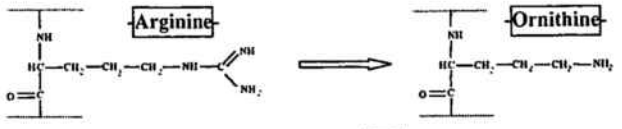
Incomplete removal of blocking groups lead to the formation of chains from which one of the amino acid residues is absent. Such materials have been designated "failure sequences" or "deletion sequences" [39]. Impurities $I5_3$, I6, $I8_1$ and $I10_2$ (chromatographic peaks I5, I8 and I10 show the co-elution of several impurities) have been attributed

ARGININE SIDE CHAIN REACTIVITY

•I1, I3, I4($I4_1$), C Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2

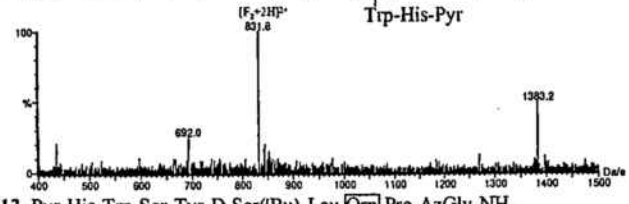


Ornithine derivatives



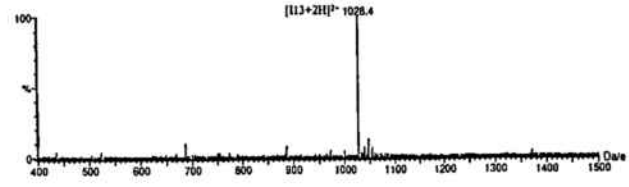
•F: F_2 Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Orn-Pro-AzGly-NH_2

Trp-His-Pyr



•I13 Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Orn-Pro-AzGly-NH_2

D-Ser(^{t}Bu)-Tyr-Ser-Trp-His-Pyr



•$I8_2$ Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Orn-Pro-AzGly-NH_2

Pyr

•$I5_2$ Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Orn-Pro-AzGly-NH_2

His-Pyr

•$I10_1$ Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Orn-Pro-AzGly-NH_2

Ser-Trp-His-Pyr

•I11 Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Orn-Pro-AzGly-NH_2

Tyr-Ser-Trp-His-Pyr

Fig. 7. Electrospray mass spectra associated with the chromatographic peaks indicated and structures associated with the products of arginine side chain reactivity. Da/e = m/z.

Table 2
Measured relative molecular masses (M_r) and proposed sequences associated with the TIC peaks in Fig. 4

Chromatographic peak	Impurity	t_R (min) 27% MeCN	Observed m/z, $[M+2H]^{2+}$ $[M+H]^+$	Measured M_r	ΔM	Proposed sequence
I1	I1	8.10	643.4 1285.9 $[M(O)+2H]^{2+}$ $[M(O)+H]^+$ 651.4 1302.0	1284.9	+15.5	Addition of a NH_2 group to the side chain of arginine residue
I2+I3	I2	9.34	713.7 1426.2	1425.3	+155.9	Goserelin + arginine
	I3	9.34	643.2 1285.7	1284.5	+15.1	Addition of a NH_2 group to the side chain of arginine residue
I4	$I4_1$	10.40	643.2 1285.7	1284.5	+15.1	Addition of a NH_2 group to the side chain of arginine residue
	$I4_2$		704.3 1407.2	1406.4	+137.0	Goserelin + histidine
I5	$I5_1$	11.75	635.8 1270.3 $[M+NH_4+H]^{2+}$ $[M+NH_4]^+$ 644.4 1287.2	1269.5	−0.1	Goserelin racemate
	$I5_2$		738.6 1476.6	1475.4	+206.0	Ornithine derivative + (His–Pyr)
	$I5_3$		580.1 1159.2	1158.2	−111.2	Goserelin–pyroglutamic acid
I6 +	I6	13.27	587.1 1173.2	1172.2	−97.2	Goserelin–proline
Goserelin +	Goserelin	13.27	635.5 1270.1	1269.1	−0.3	
I7	I7	13.27	684.2 1367.3	1366.4	+97.0	Goserelin + proline
I8	$I8_1$	16.50	606.4 1212.2	1211.0	−58.4	Goserelin–(AzGly)
	$I8_2$		669.9 1339.2	1338.0	+68.6	Ornithine derivative + (Pyr)
A	A	18.48	717.1 1433.3	1432.3	+162.9	Goserelin + tyrosine
B	B	21.52	716.9 1433.3	1432.0	+162.6	Goserelin + tyrosine

Table 2. Continued

Chromatographic peak	Impurity	t_R (min) 27% MeCN	Observed m/z, $[M+2H]^{2+}$ $[M+H]^{+}$	Measured M_r	ΔM	Proposed sequence
I9	I9	23.12	620.5 1240.2	1239.1	−30.3	Amidation of the acid precursor
I10	$I10_1$	25.04	875.2 –	1748.4	+479.0	Ornithine derivative+(Ser–Trp–His–Pyr)
	$I10_2$		567.1 1133.1	1132.2	−137.2	Goserelin–histidine
C	C	26.80	643.1 1285.2	1284.2	+14.8	Addition of a NH_2 group to the side chain of arginine residue
E	E_1	34.04	692.0 1383.5	1382.3	+112.9	Goserelin+leucine
	E_2		614.1 1226.9	1226.0	−43.4	Formation of an acylhydrazyn
F	F_1	37.72	692.0 1383.2	1382.1	+112.7	Goserelin+leucine
	F_2		831.8 –	1661.6	+392.2	Ornithine derivative+(Trp–His–Pyr)
I11	I11	43.12	956.8 –	1911.6	+642.2	Ornithine derivative+(Tyr–Ser–Trp–His–Pyr)
I12	I12	51.44	640.4 1280.2	1279.0	+9.6	Goserelin+proline–serine
I13	I13	50.7 (29% MeCN)	1028.4 –	2054.8	+785.4	Ornithine derivative+[D-Ser(^{t}Bu)–Tyr–Ser–Trp–His–Pyr]

Not identified: D

to deletion sequences. As an example, spectra of I6 and I10 are shown in Fig. 5. The spectrum associated with impurity I6 exhibits m/z of 1173.2 $[M+H]^+$ and 587.1 $[M+2H]^{2+}$, yielding the molecular mass of 1772.2. The difference between its molecular mass and the mass of goserelin is −97.2, corresponding to the proline residue. This difference indicates the presence of a modified goserelin chain due to the absence of a proline.

In the same way, chromatographic peak I10 contains the ions of m/z 1133.1 $[M+H]^+$ and 567.1 $[M+2H]^{2+}$ in its spectrum, corresponding to a molecular mass of 1132.2 ($\Delta M = -137.2$), which indicates the absence of a histidine residue. Chromatographic peak I5 contains a deletion sequence that implies the loss of the Pyr residue, and impurity $I8_1$ is attributed to a deletion sequence with the loss of the –AzGly group (spectra not shown). On the other hand, the loss of the C-terminal semicarbazide group ($\Delta M = -43$) leads to the formation of the acylhidrazine, impurity E_2. This side-product has also been observed by other authors as a degradation product of the goserelin analog [36].

3.2.3.3. Amino acidic insertions

The use of an excess of equivalents in the coupling step to ensure the maximum coupling efficiency leads to the occasional insertion of an

additional amino acid in the sequence. This is the case of impurities I2 (additional arginine), A and B (additional tyrosine, isomers) and I7 (additional proline). The chromatographic peak I4 also contains a side-product which corresponds to the insertion of an additional histidine ($I4_2$) and chromatographic peaks E and F contain impurities attributed to the additional presence of a leucine (E_1 and F_1). Fig. 6 shows the spectra of some selected side-products associated with peaks A, B and I7 and the structures of all the impurities associated with a insertion sequence. Impurity I12 has been tentatively attributed to a sequence that contains an additional proline but lacks a serine residue.

3.2.3.4. Acid precursor

Before the synthesis of goserelin is completed by means of an amidation, the acid precursor was cleaved from the resin. This substance has not been found as impurity in our mixture but a derivative of the acid precursor has appeared in the chromatographic peak I9. Its spectrum, shown in Fig. 6, contains ions m/z 1240.2 $[M+H]^+$ and 620.5 $[M+2H]^{2+}$, corresponding to a molecular mass of 1239.1. The difference between this mass and the molecular mass of the acid precursor ($M=1212.4$) is of 26.6, attributed to the formation of the dimethylamide from the acid precursor. The $-N(Me)_2$ group is supplied by the dimethylformamide or the DIEA (*N,N*-diisopropylethylamine) used in the synthesis procedure.

3.2.3.5. Arginine side chain reactivity

Individual amino acid residues can undergo undesired reactions in their side chains such as alkylations, oxidations, cyclizations, and substitutions [36,40]. Chromatographic peaks I1, I3 and C show similar mass spectra, Fig. 7, with m/z ratios $\approx$1285.5 $[M+H]^+$ and 643 $[M+2H]^{2+}$ yielding a molecular mass of approximately 1284.5, that differs from goserelin molecular mass by about 15. Impurity $I4_1$ also shows this charge-state distribution. This difference has been attributed to the addition of a NH_2 group to the side chain of arginine residue. The singly and doubly charged ions 1302.0 and 651.4, respectively, correspond to the oxidation of this impurity in the electrospray source.

3.2.3.6. Ornithine derivatives

Among the side reactions that arginine can undergo, there is a particular reaction that gets great importance in our case. Fragmentation of the side chain of arginine takes place, and it is transformed into the ornithine residue (Fig. 7), with an amino group unprotected that allows the growth of the peptide sequence in two places. In each coupling step the amino acid can be linked to the main sequence and to the side chain of ornithine. Up to six impurities ($I8_2$, $I5_2$, F_2, $I10_1$, I11 and I13) can be attributed to this side reaction. Some selected spectra together with the structure of these derivatives are shown in Fig. 7.

4. Conclusions

The separation between the target peptide goserelin and undesired side products in the crude of synthesis has been optimized applying the LSER method. The assessment of the analytical scale conditions allows preparative scale purification of the target peptide. On the other hand, LC–ES-MS provides an efficient analytical tool for reliable characterization of the target peptide and associated impurities. The knowledge of the side-products identity allows the chemist to improve the synthetic procedures by suitable modification of conflictive steps and to fulfil the necessary requirements for goserelin commercialization as a peptide of therapeutic interest.

Acknowledgements

Financial support by the DGICYT (Project PB95-0966) is gratefully acknowledged. F.B. also thanks the Generalitat de Catalunya for an F.I. Grant.

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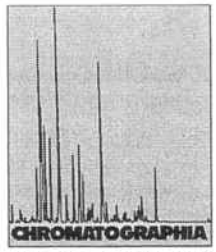
Optimization of HPLC Conditions for the Separation of Complex Crude Mixtures Produced in the Synthesis of Therapeutic Peptide Hormones



2001, 53, Suppl., S-167–S-173

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Key Words

Column liquid chromatography
Linear solvation energy relationships
Peptide hormones

Summary

The solid phase peptide synthesis (SPPS) method is usually used for the synthesis of new peptides of pharmaceutical interest. However, the final drug product commonly results in complex mixtures, where the target peptide must be separated from the undesired impurities. The linear solvation energy relationships method (LSER) is used here in order to rapidly optimize the separation of the analytes present in the complex crude mixtures which are formed during the synthesis of peptide hormones with therapeutic interest, such as: carbetocin, eledoisin, leuprolide, goserelin and triptorelin. Resolution data for adjacent solute pairs may easily be predicted from these relationships. Separation under optimum conditions provides useful information about the purity of the target peptide and allows characterization of the mixture components by LC-ES-MS. In addition conditions for the preparative purification of the candidates could be extrapolated from these analytical data.

Introduction

In the past few years pharmaceutical companies have invested record amounts in biotechnology. In this way, proteomics has emerged as a huge challenge for scientific working in many different areas [1–5]. A growing number of firms have perceived the value of proteomics as a new tool in drug development processes. The development of new and more powerful synthetic peptides and peptidomimetics for therapeutic purposes must be paralleled by proteomic data collection [6].

Carbetocin is a structural analogue to oxytocin used for stimulation of uterin contractions and for the promotion of milk-let-down as a lactation agent. Eledoisin has complex pharmacological properties, being a powerful vasodilator, with hypotensive action and a stimulator of extravascular smooth muscle. Leuprolide, goserelin and triptorelin are structural analogues of gonadotropin-releasing hormone (GnRH), previously termed luteinizing hormone-releasing hormone (LHRH), used for the treatment of a large number of diseases related with sex hormone irregularities, such as: masculine and feminine infertility, uterine myomas and prostatic and mammalian tumors.

The SPPS method, introduced in 1962 by Merrifield [7], has been widely used in the synthesis of peptides and small proteins. Inspite the continuous modifications and improvements these procedures commonly result in complex crude mixtures. The final product not only contains the target peptide but also contaminating sequences that differ in subtle ways, such as minor deletions, incomplete deprotected side chains or amino acidic insertions [8]. Analytical and preparative liquid chromatography (LC) have been routinely used for purification and fingerprinting of new peptide products [9–11]. Furthermore, liquid chromatography coupled to mass spectrometry using electrospray ionization interfaces (LC-ES-MS) has proved to be highly efficient for fast and unambiguous characterization of peptide crude mixtures [12–16]. However, because of the complexity of the crudes of synthesis, the preparation of significant amounts of purified peptides and ther impurity characterization requires firstly the development of analytical scale separation conditions. The information compiled finally allows the optimization of the synthesis process, to make it economically profitable.

Time consuming and non-systematic LC separations are still being developed. Moreover, in most cases, the final experimental conditions are not the optimum ones. A fast optimization of the separa-

Presented at: 23rd International Symposium on Chromatography, London, UK, October 1–5, 2000

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tion conditions becomes the first challenge in the purification and characterization of new peptide products, since this will lead to significant increases in throughput. Hence, the prediction of the elution profile of analytes on the basis of accurate quantitative relationships can be the basis for subsequent optimization of the chromatographic conditions [17]. The retention behaviour of a solute in liquid chromatography is the result of complex interactions with both stationary and mobile phase. Undoubtedly, the stationary phase plays a major role in the separation process, but most researchers have focused attention on mobile phase optimization, since this is the easiest way to control retention and selectivity in LC. In previous studies the LSER method [18–21], based on the Kamlet-Taft multiparameter solvent scales, has been used to predict the chromatographic retention of series of peptides [22], quinolones [23], diuretics [24] and anabolic steroids [25] in LC, taking into account dependence of retention with chromatographic characteristics of solute, mobile phase and stationary phase:

$$\log k = (\log k)_0 + M(\delta_s^2 - \delta_m^2)\overline{V}_2/100 + S(\pi_s^* - \pi_m^*)\pi_2^* + A(\beta_s - \beta_m)\alpha_2 + B(\alpha_s - \alpha_m)\beta_2 \quad (1)$$

k is the retention factor; $(\log k)_0$ is an independent term; M, S, A and B are the correlation coefficients being independent of the solute; $\overline{V}_2$ is the molar volume of the solute multiplied by 1/100 so that the values of this term cover the same range as the other terms; δ is the Hildebrand solubility parameter that measures the solvent cohesive energy [26]; the π^* parameter is used to evaluate solvent dipolarity/polarizability [27]; and α and β scales evaluate solvent hydrogen bond acidity [28] and solvent hydrogen bond basicity [29], respectively. The subscripts s and m denote the stationary and the mobile phase, respectively, and the subscript 2 refers to the solute properties. This relationship can be reduced to an easier expression when a fixed solute-stationary phase pair is considered, and assuming the invariance of the properties of the stationary phase with changes in mobile phase composition [18, 30]. Also a linear relationship between the eluent δ^2 and the solvatochromic parameters π^*, β and α of the mobile phase is assumed [26, 30]. In this way, Eq. (1) is reduced to:

$$\log k = (\log k)_s + s\pi_m^* + a\beta_m + b\alpha_m \quad (2)$$

The independent term and the coefficients in Eq. (2) depend on the solutes and stationary phase parameters and on a correlation between δ^2 and π^*, β and α parameters for the mobile phases studied. In previous studies, factor analysis was applied to Eq. (2) in order to establish the influence of the polarity and hydrogen bonding capacity terms of this equation, in standard pH and pK_a values, in the hydroorganic mixture used in LC [31, 32].

Multiparametric correlations between the retention of the solute considered ($\log k$) and the solvatochromic parameters of the mobile phases used (π_m^*, β_m and α_m) could be obtained by multiple linear regression [22–25]. However, taking into account that β_m values for acetonitrile water-mixtures are nearly constant [33–34] and the observed correlation between the normalized Dimroth and Reichardt polarity [35], E_T^N, and π_m^* and α_m solvatochromic parameters, $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha_m$ [36], then Eq. (2) can be reduced to a single solvent parameter dependent expression:

$$\log k = C + eE_T^N \quad (3)$$

Eq. (3) has been widely used to correlate the chromatographic retention of different types of analytes with polarity of the mobile phases [22–25]. The good linearity obtained indicate that E_T^N provides a useful tool for prediction of retention for a specific solute, in a fixed stationary phase, from only two experimental measurements of retention factor (k) at two mobile phase compositions. In a complex mixture, the optimization of the eluent composition for the best separation is easily conducted by analyzing the linear correlations for all the solutes in the mixture. Because of its accuracy and simplicity we judged it to be the best available as descriptor of chromatographic retention as a function of percentage of organic solvent in the mobile phase. Moreover, E_T^N values are obtained from an unique solvatochromic indicator, thus its values are unambiguous. The analysis of compounds with acid-base properties, introduces a new variable in the optimization of mobile phase composition. However, the LSER approach provides no information about the eluent pH. A model that accurately describes retention in LC as a function of pH and solvent composition has been previously developed for diuretics [37]. Peptides have been widely analized in acidic media, in the presence of ion pairing reagents, such as TFA [22, 38]. Furthermore, the chromatographic retention of compounds studied here is not strongly pH dependent, so variations in pH do not result in improvement of the chromatographic separation.

This study is focused on the fast and accurate optimization of separation of complex crude mixtures originated during the development of SPPS procedures for the synthesis of several peptide hormones of therapeutic interest, by the use of LSER methodology. Linear relationships between Reichardt's E_T^N parameter of solvent polarity and retention data were established in order to predict the retention behavior of peptides present in each complex crude mixture. This prediction allows the rapid selection of the optimum separation conditions for preparative purification of the target peptide and characterization of the mixture components by LC-ES-MS [16, 39–41] from few experimental data.

Experimental

Chemicals and Reagents

Water with a conductivity lower than 0.05 mS cm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Acetonitrile (MeCN) (Merck, Darmstadt, Germany) was of LC grade. Trifluoroacetic acid (TFA), potassium bromide and potassium hydrogenphthalate (dried at 110 °C before use) were all analytical grade obtained from Merck. Crudes of synthesis of carbetocin (Tyr(OMe)-Ile-Gln-Asn-Cys(CH_2-CH_2-CH_2-CO)-Pro-Leu-Gly-NH_2), eledoisin (pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2), leuprolide (Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt), goserelin (Pyr-His-Trp-Ser-Tyr-D-(^{t}Bu)Ser-Leu-Arg-Pro-AzGly-NH_2) and triptorelin (pGlu-Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2) used in this study were supplied by Lipotec (Barcelona, Spain). Working solutions were prepared by dissolving each crude in mobile phase at a concentration of 1 mg mL^{-1}. The synthesis crudes and the solutions were stored in a freezer at 0 °C when not in use. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by bubbling helium. The samples were passed through a 0.45-μm nylon filter (MSI).

Apparatus

An ISCO (Lincoln, NE, USA) Model 2350 chromatographic pump with a Valco (Houston, TX, USA) injection valve with a 20-µl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) were used. The chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller Software (ISCO) running on a personal computer. A 5 µm Kromasil C_8 column (250 × 4.6 mm I. D.) (BC Aplicaciones Analíticas, Barcelona, Spain) was used at room temperature.

The electromotive force (e. m. f) values used to calculate the pH of the mobile phase were as in previous works, measured with a potentiometer Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA) with a precision of ± 0.1 mV. The potentiometric system was calibrated using a standard reference solution of potassium hydrogenphthalate 0.05 mol/kg [23] whose reference pH values in the acetonitrile-water mixtures studied were previously assigned [42].

Procedures

For the optimization of mobile phase composition, mobile phases were made of different acetonitrile-water mixtures containing 0.1% (*v/v*) TFA. The acetonitrile percentages studied in each synthesis crude are shown in Table I. The flow-rate of mobile phase was 1 mL min^{-1}. All chromatograms were measured at room temperature. Retention factors were calculated from $k = (t_R - t_0)/t_0$, where t_0 is the hold-up time, established for every mobile phase composition using 0.01% (*w/v*) bromide solution in water, and t_R is the retention time of the target peptide and the associated products. The retention times and the capacity factors of the solutes were determined from three injections of 1 mg mL^{-1} solution of the crudes at each mobile phase composition considered. The signal was monitored at 220 nm. The pH was measured in the mixed mobile phases in which the chromatographic separations took place, and ranged from 1.9 to 2.1.

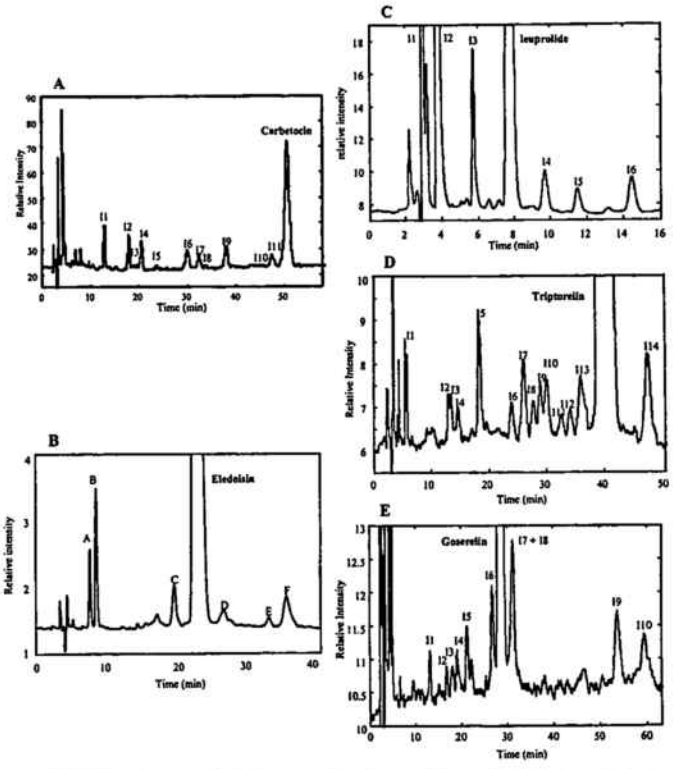

Figure 1. UV chromatograms of solutions containing 1 mg mL^{-1} of crudes of synthesis of **A)** carbetocin. Mobile phase: 24:76 MeCN:water, **B)** eledoisin. Mobile phase: 25:75 MeCN:water, **C)** leuprolide. Mobile phase: 31:69 MeCN:water, **D)** triptorelin. Mobile phase 25:75 MeCN:water and **E)** goserelin. Mobile phase 25:75 MeCN:water.

Results and Discussion

Acetonitrile-water mixtures containing TFA at acidic pH have been shown to be suitable for the chromatography of peptide hormones with a wide range of acid-base properties [9–11, 43]. Therefore, separations developed under fixed TFA concentration and acidic pH can be optimized by studying the acetonitrile composition of the mobile phase employed for the separation [16]. All the synthetic peptide products studied were examined under these experimental conditions by analytical LC. The resulting chromatograms contain a major peak, the target peptide, as well as a number of minor peaks corresponding to different peptide impurities (Figure 1). All the non-desired products are strongly related with the reaction steps involved in the synthesis, thus they usually differ from one crude mixture to another. Prior characterizations by means of LC-ES-MS have commonly shown: deletion sequences, partially protected products, amino acidic insertions sequences, products from side chain reactivity and truncated sequences [16, 39–41]. The retention factors (k) for all the labelled peaks were obtained at different percentages of acetonitrile in the mobile phase. The composition range of acetonitrile studied in each case is shown in Table I. In leuprolide and goserelin crudes large retention times for some of the substances make it impracticable to obtain their retention factors in the whole acetonitrile-water mixtures range studied.

In order to optimize the organic composition of the mobile phase, the LSER approach, based on the Kamlet-Taft multiparameter scale, was used in the same way as in previous works [22–25]. The original multiparametric correlation between the logarithm of retention factor and Kamlet-Taft solvatochromic para-

Table I. Liquid chromatographic separation conditions for the analysis of synthetic crudes of carbetocin, eledoisin, leuprolide, goserelin and triptorelin.

Target peptide	CAS number	% MeCN (v/v)		
		Range studied	Preparative scale	Characterization by LC/ES-MS
Carbetocin ^{1}CO-CH$_2$-CH$_2$-CH$_2$$_{1}$ Tyr(OMe)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH$_2$	37025-55-1	24–30	30	25
Eledoisin pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH$_2$	69-25-0	24–28	25	26
Leuprolide Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt	53714-56-0	25–35	31	28 and 33
Goserelin Pyr-His-Trp-Ser-Tyr-D-(^{t}Bu)Ser-Leu-Arg-Pro-AzGly-NH$_2$	65807-02-5	24–30	24	27 and 29
Triptorelin pGlu-Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH$_2$	57773-63-4	23–29	25	29

Table II. Schematic representation of the structure of the structure of acetonitrile-water mixtures at different percentages of acetonitrile and Reichardt's E_T^N solvent polarity values for acetonitrile-water mixtures.

Structural region	X_{MeCN}	% MeCN (v/v)	E_T^N
Rich in H_2O			
	0.018	5	0.98
	0.037	10	0.95
$X_{MeCN} \cong 0.1$	0.079	20	0.90
	0.128	30	0.86
Microheterogeneity	0.186	40	0.83
	0.255	50	0.81
	0.339	60	0.79
	0.444	70	0.77
$X_{MeCN} \cong 0.7$	0.578	80	0.75
	0.755	90	0.71
Rich in MeCN	0.867	95	0.63

meters in Eq. (1) can be reduced to the single solvent parameter-dependent expression defined by Eq. (3). Therefore, the normalized E_T^N scale of solvent polarity proposed by Reichardt [35] allows accurate prediction of chromatographic retention. Moreover, E_T^N values are measured with a unique solvatochromic indicator and, hence, they are unambiguous. Values of E_T^N at each acetonitrile percentage in the mobile phase are shown in Table II. Separate plots of log k of the substances present in each peptide crude versus E_T^N values of acetonitrile-water systems are shown in Figure 2. According to Eq. (3), and as can be observed in Figure 2, logarithms of the retention factor correlate linearly ($r^2 > 0.99$) with the polarity of the mobile phase, in the composition ranges considered. This linearity allows suitable prediction of the elution behavior of peptide derivatives and hence optimization of eluent composition for the best separation from only two experimental measurements of k values for each analyte.

In previous works, linear relationships between the log k and E_T^N parameters showed two straight lines with different slopes, which intersect at acetonitrile percentages approximately between 15 and 25% [22–25]. This behaviour has been explained by the structural features of acetonitrile-water mixtures [34, 44] and by preferential solvation [45–47]. On the water-rich side, when $X_{MeCN} \leq 0.1$, the water structure remains more or less intact and acetonitrile molecules occupy the cavities between the water structures with little disruption. In the acetonitrile rich region, when $X_{MeCN} \geq 0.7$, individual water molecules interact with individual acetonitrile molecules with little disruption of the weak dipole-dipole interaction of the main component. In the middle range of composition, when $0.10 < X_{MeCN} < 0.70$, the acetonitrile-water mixtures show microheterogeneity: the two components of the mixture prefer molecules of their own type [34]. The limits of the MeCN-water region are not narrow and depend on the LC experimental pressure [44]. Table II shows a schematic representation of the acetonitrile-water mixtures in the whole range of compositions. Different slopes observed in previous works [22–25], correspond to different structural regions of MeCN-water mixtures. In all the synthetic crudes investigated, the range of organic modifier studied was between 24% and 35% (v/v) of acetonitrile (Table I). Single-sloped lines shown in Figure 2 for each analyte confirm that all the data were obtained in one of the structural regions. Thus, the use of Eq. (3) in one of the structural regions of the MeCN-water mixtures involves an important reduction of experimental retention data for optimizing chromatographic separation of peptides presents in each synthetic crude. It is recommended that experimental data at at least three mobile phase compositions be collected if optimization is being developed in a border region. Otherwise, only two experimental measurements of retention factors for each compound considered at two different mobile phase compositions within one of the structural regions of the acetonitrile-water mixtures are enough for predicting their retention behaviour and hence for optimizing their chromatographic separation in a fixed stationary phase.

In order to examine the accuracy of retention prediction by Eq. (3), the selectivity for adjacent solute pairs in some of the studied crudes was calculated in the usual way, $\alpha = k_i/k_j$ (being $k_i > k_j$). Plots in Figure 3 show variation of solute selectivities in each crude versus percentage of acetonitrile in the mobile phase. Only selectivity between solutes whose separation is not adequate have been considered in order to simplify the graphical representations. Solid lines indicate theoretical selectivity values obtained using predicted k, calculated by means of Eq. (3) from only two experimental points, corresponding to the lowest and the highest acetonitrile percentage of the interval studied in each case. Points represent experimental selec-

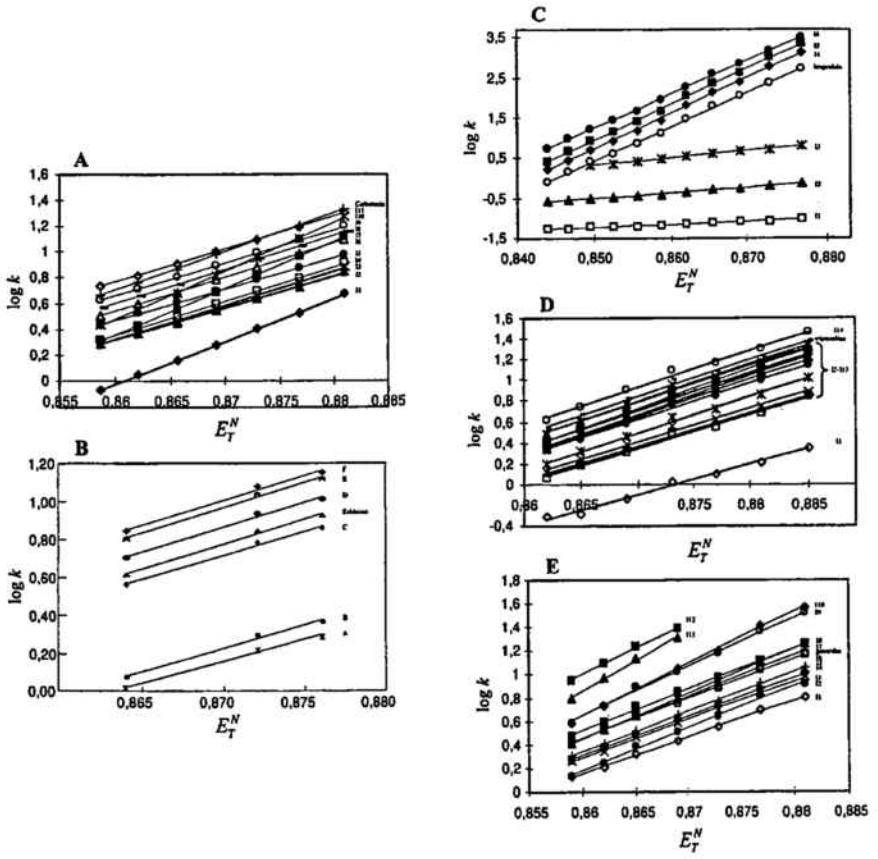

Figure 2. Plots of log k of target peptides and impurities versus E_T^N parameters of the mobile phase for the products of crude mixtures: **A)** carbetocin, **B)** eledoisin, **C)** leuprolide, **D)** triptorelin and **E)** goserelin.

tivity values . As can be observed in Figure 3, there is good concordance of both selectivity values in the whole acetonitrile ranges for all the crudes studied. Thus, accurate prediction of chromatographic retention and hence separation optimization is possible by Eq. (3), using only two experimental measurements.

Otherwise, achieving good resolutions between all the analytes of interest is the main goal of a chromatographic separation. In this way, in terms of fundamental chromatographic parameters, the resolution, R_s, between two adjacent peaks is given by:

$$R_S = \frac{1}{4}\sqrt{N}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{1+k-2}\right) \quad (4)$$

efficiency / selectivity / retention

where N is the number of theoretical plates ($N = 16(t_R/w)^2$). Although the selectivity term is generally regarded as the most important in LC, full attention must be given to all the terms in Eq. (4). Figure 4 shows variations of R_s for adjacent solute pairs in some of the analyzed crudes with the percentage of acetonitrile in the mobile phase. Solid lines indicate resolution values obtained from two retention measurements using Eq. (3) and Eq. (4), and points represent experimental resolution values obtained from the usual relation, $R_S = 2(t_{R2} - t_{R1})/(w_2 - w_1)$, where w_2 and w_1 are peak widths.

The concordance of the two sets of R_s values in Figure 4 confirms accurate estimations of resolution via Eqs (3) and (4) from only two retention measurements per compound. From Figures 3 and 4 we can deduce the acetonitrile percentages in the mobile phase for optimum separations between the target peptide and the side products present in each crude of synthesis. The concordance with the conclusions deduced for leuprolide crude by analyzing the graphics of Figures 3 and 4, confirms the importance of the selectivity term in Eq. (5). It is to be noted that there is a difference between the separation optimization used in the preparative scale purification of the target peptide, and the one conducted to achieve the best separation conditions for all the analytes in the complex peptide mixture, facilitating their subsequent characterization by LC-ES-MS [16, 39–41]. In the first case, the highest resolution between the target peptide and the adjacent impurities is desirable. In the second case, better prior separations

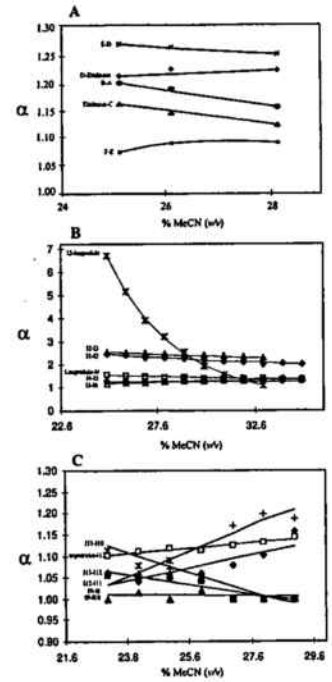

Figure 3. Plots of selectivity between adjacent pairs of solutes presents in crude mixtures versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of selectivity and points represent experimental values of selectivity: **A)** carbetocin, **B)** leuprolide and **C)** triptorelin.

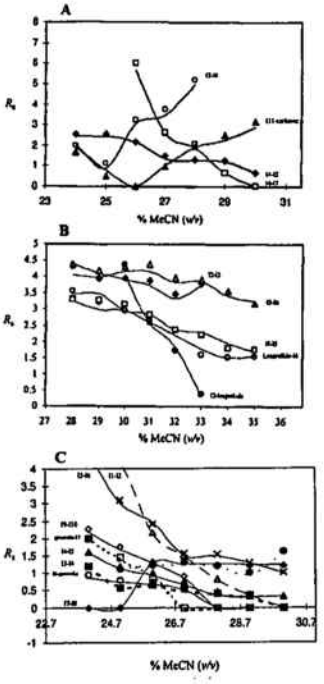

Figure 4. Plots of resolution between adjacent pairs of solutes in crudes mixtures versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of resolution and points represent experimental values of resolution: **A)** eledoisin, **B)** leuprolide and **C)** goserelin.

facilitate the on-line characterization by ES-MS without confusing overlaps. Nevertheless, in both cases a compromise between analysis time and final goal must be accepted. Table I shows the organic modifier percentage in the mobile phase recommended for the preparative scale purification of each target peptide and for the mixture components characterization by LC-ES-MS. In general, mobile phases with the lowest contents of acetonitrile are selected for preparative purification of the target peptides because they assure higher baseline resolution between the target peptide and the adjacent impurities. However, sometimes, as in leuprolide crude, a mobile phase with higher content of acetonitrile is chosen, because a reduction in analysis time is achieved whilst at the same time maintaining suitable resolution values. Otherwise, a small range of acetonitrile composition in the mobile phase is often selected for the characterization of the peptide mixtures by LC-ES-MS (Table I). Mobile phases with higher contents in acetonitrile are frequently preferred in LC/ES-MS owing to the improvement of the MS analytical response [48].

Conclusions

From this study it can be concluded that linear relationships between log k and Reichardt's E_T^N parameter of mobile phases, in a structural region of the acetonitrile-water mixtures, allow the prediction of chromatographic behavior of peptides present in complex synthetic mixtures from only two retention measurements for each analyte. Furthermore, this accurate prediction could be profitably applied to the rapid and simple optimization of conditions for the separation of synthetic peptide mixtures prior to preparative purification or characterization by LC-ES-MS. The optimization procedure proposed could find an immediate application in proteomic research if focused on the rapid and reliable analysis, by LC-ES-MS, of the complex peptide collections generated by enzimatically degrading complex protein mixtures [1]. Its combination of high-speed and simplicity in selecting optimum conditions for the separation of complex mixtures make it ideal for improvement in the throughput of those LC-ES-MS methods currently in use in this area.

Acknowledgements

Financial support of this project by DGICYT (Project PB98-1174) is gratefully acknowledged. The authors thank Lipotec, S. A., for the supply of the peptide crudes Fernando Benavente also thanks the Generalitat de Catalunya for an F. I. Grant.

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Received: Nov 23, 2000
Revised manuscript received: Jan 29, 2001
Accepted: Feb 8, 2001

SEPARATION AND CHARACTERISATION OF COMPLEX CRUDE MIXTURES PRODUCED IN THE SYNTHESIS OF THERAPEUTIC PEPTIDE HORMONES BY LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY MASS SPECTROMETRY (LC-ES-MS)

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Abstract

Native peptides and peptidomimetics can be synteshized in a routine way by rapid and efficient procedures. However, the final products always result in complex mixtures, in which the target peptide is contaminated with undesired side products and other impurities. Thus, it is imperative to develop analytical methods for the evaluation of the target peptide's purity in order to obtain an effective, safe and legal pharmaceutical product. LC-ES-MS is used here in order to separate and characterise the side-products associated with several synthetic hormones with therapeutic interest: carbetocin, eledoisin, leuprolide, goserelin and triptorelin. General directions for LC-ES-MS analysis of the synthetic peptide mixtures are established. Mass information obtained offers a significant advantage for the purity assessment of therapeutic hormones and gives a key tool to enhance their process of synthesis.

Keywords: SPPS / peptide hormones / LC-ES-MS

1. Introduction

Synthetic peptides are of growing importance in the pharmaceutical industry [1-4], despite they are generally considered as poor drug candidates, because of their low oral bioavailability and propensity to be rapidly metabolized. However, new synthetic strategies for limiting metabolism and alternative administration routes have been evolved and a number of peptide based drugs are being marketed as nasal sprays (calcitonin, nafarelin), sustained release injectables (carbetocin, leuprolide, goserelin, triptorelin) and orally active formulations (cyclosporin) [1,4]. In the next years, combined efforts in proteomic research and combinatorial chemistry may drive the increasing activity in discovery, design, and screening of new therapeutic synthetic peptides and peptidomimetics [1-3,5-6].

Since Merrifield introduced solid-phase peptide synthesis (SPPS) [7], new synthetic protocols have been derived that enable rapid and efficient preparation of peptide and non-peptide based molecules [5-6,8-10]. Despite its widespread success, the final synthetic products commonly result in complex reaction crudes, in which the target molecule is mixed with undesired by-products and other impurities, that differ in subtle ways from the target peptide, such as minor deletions, incomplete deprotected side chains or amino acidic

insertions [8-10]. In order to assure the quality and safety of the target synthetic products, they must be identified and purified [11]. Furthermore, the characterisation of these related side products may allow control and optimization of the reaction conditions before scaling-up the synthetic process and discovery of novel drug candidates.

Liquid chromatography (LC) have demonstrated its versatility for separation and purification of peptides in complex mixtures [11-14]. Furthermore, the coupling with electrospray mass spectrometry (LC-ES-MS) has allowed rapid and efficient separation and reliable characterisation of such complex samples [15-24]. In the case of synthetic peptides, product fingerprint and purity determinations have been traditionally performed by reversed-phase LC with UV detection [11-12]. MS and LC-ES-MS are also used for complementary separation and characterisation of complex crude mixtures in the pharmaceutical industry, however only a few examples have been published with regard to a systematic evaluation of impurity profiles during drug development or synthesis [16-24].

In this study, conditions for separation and characterisation by LC-ES-MS of crude mixtures generated during SPPS of several peptide hormones with widespread therapeutic use are compiled. Carbetocin is a structural analogue to oxytocin used for stimulation of uterin contractions and for promotion of milk-let-down as a lactation agent. Eledoisin has complex pharmacological properties, being a powerful vasodilator, having hypotensive action and permiting stimulation of extravascular smooth muscle. Leuprolide, goserelin and triptorelin are structural analogues of gonadotropin-releasing hormone (GnRH), used for the treatment of a large number of diseases related with sexual hormones regulation, such as: masculine and femenine infertility, uterine myomas and prostatic and mammalian tumors. LC-ES-MS experimental conditions are described in each case, in order to obtain optimum chromatographic separations and mass spectrometric sensitivity. Using the mass spectral information, the target peptides and the unknown impurities were identified on the basis of their calculated molecular mass, taking into account the SPPS procedure followed in each case to make reliable structural assignments.

2. Experimental

2.1 Chemicals and Reagents

Water with a conductivity lower than 0.05 mS cm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Acetonitrile (MeCN) (Merck, Darmstadt, Germany) was of LC grade. Trifluoroacetic acid (TFA) and potassium hydrogenphthalate (dried at 110 °C before use) were all analytical grade obtained from Merck. Crudes of synthesis of carbetocin, eledoisin, leuprolide, goserelin and triptorelin used in this study were supplied by Lipotec (Barcelona, Spain). Working solutions were prepared dissolving each crude in mobile phase at concentrations ranging from 0.05 to 3 mg mL^{-1}. The synthesis crudes and the solutions were stored in a freezer at 0 °C when not in use. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by

bubbling helium. The samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Apparatus

The instrumental set-up used to perform LC-UV analysis was described in detail elsewhere [13,19-24]. LC-ES-MS experiments were performed using two Phoenix 20 syringe pumps (CE Instruments, Milan, Italy) with a Rheodyne 7125 injection valve (Cotati, CA, USA) with interchangeable sample loops. The pumps were coupled to a VG Platform II quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with a nebulizer assisted electrospray source, working in positive mode. The high-flow nebulizer operated with N_2 as both nebulizing and drying gas. Calibration of the mass spectrometer was made using NaI. After calibration, standards of carbetocin and leuprolide were injected 8 times to test the accuracy and precision of the mass measurements. The molecular masses obtained were in good agreement with the expected ones, and the percentage of relative standard deviation was aproximately 0.05%. All the separations were performed at ambient temperature on a 5 μm Kromasil C8 column (250 x 4 mm i.d.) at 1ml/min flow rate. Only eledoisin crude mixture was separated at the same flow rate on a 5 μm LiChrospher 100 RP-18 column (250 x 4 mm i.d.). For the analysis of leuprolide and goserelin crude mixtures 0.3 ml/min of dioxane were added post-column to improve sensitivity. The total flow was always splitted to allow an effective flow between 20 and 100 μl/min into the source of the spectrometer. Experimental details are given in each case in Table 1. Instrument control and data analysis were performed using Masslynx application software from Micromass (Manchester, UK).

The electromotive force (e.m.f) values used to calculate the pH of the mobile phase were measured with a potentiometer Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA) with a precision of ±0.1 mV. The potentiometric system was calibrated using a standard reference solution of potassium hydrogenphthalate 0.05 mol/kg whose reference pH values in acetonitrile-water mixtures studied were previously assigned [26].

2.3. Procedures

The LC-UV procedure was described in detail elsewhere [13,20-24]. The source and the mass spectrometer parameters were optimised introducing directly into the electrospray ionisation source a 50 or 100 mg/ml crude solution in a mobile phase similar to the one used in LC-ES-MS experiments (Table 1). They were sequentially evaluated in order to obtain the best stability of the signal, the highest sensitivity and the lowest fragmentation for the target peptides. Working conditions in each case are shown on Table 1.

For the identification of the components of the crude mixtures, 3 mg/ml solution of each synthetic crude were injected into the LC-ES-MS system, using as mobile phase an appropriate acetonitrile-water mixture containing a 0.1 % (v/v) of TFA (Table 1). The pH measured in these mixtures ranged between 1.9 and 2. Positive ES spectra of the target

Table 1. Experimental conditions used for LC-ES-MS analysis of the peptide crude.

Target peptide	N_2 (L/h) (drying)	N_2 (L/h) (nebul.)	Sample cone Voltage[1] (V)	Capillary Voltage (kV)	Counter electrode Voltage (kV)	Ion Energy (V)	Source T (°C)	m/z range (Da/e)	LC Column	Mobile Phase (MeCN:H_2O (v/v) with 0.1 % (v/v) TFA)	Vinj (µL)	Flow[2]		Post-column addition (mL/min)
												Total (mL/min)	Effective (µL/min)	
Carbetocin [19,21]	400	20	30	4.25	0.5	5	70	250-1200	Kromasil C_8, 5 µm, 250 x 4.6 mm I.D	25:75	20	1	20	-
Eledoisin [20]	300-400	10-20	25	3.5	0.5	3.5	80	200-1300	LiChrospher 100 RP-18, 5 µm, 250 x 4 mm I.D	27.5:72.5	50	1	20	-
Leuprolide [22]	400	15-20	70 40	4	0.25	3	90	400-1500	Kromasil C_8, 5 µm, 250 x 4.6 mm I.D	28:72 33:67	100	1	60	0.3 Dioxane
Goserelin [23]	400	15-20	90	4	0.25	3	100	400-1500	Kromasil C_8, 5 µm, 250 x 4.6 mm I.D	27:73 (I13 at 29:71)	100	1	60	0.3 Dioxane
Triptorelin [24]	400	20	110	4.25	0.5		90	500-1500	Kromasil C_8, 5 µm, 250 x 4.6 mm I.D	29:71	100	1	50	-

[1]Leuprolide crude mixture was analyzed by LC-ES-MS using two different cone voltages for each mobile phase composition. Further explanations are given in the text.
[2]The total flow was splitted to allow the effective flow used in LC-ES-MS experiments

peptides and the associated impurities were obtained at the operating conditions indicated in Table 1, working at full scan mode. In order to further improve the analytical signal obtained in LC-ES-MS analysis, post-column additions of several solvents at different flow-rates were made. Better signal was obtained for goserelin and leuprolide crude mixtures using dioxane at 0.3 mL/min.

3. Results and Discussion

In order to reduce the cost and speed up the LC-ES-MS method development, the synthesis crude mixtures were first examined by analytical LC with UV detection [13,20-24,27]. The LSER (Linear Solvation Energy Relationships) method was used to select the optimum mobile phase composition for their LC-ES-MS analysis, avoiding a time consuming and non-systematic trial and error optimisation procedure [13,20-24]. In Table 1 the mobile phases selected for the analysis by LC-ES-MS of the peptide crude mixtures are shown. Figure 1 shows the LC-UV chromatograms corresponding to the analysis of the peptide crude mixtures. The resulting chromatograms contained a major peak corresponding to the target peptide, as well as a number of peaks of unidentified substances. Mobile phase compositions permitting maximum resolution between adjacent peaks were preferred, because a more reliable identification by LC-ES-MS was made if peaks were non-overlapped. In the case of leuprolide and goserelin crude mixtures a mobile phase with higher acetonitrile content was also used in order to reduce the elution times of more hydrophobic impurities (Table 1). At this point, the optimum separation conditions established by LC-UV may be implemented in the LC-ES-MS system, where several parameters may need optimization.

3.2. LC-ES-MS analysis

3.2.1. ES-MS conditions

In general, the effect of mobile phase composition on ES-MS detection is critical [28-30]. First of all, it has to be taken into account that mobile phases with higher water contents are more difficult to spray than pure organic solvents, because water has larger surface tension and allows a less efficient droplet charging [28-30]. Mobile phases consisting in acetonitrile-water mixtures containing strongly acidic ion-pairing reagents – such as trifluoroacetic (TFA) or heptafluorobutyric (HFBA) acids - have demonstrated to be suitable for the analysis of peptide hormones by reverse phase LC-UV. Formation of ion-pairs results in peptides with enhanced hydrophobicity, affecting the selectivity of the chromatographic separations. Nevertheless, the presence of TFA represent a drawback for LC-ES-MS sensitivity, because ionization of peptides may decrease due to ion-pairing with TFA [30]. In this work, it has been demonstrated that good ES-MS detection sensitivity was obtained using hydroorganic mobile phases with acetonitrile contents ranging from 25 to 33 % (v/v), and 0.1% of TFA (v/v) (Table 1).

The operating parameters affecting the ES-MS detection sensitivity and stability were sequentially optimized, taking into account the intensity differences observed in the sample signal. In

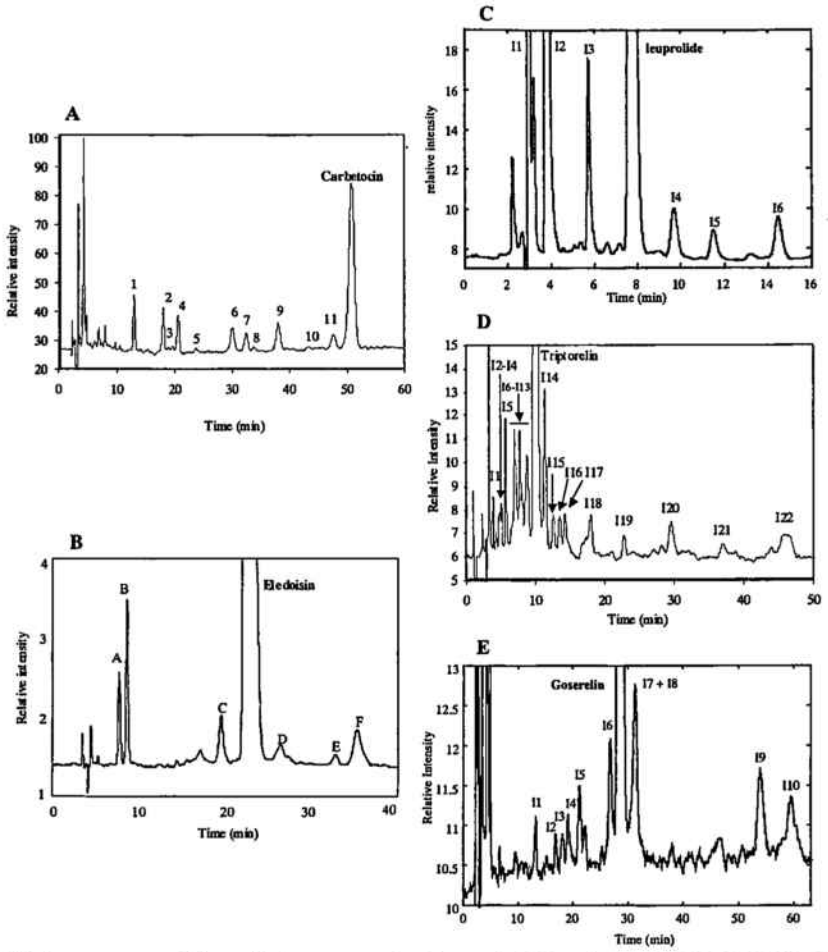

Figure 1. UV chromatograms of the peptide hormones crude mixtures. a) Carbetocin using MeCN:H_2O 25:75, 0.1% (v/v) TFA, b) Eledoisin using MeCN:H_2O 26:74, 0.1% (v/v) TFA, c) Leuprolide using MeCN:H_2O 31:69, 0.1% (v/v) TFA d) Triptorelin using MeCN:H_2O 29:71, 0.1% (v/v) TFA and e) Goserelin using MeCN:H_2O 25:75, 0.1% (v/v) TFA. Experimental details are given in Table 1.

general, fragmentation was negligible under these working conditions. Nevertheless, for the analysis of carbetocin, eledoisin and leuprolide crude mixtures, lower sample cone voltages were preferred, because significant fragmentation was observed at the optimum value (Figure 2). This readjustement promoted a decrease in sensitivity that was partially counteracted in carbetocin and eledoisin crude mixtures, using higher source temperature and ion energy (Table 1).

For a similar reason, 40 V were used for the analysis of leuprolide crude mixtures with a mobile phase containing a 33 % (v/v) of acetonitrile. Conversely, despite of the significant fragmentation observed in the ES-MS spectra (Figure 2-a), the optimum sample voltage value (70 V) was used to analyse this mixture with a mobile phase containing a 28 % (v/v) of acetonitrile, due to the important reduction in sensitivity observed at this lower acetonitrile composition. In Figure 2 can be observed that higher sample

Leuprolide

Pyr – His – Trp – Ser – Tyr – D- Leu – Leu – Arg – Pro – NH – CH_2CH_3

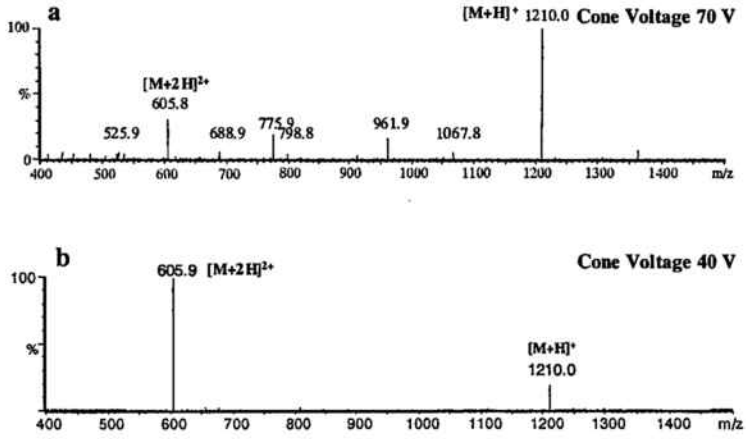

Figure 2. Electrospray mass spectra of leuprolide at different sample cone voltages a) 70 V (MeCN:H_2O 28:72, 0.1% (v/v) TFA) and b) 40 V (MeCN:H_2O 33:67, 0.1% (v/v) TFA). Experimental details are given in Table 1.

cone voltages and higher contents of MeCN in the mobile phase also favor the formation of lower charge states values (which are found at higher m/z values). This may be tentatively due to a less efficient ionization process [16].

In order to further improve the analytical signal obtained, post-column additions of several solvents (acetonitrile, propionitrile, ethanol, methanol, dioxane and tetrahydrofuran) were made. For leuprolide and goserelin crude mixtures [22,23], the addition of 0.3 ml/min of dioxane provided a signal enhancement, whereas no net improvement was observed with the other solvents or synthesis mixtures.

3.2.2. Total Ion Chromatograms (TIC)

Figure 3 shows the Total Ion Chromatograms (TIC) corresponding to the analysis of each crude of synthesis under working conditions. Separation profiles were similar to the ones obtained by LC with UV detection (Figure 1). Nevertheless, differences in the relative signal and the resolution of each analyte were observed when UV (Figure 1) and TIC (Figure 3) data were compared (i.e. triptorelin crude mixture in Figures 1-d and 3-f). The small differences in resolution were mainly due to the use of more concentrated solutions in LC-ES-MS analysis and the differences in the void volums between both instrumental set-ups. In some cases, as in leuprolide crude mixture, there are substances in the mixtures that showed a strong UV absorption but were not efficiently ionized (i.e. I1, I2, I3 in Figures 1-c and 3-c). They may be non-peptidic substances with good chromophor groups, such as a solvent or an auxiliary reagent involved in the synthesis.

3.2.3. LC-ES-MS spectra of the target peptides

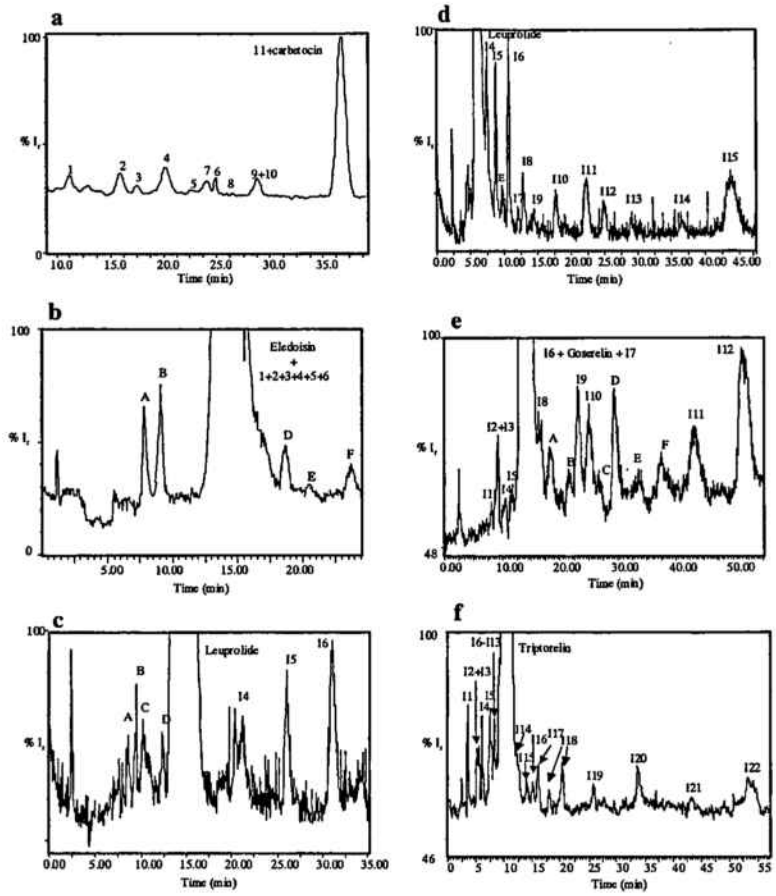

Figure 3. Total ion current (TIC) chromatograms of the peptide hormones crude mixtures. a) Carbetocin b) Eledoisin c) Leuprolide (MeCN:H_2O 28:72, 0.1% (v/v) TFA) d) Leuprolide (MeCN:H_2O 28:72, 0.1% (v/v) TFA) d) Goserelin and e) Triptorelin. Experimental details are given in Table 1.

Figure 2 and 4 show the ES mass spectra associated with the chromatographic peaks of leuprolide, carbetocin, eledoisin, goserelin and triptorelin in their corresponding TIC of Figure 3. Average molecular masses were easily calculated from both their singly and doubly charged molecular peaks present in the spectra and are summarised in Table 2 [16-24]. Some comments about fragmentation can be made in the spectra corresponding to eledoisin and carbetocin. In Figure 4-a, the ion of m/z 485.4 was attributed to a fragment of carbetocin, Asn-Cys-Pro-Leu-Gly-NH_2, which has lost the α-NH_2 group of the Asparagine. In Figure 4-b, the ion of m/z 586.8 yielded a molecular mass of 1171.6 that was attributed to the loss of an ammonia molecule from eledoisin. Several fragments could be also observed at much lower intensities, and their m/z values were interpreted using the nomenclature presented by Biemann [31] (Figure 4-b).

3.2.4. Identification of the impurities

Average molecular masses for each impurity in each peptide crude mixture

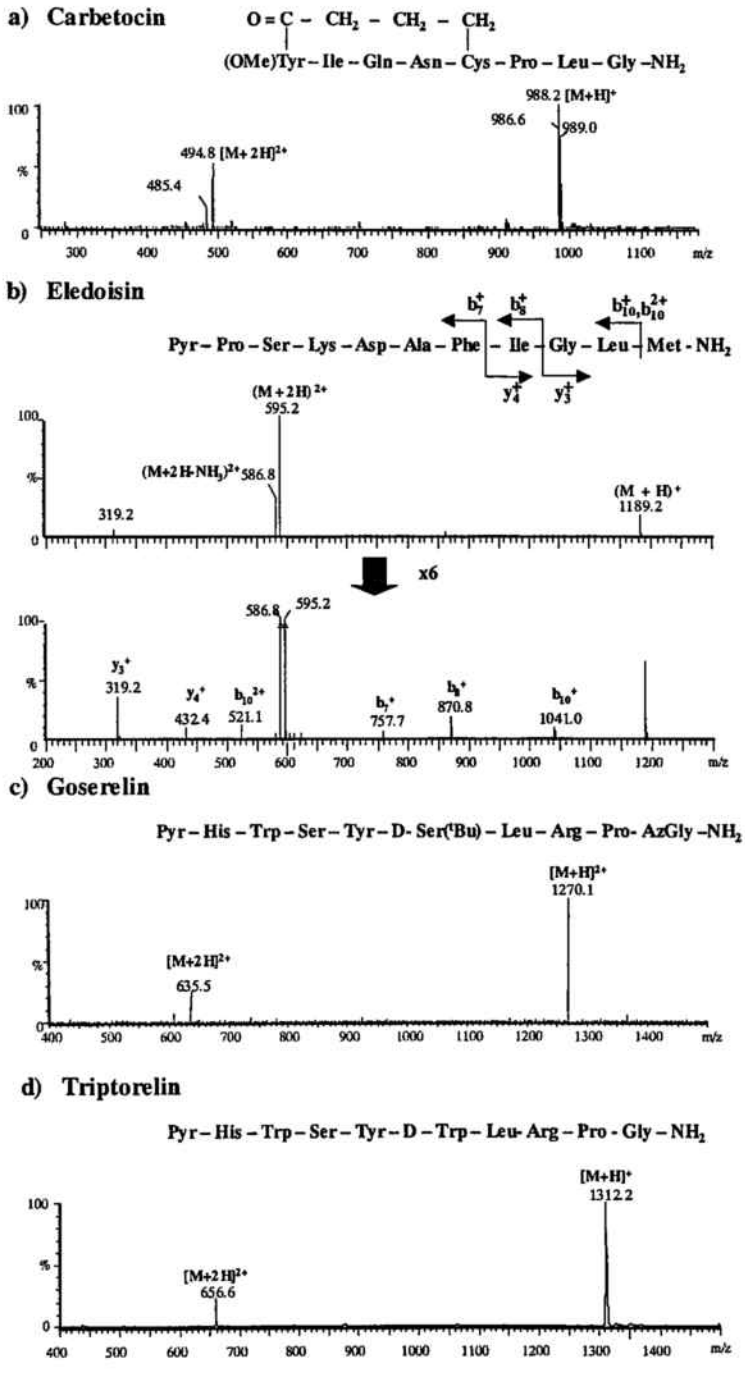

Figure 4. Electrospray mass spectra associated with the TIC peaks corresponding to the target peptides a) Carbetocin, b) Eledoisin c) Goserelin and d) Triptorelin. Experimental details are given in Table 1.

were calculated for their spectra as for the target peptides. Fragmentation was difficult to detect and interpret in complex mixtures of low-concentrated analytes, such as synthetic crudes, even more when several of them coeluted in the same chromatographic peak. Thus, the identification of the impurities was mainly based on the observed differences between the experimental average molecular mass of each target peptide and the one of each impurity [16-17,19-24].

Nevertheless, in order to add reliability to the identification of the impurities, it is desirable to know details about the synthetic process followed in each case. In SPPS, protection of the α-amino group of the amino acid that has to be coupled is mandatory in order to avoid consecutive additions [8-9]. Similarly, the amino or other nucleophilic groups in the side chains of the coupled residues, may be blocked in order to avoid their reactivity. In this work, all the target peptides were prepared using the Fmoc (9-fluorenylmethoxycarbonyl) group to protect the α-amino group of the amino acids in the coupling steps. Details about protecting groups and additional steps used in the process of synthesis are summarized in Table 3. In all the cases the sequence was completed with the coupling of the last amino acid, excepting for carbetocin where a Cl-CH_2-CH_2-CH_2-COOH was introduced. The peptide was then cleaved from the polymeric support with simultaneous deprotection of the side chains using TFA. The acidic precursor was amidated with the corresponding reagent to obtain eledosin, leuprolide, goserelin and triptorelin (Table 3). Carbetocin was obtained after cyclization of the precursor with DIEA (N-ethyldiisopropylamine) [8-9].

Table 2 summarizes the experimental average molecular masses calculated for each target peptide, and the differences between these and the experimental molecular mass of their impurities. Molecular masses of impurities were calculated from mass spectra data in the same way as with the target peptides [19-24]. Details on the assigned structures are also given on Table 2. The most frequent impurities which can be found in the crudes of synthesis of these peptides are:

Diastereoisomeric products

Diastereoisomers with approximately the same molecular mass of the target peptides have been extensively originated during their synthesis (Table 2). The isomerization of the amino acids is frequently observed during the activation and the couplings steps [32]. This undesired side reaction is difficult to control, but can be minimized using a high concentration of reactives to secure a high coupling rate. Furthermore, some additives - such as 4-dimethylaminopyridine (4-DMAP) or 1-hydroxybenzotriazole (HOB_t) - are extensively used for the same purpose [33].

Protected sequences

Problems during the deprotection of the Fmoc group or the side-chains blocking groups (Table 3) often originate incomplete protected sequences [9]. This is the case for the Fmoc derivatives (Table 2) associated with impurities 7 and $I4_2$ in carbetocin and leuprolide crude mixtures. In our case, deprotection

Table 2. Experimental average molecular masses for the target peptides (M_r) and differences with the experimental average molecular masses of their impurities (ΔM). Proposed sequences associated with the TIC peaks in Figure 4.

Target peptide	M_r (exp)	Diastereoisomers		Protected sequences			Deletions			Insertions			Incomplete sequences			Oxidations and reductions			Side chain reactivity		
		Impurity	ΔM	Impurity		ΔM	Impurity		ΔM	Impurity		ΔM	Impurity		ΔM	Impurity		ΔM	Impurity		ΔM
Carbetocin	987.4	2a	-1.5	Fmoc-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	7	-25.9	-Gln	11	-127.6	+Asn (before cyclization)	8a	+114.6	Tyr(OCH_3)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	1	-69.6	[Cys(O)[11]]	2b 3 4	+15.8 +15.8 +15.8			
													Cl-$(CH_2)_7$-CO-NH-Tyr(OCH_3)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	8b	+35.8	Sulfonate of 8b Sulfone of 8b	6 9	+(35.8+48) +(35.8+31.7)			
													(10) 8b-Gln	10	+(35.8-127.2)	[Cys(O)[11]]-Gln	5	+(-127.6+15.3)			
Eledoisin	1188.2	B2	+0.2	+^{t}Bu	B1	+57.2	-Pro	4	-97.1	+ Lys	1	+128.2				[Met(O)[11]]	A	+16.2			
							-Pyr	5	-111.2												
		E	0.0	+^{t}Bu	3	+57.7	-Ser	6	-87.2							[Met(O)[11]]+ Lys	2	+(128.8+16.2)			
				+^{t}Bu	F	+57.3	-Lys	D	-128.2												
Leuprolide	1209.3	$I4_1$	-0.2	+^{t}Bu	E	+56.4				+Trp	I9	+186.2									
				+^{t}Bu	I11	+56.2										Reduced Trp	A	+1.8			
				+^{t}Bu	I12	+56.4	-Ser	I6	-84.9												
				+^{t}Bu	I14	+56.2				+Arg	B2	+156.2					B_1	+1.8	Substituted guanidine in His	I5	+126.2
				+^{t}Bu	I15	+56.3															
				+2^tBu	I8	+112.6										Oxidized Trp	B_3	+15.6			
				Fmoc-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-CO-$NHCH_2CH_3$	$I4_2$	+111.1	-Arg	I7	-156	+ His	C	+136.6									

Table 2 (continue)

Target peptide	M, (exp)	Diastereoisomers		Protected sequences		Deletions			Insertions			Incomplete sequences			Oxidation	Side chain reactivity		
		Impurity	ΔM	Impurity	ΔM	Impurity		ΔM	Impurity		ΔM	Impurity		ΔM	ΔM	Impurity		ΔM
Goserelin	1269.1	$I5_1$	-0.4			-Pyr	$I5_3$	-110.9	+Arg	I2	+156.2					Amination in Arg	I1	+15.8
																	I3	+15.4
									+Tyr	A	+163.2						C	+15.1
										B	+162.9						$I4_1$	+15.4
						-Pro	I6	-96.9								Gos(Orn)+Pyr	$I8_2$	+(-40+108.9)
									+Pro	I7	+97.3	Gos. acid-N(Me)$_2$	I9	+(-56.7+26.7)		Gos(Orn)+His +Pyr	$I5_2$	+($I8_2$+137.4)
									+His	$I4_2$	+137.3					Gos(Orn)+Trp +His+Pyr	F2	+($I5_2$+186.2)
						-AzGly	$I8_1$	-58.1	+Leu	E_1	+113.2					Gos(Orn)+Ser +Trp+His+Pyr	$I10_1$	+(F2+86.8)
										F_1	+113					Gos(Orn)+Tyr-Ser+Trp+His+Pyr	I11	+($I10_1$+163.2)
						Acylhidrazine of Gos.	E2	-43.1										
						-His	$I10_2$	-136.9	+Pro-Ser (I6+Pro)	I12	+(96.9-87.0)					Gos(Orn)+D-Ser(^{t}Bu)-Tyr-Ser+Trp+His+Pyr	I13	+(I11+143.2)
Triptorelin	1311.2	I2	+0.1	^{t}Bu	$I17_1$	+56.7	-(Pyr+Pro)	$I19_2$	-(110.9+97.6)	+His	$I5_1$	+137.5	Deamidated triptorelin	I1	+1.1				
		I3	+0.4		$I18_2$	+56.6					I6	+137.4						$I13_2$	+14.9
		I7	+0.1		$I19_1$	+56.9					I8	+137.5							
					I20	+55.8					I12	+137.6					Amination in Arg	$I16_1$	+14.9
		I9	0		$I21_1$	+56.0					$I13_1$	+137.5							
		I10	-0.1		$I22_1$	+56.0			+Tyr	$I5_2$	+163.5		I4	+0.8				$I17_2$	+14.6
					$I22_2$	+56.9				I15	+163.5								
		I11	+0.1							$I16_2$	+163.5							I18	+14.6
		I14	+0.4							$I21_2$	+163.5								

The original nomenclature found in [19-24] for the cromatographic peaks and the coeluting impurities is followed.
The mass data represent the average of three separate measurements.

of reactive side-chains have been successful excepting for *tert*-butylated sequences. The main group of impurities in eledoisin, leuprolide and triptorelin crude mixtures correspond to *tert*-butylated sequences ($\Delta M \cong +56$) (Table 2). Even a doubly *tert*-butylated sequence ($\Delta M \cong +112.6$) have been identified as impurity I8 in leuprolide crude mixture, corresponding to the presence of *tert*-butylated hydroxyl groups in Serine and Tyrosine side-chains (Figure 2). Obviously, no *tert*-butylated sequences have been found in carbetocin and goserelin crude mixtures because other protection schemes were used.

Deletion sequences

The incomplete removal of blocking groups also leads to the formation of chains from which one of the amino acid residues is absent. Such impurities have been designated as "failure sequences" or "deletion sequences" [9,34]. The presence of modified eledoisin, goserelin and triptorelin sequences due to the absence of Pro and/or Pyr have been observed. The lack of a Ser residue originated failure sequences in leuprolide and eledoisin crude mixtures. His and AzGly residues were also absent in the sequence of impurities $I10_2$ and $I8_1$ respectively, detected in goserelin crude mixtures. As Gln, Lys and Arg residues in impurities 11, D and I7 in carbetocin, eledoisin and leuprolide crude mixtures, respectively. On the other hand, in goserelin crude mixtures the loss of the C-terminal semicarbazide group ($\Delta M \cong -43$) leads to the formation of the acylhidrazine, impurity E2, that has been previously observed by other authors in degradated goserelin [16,35].

Amino acidic insertions

The use of an excess of equivalents in the coupling steps to ensure the maximum coupling efficiency may lead to the insertion of an additional amino acid in the target peptide sequence [9]. All the studied crude mixtures show this kind of impurities (Table 2), particularly in goserelin crude mixture until 8 different structures produced by amino acidic insertions have been identified. In triptorelin crude mixture until five impurities – $I5_1$, I6, I8, I12, $I13_1$ – have been associated with diastereoisomeric forms of endo-His-triptorelin. In leuprolide and goserelin crude mixtures, sequences incorporating an additional Histidine or Arginine residue have been detected (Table 2). In some cases, as it can be observed in eledoisin crude mixture, the sequence corresponding to the additional coupling of Lysine to the target peptide, coexists with the equivalent deletion sequence. This also happens with Arginine and Proline residues in leuprolide and goserelin mixtures, respectively, and can be temptatively explained due to both type of by-products are expected to be obtained under different reaction conditions.

Incomplete sequences

In all the cases, before completion of the synthesis, the acidic precursor was cleaved from the solid support with simultaneous deprotection of the side chains using TFA. The acid precursor has not been detected in the analyzed mixtures, but in goserelin crude mixture a derivative of the acid precursor was identified as I9. The difference between its molecular mass (1239.1) and the molecular mass of the acid precursor

Table 3. Protection groups and additional steps after the SPPS procedure used for the synthesis of the target peptides.

Protection schemes used in linear SPPS procedures[1]							Additional steps after the coupling of the last amino acid
Target peptide	**α-amino group**	**α-amino group (Arg)**	**Hydroxyl groups (Ser and Tyr)**	**Sulfhydril groups (Cys or Met)**	**NH group in the imimidazole ring (Trp)**	**NH group in the imimidazole ring (His)**	
Carbetocin	Fmoc	Pmc		Trt			Last coupling of Cl-CH_2-CH_2-CH_2-COOH, acydolitic treatment (cleavage and deprotection), DIEA (cyclization) and Amidation
Eledoisin	Fmoc		^{t}Bu	^{t}Bu			Amidation of the eledoisin acid
Leuprolide	Fmoc	Pmc	^{t}Bu		Trt		Amidation with $CH_3CH_2NH_2$ of the leuprolide acid
Goserelin	Fmoc		2-Cl-Trt			Mmt	Amidation of the goserelin acid
Triptorelin	Fmoc	Pmc	^{t}Bu			Trt	Amidation of the triptorelin acid

[1] Fmoc (9-fluorenylmethoxycarbonyl), ^{t}Bu (tert.-butyl), Trt (triphenylmethyl), 2-Cl-Trt (2-chloro-triphenylmethyl), Pmc (2,3,5,7,8-pentamethylcroman-6-sulfonyl), Mmt (p-methoxytriphenylmethyl).

(1212.4) is 26.7. This is attributed to the formation of a dimethylamide from the acid precursor, by reaction with the dimethylformamide or the DIEA (N,N-diisopropylethylamine) used in the synthesis procedure. Similarly, in carbetocin crude mixtures, some of the identified impurities have been originated during an inefficient cyclization of the acidic precuror. This is the case of impurity 1 (Table 2) that corresponds to a precursor of carbetocin, before the coupling of Cl–CH_2-CH_2–CH_2-COOH, or impurity 8b, a by-product originated because of an incomplete cyclization, after these bridge has been inserted. Sequence assigned to impurity 10 is the same as for impurity 8b, but with the absence of a Glutamine residue.

Products of side chain reactivity

Amino acid residues can undergo undesired reactions in their side chains such as alkylations, oxidations, cyclizations and substitutions, even if they are conveniently protected. Thus, in goserelin and triptorelin crude mixtures impurities with molecular mass differing aproximately between 14 and 15 Da from the molecular mass of the target peptide have been identified, and this suggests the addition of an -NH_2 group to the side chain of the Arginine residue. In goserelin synthesis there is a particular side reaction of Arginine residue that gets great importance in our case. Exposure of the side chain of Arginine residue to alkali may produce its conversion into ornithine (Figure 5) [10], with an unprotected amino group that enables another potential coupling-site. In each successive step the amino acid can be linked to the main sequence and to the side chain of ornithine. Up to six impurities ($I8_2$, $I5_2$, F2, $I10_1$, I11 and I13) can be attributed to this side reaction. Deamidation of the C-terminal amide side chain of an Asparagine residue to form a free carboxylic acid may occur via either direct hydrolysis or via cyclic imide formation [36]. The change of one -NH_2 group to an –OH group resulted in a molecular mass measured difference of approximately 1 Da [45]. Deamidated triptorelin ($\Delta M \cong +1$) have been identified in impurities I1 and I4 of triptorelin crude mixture. Histidine is another residue with reactive side chains and in general have been conveniently protected (Table 3). However, impurity I5 in leuprolide crude mixture has been identified as a substituted guanidine originated by reaction of the imidazol ring of the Histidine side chain when a carbodiimide is used in the coupling step

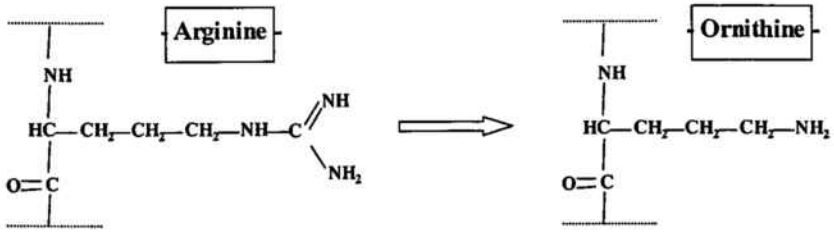

Figure 5. Conversion of Arginine into Ornithine.

(Figure 6) [9].

Oxidations and reductions

The side chain of Triptophan is quite reactive in acidic conditions, and it can undergo oxidations, dimerizations or reductions [37]. Impurity B3 in leuprolide crude mixture is identified as the leuprolide sequence with the indole ring of Triptophan residue oxydized ($\Delta M \cong +15.7$). Conversely, impurities A and B1 in the same crude mixture have been associated with leuprolide diastereoisomers with the indole ring of Tryptophan residue reduced. This reduction may be promoted by the presence of triisopropylsilane during the cleavage of the peptide-resin bond [38]. Cysteine and Methionine residues tend also to experiment oxidations during the synthesis procedure. The oxidation to sulfoxide derivatives is simply promoted by prolonged exposure to air [8]. Thus, molecular masses of diastereoisomeric impurities 2b, 3 and 4 in carbetocin crude mixtures differ approximately +16 Da from the molecular mass of carbetocin, and this can be attributed to the oxidation of the sulfhydryl group of the Cysteine residue. Similarly, impurity A in eledoisin crude mixture ($\Delta M \cong 16.2$ Da) corresponds to the oxydized Methionine residue. In carbetocin and eledoisin crude mixtures have been found by-products originated by oxidation of Cysteine residue in some of the identified impurities. Impurities 9 and 5 in carbetocin crude mixtures are the sulfonate and sulfone of impurity 8b –an incomplete sequence of carbetocin- and the sulfoxid of impurity 11 –a deletion sequence-, respectively. Impurity 2 in eledoisin crude mixture is identified as impurity 1, an eledoisin with an additional Lysine, that has been oxydized in the thioether group of Methionine residue .

4. Concluding remarks

LC-ES-MS analysis of peptide crude mixtures have demonstrated to be an efficient way to furnish fast and reliable molecular mass information of target peptides and their side-products obtained during SPPS procedures. Separation and identification of these side products may be regarded as a key tool for the optimization of the synthetic procedures as well as to fulfill with regulatory agencies before commercialization as a safe and effective peptide-based pharmaceutical drug. However, in order to achieve a deeper characterisation of the side products in the peptide complex mixture, LC-ES-MS-MS analysis may be performed. Alternatively, the

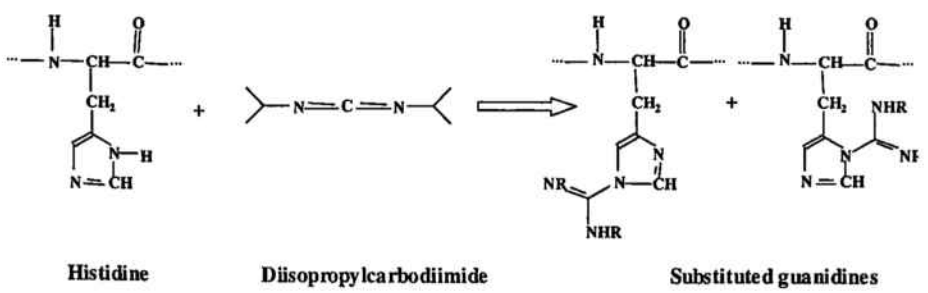

Figure 6. Reaction of formation of a substituted guanidine.

combined use of LC-ES-MS and CE have demonstrated to be a complementary and efficient way to confirm the structural assignments made solely based on the measured molecular masses [39].

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JOURNAL OF CHROMATOGRAPHY A

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Liquid chromatography–mass spectrometry and capillary electrophoresis combined approach for separation and characterization of multicomponent peptide mixtures Application to crude products of leuprolide synthesis☆

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Received 12 June 2001; received in revised form 31 December 2001; accepted 31 December 2001

Abstract

A sequential combination of reversed-phase liquid chromatography–mass spectrometry (LC–MS) and capillary electrophoresis (CE) has been explored in order to perform separation and characterization of a multicomponent peptide mixture from the synthesis of leuprolide. The mixture was first analyzed and fractionated by LC–MS, and the collected fractions were subsequently separated by CE. Unambiguous identification of the electrophoretic peaks was achieved by injecting the collected fractions separately and spiking the leuprolide crude mixture. Furthermore, structural information about the components of the mixture provided by several semi-empirical migration models has been used to check the accuracy of the structures previously proposed by LC–MS. Combination of the two orthogonal techniques results in an enhancement of their individual selectivity characteristics. © 2002 Published by Elsevier Science B.V.

Keywords: Peptides; Leuprolide

1. Introduction

The novel analytical needs of researchers working in pharmaceutical, clinical, environmental and biotechnological sciences often require the analysis of complex mixtures of structurally-related compounds. Moreover, in many cases some of the analytes may be present at very low concentration [1,2]. The development of efficient, selective and sensitive methods is necessary to separate and characterize these complex samples. However, single separation methods often lack the peak capacity required for a complete separation and quantitation of all the mixture components and, as a consequence, complementary separation techniques must be employed [3–7]. The application of multidimensional separation approaches, with enhanced separation efficiency, may permit the unambiguous characterization of complex mixtures. Moreover, with a suitable technical implementation, fully-automated, high-throughput multidimensional separation systems are possible [5–7].

☆Presented at the 30th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques/1st Meeting of the Spanish Society of Chromatography and Related Techniques, Valencia, 18–20 April, 2001.

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00025-0

Several multidimensional separation approaches have been described in the literature for the analysis of complex protein or peptide mixtures [3–7]. Two-dimensional (2D) gel electrophoresis with off-line mass spectrometry has been the method traditionally employed in this field [4]. However, this technique often results in slow, non-reproducible and labour intensive analysis. Reversed-phase liquid chromatography (LC) using ionic-pair reagents, such as trifluoroacetic acid, in the mobile-phase, has been widely used for the separation of peptides and proteins [8,9]. Furthermore, LC coupled to mass spectrometry (MS) leads to fast and unambiguous characterization of complex mixtures [7,10,11]. A two-dimensional LC system with tandem mass spectrometric detection (LC–LC–MS–MS) has been recently presented as an alternative approach for structural proteomics [7]. Although these two-dimensional systems are widely accepted because they provide valuable information, the mechanisms of the coupled separation techniques are based on the same principles. It is expected that the more orthogonal the mechanisms of the coupled separation techniques are, the better the combined resolving power.

Capillary electrophoresis (CE) has demonstrated its ability to perform efficient and selective separation and characterization of peptides and protein mixtures [12–14]. The mechanism of CE separations based on the charge-to-mass ratio can be considered orthogonal to the partitioning mechanism of LC separations, which is mainly based on the hydrophobic character of the molecules. Combined separation approaches exploiting this orthogonality can be advantageous to obtain separations with enhanced selectivities.

The off-line combination of LC with CE has been extensively explored to perform tryptic mapping of certain proteins [15–21]. The tryptic digests are firstly manually fractionated, according to hydrophobicity, by LC, and the components of the fractions are then separated, according to their charge-to-mass ratio, by CE. Moreover, LC fractions can be easily concentrated prior to the second separation step, which leads to better limits of detection. The characterization capability of these LC–CE systems is increased when on-line MS detection is incorporated in any of the dimensions [20]. On-line or 'comprehensive' LC–CE instruments have also been developed [5,22–24]. In these systems the continuous sampling of the chromatographic eluent and its sequential analysis by CE represent an important throughput improvement as compared with the fraction collection mode. However, reported limits of detection are rather poor, because the sample is diluted in the first chromatographic dimension. To overcome this problem, micro-LC–CE systems with laser-induced fluorescence (LIF) detection are being explored for the analysis of diluted analytes [23,24]. As part of these attempts to develop high-throughput and sensitive methodologies for peptide and protein multidimensional analysis, a few microchip devices have been recently described [25], that are able to perform 'comprehensive' two-dimensional open tubular capillary electrochromatography and CE (OTCEC–CE), but these laboratory-on-a-chip technologies are still in their early development [26].

The study of the migration behaviour of peptides and proteins in CE may also provide valuable structural information. For example, several semi-empirical approaches have been proposed to relate electrophoretic mobility to the structural parameters of peptides and proteins. These semiempirical models may be very useful to predict electrophoretic mobility and to perform separation optimizations [27,28]. They also offer a valuable characterization tool, particularly useful when on-line MS detection is not available. Several CE–MS interfaces have been described [29], but only a few CE–MS instruments are commercially available [30]. Several years will pass before CE–MS becomes widely used in analytical laboratories.

Solid-phase peptide synthesis (SPPS) procedures are routinely used to obtain new peptide-based drugs. However, these synthetic procedures often result in complex mixtures [31] that need further separation and characterization [11,32]. LC and CE have proved their suitability for the analysis of multicomponent peptide mixtures, but they are often unable to resolve and identify all the components [11,32,33]. In this work, a combination of LC–MS and CE has been used for the separation and characterization of a complex crude mixture obtained from the synthesis of leuprolide, a widely used therapeutic hormone, structurally analogous to the luteinizing hormone-releasing hormone (LHRH). Crude leuprolide was first fractionated and characterized by LC and LC–

MS, then it was analyzed by CE. In order to obtain an unambiguous identification of the electrophoretic peaks, it was necessary to first inject individual fractions previously collected by LC and then the leuprolide crude spiked with these fractions. The combination of the two orthogonal techniques results in an enhancement of their individual selectivity characteristics. Additionally, several semi-empirical approaches relating the electrophoretic mobilities and structural parameters of peptides and proteins have been tested in order to confirm identity assignments made by LC–MS and CE, according to molecular mass (M_r) and to charge-to-mass ratio, respectively.

2. Experimental

2.1. Chemicals and reagents

All the chemicals used in the preparation of buffers and solutions were analytical reagent grade. Crude leuprolide (Pyr–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–NHEt) used in this study was supplied by Lipotec (Barcelona, Spain). Leuprolide crude was dissolved in water at a concentration of 1 mg/ml, and was stored in a freezer at 0 °C when not in use. Water, with a conductivity lower than 0.045 mS/cm, was obtained by a Milli-Q water purification system (Millipore, Molsheim, France). Acetonitrile (MeCN), trifluoroacetic acid (TFA), formic acid 90%, ammonia solution 25% and acetone were supplied by Merck (Darmstadt, Germany). All samples and eluents were passed through 0.22 μm nylon filters (MSI, Westboro, MA, USA).

2.2. Appàratus

For the LC experiments an ISCO (Lincoln, NE, USA) Model 2350 chromatographic pump with a 100-μl sample loop in a Valco injection valve and a variable-wavelength V^4 absorbance detector (ISCO) operating at 220 nm was used. The chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller Software (ISCO) running on a personal computer. A 5 μm Kromasil C_8 column (250×4.6 mm I.D.) (BC Aplicaciones Analíticas, Barcelona, Spain) was used at room temperature. An LKB (Bromma, Sweden) time-programmable fraction collector was used to isolate LC fractions.

CE experiments were performed at 25 °C on a P/ACE System 5500 (Beckman Instruments, Palo Alto, CA, USA) equipped with a photodiode array detector (operating between 190 and 230 nm). An untreated fused-silica capillary of 57 cm (50 cm to the detector)×75 μm I.D., purchased from Polymicro Technologies (Phoenix, AZ, USA), was used. Samples were hydrodynamically injected for 3 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). The working voltage was 15 kV. Data were recorded on a personal computer-based data system (P/ACE Station 1.0 interfaced to the Gold System) supplied by Beckman.

2.3. Procedures

2.3.1. LC procedure

Mobile phase composition was optimised at 1 ml/min as described previously [11]. The solutions tested as mobile phases consisted of different MeCN–water mixtures, ranging from 25% to 35% MeCN (v/v) containing a 0.1% (v/v) of TFA. Two of them, containing 31% and 35% (v/v) MeCN, respectively, were selected to fractionate crude leuprolide.

Before beginning fraction collection, three LC–UV analyses were made to check the reproducibility of the separation profile and retention times. In these experiments, a large sample (100 μl of 1 mg/ml leuprolide crude) was injected. The LC column was then directly connected to the fraction collector with a PTFE tube of the same length and diameter as the tube used to connect it to the UV detector cell. Fractions corresponding to single peaks, previously characterized by LC–electrospray ionization (ESI)–MS [11], were automatically collected. The fractions from 10 separate runs were pooled together and lyophilized in plastic vials. This process was repeated in order to obtain two sets of samples; the lyophilisate fractions from the first set were later reconstituted with 50–75 μl of water (except from that corresponding to leuprolide which was redissolved with 375 μl of water) and those from the second set were used to spike equivalent volumes of a 1 mg/ml leuprolide crude mixture. The two sets of fractions will be called standard and spiked set, respectively.

2.3.2. CE procedure

The influence of pH on CE separations was studied using a 75 m*M* formic acid running buffer adjusted to the appropriate pH with 25% ammonia solution. The capillary was rinsed each day, before starting the analysis, with 0.1 *M* aqueous NaOH solution (10 min), water (20 min) and running buffer (30 min) and, finally a 15 kV voltage was applied for 15 min at 25 °C. The capillary was also flushed with buffer for 3 min before each sample injection and was stored overnight filled with running solution.

Peak identification in the electropherogram of leuprolide crude mixture was performed by injecting 1 mg/ml leuprolide crude, as well as both LC fraction sets, using 75 m*M* formic acid at pH 4. A 1 mg/ml solution of leuprolide crude containing 3% (v/v) acetone as electroosmotic flow (EOF) marker was also injected under the same conditions to obtain effective electrophoretic mobilities (m_e) [14]. m_e was calculated as the difference between the apparent mobility of each peptide and the mobility of acetone [14]. Each m_e value was obtained as the average of three replicates.

3. Results and discussion

3.1. CE analysis. Influence of pH of the running electrolyte

Highly efficient and selective peptide separations have been achieved by CE under acidic conditions in unmodified bare fused-silica capillaries [12–14]. Leuprolide crude mixture was analysed using a 75 m*M* formic acid buffer adjusted to different pH values within the range 2–4.5 (Fig. 1). The resulting electropherograms contain a major peak corresponding to the target peptide leuprolide, as well as a number of peaks of unidentified peptide substances.

Except for the peak indicated in Fig. 1, the migration behaviour of the mixture components does not show a great dependence on pH in the studied

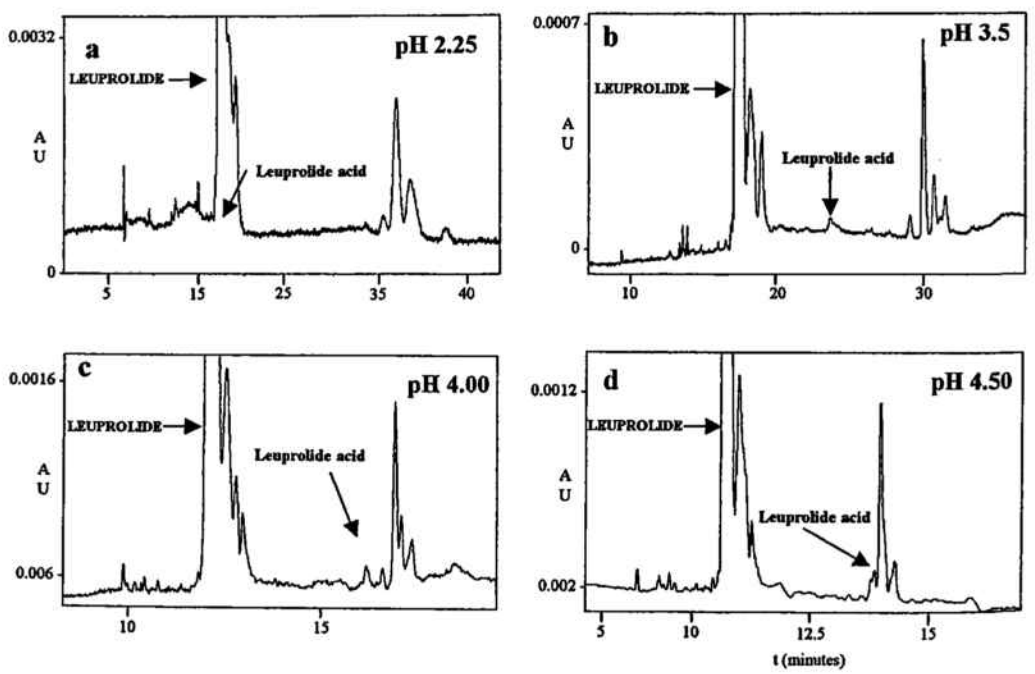

Fig. 1. Effect of buffer pH on leuprolide crude mixture CE separations. pH values (a) 2.25, (b) 3.5, (c) 4.00 and (d) 4.50.

range. This electrophoretic behaviour can be explained by the number and type of ionizable residues present in each peptide. In leuprolide, there are two basic residues—His and Arg—and an acidic phenolic group in Tyr. In a previous work, the pK values associated with His and Tyr side chains in triptorelin and buserelin were determined by CE [34]. Experimental pK values of Tyr and His in triptorelin and buserelin are about 9.75 and 6.13, respectively. The dissociation of the Arg guanidine group was not determined, but the value given by Lehninger for free Arg is 12.40 [35]. Therefore, the three residues must be fully protonated over the pH range studied and no changes of the peptide net charges are to be expected if only these ionizable groups are present. However, an increase in pH provoked a considerable reduction in the m_e of the peak indicated in Fig. 1, which probably can be attributed to a change in the ionization state. Within the pH range considered, this can only be explained by the deprotonation of a carboxyl group. Lehninger's pK values for carboxyl groups range from 2.34, for a free α-COOH, to 4.25, in a Glu residue [35]. In our case, this extra carboxyl group should be in a terminal position, because no glutamic or aspartic acids are expected in leuprolide impurities. In fact, in the last step of the synthesis of leuprolide, leuprolide acid is amidated to give the final leuprolide ethylamide. The presence of leuprolide acid in leuprolide crude mixture is verified by injecting leuprolide crude spiked with a leuprolide acid standard at pH 4 (data not shown). CE at pH 3.5–4 using a 75 mM formic acid buffer (Fig. 1b and c) is therefore a useful tool for the simple and rapid monitoring of the final amidation step of leuprolide synthesis. This control cannot be made by LC, because the chromatographic retention of leuprolide acid is not pH-dependent and leuprolide acid co-elutes with leuprolide under the pH conditions studied [11]. Optimum CE separations of the leuprolide mixture components are obtained using a 75 mM formic acid buffer at pH 4 (Fig. 1c).

3.2. LC analysis. LC–MS characterization

The separation and characterization of the components of the leuprolide crude mixture was previously performed by LC and LC–MS [11]. The composition of the mobile phase was optimized using the LSER (linear solvation energy relationships) method [8,11,32], and then, characterization by LC–MS was conducted under optimum separation conditions [11]. Typical UV-chromatograms obtained by 31% (v/v) or 35% (v/v) of MeCN mobile phases are shown in Fig. 2a and b, respectively. Masses measured by LC–ESI–MS and structures proposed for labelled peaks in Fig. 2a and b are shown in Table 1. Although characterization was possible by means of LC–ESI–MS [11], the components of the mixture were not fully-resolved. Further separation is therefore desirable and CE can be a useful alternative, as it utilizes a different separation mechanism.

3.3. LC fractionation of leuprolide crude mixture

In order to identify the peaks observed in the electropherogram of leuprolide crude mixture shown in Fig. 1c, labelled chromatographic peaks in Fig. 2a and b were collected [15–21]. A mobile phase with an MeCN percentage of 31% (v/v) was selected to isolate the peaks that elute close to leuprolide (Fig. 2a), while a mobile phase with 35% (v/v) of MeCN permits a rapid collection of the more hydrophobic substances at higher retention times (Fig. 2b). Fractions from several runs were pooled, lyophilized and redissolved in a smaller volume, to concentrate the impurities. A direct identification from the injection of the standard set of fractions is difficult, because migration times are not reproducible enough between successive runs. This could be attributed to slight changes in the electroosmotic flow between different runs, which arises from the reversible modification of the capillary inner wall. Constant electrophoretic mobilities could be obtained by sequential injection of leuprolide crude sample (three runs), but migration times always show small differences that preclude an automatic and unambiguous peak identification in such a complex sample from the fractions of the standard set. Therefore, a second set of spiked samples had to be prepared to identify the peaks in the electropherogram of the leuprolide crude. Nevertheless, co-migration of some of the impurities and their heterogeneous concentrations in leuprolide crude mixture made the information obtained from the injection of the fractions of the standard set very helpful.

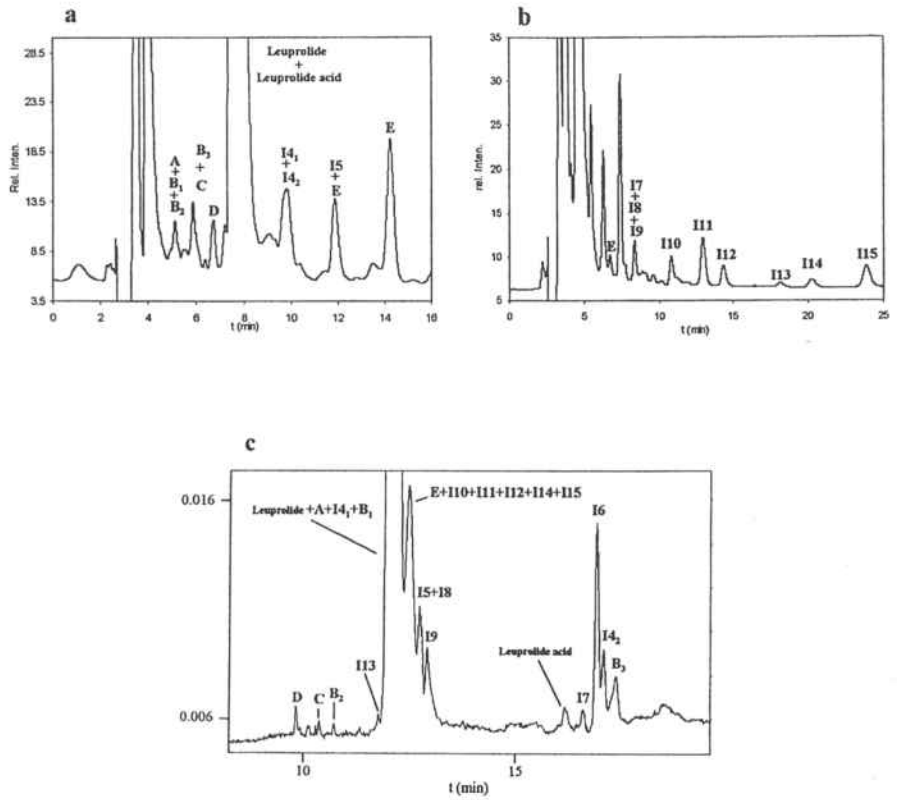

Fig. 2. Comparison of (a and b) LC and (c) CE separations of leuprolide crude mixture. LC mobile phases: (a) water–MeCN (69:31, v/v) with 0.1% (v/v) TFA and (b) water–MeCN (65:35, v/v) with 0.1% (v/v) TFA. CE running buffer: 75 m*M* formic acid, adjusted to pH 4 with NH_3. Other conditions are given in Section 2.

3.4. Identification of CE peaks

Standard and spiked sets of fractions collected by LC were separated under optimal conditions by CE (Figs. 1c and 2c). Peak assignment was made according to these CE results and structures previously proposed by LC–MS [11]. There are great differences in selectivity between the two techniques; for example the elution orders change and some impurities that were previously separated by LC (i.e. *tert.*-butylated impurities) comigrate, as can be seen in Fig. 2c. The partitioning mechanism in LC, mainly based on the hydrophobic character of the analyte, is complementary to the charge-to-mass ratio-based separation mechanism of CE. Moreover, they are considered orthogonal because correlation between capacity factors in LC and mobilities in CE are very low [15]. Thus, impurities $I4_1$ and $I4_2$, that co-elute by LC, can be baseline resolved by CE as can be seen in Fig. 3a (standard set injection of LC fraction $I4_1+I4_2$) and Fig. 3b (spiked set injection of the same fraction). One of the impurities co-migrates with the target peptide, and as a consequence, the leuprolide diastereoisomer $I4_1$ is assigned to the first peak separated by CE. In Fig. 3c the electropherogram corresponding to the leuprolide crude mixture spiked with impurity I13 is shown. Elution time for I13 is more than 10 min higher than the time for

Table 1
LC data obtained from the analysis of leuprolide crude mixture. Proposed structures by LC–MS [11]

Impurity	LC analysis		Measured M_r	Proposed sequence [11]
	t_R (min)			
	31% MeCN	35% MeCN		
A			1211.1	Leuprolide with a reduced tryptophan
B_1	5.11		1211.1	Leuprolide with a reduced tryptophan
B_2			1365.5	Additional arginine insertion
B_3	5.87		1225.0	Leuprolide with an oxidized tryptophan
C			1346.1	Additional histidine insertion
D	6.73		1105.2	Not identified
Leuprolide	7.39		1209.3	[a]Pyr–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–NH–CH_2–CH_3
Leup. acid			1182.3	Pyr–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–COOH
$I4_1$	9.87		1209.2	Leuprolide diastereoisomer
$I4_2$			1320.4	[b]Fmoc–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–NH–CH_2–CH_3
I5	11.85		1335.5	Formation of a substituted guanidine
E		6.80	1265.7	*tert.*-butylated leuprolide
I6	14.20		1124.4	Lack of a serine
I7			1053.3	Lack of an arginine
I8		8.35	1321.9	Doubly *tert.*-butylated leuprolide
I9			1395.5	Additional tryptophan insertion
I10		10.80	1308.8	Not identified
I11		12.96	1265.5	*tert.*-butylated leuprolide
I12		14.34	1265.7	*tert.*-butylated leuprolide
I13		18.35	1088.0	Not identified
I14		20.25	1265.5	*tert.*-butylated leuprolide
I15		23.84	1265.6	*tert.*-butylated leuprolide

[a] Pyr: pyroglutamic acid or 5-oxo-proline.
[b] Fmoc: 9-fluorenylmethoxycarbonyl.

leuprolide (Table 1), but these compounds are not baseline resolved in the electrophoretic conditions used. Fig. 2c shows an electropherogram of leuprolide crude mixture under optimal CE separation conditions. Identified electrophoretic peaks have been labelled according to their original LC denomination. Table 2 summarizes the electrophoretic mobilities (m_e) and identities of the peaks resulting from CE analysis of leuprolide crude mixture.

3.5. Study of the electrophoretic behaviour using semiempirical models

There are several semi-empirical approaches that relate electrophoretic mobility in free solution CE to structural parameters of proteins and peptides [27,28]. These models can be used to predict electrophoretic mobilities but also for optimizing CE separations, studying structural modifications, charge characteristics and conformations [27,28,36].

In general, the electrophoretic mobility (m_e) of a peptide is proportional to its charge (q) and inversely proportional to its Stoke's radius (r). The r is generally expressed in terms of molecular mass (M_r), because the volume of a molecule is proportional to its mass if the density is constant [37]. M_r can be easily determined from the amino acid sequence of the peptide or, as in this case, measured by MS [27,28]. The classical equations describing semi-empirical models are deduced from assumptions concerning the peptide shapes and the forces that they experience during electrophoretic motion [27,28]. The general form of the equation relating electrophoretic mobility, molecular mass, and molecular charge is as follows:

$$m_e = A \cdot \frac{q}{M_r^{\alpha}} \quad (1)$$

where A is a constant, q is the peptide charge and,

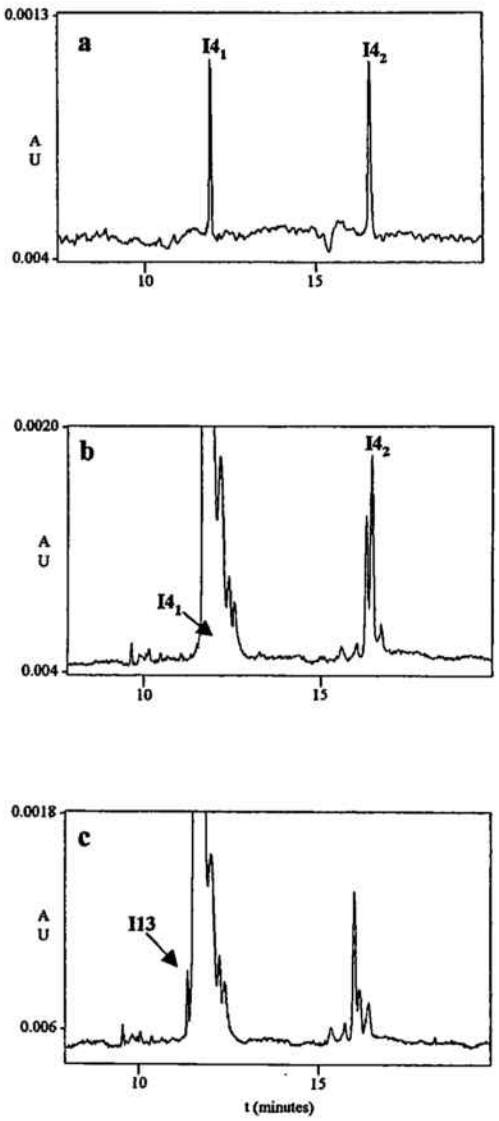

Fig. 3. Electropherograms of several LC fractions corresponding to: (a) I4 (standard set), (b) I4 (spiked set) and (c) I13 (spiked set).

for the various empirical models, $\alpha = 1/3$ for the Stoke's law, $\alpha = 1/2$ for the classical polymer model, and $\alpha = 2/3$ for Offord's surface area law [27,28,37]. In general, for peptides, α approaches 1/3 when peptides are modelled as spherical particles, that have high charge densities; α approaches 1/2 when the peptide is considered as a classical polymer with a lower charge density; and, α approaches 2/3 for larger and more rigid structures, which experience frictional forces that are proportional to the surface area of the molecule during electrophoretic motion.

The molecular charge is an important variable in estimating the electrophoretic mobility. A simple estimation of peptide charge can be obtained at any pH using the peptide pK values and Sillero and Ribeiro expression [38], based on the Henderson–Hasselbach equation:

$$q = \sum_{n=1-4} \frac{P_n}{1 + 10^{\mathrm{pH} - \mathrm{p}K(P_n)}} - \sum_{n=1-5} \frac{N_n}{1 + 10^{-\mathrm{p}K(N_n) - \mathrm{pH}}} \quad (2)$$

where P_n and N_n are the number of cationic (i.e. P_1 = tNH$_2$, P_2 = His, P_3 = Arg and P_4 = Lys) and anionic (i.e. N_1 = tCOOH, N_2 = Asp, N_3 = Glu, N_4 = Cys and N_5 = Tyr) amino acid residues, respectively, and p$K(P_n)$ and p$K(N_n)$ are the negative logarithms of the ionization constants of these amino acids. The accuracy of the charge calculated in this way directly depends on the reliability of the pK_a values employed. In this work, Lehninger's pK values for the individual amino acid residues have been used, instead of the real pK values, which were not available [35]. This fact does not affect the accuracy of the charge calculation because pK values of His, Tyr and Arg are very high, and consequently, these residues are fully protonated at the experimental conditions used. The calculated charge values for the studied peptides at pH 4 are shown in Table 2.

3.5.1. Confirmation of structures proposed by LC–MS

Proposed structures for leuprolide crude mixture components, based on experimental M_r values obtained by LC–MS [11], can be confirmed by studying the structural information provided by the CE analysis. The simple estimation of charge provided by Eq. (2) was used in conjunction with the three semiempirical models previously mentioned, to correlate m_e and q/M_r^{α} according to Eq. (1). m_e values for leuprolide and its associated side products (Table

Table 2
CE data obtained from the analysis of leuprolide crude mixture. Calculated charge*. New proposed structures

Impurity	CE analysis			
	t_m (min)	Mobility (10^{-5} cm^2 V^{-1} s^{-1})	q (pH 4)	New proposed sequences
D	9.617	21.1	Not identified	
C	10.133	19.7	2.98	
B_2	10.450	18.5	2.99	
I13	11.316	16.2	Not identified	
Leuprolide			1.99	
A	11.617	15.5	1.99	
$I4_1$			1.99	
B_1			1.99	
E			1.99	
I10			Not identified	
I11	12.117	14.4	1.99	
I12			1.99	
I14			1.99	
I15			1.99	
I5	12.367	13.8	1.99	
I8			1.99	
I9	12.517	13.5	1.99	
Leup. acid	15.550	8.59	1.01	
I7	15.967	8.06	0.99	
I6	16.250	7.71	1.99[b] 0.99[c]	Lack of a serine and arginine is degradated to citrulline
$I4_2$	16.400	7.54	1.99[b] 1.00[c]	A substituted guanidine loses an amino group
B_3	16.667	7.23	1.99[b] 1.00[c]	Leuprolide with an oxidized histidine

* Charge calculation was performed using Sillero and Riberio equation (Eq. (2) [38]) and Lehninger's pK values: His (6.00), Tyr (10.00) and Arg (12.40).

[b] These charges were calculated taking into account the sequence proposed by LC–MS [11].

[c] These charges were calculated by taking into account the new proposed sequences.

2) were plotted versus $q/M_r^{1/3}$, $q/M_r^{1/2}$ and $q/M_r^{2/3}$ in Fig. 4a–c, respectively. According to these representations, there is a linear correlation between m_e and q/M_r^{α}, except for impurities I6, $I4_2$, B3. Charge calculations are based on the amino acidic sequence proposed after separating and measuring M_r by LC–MS. An erroneous sequence assignment of a mass measured by LC–MS can lead to an erroneous charge estimation. This is particularly important when ionisable residues are involved. Thus, at pH 4, the migration behaviour of I6, $I4_2$ and B3 does not agree with the doubly-charged structures first proposed. In order to obtain good correlations between their m_e and their charge-to-mass ratio, they must be single charged at pH 4. New sequences for these impurities are proposed according to the observed migration behaviour. I6 was first interpreted as lacking a serine with respect to the sequence of leuprolide. The new structure proposed for I6 also suggests the degradation of arginine to citrulline, which is a non-ionizable residue with the same mass as arginine [32]. $I4_2$ has been related with I5 and it has been attributed to a substituted guanidine which has lost an amino group, and as a consequence is singly charged at pH 4. On the other hand, the oxidation of tryptophan in leuprolide was originally proposed to explain the molecular mass of B3. However, according to CE data, an ionizable residue must be oxidized instead of tryptophan. Oxidation of histidine is also common during SPPS procedures [31]. Table 2 shows the new proposed sequences and calculated charges for I6, $I4_2$, B3. This new set of proposed structures, with known charges and masses, is used to recalculate correlations using the semiempirical models (Eq. (1)). Fig. 4d–f show the new linear correlations between m_e and $q/M_r^{1/3}$, $q/M_r^{1/2}$ and $q/M_r^{2/3}$ for leuprolide and its associated side products. Correlation coefficients (r^2) are given in

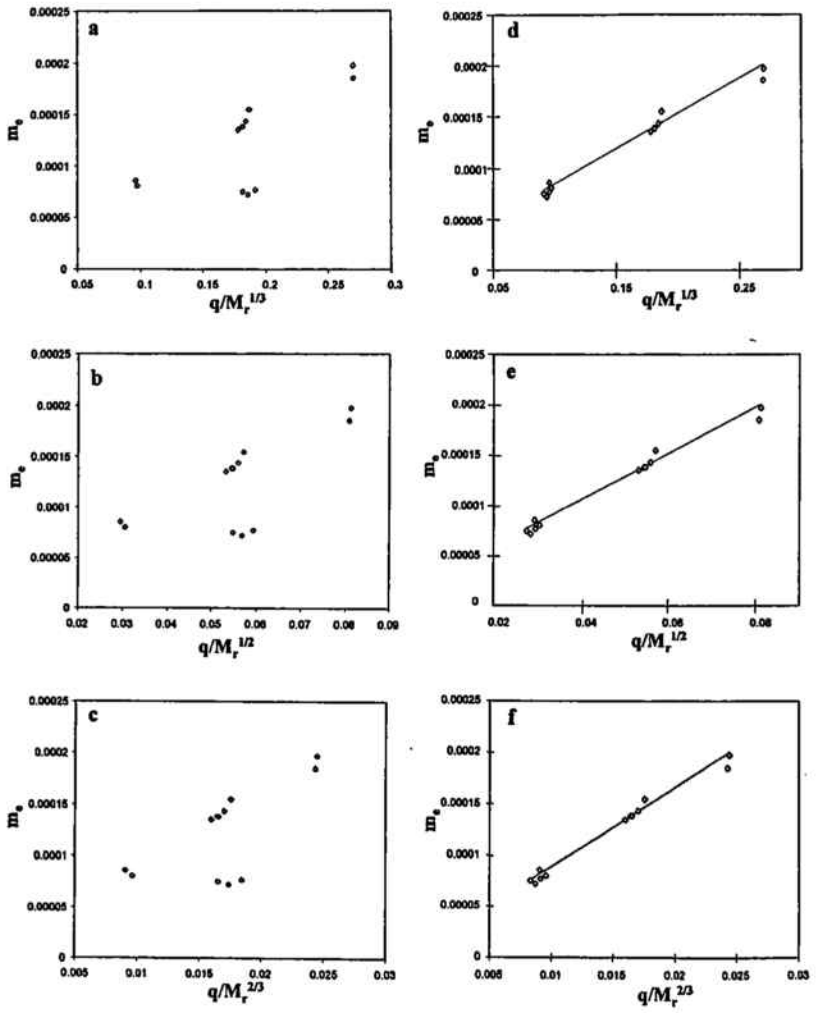

Fig. 4. Correlation of electrophoretic mobility (m_e), before and after proposing new structures, with (a and d) $q/M_r^{1/3}$ (Stoke's law), (b and e) $q/M_r^{1/2}$ (classical polymer equation) and (c and f) $q/M_r^{2/3}$ (Offord's surface area). The equation parameters and the correlation coefficients for the linear least squares fit are given in Table 3.

Table 3. The good correlations observed confirm the validity of the new proposed structures.

3.5.2. Testing semiempirical models for peptide migration behaviour

As can be observed from a comparison of the correlation coefficients given in Table 3, a slightly better linear correlation is observed when Offord's surface area model is applied. This suggests rigid peptide structures which undergo electrophoretic motion and experience frictional forces proportional to the surface area of the molecule [39]. Janini et al. already showed that these three models will yield comparable correlations for subsets of peptides with

Table 3
Summary of linear least squares parameters obtained from application of classical semi-empirical models in leuprolide crude mixture, uncorrected and corrected for charge suppression

Linear parameters $m_e = a + bX$	Stoke's law		Polymer law		Offord's law		Grossman's law
	$q/M_r^{1/3}$	$\ln(1+q)/M_r^{1/3}$	$q/M_r^{1/2}$	$\ln(1+q)/M_r^{1/2}$	$q/M_r^{2/3}$	$\ln(1+q)/M_r^{2/3}$	$\ln(1+q)/n^{0.435}$
a	1.715×10^{-5}	-4.741×10^{-5}	1.449×10^{-5}	-5.288×10^{-5}	1.187×10^{-5}	-5.795×10^{-5}	-4.679×10^{-5}
b	6.826×10^{-4}	1.910×10^{-3}	2.297×10^{-3}	6.470×10^{-3}	7.723×10^{-3}	2.184×10^{-2}	4.614×10^{-4}
r^2	0.9653	0.9848	0.9716	0.9880	0.9766	0.9873	0.9723

narrow molar mass ranges, as is our case. Nevertheless, it was also observed that better correlations were obtained when Offord's model was applied to a large set of peptides with a wide molar mass range [39].

Charge calculation using Sillero and Ribeiro's equation (Eq. (2)) is likely to result in an overestimation of q under conditions where the protein has an appreciable charge [27,28]. As the total charge on the peptide increases, the effect of other additional charges on its mobility decreases, resulting in an 'ineffectiveness' of a part of the peptide charge (charge suppression effect). The semi-empirical models described above can be improved if the electrostatic charge suppression effect is considered in Eq. (1). This means substituting the direct proportionality by a logarithmic dependence. Thus, Eq. (1) can be re-written as Eq. (3):

$$m_e = A \cdot \frac{\ln(1+q)}{M_r^{\alpha}} \tag{3}$$

This simple compensation for charge suppression is inadequate for proteins, where the magnitude of charge suppression is greater and the mechanisms are more complex [27,28]. Cifuentes and Poppe considered that an adjustable curvature better describes the charge suppression effect when charge increases, and they proposed a model corrected as $\ln(1+Bq)$ [40]. Fig. 5a–c shows plots of m_e versus $\ln(1+q)/M_r^{1/3}$, $\ln(1+q)/M_r^{1/2}$ and $\ln(1+q)/M_r^{2/3}$, respectively. Correlation coefficients (r^2) are given in Table 3. Linear correlation coefficients r^2 are slightly better when models corrected for charge suppression are used (Table 3).

3.5.3. Characterization of impurities unidentified by LC–MS

m_e can be estimated for impurities with known molecular mass using the improved semi-empiricals models. Conversely, molecular charge can be estimated for the unidentified impurities whose M_r has been previously measured by MS. Table 4 shows calculated charge values for impurities D, I13 and I10. At this point Grossman's semi-empirical relationship [27,28,41] can be introduced, where the number of amino acid residues of the peptide structure (n) is taken into account:

$$m_e = A \cdot \frac{\ln(1+q)}{n^{0.43}} \tag{4}$$

where A is again an adjustable constant. A good linear correlation between m_e and $\ln(1+q)/n^{0.43}$ is shown in Fig. 6. Better correlations are observed using models that consider molecular mass (Table 3), but Grossman's relationship is useful to estimate the number of amino acid residues in the unidentified D, I10 and I13 impurities. Table 4 shows charge values obtained for D, I10 and I13, using the classical models corrected for charge suppression, and their number of amino acids calculated from Grossman's equation. The information obtained about the ionizable groups, estimating the charge with the first type of models, and the number of amino acid residues calculated with Grossman's relation, can be used to propose a sequence for D, I10 and I13. This completes the characterization of leuprolide crude mixture. According to this information, D would have a charge of about 3 and contain 8 amino acid residues, which agrees with an additional His insertion in a leuprolide structure without the Pro and Tyr residues. For I10, a leuprolide with an additional Pro residue agrees with a doubly charged decapeptide. However, further information is needed to propose a structure for the doubly charged I13 octapeptide. An unambiguous

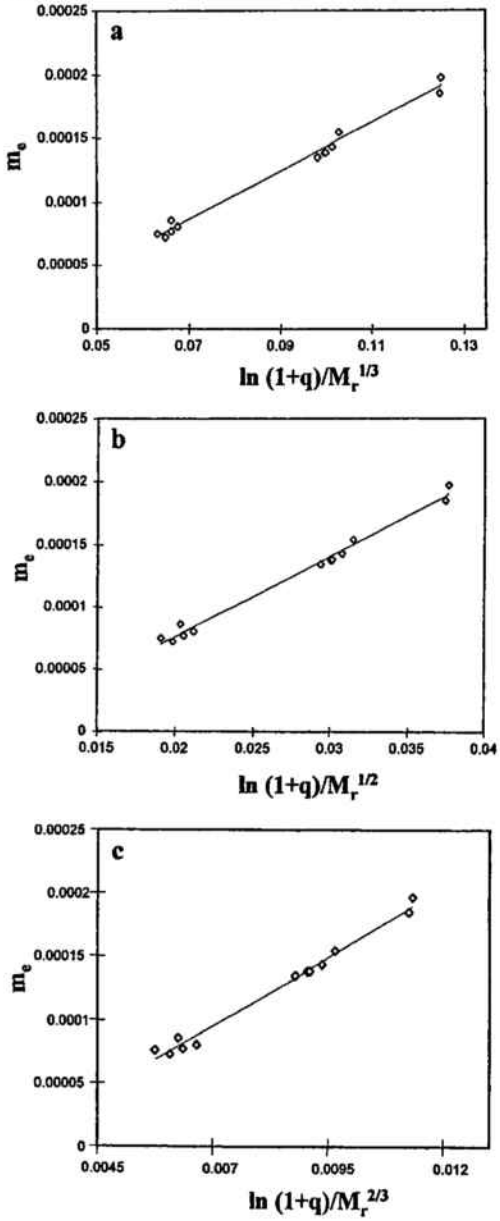

Fig. 5. Charge suppression effect is taken into account in plots of m_e correlations with (a) ln $(1+q)/M_r^{1/3}$, (b) ln $(1+q)/M_r^{1/2}$ and (c) ln $(1+q)/M_r^{2/3}$. The equation parameters and the correlation coefficients for the linear least squares fit are given in Table 3.

Table 4
Calculated charge and amino acid residue numbers for the unidentified impurities

Stoke's law		D	I13	I10
Stoke's law	q	3.06	2.09	1.99
	n	8.24	8.15	9.40
Polymer's law	q	2.89	1.99	2.00
	n	7.68	7.63	9.50
Offord's law	q	2.74	1.90	2.02
	n	7.17	7.15	9.60

q is calculated using each classical model corrected for charge suppression. n is calculated using Grossman's equation.

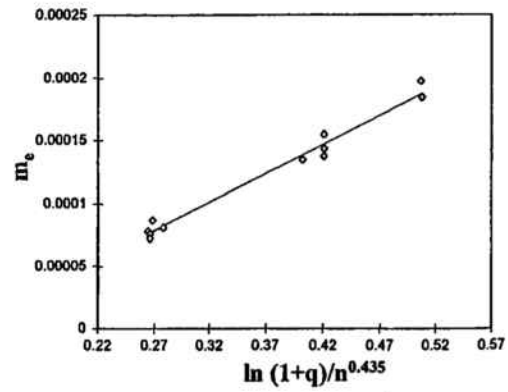

Fig. 6. Correlation of electrophoretic mobility (m_e) with ln $(1+q)/n^{0.435}$ (Grossman's law). The equation parameters and the correlation coefficient for the linear least squares fit is given in Table 3.

characterization of complex peptide mixtures would be only possible if separated peptides could be sequenced by means of MS–MS. A sequential automated combination of LC–MS–MS and CE–MS–MS may provide maximum separation and characterization capabilities to perform high-throughput analysis of complex, diluted mixtures. The performances of these combined techniques should be extensively explored in the future for the advantage of many people working in proteomic, biotechnological, environmental and pharmaceutical research.

4. Concluding remarks

Combination of LC–MS and CE has been successfully applied for the separation and characterization of a leuprolide crude mixture. The orthogonal separation mechanisms that are involved in both techniques result in complementary separation selectivities. CE analysis of collected LC fractions leads to the identification of the electrophoretic peaks. The applicability of semi-empirical models that describe the migration behaviour in CE has been assessed and the additional structural information obtained has been used to confirm structural assignments made on the basis of molecular mass measured by LC–MS. Furthermore, new sequences have been proposed for several impurities that remained unidentified.

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Capítulo 4. Separación y caracterización de hormonas peptídicas de bajo peso molecular mediante CE-ESI-MS

En el primer artículo de este capítulo se utiliza la CE-ESI-MS para separar y caracterizar una mezcla de hormonas peptídicas de gran interés terapéutico, que previamente había sido analizada por CE-UV. El instrumento empleado para realizar los análisis por CE-ESI-MS se compone de una CE convencional y un espectrómetro de masas con un analizador TOF, acoplados mediante una interfase comercial que emplea un líquido auxiliar coaxial al electrolito de separación, para asistir la formación del spray. El objetivo fundamental es optimizar de manera rigurosa los parámetros que afectan a la detección y a la separación por CE-ESI-MS cuando se emplean este tipo de interfases y comprobar la operatividad del acoplamiento cuando se emplea un analizador de masas de este tipo, que ha de proporcionar *a priori*, mayor exactitud y resolución de las masas medidas.

En el segundo artículo de este capítulo, se describe el uso de la SPE-CE y la SPE-CE-ESI-MS, utilizando fases estacionarias no selectivas, para mejorar los límites de detección en CE cuando se separan y caracterizan mezclas peptídicas en muestras diluidas. Se estudian los parámetros que afectan al diseño y construcción de los cartuchos de extracción y a las columnas de separación modificadas, y también los que afectan a los procesos de extracción y separación. La finalidad buscada es la de definir unas pautas que sistematicen y faciliten la forma de operar, cuando se emplean este tipo de novedosas metodologías. Ambos artículos se reproducen a continuación:

- ***Capillary Electrophoresis Coupled to Time of Flight-Mass Spectrometry of Therapeutic Peptide Hormones***
  V. Sanz-Nebot, F. Benavente, E. Balaguer, J. Barbosa
  Electrophoresis, 24 (2003) 883-891.

- ***Lowering the Concentration Limits of Detection of Analytes at the Nanogram per Milliliter Levels by on- line Preconcentration Capillary Electrophoresis***

  F. Benavente, M. C. Vescina, V. Sanz-Nebot, J. Barbosa, R. J. Stubbs, N. A. Guzmán

  J. Chromatogr. A, (2003) enviado.

Electrophoresis 2003, *24*, 883–891

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Capillary electrophoresis coupled to time of flight-mass spectrometry of therapeutic peptide hormones

We have established a method for separation and characterization of a series of peptide hormones of pharmaceutical interest and wide therapeutical use by capillary electrophoresis-electrospray-mass spectrometry (CE-ES-MS) using a sheath flow interface. Several parameters were systematically investigated, such as concentration of the electrolyte, organic solvent and sheath liquid composition, gas flow rates and capillary position. Moreover, limits of detection, linearity, repeatability and day-to-day reproducibility of the proposed method were studied in order to obtain the main quality parameters.

Keywords: Capillary electrophoresis / Hyphenated techniques / Peptide hormones / Time of flight-mass spectrometry
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1 Introduction

Capillary electrophoresis (CE) has become a powerful analytical technique with high analysis speed, high separation efficiency and low sample amounts needed [1]. This technique is widely used for analysis of a high number of biomolecules as peptides and proteins [2–5]. The coupling of CE with a mass spectrometer (CE-MS) provides a powerful system for the analysis of complex biological mixtures, replacing or complementing other conventional detection methods such as absorbance, electrochemical detection or laser-induced fluorescence, which prove to be less informative and universal [1, 6].

The use of a mass spectrometer as a detector enhances the utility of the CE and allows an efficient separation and identification of components in complex mixtures, obtaining structure and/or molecular mass information [1]. Among all kinds of mass spectrometers that can be used in combination with CE, a time-of-flight (TOF) analyzer is an ideal detector [1, 7, 9]. One advantage of this kind of analyzer is the fast acquisition rates (0.1 s) that provides an extremely short time to produce a full mass spectrum [7], allowing a high number of spectra for time unit and the complete characterization of the peak profile. Other advantages of a TOF analyzer are good mass resolution and sensitivity because most of the ions introduced into the vacuum system reach the detector [8].

The most used ionization technique for coupling CE and MS is electrospray (ES). Advantages of this technique are its high ionization efficiency, the soft nature of the ionization process, which avoids undesired fragmentations, the multiple charged ions obtained, which provides an *m/z* range compatible with most of the analyzers used, and the direct transference of molecules from the liquid phase to the gas phase *via* a CE-ES-MS interface, which provides a stable spray and current [6, 9–10]. Although three kinds of interfaces, sheathless, liquid-junction, and sheath-flow interface, have been used for CE-ES-MS coupling, sheath-flow interface has predominately been applied because it has an easy and reproducible construction, and allows a great versatility for buffer choice [6, 8, 10]. In this design the CE capillary is introduced in a steel tube, where the sheath liquid, usually a hydroorganic mixture, is also delivered. The tip of the tube is where the mixing of the sheath liquid and the separation buffer takes place. Thus, the CE flow rate is increased in several microliters per minute, improving spray stability but diluting the capillary eluent [8]. The sheath liquid must contain an electrolyte because it also acts as a CE terminal buffer allowing the electrical continuity and an efficient electrophoretic separation [6, 10].

In the last years, CE-ES-MS has been principally used for the analysis of peptides, peptide hormones and proteins, drugs and drug metabolites, biological extracts and for environmental analysis [1, 6]. Among these wide application fields, peptides represent a numerous class of important and widely spread biomolecules. Acting as hor-

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Abbreviations: MeCN, acetonitrile; **MeOH**, methanol; **TIE**, total ion electropherogram; **XIE**, extracted ion electropherogram

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mones, neurotransmitters, immunomodulators, enzymes or enzyme inhibitors, drugs, toxins and antibiotics, they are involved in control and regulation of many vitally important processes in living organisms [4, 11]. Therefore, development of separation, analysis and characterization methods remains one of the most challenging tasks to develop. Previously, methods based on CE and liquid chromatography (LC) with ultraviolet (UV) detection [12–14], and LC coupled to MS [15, 16] were proposed for the separation and characterization of a series of peptides and related compounds, such as peptide impurities in crudes of reaction. In this work, we have established a method for separation and characterization of a series of peptide hormones of pharmaceutical interest and wide therapeutical use by CE-ES-MS using a sheath-flow interface. Several parameters were systematically investigated, such as concentration of the electrolyte, organic solvent and sheath liquid composition, gas flow rates and capillary position. Moreover, limits of detection, linearity, repeatability and day-to-day reproducibility of the proposed method were studied in order to obtain the main quality parameters.

2 Materials and methods

2.1 Chemicals

All chemicals used were of analytical reagent grade. Acetonitrile (MeCN), methanol (MeOH), 2-propanol, acetic acid (glacial), and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Ammonia (30%) and formic acid (98%) were supplied by Panreac (Barcelona, Spain). Water with a specific conductivity lower than 0.05 μScm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Bradykinin, Leu-enkephalin, Met-enkephalin and oxytocin were purchased from Sigma Química (Alcobendas, Madrid, Spain), buserelin from Hoechst Ibérica (Barcelona, Spain), triptorelin from Lasa (Barcelona, Spain) and eledoisin from Lipotec (Barcelona, Spain). All peptides were stored in a freezer at –4°C when not in use. Sequences and molecular mass are shown in Table 1.

2.2 Electrolyte, sheath liquid and sample solutions

The electrolyte solution for the CE separation was optimized in a previous work [13] and contained 50 mM of acetic acid and 50 mM of formic acid at pH 2.85 adjusted with NH_3. In this work, we have evaluated the use of different sheath liquid solutions consisting of hydroorganic mixtures with MeOH, MeCN or 2-propanol, ranging from 60% to 100% v/v. Acetic or formic acids were added to the mixtures at concentrations ranging from 0.05% to 0.5% v/v. All solvents and solutions were filtered through a 45 μm Nylon (MSI) membrane. The sheath liquids were degassed for 15 min by bubbling helium before use. Stock solutions of 500 μg/mL of the studied peptide hormones were prepared by dissolving in water. Solutions used in the determination of the quality parameters were made by dilution of the stock solutions with water.

2.3 Instrumentation

All CE experiments were performed in an Agilent Technologies $HP^{3D}CE$ system (Waldbronn, Germany) with an on-column diode-array detector. For CE-ES-MS experiments a Mariner TOF mass spectrometer (PerSeptive Biosys-

Table 1. Sequence, basic ionizable groups and monoisotopical molecular mass of the studied peptide hormones

Peptide	Sequence	Basic ionizable groups	Molecular mass (Da)
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	1 Amino group (-Arg) 2 Guanidine groups (Arg-/-Arg)	1059.56
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	1 Amino group (Cys-)	1006.44
Eledoisin	Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2	1 Amino group (-Lys-)	1187.60
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	1 Amino group (Tyr-)	555.27
Met-enkephalin	Tyr-Gly-Gly-Phe-Met	1 Amino group (Tyr-)	573.23
Triptorelin	Pyr-Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2	1 Imidazole group (-His-) 1 Guanidine group (-Arg-)	1310.63
Buserelin	Pyr-Pro-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-NHC_2H_5	1 Imidazole group (-His-) 1 Guanidine group (-Arg-)	1238.66

tems, Framingham, MA, USA) was coupled to the CE system by an electrospray G1603A Agilent Technologies sprayer, based on a sheath flow CE-ES-MS interface. The separation was performed at 25°C in a 1 m × 75 μm ID × 360 μm OD fused-silica capillary purchased from CS-Chromatographie (Langerwehe, Germany). The UV window was placed at 21 cm from the capillary inlet and detection was made at 195 nm. The samples were hydrodynamically injected at 3448 Pa (0.5 psi) for 3 s. The CE separation voltage applied was 25 kV in normal polarity (anode in the inlet). For CE-ES-MS measurements the sheath liquid was delivered by an Agilent 1100 series isocratic LC pump and splitted to allow an effective flow from 1 to 10 μL/min with an ICP-50–150 splitter (LC Packings, Amsterdam, The Netherlands). This LC pump provided a more consistent sheath flow than an infusion pump allowing a stable spray formation and hence a more stable signal [17]. All CE-ES-MS experiments were done in positive ion mode. The mass spectrometer was operated in an *m/z* range from 200 to 1400 obtaining 1 spectrum every 5 s. pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

2.4 Procedures

2.4.1 CE

All capillary rinses were performed off-line at high pressure (1.03 MPa, 15 psi) with the nebulizer gas and ES voltage switched off to avoid spray formation and the unnecessary entrance of NaOH and buffer solution in the MS. New capillaries were activated by flushing for 10 min with 1 M NaOH, followed by 10 min with water and 20 min with buffer solution. Between days, the capillary was conditioned by rinsing for 5 min with 1 M NaOH, 5 min with water and 10 min with buffer solution. Between runs, the capillary was rinsed 3 min with buffer solution. When not in use the capillary was rinsed 10 min with water and 5 min with air to avoid salt crystallization in the capillary outlet.

2.4.2 ES-MS

The sheath-flow interface was located at a distance long enough to the mass spectrometer entrance to avoid charge discharge at the tip of the electrode but close enough to obtain an intense signal [18]. The electrode was placed at 45° with regard to the curtain plate of the mass spectrometer and it was pointed to the left side of the orifice entrance. A stable spray formation depends on a suitable capillary outlet cut. An irregular profile prevents a perfect spray, and the capillary edge may act as an adsorptive site for sample components [19]. In this way the last 2 cm of the nonconductive polyimide coating were removed from the capillary to obtain a better mixture with sheath liquid and maintain a stable spray [20]. During sample injection some important factors that affect the sample amount injected had to be considered. The positive spray voltage was switched off to avoid the tendency of the loaded sample to migrate back out of the capillary entrance and be diluted in the running buffer [21]. The nebulizer gas was also disconnected during injection because it had an aspirating effect that increased the sample amount injected [22]. Similarly, the capillary inlet and outlet were placed at the same level to avoid the siphoning effect that influenced the amount of sample introduced into capillary [20]. Optimization of the CE-ES-MS signal was performed using a 500 μg/mL solution of three peptide hormones: bradykinin, triptorelin and Leu-enkephalin. The interface parameters, sheath liquid composition and its flow rate, nebulizer gas and curtain gas flow rates, relative capillary position, spray tip potential, nozzle potential and nozzle temperature, were sequentially optimized measuring the total ion electropherogram (TIE) areas of the electrophoretic peaks corresponding to each peptide.

2.4.3 Quality parameters

Repeatability (n = 12) and long-term reproducibility (n = 36) were determined under the above optimized conditions, with a solution of the seven peptide hormones studied, containing bradykinin, triptorelin, buserelin, Leu-enkephalin, oxytocin, Met-enkephalin and eledoisin at individual concentrations ranging from 46 to 80 μg/mL, depending on each peptide. Repeatability and reproducibility have been calculated as a percentage of the relative standard deviation (RSD %) of migration times and peak areas obtained in the extracted ion electropherogram (XIE) corresponding to each peptide hormone. In order to set up the sensitivity of the method, the limit of detection (LOD) for each peptide was established analyzing low-concentration solutions of each peptide (near of the LOD level as estimated from the approach based on the peak height). Replicate analyses were performed and the concentration corresponding to three times the standard deviation of the peak areas was then considered as the LOD [23]. The linearity range was obtained by injection of the peptide hormone mixture at concentrations from 1.46 to 160 μg/mL depending on the peptide, and measuring the area on the XIE.

3 Results and discussion

The migration behavior of the studied peptide hormones was previously studied by our research group using CE with UV detection [13]. Their ionization constants were

calculated and experimental conditions to obtain an optimum separation were established, mainly based in selection of an electrolyte solution containing 50 mM acetic acid and 50 mM formic acid adjusting the pH at 2.85 with ammonia. The UV electropherogram obtained under these conditions is shown in Fig. 1. In addition to the excellent separation observed, these conditions are potentially compatible with on-line MS detection. The use of separation buffers containing volatile components such as acetic or formic acid, is mandatory to generate an efficient spray, improve detectability and avoid formation of salt deposits in the interface and the entrance of the mass spectrometer [24]. Nevertheless, several operational parameters must be taken into account in order to achieve good detection sensitivities and reproducibilities using CE-ES-MS. In this work, CE was coupled to MS with a commercial sheath-flow interface. The most important parameters to optimize in this instrumental setup, are the composition and flow rate of sheath liquid, the gas flow rates, the capillary position, the applied voltages and the temperature.

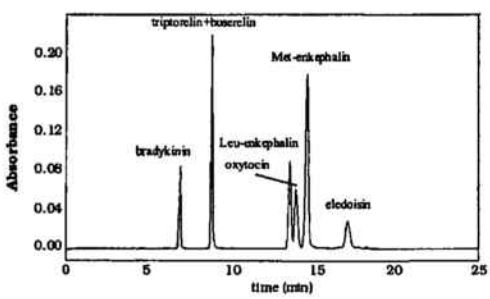

Figure 1. UV electropherogram obtained at 195 nm of a sample containing 250 ppm of each peptide hormone. Separation was performed at 25°C in a bare fused-silica capillary (57 cm × 75 μm ID), 50 cm effective length. Buffer, 50 mM acetic acid and 50 mM formic acid. The sample was hydrodynamically injected at 0.5 psi for 3 s and separated at 25 kV [14].

3.1 Optimization of ES-MS parameters

3.1.1 Sheath liquid

The sheath liquid composition is one of the most important parameters to be optimized because of its influence on signal intensity and spray stability [20, 25, 26]. Variation of the nature and the percentage of the organic solvent and of the electrolyte added in the hydroorganic mixture used as sheath liquid has been investigated. The role of the organic modifier is to decrease surface tension of the sheath liquid [26] and to help the gas-phase ions formation [25]. The electrolyte allows the sheath liquid to work as a CE terminal buffer reservoir enabling the electrical contact in the ES tip [6]. In this work, the organic modifiers were MeOH, 2-propanol and MeCN at percentages of 60, 80 and 100% v/v. Figure 2a shows peak areas obtained in the TIE for bradykinin and it reveals that an increment in the MeOH proportion increases the signal intensity. In contrast, increasing 2-propanol percentage decreases the signal intensity. MeCN produces the lowest signal intensity and shows slight variations on peak areas. The best signal intensity was obtained with a 100% v/v of MeOH. However, working with a high percentage of organic solvent gave an unstable CE current owing to the bad electrical contact in the ES tip. Therefore, a compromise was necessary between signal intensity and system stability. Sheath liquids with 80% v/v MeOH and 60% v/v 2-propanol, which provided the best results, were selected to study the electrolyte type and composition. These results agree with other similar CE-ES-MS optimization studies [20, 22, 27]. By keeping the

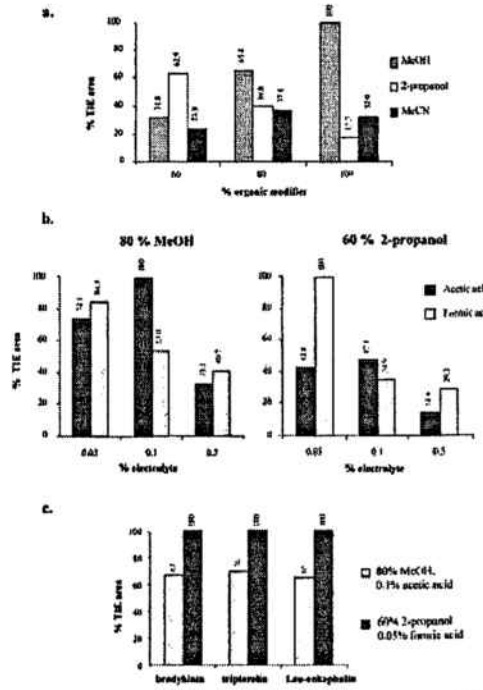

Figure 2. Influence of sheath-liquid composition on CE-ES-MS signal. (a) Effect of the organic modifier type and proportion for bradykinin. (b) Effect of electrolyte, type and proportion for bradykinin. (c) Comparison between the two sheath liquid compositions providing optimum signal results.

organic solvent percentage constant, different electrolytes as acetic and formic acids were investigated in a range from 0.05 to 0.5% v/v. When MeOH was used, the best results were obtained with 0.1% v/v acetic acid and, in the case of 2-propanol, 0.05% v/v formic acid resulted in a higher signal intensity (Fig. 2b). From comparison between these both compositions, an aqueous solution of 60% v/v 2-propanol in the presence of 0.05% v/v formic acid was selected as sheath liquid giving the highest signal intensity (Fig. 2c).

Once the composition of the sheath liquid was optimized, the influence of its flow rate upon signal intensity and stability was investigated. The sheath liquid flow rate was studied in a range from 1 to 10 μL/min. As sheath liquid flows through the ES needle, the separation electrolyte rising from capillary is diluted [7, 9, 20]. Therefore, if the sheath liquid flow rate was increased, the signal intensity was attenuated as shown in Fig. 3. However, very low flow rates turned the spray unstable. Thus, the flow rate was set at 2 μL/min, the lowest flow rate that provided a stable signal.

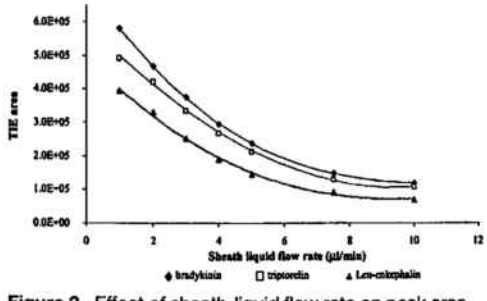

Figure 3. Effect of sheath-liquid flow rate on peak area.

3.1.2 Gas flow rates

The curtain gas serves to accelerate and assist droplet desolvation and to avoid the entrance of unfiltered air into the mass spectrometer [20, 28]. The effect of curtain gas flow rate was studied from 1.5 to 3.5 L/min. The highest peak areas were obtained with 2.5 L/min, although the curtain gas flow rate variation did not considerably affect the signal intensity. The nebulizer gas assists stable spray formation and helps solvent evaporation [8, 19]. The nebulizer flow rate was optimized in the range between 0.2 and 0.8 L/min. Gas flow rate modification did not cause important variation in signal intensity although high values such as 0.8 L/min should be avoided, because the electrical contact in the ES tip was difficult and a stable spray formation was prevented [26]. Otherwise the nebulizer gas had an aspirating effect into the capillary which substantially modify migration times and separation resolution (Fig. 4) [20, 29]. The nebulizer gas flow rate was set at 0.6 L/min.

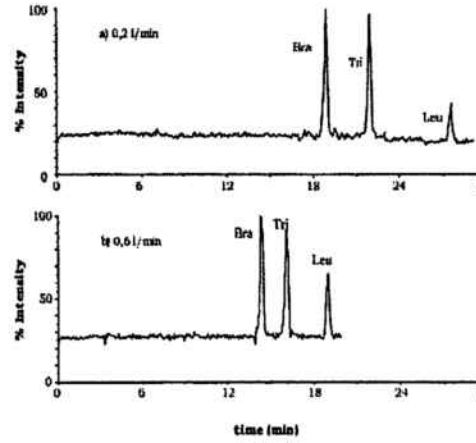

Figure 4. Effect of the nebulizer gas flow rate on the TIE for a mixture of bradykinin (Bra), triptorelin (Tri) and Leu-enkephalin (Leu). (a) 0.2 L/min, (b) 0.6 L/min.

3.1.3 Other parameters optimized

The capillary position from the ES needle is the most important physical parameter to adjust in the sheath-flow interface [21]. The capillary must protrude enough from the ES needle so that the spray formation process is not interfered and the dead volume at the capillary end is minimized [9, 21]. The capillary position was optimized from 0.0 to 0.2 mm and the highest signal intensity was obtained at 0.1 mm. The electrospray voltage or spray tip potential has a great influence on sample ionization, signal intensity and spray stability [9, 22]. The effect of the spray tip potential in signal intensity was studied in the range between 2500 and 4000 V (typical values in positive ion mode) and the best results were obtained at 3000 V. The nozzle voltage affects sample ionization and hence, signal intensity [28]. It was optimized from 40 to 150 V and the best results were obtained at 100 V. Under these conditions no fragmentation was observed on the analyzed peptides. Finally, the influence of nozzle temperature was studied. The nozzle temperature helps sample desolvation, but too high temperature can cause sample degradation [28]. In our case, practically no improvement was observed during its optimization, and hence it was set at 90°C.

3.2 Separation of the series of peptide hormones studied

Using the optimized conditions, a solution of the seven peptide hormones considered was injected into the CE-ES-MS system and the TIE of Fig. 5a was obtained. Comparison of the TIE (Fig. 5a) and the UV electropherogram (Fig. 1) reveals a loss of resolution of peptides Leu-enkephalin, oxytocin, Met-enkephalin and eledoisin. In general, it is easier to establish first the separation method using UV detection, and then carry out a subsequent optimization of the most important parameters of the ES-MS interface. Nevertheless, as shown in Fig. 5a, it is difficult to reproduce the separation obtained in CE-UV using an

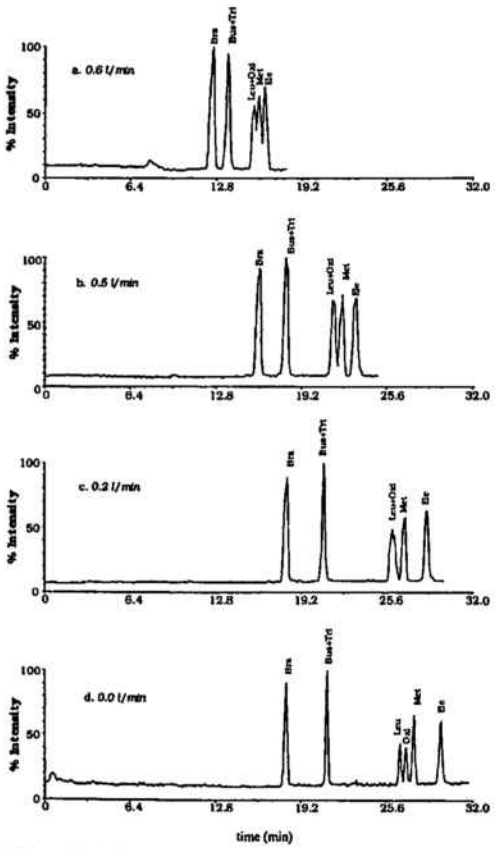

Figure 5. Effect of the nebulizer gas flow rate on separation resolution injecting the seven peptide hormone mixture of bradykinin (Bra), buserelin (Bus), triptorelin (Tri), Leu-enkephalin (Leu), oxytocin (Oxi), Met-enkephalin (Met) and eledoisin (Ele). (a) 0.6 L/min, (b) 0.5 L/min, (c) 0.2 L/min, (d) 0.0 L/min.

MS detector, because there are several differences between the two instrumental setups. In CE-ES-MS mode (i) a longer capillary is necessary, (ii) the positive ES voltage decreases the total electric field in the capillary applied by the CE instrument [22], (iii) a nebulizer gas is applied in the capillary end and an aspirating effect is produced which influences separation resolution [20], (iv) the whole capillary cannot be thermostated, (v) the terminal buffer solution is different in both capillary ends, thus, sheath-liquid ions can diffuse into CE capillary and can produce a loss of separation resolution and alterations in migration times [10], and (vi) the area where sheath liquid and separation buffer are mixed has a little dead volume than can produce band broadening [8].

Among all the features above described, the nebulizer gas seems to be the easiest parameter to modify. The solution of the seven peptides studied was injected and the effect of nebulizer gas on separation was studied. As shown in Fig. 5, resolution increases as nebulizer gas decreases. Only in the absence of nebulizer gas the separation had a resolution similar to the one obtained with CE with UV detection, but this involved higher migration times and an unstable spray formation. Thus, the nebulizer gas flow rate was set at 0.5 L/min (Fig. 5b). The final optimum CE-ES-MS parameter values are summarized in Table 2. The order of migration is: bradykinin, buserelin, triptorelin, Leu-enkephalin, oxytocin, Met-enkephalin and eledoisin. Good separation is achieved in all cases except between the pairs triptorelin/buserelin and Leu-enkephalin/oxytocin. In order to quantify peptides in unresoluted peaks, XIEs at the corresponding *m/z* values can be used.

Table 2. Final optimized CE-ES-MS parameters

Optimized parameters	
Sheath-liquid composition	2-Propanol-water/ 0.05% formic acid (60:40 v/v)
Sheath-liquid flow rate	2 μL/min
Nebulizer gas flow rate	0.5 L/min
Curtain gas flow rate	2.5 L/min
Relative capillary position	0.1 mm
Spray tip potential	3000 V
Nozzle potential	100 V
Nozzle temperature	90°C

3.3 CE-ES-MS spectra

Spectra for each electrophoretic peak of the TIE of Fig. 5b were obtained. As an example, Fig. 6a shows the spectrum of badykinin. Molecular peaks with single, double or triple charge are the most important ones in spectra and no fragmentation was observed. The number of basic

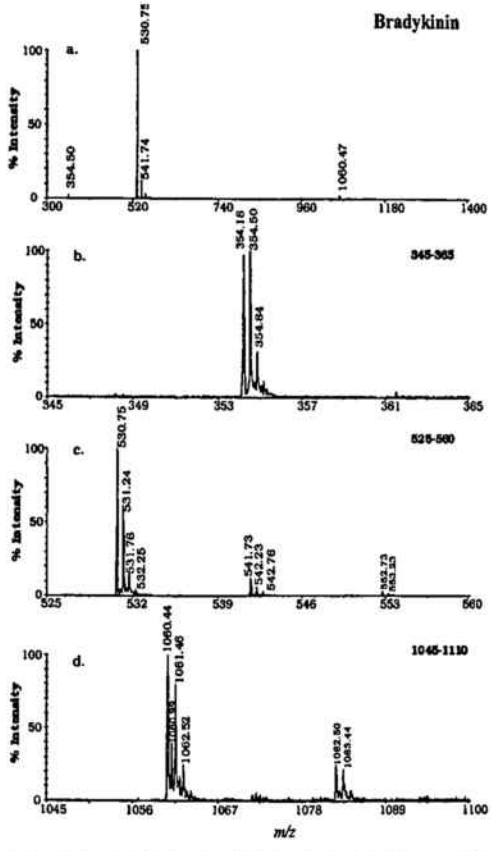

Figure 6. (a) Spectrum obtained for bradykinin, and its respective extended spectra, in the *m/z* ranges (b) 345–365, (c) 525–560 and (d) 1045–1110.

residues present in the primary structure of each peptide is related to the charge of the most intense molecular peaks observed in spectra. Table 3 shows the *m/z* values corresponding to molecular ions obtained. From these values, the monoisotopic molecular mass has been calculated, obtaining a relative error (% Er) *versus* theoretical molecular mass between 0.005 to 0.070. The high resolution of the TOF analyzer allows the detection of the Na^+ adducts and the ^{13}C isotopic profile as shown in the extended spectra for bradykinin (Figs. 6b–d). The XIEs, obtained from the most intense *m/z* peak of each peptide hormone spectrum, allow the quantification analysis of each peptide, even those not resoluted in the TIE.

3.4 Quality parameters

The suitability of the method for quantitative analysis was studied by testing linearity, LODs, reproducibility and repeatability. Signal *vs.* concentration regression lines were calculated in a range between 1.46 and 160 μg/mL depending on the peptide, and linearity was observed over the whole concentration range studied, as shown in Table 4 ($r > 0.99$). LODs obtained as indicated in Section 2.4 varied between 1 to 4 μg/mL. Figure 7 shows the XIE obtained for each peptide at these concentrations.

Repeatability studies were performed by 12 replicate analyses under the optimum conditions using a standard solution of a peptide hormone mixture with individual concentrations from 46 to 80 μg/mL depending on the peptide. The RSDs obtained from the migration time ranged between 1.2 and 2.2%, and peak area measurements obtained in the XIE of five majority peaks from each peptide were between 8 and 13%. Day-to-day reproducibility studies were performed by 36 replicate anal-

Table 3. Calculated and theoretical molecular masses, *m/z* ratios and the respective charged forms for the studied peptide hormones

m/z			Molecular mass			
Peptide	$[M+3H]^{3+}$	$[M+2H]^{2+}$	$[M+H]^+$	Calculated mass	Theoretical mass	% Er
Bradykinin	354.18 (2.01)	530.76 (100)	1060.49 (2.56)	1059.50	1059.56	0.006
Buserelin		620.30 (100)	1239.60 (4.30)	1238.56	1238.66	0.008
Triptorelin		656.28 (100)	1311.54 (2.69)	1310.54	1310.63	0.007
Leu-enkephalin			556.24 (100)	555.24	555.63	0.070
Oxytocin		504.20 (100)	1007.39 (38.67)	1006.38	1006.20	0.017
Met-enkephalin			574.20 (100)	573.20	573.23	0.005
Eledoisin		594.77 (100)	1188.54 (2.53)	1187.53	1187.60	0.006

Table 4. Calibration data and LODs for the studied peptide hormones

Peptide	Conc. range (μg/mL)	Regression line	Correlation coefficient	LOD (μg/mL)
Bradykinin	1.46–73.0	$A = 187 \cdot C - 31.5$	0.999	1.46
Buserelin	2.45–98.0	$A = 160 \cdot C - 35.1$	0.999	0.98
Triptorelin	3.65–146	$A = 65.3 \cdot C - 112$	0.998	3.65
Leu-enkephalin	2.30–92.0	$A = 76.1 \cdot C - 169$	0.996	2.30
Oxytocin	2.62–106	$A = 52.9 \cdot C + 32.7$	0.998	2.62
Met-enkephalin	4.00–160	$A = 73.8 \cdot C - 420$	0.992	4.00
Eledoisin	3.00–120	$A = 124 \cdot C - 414$	0.998	1.20

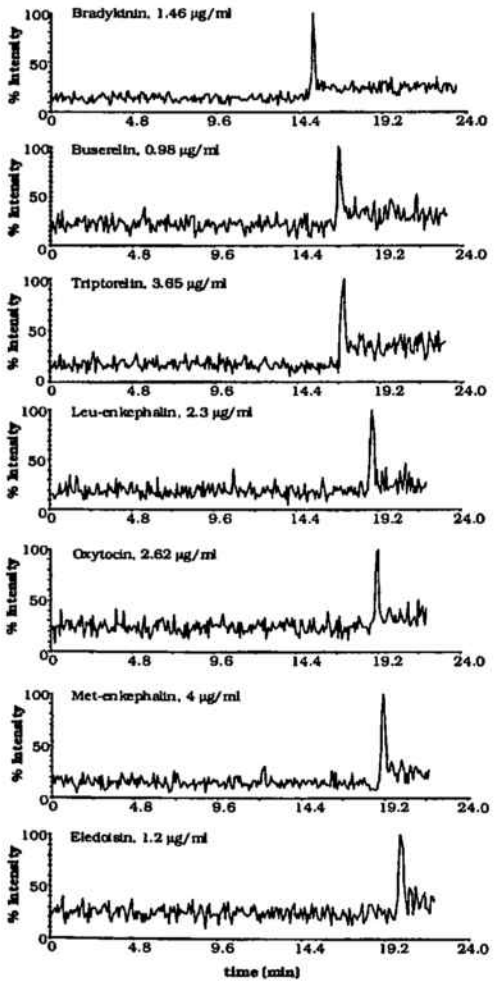

Figure 7. XIEs obtained at the LOD for each peptide hormone.

yses under the same conditions on three different days. The relative RSDs were 1.7–2.3% for migration time and 9–15% for peak area measurements (Table 5).

Table 5. Peak area and migration time repeatability and reproducibility

	Repeatability ($n = 12$)		Reproducibility ($n = 36$)	
	Peak area (% RSD)	Migration time (% RSD)	Peak area (% RSD)	Migration time (% RSD)
Bradykinin	13	1.3	15	1.7
Buserelin	11	1.5	14	1.9
Triptorelin	11	1.2	15	1.9
Leu-enkephalin	11	1.6	10	2.2
Oxytocin	8	1.6	9	2.2
Met-enkephalin	12	1.7	12	2.2
Eledoisin	11	2.1	13	2.3

4 Concluding remarks

In this study, a successful coupling of CE with ES-TOF with a sheath-flow interface is demonstrated for a mixture of peptide hormones of therapeutical interest. The separation conditions, compatible with CE-ES-MS, were established in a previous work [13] with regular CE with UV detection. Main parameters affecting CE-ES-MS signal intensity were optimized, such as sheath-liquid composition, sheath-liquid flow rate, nebulizer gas and curtain gas flow rates, capillary position, and voltages and temperatures applied. A sheath liquid with 60% 2-propanol and 0.05% formic acid delivered at 2 μL/min provided the best results in signal intensity and spray stability. The curtain gas flow rate was set at 2.5 L/min and the nebulizer gas flow rate was optimized in order to obtain best resolution. Best signal intensity was obtained when 0.1 mm of the separation capillary protrudes from the ES needle. Finally, the spray tip potential was set at 3000 V, the nozzle potential at 100 V and the nozzle temperature at 90°C. The TOF analyzer used in this study provides

high-resolution spectra for each peptide hormone, thus allowing their characterization. The method showed good linearity in the concentration range between 1.46 and 160 μg/mL, acceptable repeatability and day-to-day reproducibility in terms of migration time and peak area, and good sensitivity with LODs from 1 to 4 μg/mL.

Received November 22, 2002

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LOWERING THE CONCENTRATION LIMITS OF DETECTION OF ANALYTES AT THE NANOGRAM PER MILLILITER LEVELS BY ON-LINE PRECONCENTRATION CAPILLARY ELECTROPHORESIS

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Abstract

The use of Solid-Phase Extraction coupled on-line to Capillary Electrophoresis (SPE-CE) and CE coupled to Mass Espectrometry (SPE-CE-MS) has been described for the analysis of peptides in dilute solutions. A disposable microextraction cartridge or analyte preconcentrator contanining C18 particles as affinity support was easily constructed near the inlet of the separation capillary using commercially available materials. The hydrophobic C18 sorbent selectively retains the target analytes, enabling large volumes of sample to be injected. The bound analytes are eluted in a small volume of an appropriate solution, resulting in sample clean-up and concentration enhancement. If analysis conditions are compatible with on-line MS detection, analytes can be identified and characterized. Special attention is paid to the systematic optimization of the operational parameters involved in SPE-CE method development, in order to facilitate a widespread use of this technique and the development of quantitative analytical SPE-CE methodologies.

Keywords: Peptides, capillary electrophoresis, sensitivity, on-line preconcentration, solid-phase extraction.

1. Introduction

Simplifying assay protocols, improving separation efficiencies, selectivities and detection sensitivities are important considerations in developing new formats, technologies, and methodologies for the analysis of selected analytes in complex samples. This was the original aim of the development of Capillary Electrophoresis (CE) first [1,2], and related techniques, including microchip-based approaches, later [3,4]. Microseparation techniques have moved to the forefront of analytical methodology, reducing the manpower required, the reagent and sample comsumption, the organic solvent waste, the analysis time and the costs. However, one of the main consequences of reduced volumes is the limited number of molecules that can be detected at any given time. This often leads to compromises between sensitivity and measuring times [5,6]. Special instrumental and/or methodologies are needed for detecting molecules at the subnanomolar concentrations utilizing CE or the related techniques. One example involving sensitivity and selectivity

issues is becoming apparent in structural proteomic research [7,8], when analyzing proteins and peptides in complex biological matrices. The ability to specifically characterize thousands of proteins and peptides found at low concentration in nature, raises an immediate challenge to protein biochemists and analytical chemists. Aproximately 50 to 80% of the gene-products (depending on the species) are found as low abundant proteins and peptides in complex mixtures.

There has been many attempts to overcome poor concentration limits of detection in CE, without using off-line approaches. Improvements focused in instrument and UV-detector modification, capillary detection-cell design and electrochemical, laser-induced-fluorescence and mass spectrometry detection, have had limited success [1,5,9-11]. Other researchers have developed alternative sample introduction methodologies that provide an on-column focusing effect, enhancing separation efficiency and allowing the use of larger sample volumes [12-14]. Nevertheless, the sensitivity improvement strongly depends on the analyte and sample matrix nature, it is limited by capillary length and it is only applicable under very specific conditions. In a parallel way, Solid Phase Extraction have been coupled on-line to Capillary Electrophoresis (SPE-CE) [15-20]. In SPE-CE, a microextraction cartridge or analyte preconcentrator is inserted near the inlet of the separation capillary. It contains a solid affinity support which selectively retains the target analyte, enabling large volumes of sample to be injected. The bound analyte is eluted in a small volume of an appropriate solution, resulting in a concentration enhancement. Sensitivity can be furtherly improved if elution plug and separation buffer composition permit analyte focusing. The selection of specific affinity stationary phases also allow removing of interferences, avoiding slow and labor-intensive sample clean-up methods, that are not recommended when analyzing dilute complex samples [15-20]. Many researchers have perceived the suitability of SPE-CE to perform selective and sensitive analysis of complex dilute samples, even in microfabricated devices [19-20]. Furthermore, it can be applied to improve the limited preparative capabilities of this microanalytical systems or as an excellent alternative to perform on-column microreactions or derivatizations with inmobilized ligands [21-26]. Nevertheless, this emerging technology is far away from its maturity, requiring the understanding of the parameters involved in analyte preconcentrator design and binding and elution chemistries. Its uselfulness for quantitative purposes has to be explored while improving analyte preconcentrator microcartridges design and performance, as in the last twenty years has been happening with off-line conventional SPE [27-29], now the preferred technique for concentration and isolation of selected analytes prior to chromatographic or electrophoretic analysis in environmental, food, forensic, pharmaceutical and biological samples. The parallel growing interest in column technology, method development, performance and troubleshooting in Capillary Electrochromatography (CEC) [30-31] may also benefit SPE-CE development.

The coupling of Capillary Electrophoresis to Mass Spectrometry (CE) has always generated a great interest [10-11], because it permits analyte identification and characterization by differences in electrophoretic mobilities and structure. The simplest way to couple both techniques is via Electrospray Ionization (ESI) interfaces. Nevertheless, the coupling still showing many challenging aspects which complicate the successful interfacing of both processes [10-11]. Considerable advances are continously made in ESI interfaces design, in order to improve ionization efficiency and robustness of detection, and some of them are already comercially available [10-11]. The on-line coupling of SPE-CE to mass spectrometry detection (SPE-CE-MS) and the development of high selective affinity supports, offer great possibilities to simply perform high specific detection and accurate structural characterization of low concentrated analytes in complex matrices, while on-line sample desalting and preconcentration enhances MS detection sensitivity [22-23,32-51].

In this study, non-specific sorbent material (C18) immobilized on glass beads, was utilized as a solid affinity support, cased in a microextraction cartridge [32,34-35,52-57], for the isolation and sensitivity enhancement of Angiotensin II and Leucine-enkephalin by CE and CE-MS. Several parameters affecting SPE-CE performance have been studied, in order to get a deeper understanding of the methodology fundamentals and facilitate the systematic development of new quantitative analytical SPE-CE methodologies.

2. Experimental Section

2.1 Chemicals and Materials

All chemicals were analytical reagent grade. Acetonitrile (MeCN) and Methanol (MeOH) were supplied by AlliedSignal, Burdick & Jackson, Muskegon, MI, USA. Sodium hydroxide was obtained from Fisher Scientific, Fair Lawn, NJ, USA. Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), glacial Acetic acid (HAc), Formic acid (HFor), 2-[N-Cyclohexylamino]ethanesulfonic acid (CHES) and N-[Carbamoylmethyl]-2-aminoethanesulfonic acid (ACES) were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Deionized water was purified with a Mili-Q-Plus Ultra-Pure water system from Millipore Corporation, Bedford, MA, USA. Samples and solutions were degassed and filtered through 0.2 mm filters (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Stock standard solutions of 1 mg/mL of Angiotensin II and Leu-enkephalin were prepared in water. Standard solutions at desired concentration were prepared by furtherly diluting with water.

2.2 Instrumentation

All the instrumental set-up was purchased from Agilent Technologies, Inc., Wilmington, DE, USA. An HP 3DCE capillary electrophoresis instrument equipped with diode array detector was used for CE analysis. For CE-MS experiments the CE system was coupled to a model 1100 MSD single quadrupole mass spectrometer. The orthogonal coaxial sheath flow electrospray ionization interface and an

special CE-MS cartridge cassette kit were also obtained from Agilent Technologies. For the delivery of the sheath liquid an HP1100 liquid chromatography (LC) pump was used. Instruments control, data acquisition and data processing were performed using ChemStation software (Agilent Technologies).

2.3 Construction of an analyte concentrator

The way we construct a microextraction cartridge is schematically described in Figure 1 and was carried out as a minor modification of a previous method [56,58]. The entire process of fabrication of the analyte concentrator was monitored under a stereomicroscope. An on-column polyethylene frit (0.1 cm) was built in a piece of bare fused-silica capillary (365 μm O.D. x 250 μm I.D. x 10 mm L_T (total length), Polymicro Technologies Inc., Phoenix, AZ, USA) packing small fragments of the original filters found in Sep-Pak Cartridges (Millipore, Marlborough, MA, USA). The body of the analyte concentrator is fused to a 9 cm piece of bare-fused silica capillary (365 μm O.D. x 75 μm I.D.) using a 0.5 cm polyethylene sleeve (610 μm O.D. , 280 μm I.D., "Intramedic" PE 10, Clay Adams division of Becton Dickinson, NJ, USA). Thus, it can be easily vaccum-filled with dry irregularly shaped (40 μm O.D., 400 Å pores) C18 end-capped silica material from Sep-Pack cartridges. When the concentrator tube was almost completly full, a second on-column 0.1 cm polyethylene frit was inserted in this end side. Care was taken to leave enough room in the tube for the insertion of this second frit without compressing the C18 particles. The column was extended to full length using another polyethylene sleeve. The tight junction provided, makes no adhesive sealing necessary and the microextraction device completly replaceable. The joining ends connected by the plastic sleeves must be cleanly cut in order to minimize dead volume and leaking at the joints.The full length column (57 cm for UV detection and 100 cm for UV and MS detection), containing the analyte concentrator at 9 cm from the inlet, was prepared to fit into a commercial cartridge-cassette device which held the capillary in a coiled configured form. The system was check for abnormal flow restriction. High flow and minimal restriction resulting from the presence of the concentrator alllow the assembly to remain on-line with the separation capillary throughout the entire analysis.

2.4. Separation by CE and CE-MS.

CE and CE-MS separations were performed at 25 °C in 57 cm x 75 μm i.d. and 100 cm x 75 μm i.d. bare fused-silica capillaries, respectively (unless anything else is indicated). In CE separations, conventional capillaries (without the analyte concentrator device) were conditioned rinsing at 930 mbar with 0.1 N NaOH (2 min), followed by H_2O (2 min) and separation electrolyte (4 min). The separation electrolytes consisted of 20 mM CHES, pH 9.5 or 20 mM ACES, pH 7.4. Samples were hydrodinamically injected at 50 mbar for 5 s (34 nL (L_T=57 cm) and 19 nL (L_T=100 cm) aproximately, using Hagen-Poiseuille equation). Modified capillaries were conditioned by successive flushing the capillaries with MeOH (2 min), H_2O (2 min) and separation electrolyte (4 min). Samples were hydrodinamically introduced at

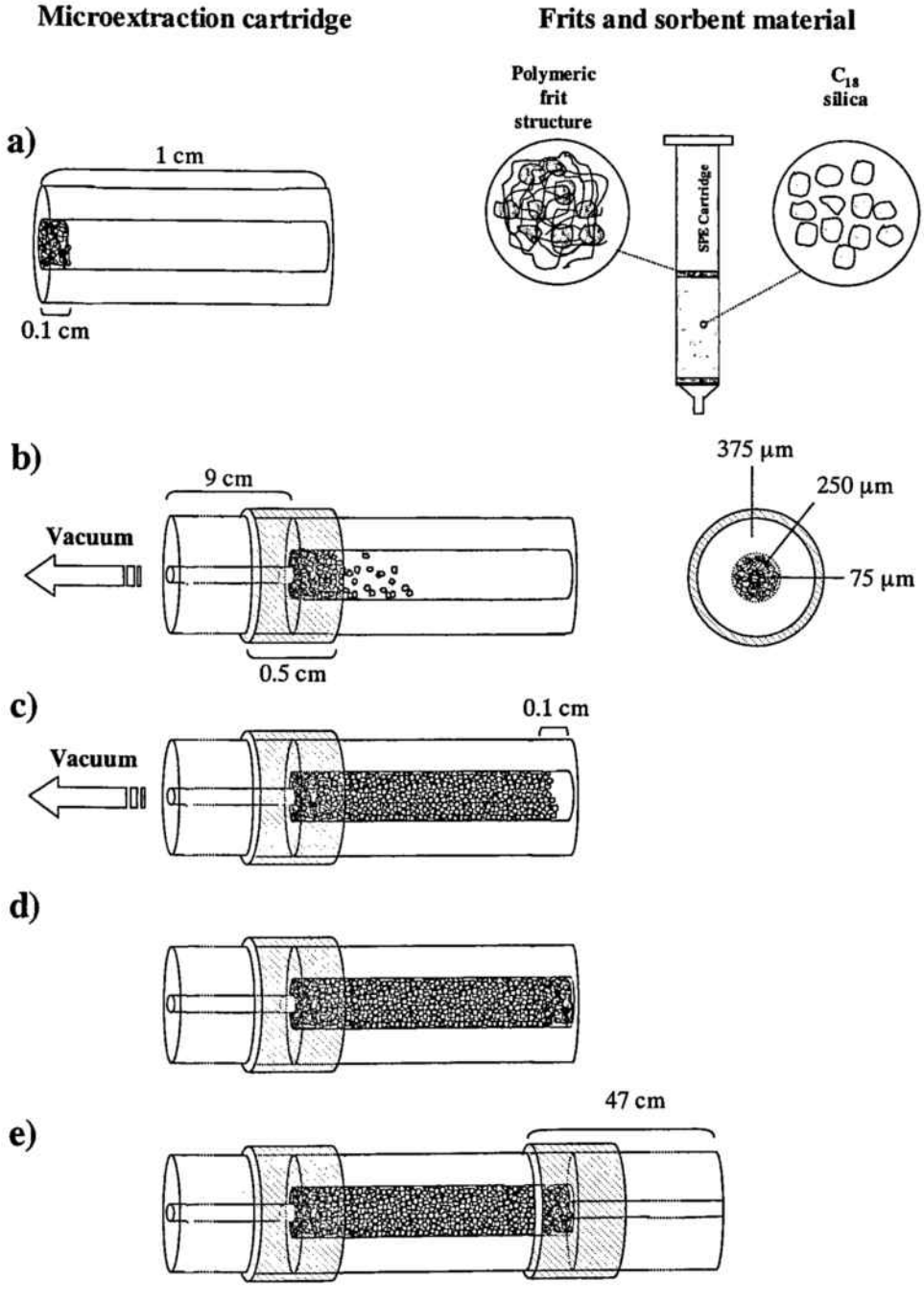


Figure 1 Schematic of the fabrication of the C18 packed microextraction cartridge coupled on-line with CE used in this work.

pressures and times that are specified in the text. A final rinse with separation electrolyte at 930 mbar for 6 minutes elmininates non-retained analytes and equilibrates capillary before elution and separation. Retained analytes were eluted injecting a solution of 80:20 v/v MeCN (HFor 25 mM):H_2O (apparent pH 3.13) at 50 mbar for 5 s. All experiments were carried out at 29 kV under normal polarity (anode at the inlet). Modified capillaries were rinsed with H_2O (2 min) and MeCN (2 min) at 930 mbar after the electrophoretic separation, in order to avoid carry over between consecutive SPE-CE analysis. In our case, no capillary regeneration of the separation capillary with 0.1 M NaOH was needed. Exposure of the C18 material to extreme pH should be avoided and, in case necessary, capillary can be regenerated rinsing it off-line with 0.1 M NaOH. Duration of conditioning, injection and postconditioning steps in CE-MS procedures, with or without analyte concentrator, were twice the indicated for CE operation. When not in use, conventional and modified capillaries were stored dried with air and filled with MeCN, respectively.

The UV detection window was always placed at 47 cm from the inlet of the capillary. The photodiode array detector was working at 195 nm. For positive electrospray ionization, the sheath liquid consisted of 20 mM HAc in 50:50 v/v MeOH:H_2O. The original flow of 0.5 mL/min provided by the LC pump was splitted to allow an effective flow of 10 μL/min into the coaxial interface. Drying and nebulisation gases were both nitrogen. The nebulizing pressure and the drying gas flow-rate were set at 10 p.s.i (1 p.s.i = 6894.76 Pa) and 4 L/min, respectively. Capillary voltage was set at 4 kV. Gas temperature was 150 °C and fragmentor voltage 110 V. Electropherograms were generated scanning from 250 to 1250 a.m.u at 1 scan/s. Mass calibation was daily performed following Agilent Technologies directions.

3. Results and Discussion

Affinity capillary electrophoresis (ACE) is a broad term referring to the separation by CE of substances - the affinity ligand/s and the target analyte/s - which participate in selective or non selective interactions during electrophoresis [15,16,18,59]. The affinity ligand can be free in solution or immobilized to a solid support. If it is immobilized to some extent near the inlet of the separation capillary, large sample volumes can be injected prior to the electrophoretic separation, performing an on-line solid phase extraction. The target analytes will interact with the bound ligand, being extracted from the sample matrix or reacting if an enzyme or a derivatizing agent are immobilized. If they are finally eluted with a volume smaller than the injected, their concentration will be enhanced and, as a consequence, also limits of detection in the subsequent on-line electrophoretic analysis. The steps involved in a SPE-CE procedure are schematically described in Figure 2 and will be explained later in detail. In Figure 3 it is shown the concentration limit enhancement achieved after preconcentrating and analyzing a standard solution of Angiotensin II by SPE-CE using a C18 sorbent from a commercial SPE cartridge. The peak at 4 min is due to the organic solvent of the elution mixture which migrates with the

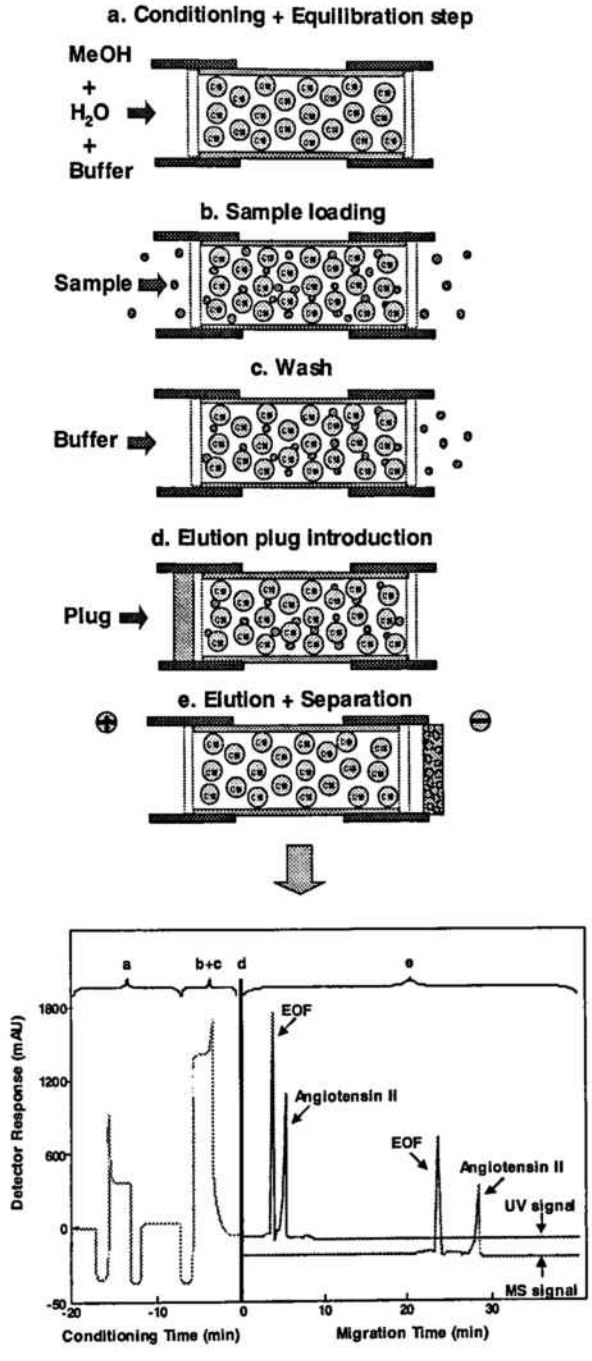


Figure 2 Schematic of a SPE-CE procedure..

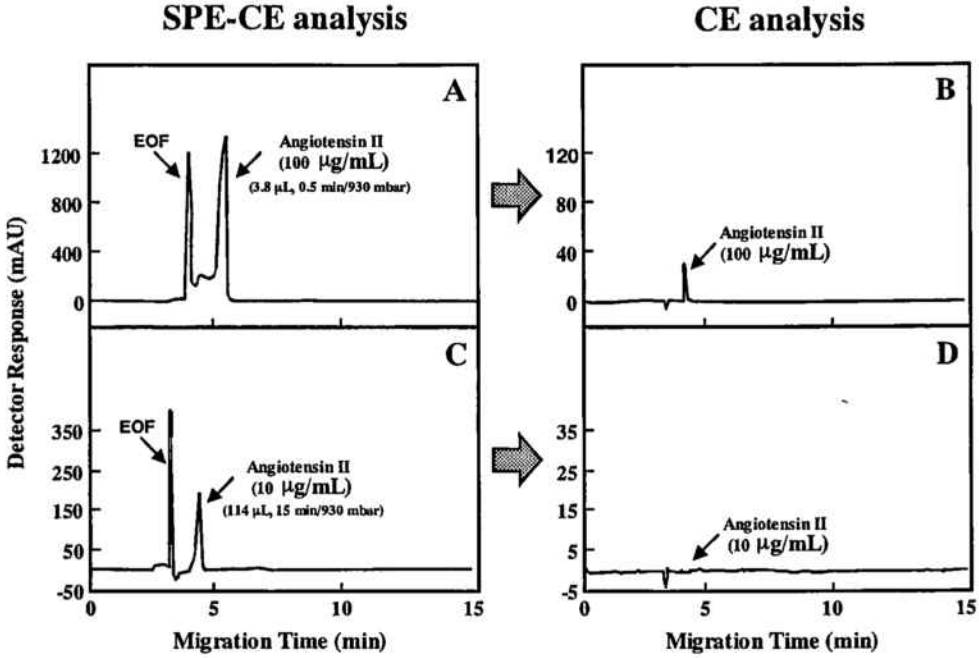


Figure 3 Analysis of Angiotensin II solutions using SPE-CE and CE, respectively. a) and b) 100 µg/mL Angiotensin II solution and c) and d) 10 µg/mL Angiotensin II solution. Separation electrolyte: 20 mM CHES, pH 9.5. The rest of the experimental conditions are detailed in the experimental section.

EOF. The SPE-CE methodology have to be optimized in order to improve these initial results.

3.1 Microextraction cartridge designs

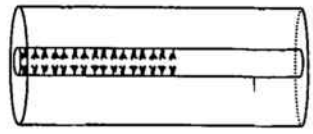
The configuration of the SPE microcartridges coupled on-line to CE and the components employed in their manufacturing have experienced many modifications since the pioneering work of Guzman et al [15-20,58]. Most of the proposed modifications are aimed to increase the binding capacity and to eliminate the frit structures used in the microextraction cartridges. Figure 4 depicts some of the configurations reported in the literature for these SPE microdevices. As in Capillary Electrocromatography (CEC) [30], these microextraction cartridges can be classified into three types: open tubular, packed, and continuous-bed or monolythic cartridges. Nowadays, despite the determined efforts by various laboratories to fabricate a reliable SPE microdevice, there is not yet a model system that can be considered ideal. Several attempts to make it commercially available failed and in most of the described applications, selection of a particular manufacturing mode was arbitrary made. Furthermore, in most cases this manufacturing is not exhaustivly described, and hence it is difficult to reproduce by non-skillful operators, limiting a widespread application of the technique.

In open tubular microextraction cartridges (Figure 4 a, b and c), the affinity ligand is bound to the inner fused silica capillary surface, and no frits are necessary to retain the affinity

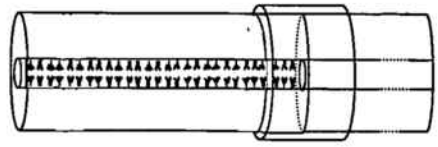
Fritless microextraction cartridges

Open Tubular mode

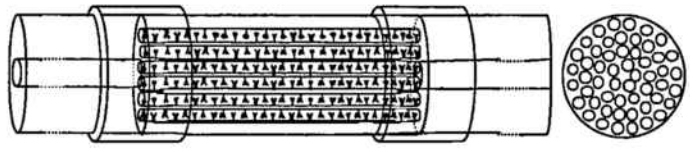
a)



b)

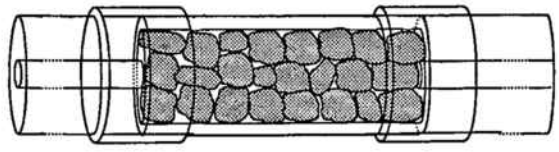


c)

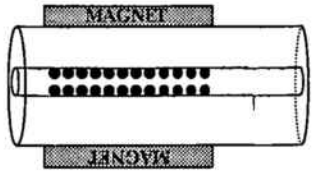


Entrapped mode

d)



e)



f)

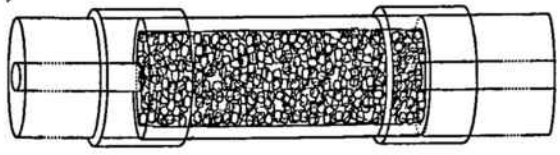


Figure 4 Schematic of the fritless configurations currently available to fabricate microextraction (or microreaction) cartridges to perform SPE-CE analysis.

be Derivatization conditions can be controlled in order to reproducibly bond the affinity ligand to a fixed lenght of the capillary inlet [15-20] (figure 4-a). In other way, an appropriate capillary piece can be off-line derivatized, and cut to obtain several cartridges that will be coupled to the separation capillary with polyethilene or teflon sleeves (figure 4-b). Open tubular microreaction cartridges have been traditionally used to perform efficient enzymatic digestions of proteins [22-26], because otherwise, small concentration factors are achieved in a microextraction mode. Improving extraction efficiency involves in many cases increasing available affinity ligand surface in the microextraction cartridge, which is limited by its length and its inner diameter. Nevertheless, getting longer and wider cartridges or using multi-bore cartridges (figure 4-c), can result in CE separations with lower efficiencies. In the near future, synthesis of porous multilayered affinity materials must be explored in order to improve extraction efficiencies and reaction yields.

Packed microextraction cartridges have higher sample capacity than open-tubular cartridges because higher affinity surface area is available. In these microextraction cartridges a fused silica capillary, PTFE or polyethilene tubing [51,60] that configures the cartridge body (Fig. 1), is homogenously packed with the affinity material (e.g. silica based C18 beads, Mollecular imprinted polymers (MIPs) or an inert material – silica, alumina or polymers - derivatized with antibodies, enzymes, lectins or other affinity ligands). We have observed that fused silica capillary is the best material to configure the microcartridge body, avoiding a system discontinuity that can result in worse performance of the SPE-CE system. The particle and pore size of the packing material beads have to be taken into account in order to avoid flow restriction and bubble formation in the microextraction cartridge. Derivatized silica particles of diameters ranging from 40 to 100 mm have been traditionally used as affinity support to avoid these problems. High porosity particles, usually 10 to 300 Å, are desirable because a larger affinity surface area is available. Nevertheless, small pores may restrict the access of macromolecules. The affinity material is usually confined between two porous frit structures (Figure 1), although some applications have described the use of fritless cartridges (figure 4 d and e) or with only the outlet frit. Fritting technologies have been improved since the early development of SPE-CE and CEC. Nevertheless, forming suitable frits in microextraction cartridges used for SPE-CE is more complicated than in CEC [30]. Since during CE separations no external pressure is applied, undesirable back pressures and bubble formation dued to very compact frits can promote current unstability and/or break-down during the electrophoretic separation. Furthermore thermolabile affinity materials can be destroyed while a sintered frit is constructed [18].

Thin frit structures consisting in multibore capillaries seems to be a good alternative to traditional porous frits in SPE-CE and CEC: sintered silica materials, porous membranes (nitrocellulose, polysulfone o PTFE), silica gels or entangled polymers. In our case, where the microcartridge body is made on fused silica capillary, on-cartridge frits have been found to be less

troublesome than frits positioned outside the packed capillary (Figure 1). Packing procedures analog to the ones used in CEC can be used in fabrication of SPE-CE microextraction cartridges [30-31]. Packed SPE-CE systems must be checked for abnormal flow restriction, electroosmotic flow behaviour and current stability before begining the analysis. Fritless packed cartridges are possible if the affinity particles have bigger diameters than the separation capillary diameter (figure 4-d), if derivatized magnetic beads are retained with a magnet (Fig 4 e) or if an affinity membrane is used. In this last case, the cartridge body is usually the teflon or polyethilene sleeve used to connect the separation capillary. Some applications using a metal casing for the membrane have been also described [19]. Such membranes have high extraction capacity and yield enhanced recoveries in a smaller volume of elution solvent than comparable SPE microextraction cartridges. Nevertheless, these SPE-CE systems are more difficult to reproducibly pack, not available with different selectivities and less robust than conventional packings, due to undesirable flow restriction originated upon fabricating the microcartridges.

Continuous-bed or monolithic CEC columns have been extensively explored in the last few years [30-31]. In these fritless columns a molded porous polymer, molded porous sol-gel or inmobilized particles in a continuous matrix are used as stationary phase (Figure 4-f). In the near future, high efficiency SPE-CE microextraction cartridges may be fabricated profiting this emerging technology to retain the affinity support. The absence of frit structures in continuous-bed CEC columns reduces bubble formation and back-pressure. Nevertheless their preparation still being difficult, labor-intensive and requires high-skilled researchers.

In this work, several SPE microextraction cartridges designs were tested in order to obtain a high efficiency, robust and durable microextraction cartridge. In the experimental section and Fig. 1 it is sistematically described the simple fabrication of the packed SPE-CE microextraction cartridge using commercially available materials. Usually, it can be used for 20-30 runs without deterioration in performance.

3.2 Microextraction cartridge affinity ligands

Many affinity ligands have been physically or chemically attached to an appropriate support to generate efficient microextraction or microreaction cartridges coupled to CE [15-20]. Selectivity strongly depends on the chemical nature of the affinity ligand and the target analyte, and may also be influenced by the experimental conditions in the binding and elution steps. Alkylsilanes, metals, proteins, monoclonal or polyclonal antibodies or their fragments, lectins and enzyme coated supports have been traditionally used. Nevertheless, researchers working in this area have encountered many diffficulties to find commercially available affinity materials compatible with SPE-CE. This forced them to develop their own affinity materials adapting various procedures commonly used in affinity chromatography, affinity electrophoresis or immunoassays. The development of reproducible

derivatization protocols and high-selective and stable affinity supports are issues currently under investigation. Meanwhile, packing SPE-CE microextraction cartridges with commercilly available derivatized silica particles, is the most widely used way to conduct simple and high efficient preconcentrations of dilute samples [15-20]. Hydrophobic C18 particles can be easily removed from conventional SPE cartridges, although they provide a limited selectivity. Nevertheless, minor differences in the chemical structure or other physicochemical properties of two commercial C18 can often translate into dramatic differences in extraction efficiency and selectivity.

The nature of the affinity phase on the SPE microextraction cartridges not only affects selectivity and efficiency of the preconcentration or microreaction, but also the electrophoretic separation of the eluted analytes. Charged or ionizable affinity materials that remain stable under an electric field have been preferred in order to generate Electroosmotic Flow (EOF) in some extent [61-64]. In conventional silica-based reversed phase affinity materials, residual silanol groups become ionized at pH greater than 2.2. Thus, the EOF generated is strongly pH dependent and it has to be taken into account in order to optimize the electrophoretic separation. Furthermore, the contribution of a C18 packed microextraction cartridge to the total EOF can be changed if packing material net charge becomes permanently modified by irreversible adsorption of the target analyte after loading the microextraction cartridge [61,64-65]. Several authors have been working with coupled switchable systems to avoid undesirable sample components come through the separation capillary [47-48].

3.3 Microextraction cartridge position

In an ideal situation the SPE microextraction cartridge may be placed in the inlet of the capillary, to avoid negative efects on the electrophoretic separation due to dispersion of the elution plug before extraction of the analytes. Commercial instrumentation currently available make this difficult, if not impossible, and capillary is frequently broken. However, several authors have positioned the SPE microextraction cartridge at 1.5-3 cm from the capillary inlet, using a Beckman Coulter cartridge [41]. We have observed that the robustness of the system is enhanced and good separations are still obtained if the SPE microextraction cartridge is placed 9 cm from the inlet of the system. This could be easily performed in commercial CE cartridge cassettes, the temperature during the extraction process is always controlled and problems with electrode/capillary configuration and automated injection are avoided.

3.4 Development of SPE-CE methods

After selecting an appropriate affinity material -according to the target analyte and the selectivity desired-, and a SPE microextraction cartridge type and design, the analysis method must be developped. The major goal of SPE-CE method development is to optimize the extraction process, maximizing the recovery of the target analyte and minimizing the amount of co-eluted impurities, under conditions that provide reproducible and efficient on-line CE separations. A generic SPE-CE protocol

consists in six basic steps [15-20] (figure 2): conditioning, equilibration, sample loading, wash, elution and electrophoretic separation, that may be adapted depending on the type of the affinity support and the target analyte. This section is focused on giving the basic directions to preconcentrate peptides from aqueous matrices using hydrophobic C18 sorbents. Similar protocols can be developped with small variations for other affinity ligands and target analytes [32-98] and for microreaction methodologies [22-26].

In the conditioning step (Figure 2-a) the SPE microextraction cartridge is typically washed with methanol, isopropanol or another organic solvent of intermediate polarity. This removes trapped air, and solvates the C18 alkylsinale chains, enabling them to interact more effectively with the target analyte. The conditioning step is often followed by an equilibration step (figure 2-a). In the equilibration step is typically employed a solution with a composition which is similar to the sample/matrix in terms of the solvent ratio, ionic strength and pH. These washes, in our case with water and separation electrolyte, help to remove residual methanol remaining from the conditioning step, and equilibrate the affinity support in an adequate medium to maximize the interactions with the target analyte. Flow rate is not particularly critical during conditioning and equilibration steps, and these capillary treatments were carried out under high pressure.

The major goal of sample loading step (Figure 2-b) is to ensure that the target analyte is quantitatively and reversibly retained by the sorbent. In general, it is a dynamic process, where the sample solution is hidrodynamic or electrokinetically introduced onto the SPE-CE system [15-20]. Nevertheless, when a microrreaction or antigen antibody interaction is involved, the sample can be incubated with the immobilized enzyme, derivitizing agent or antibody for a limited period of time according to the reaction rate or the effectivity of the interactions [23]. In general, when the C18 is exposed to the target analyte solution, an adsorbed layer will form, after the analyte molecules pass through the following steps: transport towards the particles surface, attachment and spreading [99-100]. After arriving to the affinity surface, the molecules attempt to maximize their favorable interactions by deformation or spreading out to a large extent (depends on structure and sample matrix), and there is an energy penalty in the form of the resistance to deformation. In general, for small and flexible molecules as peptides, spreading occurs very fast as compared to the experimental time scale [100]. On the contrary, as the coverage of the particles increases, so does the rate of desorption. A combined adsorption-desorption process occurs and in the equilibrium their rates become equal. In our almost neutral aqueous solutions, the retention of Angiotensin II and Leu-enkephalin in a C18 sorbent is primary afected by their hydrophobicities. Nevertheless, secondary driving forces as Coulomb and Lifshitz-van de Waals interactions (originated from interactions between fixed and/or induced dipoles) or hydrogen bonding with residual silanols in non-encapped C18 sorbents could become important under certain experimental conditions [99-100]. Furthermore, Angiotensin II and Leu-enkephalin are zwitterionic substances

with teorethical isoelectric points (pI) of 7.84 and 6.01[101], respectively, and they will be electrically charged at pH values distant from their pI.

Other processes than affinity, as sample flow or transport rate [102] may also play a major role in selection of optimum loading conditions of sample preconcentration step. This has been previously shown in some studies of polymer adsorption kinetics [102] and OTSPE-CE (Open Tubular Solid Phase Extraction Capillary Electrophoresis) [98] and is a general rule for analytical chemists working with solid phase extraction [27-29]. In figure 5 it is shown the influence of the loading pressure of a 100 μg/mL standard of Angiotensin II upon the peak area corresponding to the desorbed peptide from the C18 support. As can be seen in figure 5 a the desorbed amount first increases with time, and in the case of a loading pressure of 930 mbar levels off at a saturation value. This means that the adsorption sites become saturated with Angiotensin II molecules, and the maximum capacity of the microextraction system have been reached. Furthermore, the area of the desorbed peptide is smaller at any absolute mass of infussed Angiotensin II when the sample is supplied at a pressure of 50 mbar (fig. 5-a). Phisiadsorption in multilayer at high loading pressure, due to hydrophobic interactions and hydrogen bonding between succesive layers of peptide, can be discarded in order to explain this extra efficiency of high supplying pressures, because this softly adsorbed molecules will be eliminated during the following washing step and will not be eluted. This can be tentatively explained taking into account a balance between the rate of spreading of the molecule on the surface of the sorbent particles and differences in loading pressures. At low pressure, in certain experimental conditions the spreading time can be comparable to the time required to deposit the molecules in the surface. If the deposition is slow, each molecule can unfold and spread before it is surrounded by other molecules. The adsorbed amount will correspond to a thin layer and hence will be low. On the contrary, at higher pressure, the molecules are enclosed by neighbours before they had time to unfold. The adsorbed layer will be larger and thicker and the adsorbed amount higher. Nevertheless peptides are not supposed to present a slow spreading process, as they are less rigid molecules than proteins or other polymeric macromolecules. An alternative explanation can be that when supply pressure is 930 mbar, sample solution may flow through all the particle pores, getting access to adsorption sites that at lower supplying pressures may be inaccesible. Otherwise, it is also desirable to use high loading pressures in order to minimize time and sample volume required for preconcentration (Figure 5-b). Loading large sample volumes may promote in some cases, analyte breakthrough [35]. In Figure 5-c we plot the signal area dued to the desorbed Angiotensin II as a function of the total loaded amount at 930 mbars for two standards of 10 μg/mL and 100 μg/mL. As can be observed again the adsorbed amount first increases with time (as is to be expected for a constant flow) and then levels off. The signal area reached for a given analyte mass loaded using the 10 μg/mL solution is significantly lower. On the contrary, longer injection times (large injection volumes) are necessary in order to inject

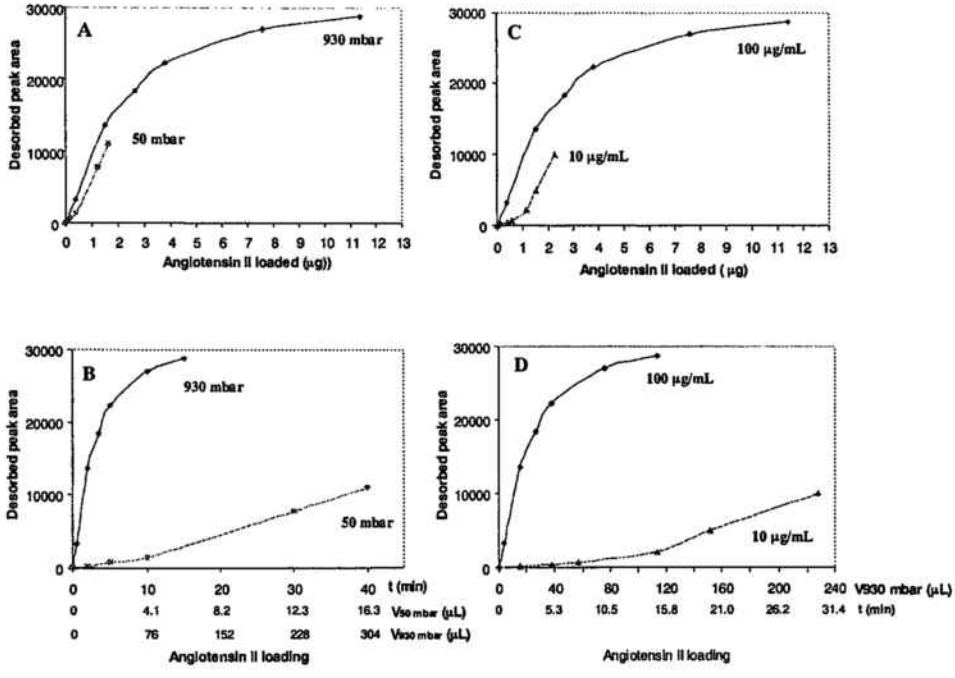


Figure 5 Peak areas of desorbed Angiotensin vs a) loaded Angiotensin II (µg) at 930 and 50 mbar, b) loaded Angiotensin II (t and V) at 930 and 50 mbar, c) loaded Angiotensin II (µg) at 930 mbar from solutions of 100 µg/mL and 10 µg/mL and d) loaded Angiotensin II (t and V) at 930 mbar from solutions of 100 µg/mL and 10 µg/mL. Separation electrolyte: 20 mM CHES, pH 9.5. The rest of the experimental conditions are detailed in the experimental section.

the same analyte mass in the capillary. This makes not feasable to arrive until saturation conditions with dilute solutions (Figure 5-d). Using 10 µg/mL solutions at high loading pressure (Figures 5-c and 5-d) and 100 µg/mL solutions at low loading pressure (Figures 5-a and 5-b), the curves seem an assimetric sigmoid, that becomes smoothed as the concentration and supplying rates raises. This sigmoidal behaviour have been shown in the case of some amino acid adsorption and must hide certain degree of cooperativity on the extraction mechanism [100,103-104]. This cooperative effect upon extraction efficiency can be also observed when analyzing mixtures of peptides. In figure 6-b it is shown the separation of a 10 µg/mL mixture of Angiotensin II and Leu-enkephalin with SPE-CE. Comparing the areas of the desorbed peaks in the mixture, with the ones obtained for SPE-CE analysis of 10 µg/mL Angiotensin II and Leu-enkephalin standards (figures 6-c and d, respectively), it can be observed that Angiotensin II retention is promoted by Leu-enkephalin presence in the mixture. In other cases, high affinity peptides may preclude preconcentration of the weakly interacting peptides. The composition of the matrix sample may also affects recoveries, as certain serum proteins which bound drugs [93]. These are important factors to study for the validation of SPE-CE in the analysis of complex matrices [84]. Saturation conditions and cooperativity must be extensively analyzed in order to evaluate the SPE-CE linearity for the analysis of mixtures of Angiotensin II and Leu-

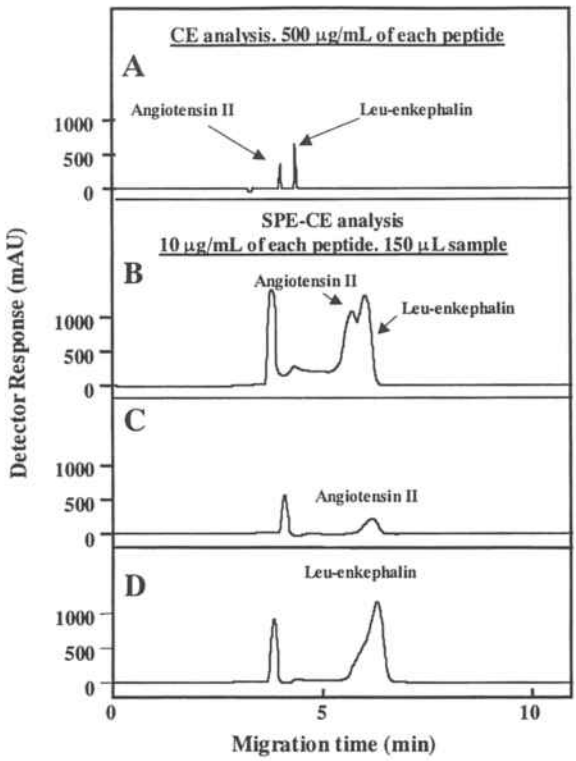


Figure 6 Analysis of Angiotensin II and Leu-enkephalin mixtures by a) CE (500 µg/mL) and b) SPE-CE (10 µg/mL). Analysis of 10 µg/mL solutions by SPE-CE of c) Angiotensin II and d) Leu-enkephalin. Separation electrolyte: 20 mM CHES, pH 9.5. The rest of the experimental conditions are detailed in the experimental

enkephalin [95]. In figure 7 it is shown the linear relationship between the area of the desorbed Angiotensin II and the concentration of the Angiotensin II solution loaded during 30 minutes at 930 mbar. This results are an indication of a linear adsorption isotherm and are in agreement with earlier observations [61,93]. It is not recommended to saturate the sorbent during the loading step, otherwise a linear relation may not be obtained.

After the loading step, the microextraction cartridge is washed under high pressure with water and separation electrolyte, to remove the impurities that are weakly retained on the C18 sorbent, and to rinse residual, unretained sample/matrix components that may remain from the sample loading step [15-20]. The washing buffer composition (pH, ionic strength and electrolyte) must be compatible with the mechanism that promotes the retention of the target analyte in order to avoid their undesired elution in this step [52,59,90]. In Figure 8 it is shown the enhancement in concentration detection limits of Angiotensin II (100 ng/mL) when the running buffer is changed from CHES at pH 9.5 to ACES at pH 7.41. The retained Angiotensin II is deprotonated and, hence eliminated in some extent, during the washing step with CHES at pH 9.5. In such cases, separation electrolyte may be changed or separations may be developped under

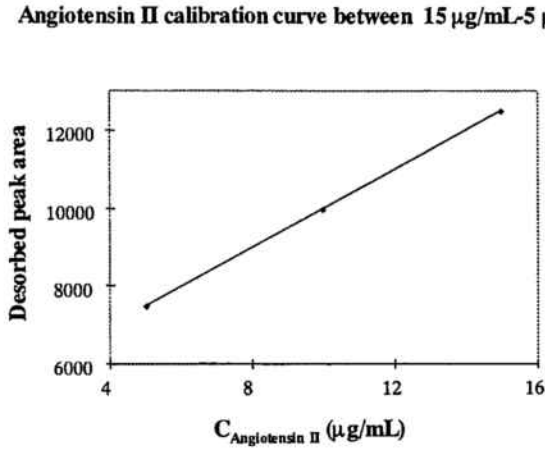


Figure 7 Graphic of peak areas of desorbed Angiotensin II vs Angiotensin II concentration obtained by SPE-CE, loading 228 mL (930 mbar/30 min). Separation electrolyte: 20 mM CHES, pH 9.5. The rest of the experimental conditions are detailed in the experimental section.

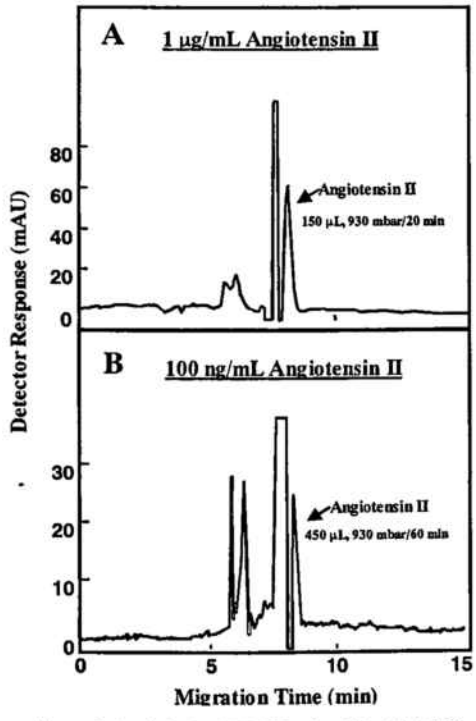


Figure 8 Analysis by SPE-CE using 20 mM ACES as separation electrolyte of a) a 1 mg/mL Angiotensin II solution and b) a 100 ng/mL Angiotensin II solution. The rest of the experimental conditions are detailed in the experimental section.228 mL (930 mbar/30 min). Separation electrolyte: 20 mM CHES, pH 9.5. The rest of the experimental conditions are detailed in the experimental section.

partial filling experiments [55].

The elution of the retained analytes occurs upon low pressure injection of an organic solvent that effectively disrupts the primary and secondary interactions between the sorbent and the target analyte. Sometimes this can be followed by a small plug of separation buffer to push the elution plug away from the solid support, in order to avoid the analyte readsorption or the dispersion back of the elution plug into the separation buffer [23,41,59,85,89,92]. In our case, with the microextraction cartridge placed 9 cm from the inlet of the SPE-CE system, we are performing an electroelution in reversed phase mode [64]. It has been demonstrated that a pure electroelution can be performed by means of the electroosmotic flow generated by the applied separation voltage without using an elution solution [96]. Further studies are currently under development in our laboratories to set the influence of the separation voltage upon elution. Optimized the previous steps and under a fixed separation voltage, recovery strongly depends on

the organic solvent content, pH, ionic strength and electrolyte composition of the elution plug. Typical elution solvents with C18 sorbents include moderately polar organic solvents such as, ethanol, isopropanol, acetonitrile, THF, acetone, and ethlyl acetate or non-polar organic solvents such as hexane and methylene chloride. These should be used either alone or in combination with each other, often with a small percentage of water or buffer containing a strong acid or a strong base. In many situations amines and other polar solutes, like peptides, may also be retained by interactions with residual silanols [27-29]. These may be disrupted by adding a low concentration acid or base, such as 0.1 or 1% concentrated HCl or ammonia in methanol. In many cases, a weak organic acid is preferred (e.g. 1-10% HOAc or HFor), since it may enhance analyte solubility and often provides good recoveries without eluting contaminants. Several authors have described sequential elution to enhance selectivity and recovery if impurities are extracted first. The elution solution composition and size can lead to a reduced EOF and, hence affect the efficiency of the electrophoretic separation [64]. Nevertheless, the presence of acetonitrile or other organic solvents in low pH elutions solutions may favour sample stacking [14,59-60]. Large elution plugs may result in a lose of efficiency and electrophoretic resolution [59-60]. Plug size may be of the same order as the ones used in CE (20-50 nL). The selection of the proper sorbent amount, and hence SPE microextraction cartridge size, is critical to determine elution plug duration because insufficient sorbent leads to column overload and low or irreproducible recoveries, while an excess of sorbent increases backpressures, solvent requirements and may also reduce robustness, recovery and separation efficiency. High capacity sorbents are desired in order to minimize the bed volume of sorbent and reduce the elution solvent required. Tomlinson et al minimized the bed volume of sorbent by using an appropriate coated membrane and that made possible to reduce the volume of elution solvent required for the efficient recovery from the affinity phase [19]. For more detailed information on capacity (total amount of analyte that can be retained by an adsorbent mass under optimum conditions) and sorbent amounts requirements, it is advisable to perform several breakthrough experiments in which either the sorbent amount is varied or, the sample/matrix volume increased. The optimum elution plug composition must be determined empirically for each particular SPE-CE methodology. In our case, using 8 mm of affinity support, the most effective elution solvent was 20-30 nL aproximately of 80:20 v/v MeCN:H_2O (25 mM HFor), apparent pH 3.13.

After elution, the extracted analytes are separated according to their different electrophoretic mobilities under conventional CZE conditions. Fused silica capillaries have been traditionally used, although some applications have been described using coated separation capillaries [61]. The presence of an on-line SPE process affects migration times [92] and separation efficiency and selectivity [59,92,94]. This can be due, as we have seen, to the elution plug size and composition, the increasing analyte-analyte and analyte-wall interactions when analyzing relatively large amounts of components mixtures, the affinity

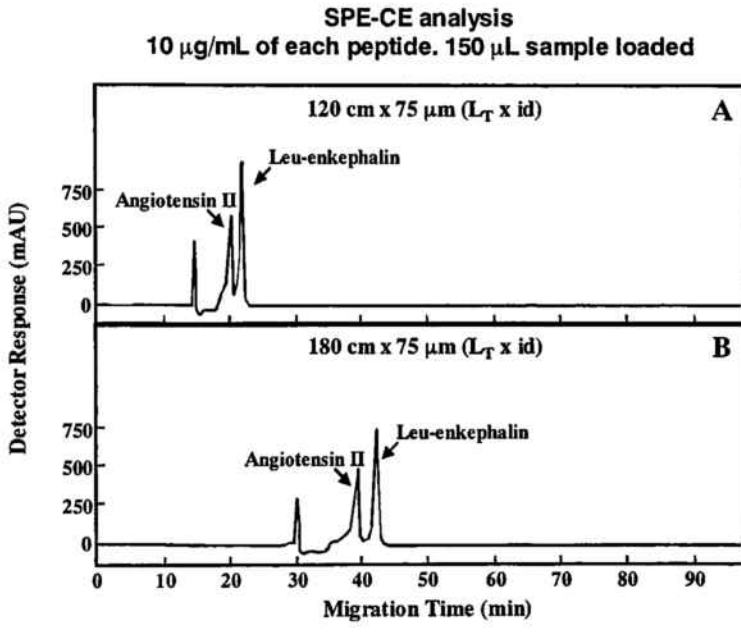


Figure 9 SPE-CE analysis of a 10 μg/mL mixture of Angiotensin II and Leu-enkephalin using 20 mM CHES, pH 9.5 in a) a 120 cm x 75 μm fused silica capillary and b) a 180 cm x 75 μm fused silica capillary. The rest of the experimental conditions are detailed in the experimental section.

sorbent type and amount, which determine the total EOF observed in the SPE-CE system and can lead to a reduced hydrodynamic flow [61,65], and the unavoidable chromatographic interactions produced in the microextraction cartridge [59]. Thus, CE separation conditions may be reoptimized in a SPE-CE mode in order to improve separation performance [86]. In Figure 9 baseline resolution of a 10 μg/mL mixture of Angiotensin II and Leu-enkephalin using SPE-CE is achieved changing the separation capillary lenght. SPE-CE experiments using conventional C18 sorbents and affinity membranes have been described where the electrophoretic separation was performed under transient isotachophoresis (tITP) conditions, resulting in enhanced selectivities, efficiencies, and concentration limits of detection [47,49-50,85,88]. Other in-capillary electrophoretic focusing approaches, as isotachophoresis, analyte stacking [80,87] or capillary isoelectric focusing (CIEF) may also have the same result.

3.5. Development of SPE-CE-MS separation methods

Since Tomlinson et al firstly explored the coupling of SPE-CE-MS and SPE-CE-MS-MS [19], several authors have shown the great potential of these methodologies to obtain structural information and perform high-throughput, sensitive and specific analysis of low concentrated analytes in complex matrices, using both coaxial and sheathless interfaces [22-23,32-52,57,81,83,85,88-89]. Recently, Guzmán have shown the first SPE-CE-MS application in a commercial CE-MS instrument using a highly selective

immunoadsorbent [33]. In figure 10 it is shown the CE-UV and the CE-MS analysis of a 500 μg/mL mixture of Angiotensin II and Leu-enkephalin. Both CHES and ACES buffers were found suitable for CE-MS analysis. Nevertheless, an ACES buffer was used throughgout the CE-MS analysis in order to improve sensitivity in SPE-CE-MS experiments. The flow and composition of sheath liquid was found to affect Electrospray stability. A solution containing 20 mM HAc in 50:50 v/v MeOH:H_2O delivered at 10 μL/min provided the best results. Sensitivity and specificity of CE-MS analysis can be improved if the $[M+H]^+$ ions of each compound are monitorized. According to the Angiotensin II and Leu-enkephalin mass spectra shown in figure 11 a and b, respectively, in figure 10 c and d are shown the electropherograms obtained for the analysis of the same 500 μg/mL mixture, but monitoring the $[Angiotensin+2H]^{2+}$ and $[Leu\text{-}enkephalin+H]^+$ ions. CE-MS sensitivity can be furtherly improved if on-line SPE is performed. SPE-CE-MS

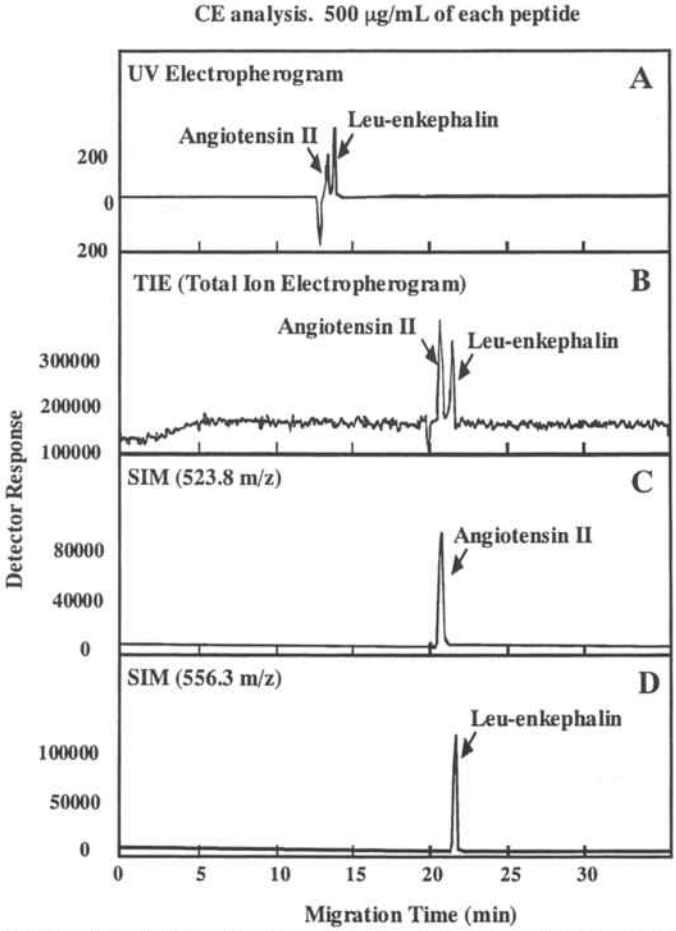


Figure 10 CE-MS analysis of a 500 μg/mL mixture of Angiotensin II and Leu-enkephalin using 20 mM ACES, pH 7.4. Selected-ion electropherograms of c) $[Angiotensin\ II+2H]^{2+}$ and d) $[Leu\text{-}enkephalin+H]^+$. The rest of the experimental conditions are detailed in the experimental section.

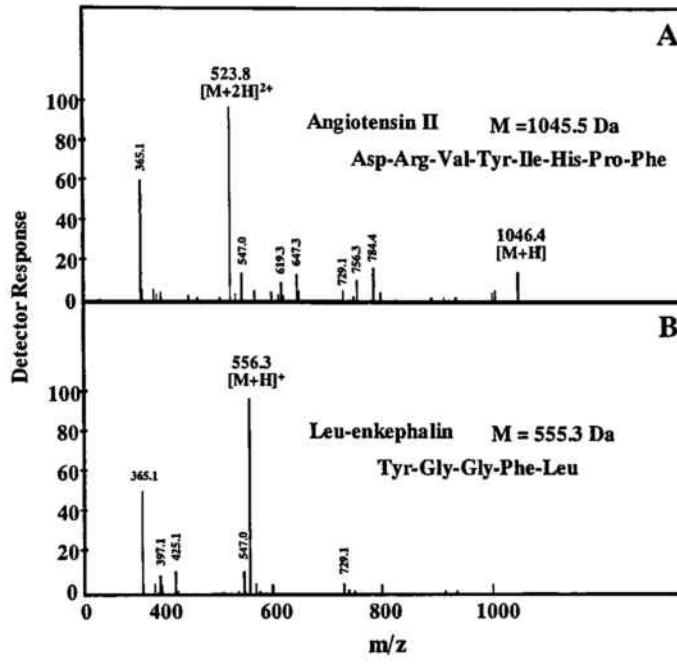


Figure 11 Mass spectra obtained in the CE-MS analysis of a 500 μg/mL mixture of Angiotensin II and Leu-enkephalin using 20 mM ACES, pH 7.4. a) Angiotensin II and b) Leu-enkephalin. The rest of the experimental conditions are detailed in the experimental section.

experiments are performed under the experimental conditions optimized for SPE-CE-UV analysis. In figure 12 a and b can be observed the electropherograms obtained for the analysis of 10 μg/mL and 100 ng/mL solutions of Angiotensin II, respectively. Analysis of 100 ng/mL Angiotensin II solutions requires injection of a large sample volume. For the analysis of furtherly diluted Angiotensin II solutions a higher binding capacity affinitty support may be selected, and the SPE-CE procedure reoptimized. Nevertheless, sensitive and selective analysis of Angiotensin II and Leu-enkephalin diluted mixtures can be performed using these SPE-CE-MS experimental conditions. In Figure 13 it is shown the electropherogram corresponding to the SPE-CE-MS analysis of a mixture of 5 μg/mL of Angiotensin II and Leu-enkephalin.

4. Conclusions

SPE-CE and SPE-CE-MS have demonstrated the ability to separate and characterize peptides at the nanogram per milliliter level, using disposable microextraction cartridges which were easily constructed using commercially available materials. In order to improve the preconcentration factors new affinity sorbents may be explored and special care must be taken to the optimization of experimental conditions. This last factor is also extremely important in order to achieve a widespread development of a great variety of quantitative applications. In proteomic studies the analyte concentrator device may become a high throughput and selective "amplification method" for enhancing detectability of proteins, peptides, their degradation products and impurities, as

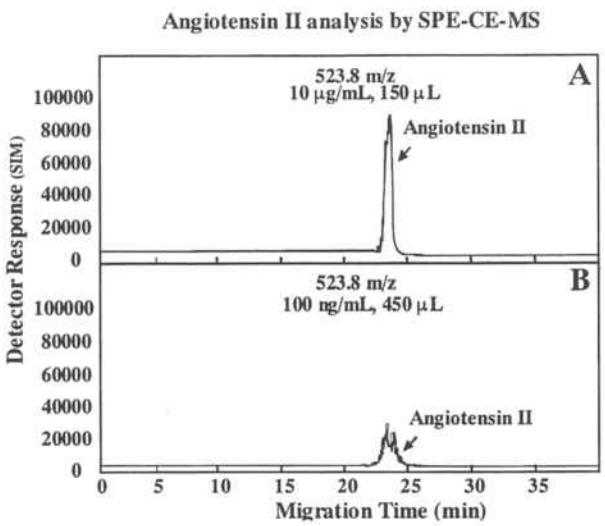


Figure 12 Selected-ion electropherograms of Angiotensin II obtained by SPE-CE-MS analysis of solutions at a) 10 μg/mL (150 μL) and b) 100 μg/mL (450 μL). Separation electrolyte: 20 mM ACES, pH 7.4. The rest of the experimental conditions are detailed in the experimental section.

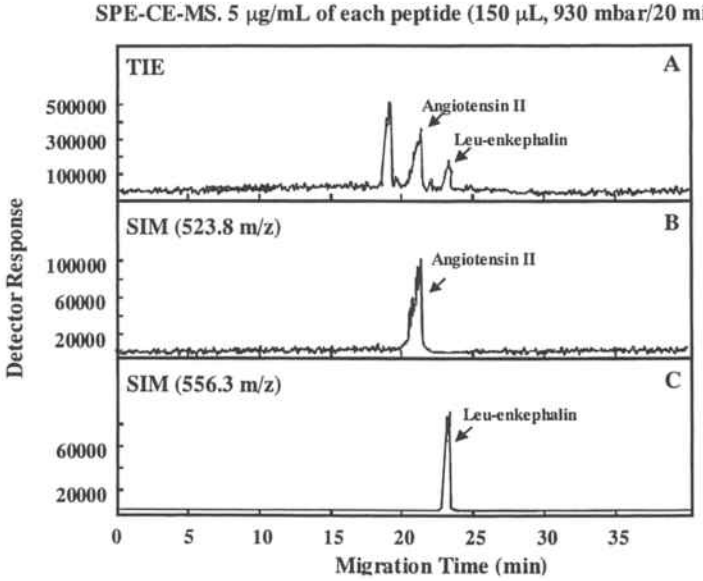


Figure 13 SPE-CE-MS analysis of a 5 μg/mL mixture of Angiotensin II and Leu-enkephalin using 20 mM ACES, pH 7.4. The rest of the experimental conditions are detailed in the experimental section.

PCR technology has been used for the amplification of DNA molecules. In the future, on-line SPE-CE-MS methodologies with high capacity and selective microextraction cartridges positioned in the capillary outlet position may be explored, in order to desalt buffer and scavenge low volatile

components, improving ES-MS sensitivity and CE-ES-MS versatility.

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Capítulo 5. Separación y caracterización de EPO mediante CE y MS

En este capítulo se describen las investigaciones realizadas para el desarrollo de métodos de separación y caracterización de glicoformas de Eritropoyetina Humana Recombinante (rHuEPO) empleando CE y MS. La Eritropoyetina (EPO), como la mayoría de las moléculas peptídicas de elevado peso molecular, tiene una gran tendencia a adsorberse irreversiblemente en las paredes internas de los capilares de sílice fundida que se emplean en CE. Una de las alternativas más eficaces para evitar este fenómeno, es emplear capilares recubiertos adecuados. En este capítulo se investiga el uso de diferentes capilares recubiertos permanentemente para el análisis de EPO humana, empleando electrolitos de separación potencialmente volátiles y compatibles con la detección en línea por MS. La composición y el pH del electrolito de separación, el voltaje, la temperatura y la longitud del capilar, son optimizados para obtener las mejores separaciones de las glicoformas de la EPO. Paralelamente la Espectrometría de Masas con Ionización por Electrospray (ESI-MS) y la Espectrometría de Masas con Analizador de Tiempo de Vuelo e Ionización por Desorción con Láser Asistida por una Matriz (MALDI-TOF) son empleadas para analizar la EPO. Más allá de las limitaciones obvias que supone el empleo de estas técnicas para analizar la mezcla de glicoformas de EPO sin separación previa, se pone de manifiesto la problemática planteada al emplearlas para caracterizar rigurosamente la masa molecular media de una molécula de tan extrema labilidad como la EPO humana.

El trabajo realizado ha conducido a la publicación que se reproduce a continuación:

- ***Separation of Recombinant Human Erythropoietin Glycoforms by Capillary Electrophoresis Using Volatile Electrolytes. Assesment of Mass Spectrometry for the characterization of EPO Glycoforms***
  V. Sanz-Nebot, F. Benavente, A. Vallverdú, N. A. Guzmán, J. Barbosa,
  Anal Chem., (2003) enviado.

SEPARATION OF RECOMBINANT HUMAN ERYTHROPOIETIN GLYCOFORMS BY CAPILLARY ELECTROPHORESIS USING VOLATILE ELECTROLYTES. ASSESSMENT OF MASS SPECTROMETRY FOR THE CHARACTERIZATION OF EPO GLYCOFORMS.

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Part of this work was presented at the Second International Symposium of Separations in the BioSciences (SBS-2001), held in Prague, Czech Republic, September 17-20, 2001.

Abstract

The separation of the glycoforms of erythropoietin (EPO) by capillary electrophoresis (CE), was recently published as a monograph by the European Pharmacopoeia (European Pharmacopoeia 4, 01/2002: 1316, 1123-1128 [1]). Although the experimental CE conditions employed a background electrolyte containing additives suitable for on-line UV-absorption detection, they were not appropriate for on-line mass spectrometry (MS) detection. In this work, an attempt was made to investigate experimental conditions employing volatile electrolyte systems to achieve the separation and characterization of EPO glycoforms using CE-ESI-MS methodology. The influence of several operating conditions, such as the coating of the internal walls of the capillary, as well as the composition, concentration, and the pH of the separation buffer were investigated. The results demonstrated that when the internal walls of the capillaries were permanently coated with polybrene, and a buffer electrolyte containing 400 mM of HAc-NH_4Ac (acetic acid:ammonium acetate), pH 4.75, was used, a significantly reproducible separation was achieved for EPO glycoforms. Intact EPO was characterized by two mass spectrometry techniques, electrospray ionization (ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-MS). The data demonstrated that MALDI-TOF-MS provided a good approximation to an average molecular mass of the EPO molecule. However, it was still necessary to carry out further separation of the intact EPO glycoforms in order to obtain molecular mass information when ESI-MS was used.

Keywords: EPO / glycoforms/ capillary electrophoresis / electrospray ionization mass spectrometry / MALDI-TOF-MS / capillary coating

1. Introduction

Erythropoietin is a glycoprotein hormone which regulates erythropoiesis and it is produced primarily in the kidney. Among the biopharmaceutical drugs currently approved [2-4], recombinant human erythropoietin (rHuEPO) is perhaps the most successful pharmaceutical product with sales reaching billions of dollars [5]. rHuEPO has been extensively used for the

treatment of several anemias associated with acute and chronic diseases [6-8]. In recent years, scientists have developed several strategies to enhance its properties and expand its applications [9-12]. Recently, it has been commercialized a hyperglycosylated EPO analogue with a long-lasting effect termed novel erythropoiesis stimulating protein (NESP) [8,11-12]. Despite the many benefits of rHuEPO and NESP in the clinic, they have been popularly known by their extensive misuse as performance-enhancing agents in endurance sports [8,13-15].

Approximately 40% of the molecular mass of human EPO (30,000 – 34,000-Da) is composed of carbohydrates, of which 17% correspond to sialic acids [6,16-17]. The sugar content of EPO has been demonstrated to be critical for its biological activity and it is influenced during its manufacturing process by the cell line and the incubation culture conditions [8, 11-12, 15,18]. The polymorphism associated with the amount, the size, and the structure of the carbohydrate chains is known as microheterogeneity, and the molecular species generated are termed glycoforms. The glycoform types and their quantitative amounts enable to differentiate each type of EPO hormone whether naturally- or artificially-produced [15,18].

The amino acid sequence and the microheterogeneity of the carbohydrate structure of digested EPO glycoforms have been extensively characterized using LC, CE and MS [18-32]. However, due to the complexity of the intact molecule, it is still difficult to achieve a detailed structural information [15,18,20,21,24,26-27,33-34].Separation of the EPO glycoforms has been difficult to perform using LC or any other hydrophobicity-based technique because they are structurally related molecular species with subtle differences [24,27]. Using electrophoretic techniques, the glycoforms of EPO are separated according to their charge-to-mass ratios, mainly due to the differences in their sialic acid content [15,18-21,24,33]. Conventional slab-gel electrophoresis, in particular the isoelectric focusing mode, has been the method of choice for several years for the direct resolution of many protein isoforms [1,15,18,21,33]. During the last decade, CE is becoming an attractive alternative for the determination of protein purity and resolution of protein isoforms, specially in production and quality control laboratory settings in the biotechnology industry [1,20,21,24,33].

In the last few years, several CE methods have been developed for the separation of rHuEPO glycoforms using UV-absorption detection systems [1,20,24,35-43]. In most cases, the use of dynamically or permanently coated capillaries was preferred to avoid any type of adsorption of proteins to the inner surface of the capillary [1,35-42]. The glycoform analysis of intact rHuEPO using putrescine-coated capillaries have been extensively studied by several authors [1,21,33,35-38,42]. The specifications recently proposed by the European Department for the Quality of Medicine in a collaborative study [35] have been included in the 2002 European Pharmacopoeia substituting the isoelectrofocusing (IEF) test for the CE method [1,37]. Nevertheless, there are still some technical diffculties in obtaining reproducible separations and

separation conditions are not compatible with CE-ESI-MS operation [36,42,44-48].

The present report focuses in the assessment of CE and CE-ESI-MS as suitable techniques for the separation and characterization of rHuEPO glycoforms. Major disadvantages of the CE methodology included in the 2002 European Pharmacopoeia have been investigated. Alternative experimental conditions that are compatible with the coupling of CE with electrospray ionization mass spectrometry (ESI-MS) were studied. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) studies were performed as a complementary procedure for the characterization of rHuEPO glycoforms.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetonitrile, acetone, methanol, isopropanol, formic acid (FA), acetic acid (HAc) (glacial), hydrochloric acid (25%), phosphoric acid (85%), trifluoroacetic acid (TFA), sodium acetate (NaAc), sodium chloride, sodium dihydrogenphosphate, sodium dodecylsulphate (SDS), ethylene glycol, and sodium hydroxide were supplied by Merck (Darmstadt, Germany). 1,4-diaminobutane (putrescine), heptafluorobutyric acid (HFBA), Brij 35, poly(diallyldimethylammonium chloride) (PDMAC, M= 100000-200000), and hexadimethrin bromide (polybrene, PB, M= 15000) were purchased from Aldrich (Madrid, Spain). 2-[N-morpholino]ethanesulfonic acid (MES), sodium dextran sulfate (M= 500000), N-tris-[hydroxymethyl]methylglycine (tricine), 2,5-dihydroxybenzoic acid (DHB), 3-hydroxypiconilic acid (HPA), 4-hydroxy-3-methoxycinnamic acid (ferulic acid, FA) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Ammonia (30%) were purchased from Panreac (Barcelona, Spain). Nonidet P40, Tween-20 and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic or sinapinic acid, SA) were supplied by Fluka (Madrid, Spain). Urea was obtained from J.T. Baker (Deventer, Holland) and from Sigma. Water with a conductivity value lower than 0.05 mS/cm was obtained using a Milli-Q water purification system (Millipore, Molsheim, France).

rHuEPO produced in a Chinese hamster ovary (CHO) cell line was provided by the European Pharmacopoeia as Biological Reference Product (BRP). Each sample vial contained 250 μg of EPO (32500 units of a mixture of Epoetin Alfa and Beta), 0.1 mg of Tween 20, 30 mg of trehalose, 3 mg of arginine, 4.5 mg NaCl, and 3.5 mg of Na_2HPO_4. Purified rHuEPO (research batch) was obtained from Johnson & Johnson Pharmaceutical Research and Development, L.L.C. (Raritan, NJ, USA). Standard solutions of rHuEPO BRP were prepared using one of the following procedures:

Unfiltered rHuEPO: The contents of each BRP vial were dissolved in Milli-Q water to obtain a 1000-ppm solution of rHuEPO. Filtered BRP rHuEPO: The contents of each vial were dissolved in Milli-Q water to obtain a 1000 ppm

solution of rHuEPO and the excipients of low-molecular mass were removed by passage through a Microcon-10 cartridge (Amicon, Beverly, MA, USA) [1,35,37,42]. The filter membrane was initially washed with Milli-Q water for 10 min in a centrifuge at 13000 g. Then the sample was centrifuged for 10 min under the same centrifugal force. The residue was washed three times, for 10 min in the same way, with 0.2 ml of Milli-Q water. The filtrates from each of the four previous centrifugation steps were discarded. The residue was recovered from the cartridge by centrifugation upside down into a new vial (3 min at 1000 g). For the CE and MALDI-TOF-MS experiments, sufficient Milli-Q water was added to adjust the rHuEPO concentration to 1000 ppm (. g/ml). For ESI-MS experiments a solution of 300 ppm (. g/ml) of rHuEPO in H_2O/MeCN (65:35 v/v) with a 0.05% v/v of TFA was prepared immediately before use, to avoid sample hydrolysis during storage.

All samples and solutions were passed through a 0.45-mm nylon filter (MSI, Westboro, MA, USA) and they were stored at 4 °C when not in use.

2.2 Capillary zone electrophoresis

An HP ^{3D}CE capillary electrophoresis instrument (Agilent Technologies, Inc., Wilmington, DE, USA) equipped with a diode array detector was used for CE analysis. Instrument control, data acquisition and data processing were performed using ChemStation software (Agilent Technologies). Fifty μm i.d. x 360 o.d. (total length, 57 cm; effective length, 48.5 cm) bare fused silica capillary columns were purchased from Polymicro Techonologies (Phoenix, AZ, USA). Polyvinyl alcohol (PVA) 50 μm i.d. x 360 o.d. (total length, 64.5 cm; effective length, 56 cm) and CEP (polymeric)-coated 75 μm i.d. x 360 o.d. (total length, 97 cm; effective length, 88.5 cm) columns were obtained from Agilent Technologies. C-8 coated columns 50 μm i.d. x 360 o.d. (total length, 100 cm; effective length, 71.5 cm) were purchased from Supelco (St. Louis, MO, USA). The detection window was placed at 8.5 cm from the outlet of the capillary. pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

2.2.1 Bare fused silica capillary

Separations were performed at 25 °C, using a 100 mM FA solution adjusted to pH 4 with ammonia, in 57 cm capillary columns. Samples were injected hydrodynamically at 33.5 mbar for 5 s. All experiments were carried out under normal polarity (with the anode at the injection end of the capillary), applying a voltage of 15 KV during the electrophoretic separations. Detection was performed at 214 nm. All capillary rinses were performed at 930 mbar. New capillaries were activated by flushing them for 20 min with aqueous 1 M NaOH, followed by 15 min with water and 30 min with working buffer. Capillaries were conditioned everyday rinsing for 5 min with water, 5 min with aqueous 0.1 M NaOH, 10 min with water and 10 min of separation buffer. After the last flush of the activation or the conditioning treatments, 15 KV were applied until a steady baseline was obtained. Between runs, capillary was

successively rinsed for 3 min with water and 3 min with running electrolyte.

2.2.2 Putrescine coated capillary

Experimental conditions have been described elsewhere [1,35-38,42]. Separations were performed using a 108.5 cm capillary column. The temperature was set at 35 °C. Samples were injected hydrodynamically at 33.5 mbar for 15 sec. A voltage of 15.4 kV was applied during normal polarity electrophoretic separations. Detection was performed at 214 nm. The separation electrolyte contained 0.01 M tricine, 0.01 M NaCl, 0.01 M NaAc, 7 M urea, and 2.5 mM putrescine. This mixture was adjusted to pH 5.5 with 2 M acetic acid and remained stable for seven days at room temperature. New capillaries were activated by flushing them for 45 min with aqueous 1 M NaOH, followed by 30 min with water and 45 min with separation electrolyte. Each capillary was conditioned every day rinsing for 5 min with water and 10 min of separation buffer. After the last flush of the activation or the conditioning methods, 15 kV were applied until a steady baseline was obtained. Between runs, each capillary was successively rinsed for 5 min with water and 10 min with running electrolyte.

Bare- and putrescine-coated columns were stored overnight filled with separation electrolyte. If a longer storage was necessary, the capillary was rinsed with water for about 10 min and dried by flushing air for 10 min.

2.2.3 Polybrene coated capillary

Each new, 57-cm capillary was rinsed for 30 min with aqueous 1 M NaOH, followed by 30 min with water. After activation, capillaries were coated and equilibrated by flushing them first for 30 min with a solution of 5 % (w/v) polybrene (PB) and 2 % (v/v) ethylene glycol [44-45,49-52], and then with separation buffer for 45 min. Between workdays or after a change of buffer, the capillary was conditioned by flushing it for 5 min with aqueous 1 M NaOH solution, followed by 5 min with water, 5 min with PB solution and 10 min with separation buffer. For coating regeneration the first flush with 1M NaOH was substituted by 1M HCl. In all cases, the system was finally equilibrated by applying the separation voltage for about 10 min, until a steady baseline was obtained. Samples were introduced into the capillary hydrodynamically at 33.5 mbar for 5 s. Separations were carried out under reversed polarity (with the cathode at the sample introduction end of the capillary). Between runs, the capillary was rinsed for 2 min with 0.1 M NaOH, 2 min with water, 2 min with PB solution and 5 min with separation buffer. For 107-cm capillaries, the duration of the rinsing steps was doubled. The procedure followed to coat capillaries with 5% aqueous solutions of poly(diallyldimethylammonium) chloride (PDMAC) was analogous to the one used with PB [53-54].

A PB coating configured in Successive Multiple Ionic-Polymer Layers (SMIL) [55] was generated by rinsing the capillary first with PB, followed by a layer of an anionic polymer, dextran

sulfate (DS), and then again with PB. New capillaries were rinsed for 60 min with aqueous 1 M NaOH, followed by 30 min with water. After the activation, 30 minutes were allowed to pass before the first layer of PB was created by rinsing with PB solution for 45 min. The layer of dextran was created by rinsing the column for 30 min with 3% (w/v) DS solution. Finally, the second layer of PB was obtained by flushing the capillary again for 45 min with PB solution. Thirty min were allowed to pass before performing the last 15-min flush with separation buffer. Between runs the capillary was flushed for 5 min with separation buffer.

Acetic acid, formic acid, tricine and MES running electrolytes covering the pH range 3.5-6.50 were prepared at different concentrations and adjusted to the appropriate pH values using ammonia or HAc. When other substances were present in the separation buffer, the pH was adjusted after preparing the mixture. They were degassed and passed through a 0.45-μm nylon filter (MSI, Westboro, MA, USA). The PB-coated capillaries were stored overnight filled with separation buffer. Storage of the capillaries for long periods of time favoured a decrease in the performance of PB-coated capillaries.

2.3 Mass spectrometry

2.3.1 Electrospray ionization mass spectrometry (ESI-MS).

ESI-MS experiments were performed using a VG Platform II single quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with a nebulizer-assisted electrospray source. Samples were injected using a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 5-μl sample loop. Two Phoenix 20 syringe pumps (CE Instruments, Milan, Italy) where used to deliver at 10 μL/min a mobile phase containing H_2O/MeCN (65:35 v/v) with a 0.05% v/v of TFA. The high-flow nebulizer was operated in a standard mode with N_2 as the nebulizing (20 l/h) and the drying (400 l/h) gas. The source and analyzer settings were as follows: source temperature, 60°C; sample cone voltage, 110 V; capillary voltage, 4.5 kV; counter electrode voltage, 0.6 kV; ion energy, 6 V; low mass resolution, 10.5; and high mass resolution, 11.0. Mass calibration was made in the m/z range 300-2000 using NaI. Instrument control and data analysis were performed using Masslynx application software from Micromass (Manchester, UK). Data obtained were analysed with the MaxEnt algorithm of MassLynx.

2.3.2 Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS).

The MALDI-TOF mass spectra were obtained using a Voyager-RP-DE system from Perseptive Biosystems (Framingham, MA, USA) equipped with a nitrogen laser (337 nm). Laser energies ranging from 60 % and 75 % of the maximum and an acceleration voltage of 25 kV were used. The voltage on the first grid was 90 % of the total acceleration voltage and the delay time between ion production and extraction was 300 ns. The guide wire voltage was set at 0.1 %. Spectra were externally calibrated using the singly and doubly charged ions from bovine serum albumin (BSA). Samples were prepared by mixing a 1 μL aliquot of the

filtered rHuEPO solution (1 mg/mL) or BSA (1 mg/mL) with 1 μL of matrix solution (10 mg/mL of sinapinic acid in MeCN:H_2O 1:1 with 0.1 % of TFA). 1 μL of a 27 mg/mL sinapinic acid solution in acetone:water 1:1 was first deposited onto the stainless steel sample plate and allowed to evaporate to dryness in air. Then, 1 μL of sample solution was deposited over this first layer and again air-dried [56]. DHB and HPA matrices were prepared and samples were deposited in a similar way. Ferulic acid matrix was prepared and samples deposited according to Sottani et al [34]. The spectra shown were acquired using the linear positive mode and represent summation of 100 consecutive laser shots.

3. Results and Discussion

3.1 Capillary Zone Electrophoresis using a putrescine dynamic coating

Figure 1-a shows the baseline resolution of eight bands of rHuEPO glycoforms following the methodology included in the 2002 European Pharmacopoeia [1,35,37]. Individual peaks do correspond to multiple glycoforms with similar overall charge-to-mass ratio [1,35,37,57-59]. The rHuEPO glycoforms migrate in order of increasing number of sialic acids, because at pH 5.5 the glycoforms are negatively charged and migrate against the EOF. Nevertheless, in addition to the lengthy analysis times, this methodology has several other drawbacks related to sample preparation, reproducibility and non-volatile additives in the separation electrolyte. The presence of low molecular mass excipients and salts in the sample have a detrimental effect on resolution of the EPO glycoforms (Figure 2-c) [22]. Optimum CE separations were obtained only after successive ultrafiltration of the sample using a centrifugal filter device with molecular mass (M) cut-off of 10000 [1,35,37,42]. We optimized the filtration method first reported [37] in order to reduce the duration of the sample preparation step without reducing desalted sample recovery or CE separations. Although good separations of rHuEPO glycoforms were obtained in this way (Figure 1-a) a lack of reproducibility was observed when the column was changed [35,37,42]. Moreover, resolution decreases irreversibly after several analyses (Figure 1-b), probably due to the non-specific adsorption of the protein resulting from the low effectiveness and/or stability of the putrescine coating [36]. Reproducibility and repeatability may be increased by adjusting the putrescine concentration [37,42] and by using the appropriate conditioning steps [34]. Nevertheless, working with running electrolytes containing putrescine and a high concentration of the nonionic chaotrope urea may hinder on-line ESI-MS detection, due to charge suppression and high chemical background [47-48].

3.2 Capillary Zone Electrophoresis using a polybrene noncovalent permanent coating

The development of a new, and robust analytical methodology for the glycoform analysis of intact rHuEPO avoiding non-volatile compounds in the running electrolyte was initially attempted using bare fused-silica capillaries. Acidic buffers composed of ammonium formate and acetate have been successfully used in many CE-ESI-

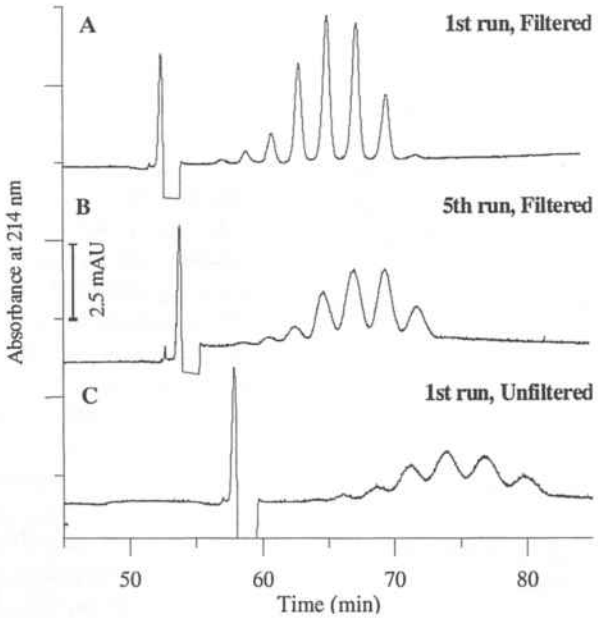

Figure 1 CE separation of rHuEPO glycoforms using a separation buffer containing putrescine and urea (Injection: 15 s, 33.5 mbar; Bare-fused silica capillary: 108.5 cm x 50 μm; Buffer: 0.01 M tricine, 0.01 M NaCl, 0.01 M NaAc, 7 M urea, 2.5 mM putrescine, pH 5.5 with 2 M HAc; Voltage: 15.4 kV; T : 35 °C, λ: 214 nm). a) First injection of a 1000 ppm rHuEPO BRP filtered b) Fifth consecutive injection of a 1000 ppm rHuEPO BRP filtered c) 1000 ppm rHuEPO BRP unfiltered.

MS experiments [23-24,44-48]. In this work, several buffers at different pH and different concentrations of FA were used. Using a 100 mM FA running electrolyte at pH 4, five bands corresponding to rHuEPO glycoforms can be observed (Figure 2). However, a long equilibration step was necessary, where repeated analysis of rHuEPO were essential to be performed before detecting any peaks. The same observation was reported by Tran et al. [43] using acetate buffers. In both cases, non-specific adsorption of the glycoprotein in each successive analysis may promote a decrease in solute-wall interaction and an EOF modification that results in improved separations [60]. However, this adsorption may be prevented in order to obtain reproducible separations. This problem has generally been overcome by using appropriate

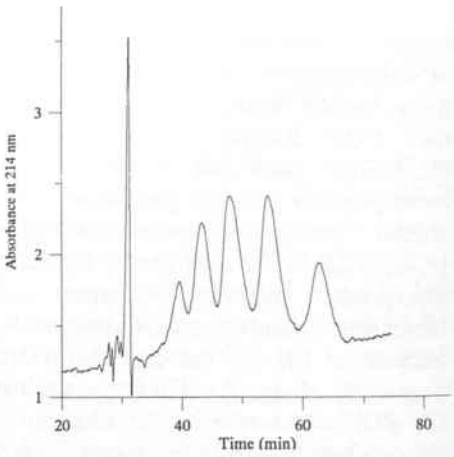

Figure 2 CE separation of rHuEPO BRP glycoforms using an uncoated capillary and a buffer containing 100 mM FA adjusted to pH 4 with ammonia (Sample: 1000 ppm rHuEPO BRP filtered; Injection: 5 s, 33.5 mbar; bare-fused silica capillary: 57 cm x 50 mm; Voltage: 15 kV; T : 25 °C, λ: 214 nm).

coated capillaries [1,21,33,35-40,42], as noted in the previous section. In this report, a comparative study was carried

out by testing several permanent coated capillaries, aiming to improve reproducibility of separations and to obtain better characterization of the glycoforms of rHuEPO. No peaks were detected using C-8, CEP and PVA covalently coated capillary columns, even when tested with different buffers, buffer concentrations and pH (data not shown). This may well indicate non-reversible adsorption of rHuEPO molecules to the inner capillary wall of the separation capillaries.

Polybrene (PB) is a polycationic polymer composed of quaternary amines that strongly adsorbs to the fused-silica inner surface and enables the EOF to be reversed towards the anode. The net positively-charged amine layer formed on the capillary wall reversed the EOF and the field polarity used for the separation must be also reversed in order to ensure that the positively-charged analytes migrated towards the detector [49-52]. Several authors have also pointed out that PB and other related compounds may act as pseudo-stationary phases promoting a reversible partitioning mechanism that would also affect selectivity of separations and the resolution of rHuEPO glycoforms [51,61]. The effects of various factors such as volatile buffer type and composition, buffer pH, voltage, capillary length and temperature on glycoform separation were studied. The influence of the type of running electrolyte used and its pH was initially investigated in the pH interval values near the pI of the EPO glycoforms (assumed to be between 3.5 and 5 [19,37]), using 100 mM FA-NH_4F, 100 mM HAc-NH_4Ac, 100 mM MES-NH_4MES and 100 mM Tricine-TricineAc. However, no significant separation of rHuEPO glycoforms was obtained under these experimental conditions. In order to improve the efficiency of the separations, the concentration of the running electrolytes was increased to 300 mM. Notable results were only obtained with separation electrolytes containing 300 mM FA-NH_4F or 300 mM HAc-NH_4Ac at pH values ranging from 4 to 5.5 (Figure 3). The electropherograms of Figure 3 show poor resolution with a single broad peak of rHuEPO glycoforms when a 300 mM FA -NH_4F buffer is used at any pH. Improved resolution was obtained by using a running electrolyte containing 300 mM of HAc-NH_4Ac at pH 4.75 and pH 5.5 . Furthermore, the separation at pH 4.75 (Figure 3-c) was slightly better than at 5.5. The glycoform electrophoretic mobility differences at pH values surrounding their pI values were maximized, induced presumably by a combination of charge differences between the glycoforms and their selective interaction with the positively charged polyamine layer of PB. Resolution was further improved by using 400 mM of HAc-NH_4Ac at pH 4.75 (Figure 3-e), without markedly reducing the EOF. Higher ionic strengths were not tested in order to avoid excessive current intensities that may have lead to peak broadening and lack of reproducibility due to excessive Joule heating. Selection of a 10 kV separation buffer, resulted in lower currents and better resolutions, with a reasonable increase in analysis times (Figure 4-a). In contrast, lowering the temperature resulted in longer analysis times, but had no significant effect upon resolution. Figure 4-a shows the optimum separation of 5 major partially-resolved peaks of rHuEPO glycoforms

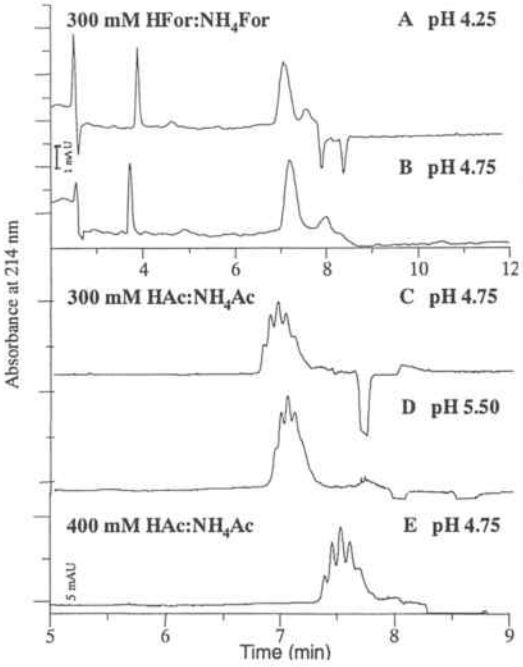

Figure 3 CE separation of rHuEPO glycoforms (J&J (Research batch)) using a capillary permanently coated with PB and a separation buffer containing 300 mM of FA-NH_4For at pH a) 4.25, b) 4.75; 300 mM of HAc-NH_4Ac at c)pH 4.75, d)5.50 and 400 mM of HAc-NH_4Ac at e)pH 4.75 (Sample: 1000 ppm rHuEPO, unfiltered; Injection: 5 s, 33.5 mbar; bare-fused silica capillary: 57 cm x 50 μm; Voltage: 15 kV (reversed polarity); T : 25 °C, λ: 214 nm).

in 15 minutes. In polybrene coated capillaries at pH 4.75, rHuEPO glycoforms carry an overall negative charge, and they pass through the detection window before the neutral substances of the sample matrix, which migrate with the EOF as a peak with negative absorbance (Figure 3). The glycoform migration order is reversed with respect to the order observed in the electropherograms obtained using uncoated capillaries (figure 2) and capillaries dynamically coated with putrescine (Figure 1) and because of this the glycoforms are numbered in reversed order. However, the partial separations obtained suggest that direct comparisons between the glycoform profiles cannot be made. Using this methodology, separation was independent of sample matrix, in contrast to the results obtained with the putrescine method, where the

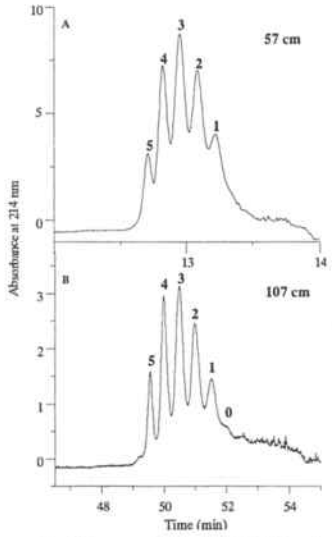

Figure 4 CE separation of rHuEPO glycoforms (J&J (Research batch)) at 10 kV (reversed polarity) and 25 °C using a capillary permanently coated with PB and a separation buffer containing 400 mM of HAc-NH_4Ac at pH 4.75 . Total capillary length was a) 57 cm and b) 107 cm. (Sample: 1000 ppm rHuEPO, unfiltered; Injection: 5 s, 33.5 mbar; 50 μm bare-fused silica capillary; λ: 214 nm).

sample might be ultrafiltered before the analysis. Similar separations were obtained under these same experimental conditions in capillaries coated with the cationic polymer PDMAC, which has similar characteristics to polybrene (data not shown) [53-54]. However, resolution of the analytes was deteriorated with successive analysis due to the lower stability of the coating.

At this point, further possibilities were explored in search of improving resolution of rHuEPO glycoforms. One strategy, was to evaluate the utility of several additives, or modifiers, present in the separation buffer. The presence of small quantities of an organic solvent, a surfactant, a liquid chromatography (LC) ion-pair agent, or another chaotrope or complexating agent in the separation may cause a differential disruption of glycoform molecular structures, resulting in enhanced separations due to maximized differences between their charge-to-mass ratios. Unfortunately, the poor volatility properties of some of these products precluded their use in this case at high concentration [21,33,62]. Acetonitrile, isopropanol, and methanol contents in the separation buffer between 5-20 % (v/v) resulted in a clear deterioration of the resolution. The same observation was noticed when Brij 35, Tween 20, SDS or Nonidet 40 were added in proportions ranging from 0.2 to 2 % (w/v). The addition of 7 M urea to either the running electrolyte or the sample did not improve the resolution of rHuEPO glycoforms. Conversely, the presence of a 0.1% of HFBA in the separation buffer led to a slight improvement in the separation of rHuEPO glycoforms and also to lower reproducibilities due to increased Joule heating. At higher concentrations of the ion-pairing reagent a single broad peak was obtained. The inclusion of the studied additives in the separation buffer was discarded and no other attempts were made in order to improve separation resolution.

The optimum separation conditions resulting in the electropherogram of Figure 4-a were evaluated for intraday and interday precision. Repeatability was determined for twenty replicate analyses of the same rHuEPO sample in a single day. The lack of baseline resolution made the use of areas from individual peaks unadvisable, and an overall integrated peak area was used. An average migration time for the five most important glycoforms was also taken into account, and it was used to correct the overall calculated area. The intra-day Relative Standard Deviation (RSD) of migration time and of the corrected overall area were 2.6 % and 3.2 %, respectively. Reproducibility was also evaluated through 5 replicate analyses of a rHuEPO sample on three different non-consecutive days. The inter-day relative standard deviation (RSD) of migration time and of the corrected overall area were 2.7 % and 3.3 %, respectively.

These results confirm the robustness of the methodology and showed it to be a good alternative for analyzing intact rHuEPO, even though the resolution was not as good as that obtained with putrescine coated capillaries. Although at pH 4.75 the rHuEPO glycoforms appeared to be negatively-charged, they were not irreversibly adsorbed upon the positively charged capillary inner wall. Several authors have shown that polybrene coated capillaries are more tolerant to inorganic anions or proteins carrying charges opposite to those on its

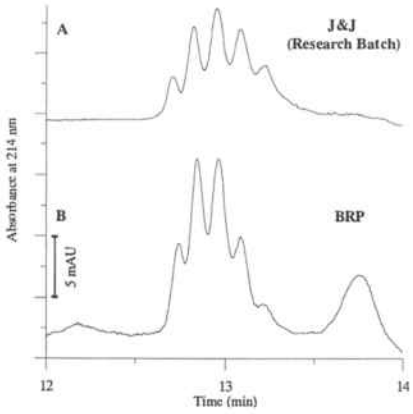

Figure 5 Comparison of two commercial 1000 ppm solutions of rHuEPO under the optimum CE separation conditions. a) rHuEPO (J&J (Research batch)) and b) rHuEPO BRP (European Pharmacopeia).

surface than are uncoated capillaries [51,63-64]. This tolerance is probably maximized at pH values of the separation electrolyte surrounding the pI of the protein, probably due that the partitioning interactions are interfering in the non-reversible adsorption processes.

The electrophoretic methodology established here is compatible with electrospray ionization [23,44-45]. Several authors have already demonstrated that good CE-ESI-MS sensitivity is achieved with HAc and FA buffers, even when a 2 or 3 M FA buffer is used [44-45]. On the other hand, the presence of PB on the capillary surface did not lead to any unusual extraneous background signal or leaching of the coating reagents during normal CE-ESI-MS operation [23,44-45]. Boss et al. [23] have shown that only partial interference due to bromine in the PB structure was found with glycopeptides obtained from digestion of rHuEPO glycoforms [23]. Furthermore, the stability shown by the PB coating prevents inclusion of the non-volatile polymer for at least 20 runs [49-51,55,65]. If a longer capillary column lifetime is desired, a SMIL coating of PB and dextran sulfate can be developed as indicated in the experimental section [55]. An SMIL coating of PB generated in this way is found to be stable for more than 600 runs [55] and separations are practically identical to the ones obtained with a sigly-layered PB coating.

Current CE-ESI-MS instrumental set-ups commonly operate with capillary columns of approximately 100 cm in length [23,44-45]. In Figure 4-b, the separation of rHuEPO is shown, employing a 50 μm x 107 cm capillary column. Resolution was slightly improved, although the long analysis time makes the use of a 57 cm capillary column preferable for faster fingerprinting of rHuEPO. A comparative analysis of two rHuEPO from different manufacturers using these conditions showed identical migration patterns with slight variations in the relative abundances of individual peaks (Figure 5). This indicates that this method can be used to differentiate individual concentrated preparations in quality assessment procedures.

3.3 ESI-MS analysis of rHuEPO

The total ion current (TIC) for the intact rHuEPO in the full mass scan acquisition (m/z 200-2000) is presented in Figure 6-a. The glycoform population of rHuEPO appears to be complex, as shown by the broadening of the single peak obtained. The ESI-MS mass spectrum corresponding to this single peak is shown in Figure 6-b. The extreme complexity introduced by the oligosaccharide microheterogeneity of

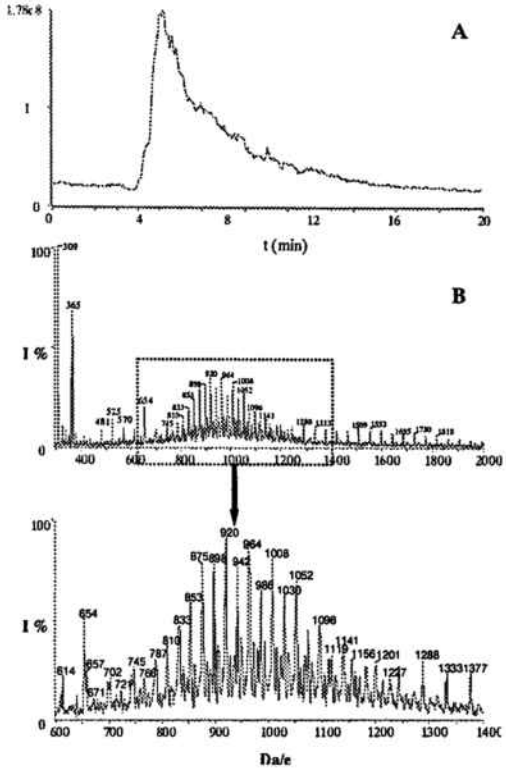

Figure 6 Direct ESI-MS analysis of rHuEPO BRP a) TIC b) ESI-MS spectrum of the glycoform mixture.

the sample and the multiple charging electrospray phenomenon give rise to a series of multiply protonated ions extending from m/z 300 to 2000 [26,44-46]. An intense peak at m/z 365 indicated the presence of [Glc-GlcNAc]$^+$ ([Glucosyl-N-acetylGlucosamine]$^+$) ion generated residues, due to some degree of in-source fragmentation, even at low cone voltage values. Yeung et al. [46] proposed that glycoforms may be specifically detected by determining the ratios between the carbohydrate ions generated during the ionization process. In addition to the problems due to spectral complexity, the high sialic acid content in rHuEPO molecules compromise the generation of charged positive ions, and hence detection sensitivity [23,26]. Spectral deconvolution to obtain reliable information about an average molecular mass of the glycoforms was not feasible with the MaxEnt algorithm of MassLynx. Thus, separation of the rHuEPO glycoforms prior to ESI-MS detection is desirable in order to obtain improved characterization.

3.3 MALDI-TOF-MS analysis of rHuEPO.

The glycosylation process of rHuEPO interferes with production of multiply charged ions in electrospray ionisation, but does not affect the desorption/ionisation in MALDI analysis. Sotanni et al [34] reported that certain degree of fine structure on the signals corresponding to the glycoforms could be obtained using ferulic acid as

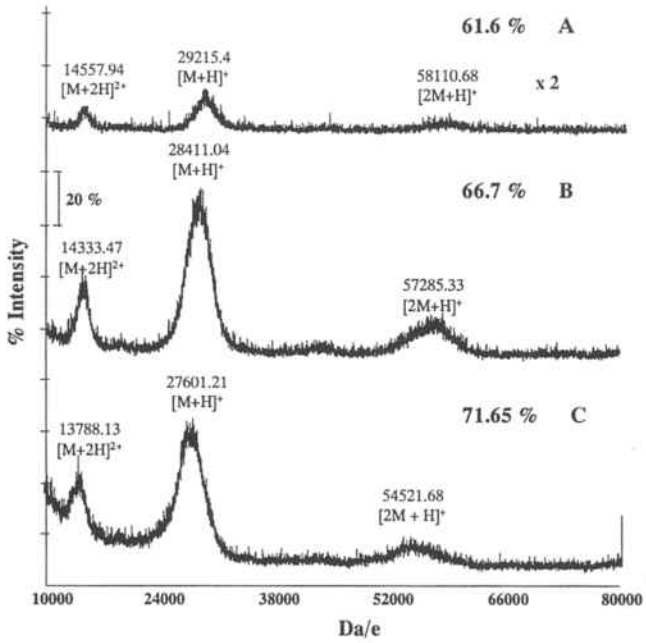

Figure 7 MALDI-TOF mass spectra of the rHuEPO BRP at different laser intensities: a) 2463 (61.6 % of the total) b) 2667 (66.7 %) and c) 2866 (71.65 %).

matrix compound. In this work, unresolved and broader peaks were observed, perhaps because the rHuEPO BRP is expected to be more heterogeneous (a mixture of α and β rHuEPO forms [31]) than the one used in the study made by Sottani et al [34] which was purified from Epogen (Amgen Inc.) (α rHuEPO). Among the matrix compounds and sample deposition methods tested in this work, sinapinic acid using the fast evaporation method described by Vorm et al [56] was found to be the most appropriate in terms of sample stability, sensitivity and peak broadening.

Figure 7 shows the MALDI-TOF mass spectra of rHuEPO obtained in the delayed extraction (DE) mode under different laser conditions. The spectra of Figure 7 show the simply and doubly-charged molecular ions of the rHuEPO glycoforms. The presence of the singly-charged dimeric forms, which are often generated in the desorption process, was also detected. As was pointed out above, the significant width of the peaks is directly related to the intrinsic microheterogeneity of the rHuEPO glycoforms.

MALDI mass spectrometry relies on the formation of intact molecular ions in order to achieve a highly-accurate molecular mass determination. However, in some cases MALDI mass spectrometry of peptides and proteins containing labile functional groups, such as sialic acids, tend to undergo fragmentation in the ion source and/or during acceleration [66-70]. Figure 7 shows the mass spectra of rHuEPO obtained under different laser intensities in the linear delayed extraction mode. As can be seen increased laser intensities

resulted in enhanced in-source sialic acid losses and/or further loss of Glc an/or GlcNAc units [67-70], which led to a peak shifting to lower masses. Thus, it may be interesting to compare the differences in spectra with changes in laser intensity, of the highly glycosylated rHuEPO and an standard and unglycosylated BSA. Figure 8 shows the peak mass/charge ratio of the singly-charged ion of the rHuEPO ($[rHuEPO+H]^+$) and the doubly-charged ion of the BSA ($[BSA+2H]^{2+}$) against the laser intensity. It can be observed that the $[rHuEPO+H]^+$ mass/charge position ranges over 2000 Da/z, while the $[BSA+H]^+$ peak only shifts a maximum of 400 Da/z in the studied interval, maybe due to loss of neutrals or a reduction in production of photochemically induced matrix adducts [66]. This experimental observation confirms the suspected extra lability of the rHuEPO molecule. Thus, low laser intensities providing good detection sensitivities are desirable in order to decrease in-source fragmentation. The mass spectrum shown in Figure 7-b has been obtained at a 66.7 % of the total laser intensity (4000 V). Using this laser conditions approximately 1 picomol of the rHuEPO

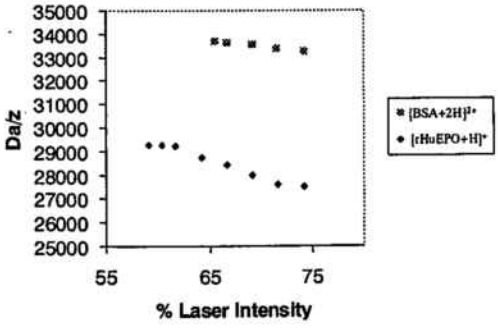

Figure 8 Influence of laser intensity on MALDI-TOF mass spectra. Variation of m/z corresponding to singly-charged rHuEPO ([rHuEPO+H]+) and doubly-charged BSA ([BSA+2H]2+) with laser intensity.

can be detected and an average M of 28443 ± 183 Da (n = 3) can be calculated. This value as the ones determined before by MALDI-TOF-MS [24,27,31,34], is slightly lower than the 30400 Da estimated by sedimentation equilibrium experiments [16] and is considerably lower than the 34000 Da estimated by slab gel electrophoresis [16,20]. In our view, although several authors have related these differences to the uncertainty and/or inaccuracy introduced in these alternative methods of determining the molecular mass [31], fragmentation of the rHuEPO glycoforms during the MALDI process should not be ruled out. Laser intensity values among different instruments must be carefully taken into account in order to achieve high robustness in the mass fingerprinting of EPO or other extremely labile proteins by MALDI-TOF-MS.

4. Concluding remarks

The versatility of CE have been investigated and a new separation methodology has been proposed for rHuEPO glycoforms. In addition, ESI-MS and MALDI-TOF-MS have been shown to be complementary ways of obtaining mass spectral information about complex intact glycoforms. A separation methodology using volatile electrolytes for the future separation and characterization of intact rHuEPO glycoforms by CE-ESI-MS has been proposed. The method shows improved reproducibility and shorter sample preparation and analysis times, although glycoforms are only partially resolved. Rapid fingerprinting and differentiation of rHuEPO products in concentrated solutions has been demonstrated. However, EPO concentration limits of detection for all this CE methodologies

must be lowered in order to determine EPO in biologicals fluids. The detection and mass spectral characterization of its separation profiles in biological samples, may prove useful in diagnosing pathological conditions, developing pharmacokinetic and pharmacodynamic studies, and establishing a more reliable direct method [14,15] for detecting its misuse in sports.

Acknowledgements

The authors thank Dr. Irene Fernández (Servei d'Espectrometria de Masses, Divisió III, Universitat de Barcelona, Barcelona, Spain) for her helpful and constructive assistance with the MALDI-TOF-MS experiments.

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Capítulo 6. Resultados y discusión

6. RESULTADOS Y DISCUSIÓN

6.1. OPTIMIZACIÓN DE LAS SEPARACIONES POR LC Y CE

En la mayoría de ocasiones, la separación de mezclas complejas empleando LC o CE requiere una cuidadosa optimización de las condiciones experimentales de separación, con el objeto de resolver el mayor número posible de componentes. Este proceso puede ser simplificado, si se describe el comportamiento cromatográfico o electroforético mediante modelos que tengan en cuenta la variación de la retención o la migración en función de las principales variables experimentales involucradas en los procesos de separación. Así, a partir de estos modelos y considerando sólo un reducido número de medidas experimentales, se pueden llegar a predecir las condiciones de separación a las que se consiguen las selectividades y resoluciones óptimas.

6.1.1. Optimización del pH del medio de separación

Para sustancias ionizables, como los péptidos y las hormonas peptídicas estudiadas en esta tesis doctoral, el pH de la fase móvil o del electrolito de separación juega un papel fundamental en la selectividad de las separaciones, ya que determina el grado de ionización de los analitos. En nuestro trabajo, se han deducido las siguientes ecuaciones generales que describen el comportamiento cromatográfico o electroforético de cualquier sustancia poliprótica H_nX^z -con n grupos ionizables y z grupos básicos- en función del pH de la fase móvil o del electrolito de separación, sus constantes de ionización (K_i) y los coeficientes de actividad (γ) [Artículos 2.3-2.4]:

$$k = \frac{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} k_{H_{n-i}X^{z-i}} + k_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}} k_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} + 1 + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}}} \quad (6.1)$$

$$m_e = \frac{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} + 1 + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}}} \quad (6.2)$$

donde k es el factor de retención en LC, m_e la movilidad electroforética en CE y K_i' es la constante de ionización aparente que está relacionada con la constante de ionización (K_i) y los coeficientes de actividad (y) mediante la expresión $K_i' = y^{z-(i-1)}/y^{z-i}K_i$. Estas ecuaciones generales permiten predecir el comportamiento cromatográfico o electroforético a partir de un número limitado de medidas experimentales y, al mismo tiempo, determinar las constantes de ionización de las sustancias. El número mínimo de medidas experimentales a realizar, depende del número de variables independientes de la ecuación, y éste viene determinado por el número de equilibrios de ionización que se consideren en el intervalo de pH estudiado y de las simplificaciones que se puedan realizar. De este modo, una vez deducidas las expresiones particulares a partir de la ecuación general para estudiar el comportamiento cromatográfico o electroforético de cada analito en un cierto

intervalo de pH, se pueden ajustar las medidas experimentales mediante una regresión no lineal, tal y como se ha demostrado en el caso de la bradicinina, la triptorelina, la buserelina, la Met-encefalina, la Leu-encefalina, la oxitocina y la eledoisina, tanto en LC [Art. 2.4] como en CE [Art. 2.3]. En el caso de la serie de péptidos más sencillos (H_2X^+), que sólo presentan dos grupos ionizables con valores de pK_a suficientemente diferenciados, la expresión particular puede ser simplificada y obtener sendas expresiones lineales [Art. 2.1-2.2]: una para la zona de pH próxima al pK_1 y otra para la zona de pH próxima al pK_2, tal y como se muestra en la Tabla 6.1. En este caso concreto, sólo son necesarios dos valores experimentales para modelar el comportamiento cromatográfico o electroforético en las zonas de pH ácido y básico, a diferencia de lo que ocurre con las hormonas peptídicas, para las que es necesario un mayor número de medidas experimentales.

Las ecuaciones establecidas en cada caso, pueden emplearse para seleccionar las condiciones de pH a las que se obtienen separaciones óptimas, bien directamente analizando las curvas de regresión ajustadas, bien calculando la resolución teórica a partir de los valores de retención o migración determinados con cada ecuación. En este último caso, se demuestra que existe una excelente correlación entre los valores de resolución teóricos y los experimentales, confirmando la validez del modelo establecido para predecir el comportamiento electroforético y cromatográfico. De esta forma, se han establecido las condiciones de pH óptimas para la separación cromatográfica y electroforética de la serie de péptidos sencillos [Art. 2.1-2.2] y la serie de hormonas peptídicas [Art. 2.3-2.4]. En la Figura 6.1 se muestran las separaciones cromatográficas y electroforéticas de ambas series a los pH de separación óptimos. Se puede observar la gran diferencia en la selectividad de las separaciones obtenidas por CE y LC, lo que pone de manifiesto las diferencias en los mecanismos de separación en que se basan ambas técnicas. Así, algunas sustancias que se separan por LC, no lo hacen por CE, y a la inversa. Esto puede resultar de utilidad, en algunos casos, para resolver un mayor

número de componentes en una mezcla compleja, mediante la combinación secuencial de ambas técnicas, tal y como se ha puesto de manifiesto en la separación de la mezcla compleja procedente de la síntesis del leuprolide [Art. 3.5].

Tabla 6.1 Linealización de la ecuación 6.1 para péptidos del tipo H_2X^+ [Art. 2.1-2.2].

Ecuación 6.1 para H_2X^+	Expresiones lineales para péptidos sencillos [3-4]
$m_e = \dfrac{\frac{a_{H^+}}{K_1'} m_{H_2X^+} + \frac{K_2'}{a_{H^+}} m_{X^-}}{\frac{a_{H^+}}{K_1'} + 1 + \frac{K_2'}{a_{H^+}}}$	**pH<<pK₂** $m_e = \dfrac{\frac{a_{H^+}}{K_1'} m_{H_2X^+}}{\frac{a_{H^+}}{K_1'} + 1} \Rightarrow \dfrac{1}{m_e} = \dfrac{1}{m_{H_2X^+}} + \dfrac{1}{m_{H_2X^+}} \dfrac{K_1'}{a_{H^+}}$ **pH>>pK₁** $m_e = \dfrac{\frac{K_2'}{a_{H^+}} m_{X^-}}{1 + \frac{K_2'}{a_{H^+}}} \Rightarrow \dfrac{1}{m_e} = \dfrac{1}{m_{X^-}} + \dfrac{1}{m_{X^-}} \dfrac{a_{H^+}}{K_2'}$

La movilidad electroforética de la especie zwitteriónica (m_{HX}) puede considerarse prácticamente nula [1-4]

$k = \dfrac{\frac{a_{H^+}}{K_1'} k_{H_2X^+} + k_{HX} + \frac{K_2'}{a_{H^+}} k_{X^-}}{\frac{a_{H^+}}{K_1'} + 1 + \frac{K_2'}{a_{H^+}}}$	**pH<<pK₂** $k = \dfrac{\frac{a_{H^+}}{K_1'} k_{H_2X^+} + k_{HX}}{\frac{a_{H^+}}{K_1'} + 1} \Rightarrow k\left(\frac{a_{H^+}}{K_1'} + 1\right) = k_{HX} + \frac{a_{H^+}}{K_1'} k_{H_2X^+}$ **pH>>pK₁** Está fuera del intervalo de pH estudiado

Por otro lado, el uso de electrolitos de separación hidroorgánicos en CE, además de permitirnos analizar sustancias poco solubles en agua o emplear electrolitos de separación de mayor fuerza iónica, también puede aprovecharse para alterar la selectividad de las separaciones, tal y como se observa en la Figura 6.1 [Art. 2.1-2.2].

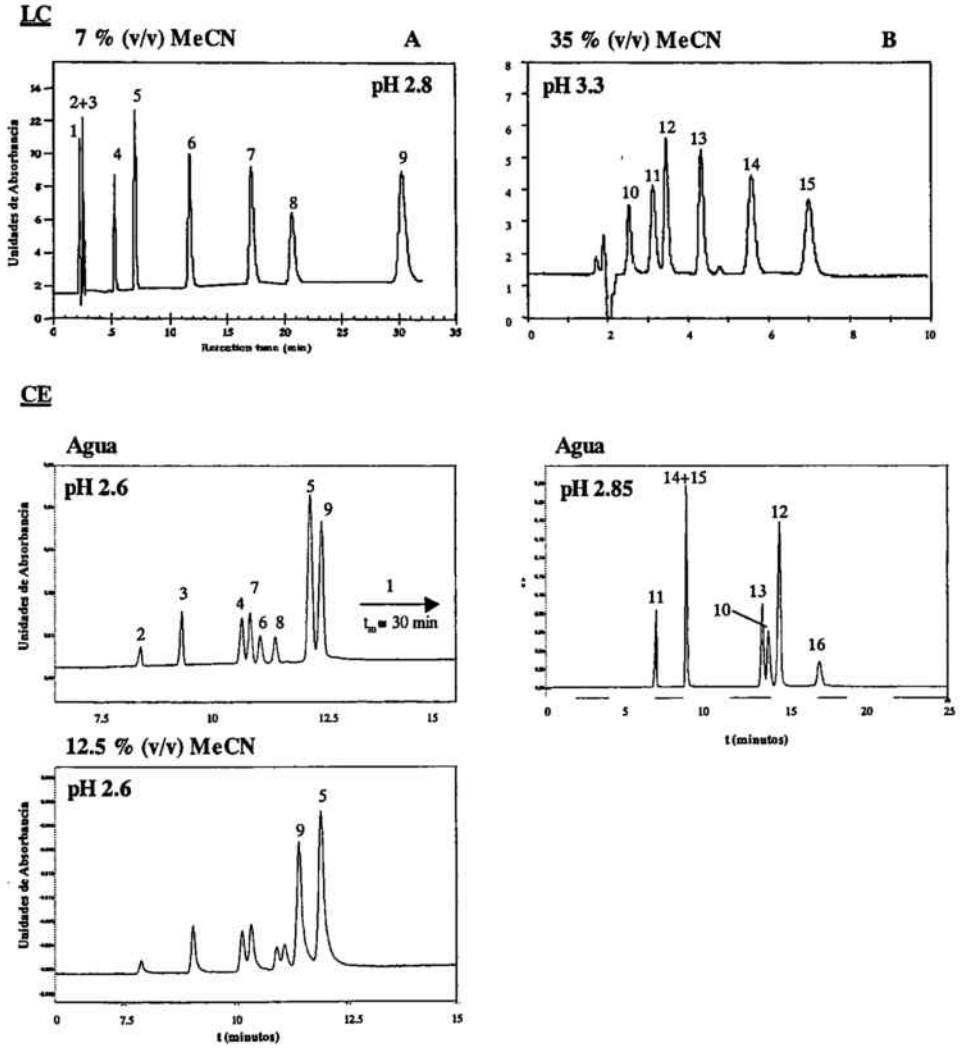

Figura 6.1 Cromatogramas y electroferogramas al valor de pH en el que se obtienen las separaciones óptimas. A) péptidos sencillos [3-4] y B) hormonas peptídicas [1-2]. 1) Citrulina, 2) Gly-Gly, 3) Gly-Gly-Gly, 4) Gly-Gly-Val, 5) Tyr-Gly-Gly, 6) Ala-Leu-Gly (I), 7) Gly-Gly-Ile, 8) Ala-Leu-Gly (II), 9) Gly-Gly-Phe, 10) oxitocina, 11) bradicinina, 12) Met-encefalina, 13) Leu-encefalina, 14) triptorelina y 15) buserelina [1-4].

6.1.2. Determinación de las constantes de ionización por LC y CE.

La obtención de las constantes de ionización de las sustancias de interés farmacológico suele ser de gran importancia para explicar sus propiedades físico-químicas y biológicas (estabilidad, solubilidad, permeabilidad a las membranas celulares, toxicidad, actividad biológica,...). A partir de las expresiones particulares para cada analito obtenidas a partir de las ecuaciones generales 6.1 y 6.2, pueden determinarse las constantes de ionización de los péptidos y hormonas peptídicas analizadas [Art. 2.1-2.4]. La LC y la CE presentan numerosas ventajas para la determinación de constantes de ionización frente a las determinaciones tradicionales empleando técnicas potenciométricas o espectrofotométricas. Entre ellas destacan las menores cantidades de muestra que son necesarias, la posibilidad de separar y determinar simultáneamente las constantes de ionización de varias sustancias en una mezcla y, como podemos observar en la Figura 6.1, la obtención de las constantes de ionización de diastereoisómeros (Ala-Leu-Gly (I) y Ala-Leu-Gly (II), picos 6 y 8 en la Figura 6.1) [Art. 2.1-2.2]. Los valores de pK_a determinados por LC o CE, correspondientes a los péptidos y las hormonas peptídicas estudiados en esta tesis doctoral, aparecen en la Tabla 6.2, indicando la técnica empleada y la composición hidroorgánica del medio en el que se han obtenido. A los datos publicados en los artículos que se recopilan en esta memoria [Art. 2.1-2.4], se añaden en la Tabla 6.2 los valores de la primera constante de ionización del leuprolide, que se determinaron siguiendo el mismo procedimiento a partir de los valores experimentales obtenidos al estudiar su retención cromatográfica en función del pH, empleando fases móviles que contenían un 27% y un 31% (v/v) de MeCN (Figura 6.2). También se han incorporado los datos bibliográficos que aparecen sombreados en la Tabla 6.2, y los datos potenciométricos obtenidos previamente por nuestro grupo de investigación [1-5].

Tabla 6.2 pK_a determinados en esta tesis doctoral [Art. 2.1-2.4].

Péptido	pK_a	0% MeCN		12.5% MeCN		7% MeCN		27% MeCN	31% MeCN	35% MeCN
		CE	Bib	CE	Pot[1]	LC	Pot[1]	LC	LC	LC
Gly-Gly	1	3.08	3.14	3.33	3.33	3.35	3.18			
	2		8.04	8.32	8.30					
Gly-Gly-Ile	1	3.26		3.53	3.55	3.59	3.54			
	2		8.09	8.11	8.09					
Gly-Gly-Gly	1	3.22	3.30	3.41	3.46	3.57	3.50			
	2		7.96	8.08	8.09					
Tyr-Gly-Gly	1	3.18		3.36	3.46	3.54	3.45			
	2			7.41	7.45					
Gly-Gly-Val	1	3.23		3.50	3.54	3.59	3.47			
	2		8.12	8.15	8.08					
Ala-Leu-Gly (I)	1	3.34		3.56	3.62	3.71	3.57			
	2			8.13	8.12					
Ala-Leu-Gly (II)	1	3.27		3.46	3.62	3.61	3.57			
	2			8.30	8.12					
Gly-Gly-Phe	1	3.06		3.33	3.33	3.30	3.21			
	2		8.04	8.01	8.02					
citrulina	1	2.30	2.40	2.60	2.39	2.37	2.57			
	2		9.69	9.44	9.58					
eledoisina	1	3.48								
	2	10.54								
oxitocina	1	6.04								
	2	9.84								
bradicinina	1	2.68								3.21
	2	6.66								
triptorelina	1	5.93								4.71
	2	9.63								
buserelina	1	5.92								5.11
	2	9.76								
Met-encefalina	1	3.17	3.45							3.96
	2	7.30	7.36							
	3	10.30	10.36							
Leu-encefalina	1	3.31	3.69							4.38
	2	7.29	7.40							
	3	10.34	10.34							
Leuprolide	1							4.74	4.79	
	2									

[1] Datos potenciométricos obtenidos previamente por nuestro grupo de investigación [1-2]

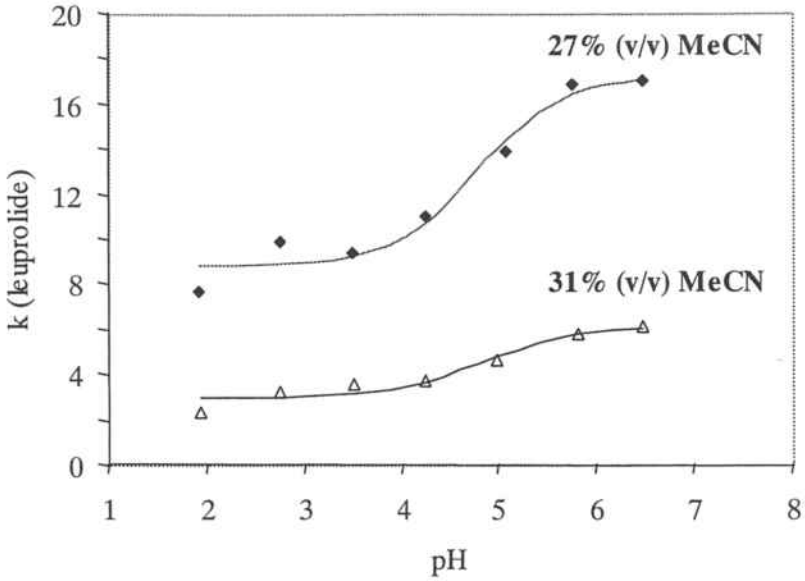

Figura 6.2 Representación de los valores experimentales (puntos) y teóricos (líneas) del factor de retención (k) del leuprolide en el crudo de síntesis, en función del pH de fases móviles que contienen un 27% y un 31% (v/v) de MeCN.

La mayoría de los datos bibliográficos corresponden a los péptidos más sencillos [1-5], lo que se debe a que, en general, las hormonas peptídicas son caras y difíciles de conseguir y para realizar valoraciones potenciométricas convencionales se necesita una cantidad considerable de muestra. Por esta razón sólo se han podido comparar, con los datos bibliográficos obtenidos mediante microvaloraciones potenciométricas, los valores de pK_a en medio acuoso obtenidos mediante CE para dos de las hormonas peptídicas: la Met-encefalina y la Leu-encefalina, observándose una concordancia razonable entre ellos [Art. 2.3-2.4]. En general, los valores del primer pK_a por LC al 7 % (v/v) de MeCN para los péptidos más sencillos son ligeramente superiores a los obtenidos potenciométricamente [Art. 2.2]. Esto podría explicarse teniendo en cuenta las interacciones no hidrofóbicas que se producen entre los péptidos estudiados y los silanoles residuales de la fase estacionaria cromatográfica. Estas interacciones aumentarían el factor de retención de los

péptidos, lo que provocaría un aumento de los valores de pK_a. En cambio, los resultados obtenidos para este primer pK_a por CE al 12.5 % (v/v) de MeCN para los péptidos más sencillos son muy similares a los valores potenciométricos [Art. 2.1-2.2]. Sólo se observan pequeñas diferencias, que pueden ser explicadas por la presencia de silanoles residuales en la superficie interna del capilar. Estas interacciones disminuirían la movilidad electroforética de los péptidos, lo que provocaría una ligera disminución de los valores de pK_a. La citrulina se comporta de forma excepcional quizá debido a que es un ácido mucho más fuerte, lo que implica una mayor dificultad de determinación experimental, ya que se debe trabajar a valores de pH mucho menores. Los valores del segundo pK_a por CE al 12.5% (v/v) para los péptidos más sencillos son muy similares a los valores potenciométricos. El grupo amino de los péptidos en medio básico está desprotonado, de forma que disminuyen las posibles interacciones con los grupos silanoles de la pared del capilar. Las diferencias tanto positivas como negativas son probablemente reflejo del error aleatorio experimental. En general, se puede considerar que existe una concordancia aceptable entre los pK_a obtenidos por LC, CE y los obtenidos mediante técnicas potenciométricas [Art. 2.2, 2.4], lo que pone de manifiesto la validez de la LC y la CE para la determinación de constantes de ionización. Además, la CE tiene la ventaja adicional respecto a la LC de permitir realizar las determinaciones de pK_a en medios acuosos. Para determinar correctamente los valores de pK_a en los medios hidroorgánicos MeCN:H_2O estudiados en esta tesis doctoral, se ha medido correctamente el pH en las mezclas hidroorgánicas utilizadas, empleando una escala de referencia externa previamente establecida en nuestro grupo de investigación [6]. En general, como puede observarse en la Tabla 6.2, si el primer pK_a corresponde a un grupo carboxilo protonado, los valores de pK_a en medio hidroorgánico aumentan cuanto más disolvente orgánico contiene el electrolito de separación, aunque estas variaciones de pK_a son menores que las

esperadas debido a la solvatación preferencial de los analitos en las mezclas MeCN-H_2O [7].

6.1.3. Optimización de la proporción del disolvente orgánico de la fase móvil en LC

En trabajos previos de nuestro grupo de investigación se utilizó el método LSER para optimizar la proporción de disolvente orgánico en las fases móviles empleadas en LC, para separar mezclas de péptidos y hormonas peptídicas [8-9]. En los artículos recopilados en esta memoria, se ha demostrado la validez del método LSER para explicar la retención cromatográfica de las sustancias presentes en las mezclas complejas obtenidas tras la síntesis de hormonas peptídicas [Art. 3.1-3.4]. Empleando una columna del tipo C8 o C18 y fases móviles de MeCN:H_2O con un 0.1% (v/v) de TFA, las excelentes relaciones lineales obtenidas entre el logaritmo del factor de retención de las sustancias presentes en las mezclas y el parámetro de polaridad E_T^N de las mezclas hidroorgánicas utilizadas como fases móviles, permiten una rápida y sencilla selección de la proporción de disolvente orgánico en la fase móvil para conseguir separaciones adecuadas, bien analizando conjuntamente las rectas de regresión ajustadas, bien calculando la selectividad o la resolución teórica a partir de los valores de retención teóricos obtenidos con cada recta (Figura 6.3). De esta forma, para los crudos de síntesis estudiados [Art. 3.1-3.4], se han seleccionado las fases móviles óptimas para poder aplicar posteriormente las separaciones a escala preparativa, o bien para separar y caracterizar las sustancias presentes en la mezcla por LC-ESI-MS, tal y como se muestra en la Figura 6.3 para el crudo de síntesis de la goserelina [Art. 3.2]. En el primer caso, cuando se desea purificar la hormona peptídica sintetizada, es necesaria la máxima resolución entre ésta y las impurezas inmediatamente adyacentes. En el segundo caso, se han de seleccionar las condiciones de separación que permiten la resolución en tiempos razonables de un

mayor número de componentes en las mezclas. En todos los crudos de síntesis analizados, las fases móviles seleccionadas con ambas finalidades contienen proporciones de MeCN comprendidas entre un 25 y un 33 % (v/v) y un 0.1% (v/v) de TFA.

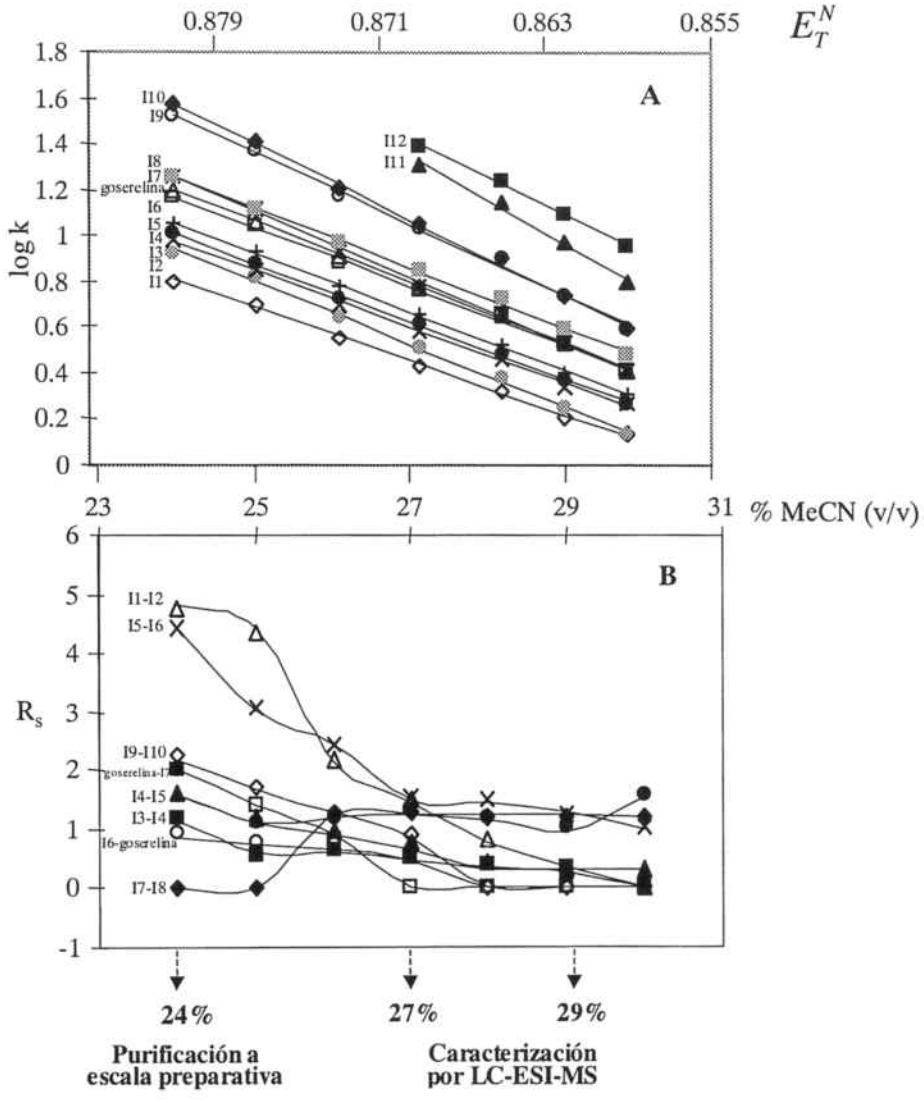

Figura 6.3 Crudo de síntesis de la goserelina, a) Log k de la goserelina y sus impurezas frente al parámetro E_T^N de la fase móvil y b) resolución entre solutos adyacentes frente al porcentaje de acetonitrilo en la fase móvil. Las impurezas que acompañan a la goserelina se han nombrado desde I1 a I12 [16,18].

6.2. SEPARACIÓN Y CARACTERIZACIÓN DE CRUDOS DE SÍNTESIS DE HORMONAS PEPTÍDICAS EMPLEANDO LC-ESI-MS Y CE

En general, en LC-ESI-MS se prefieren las fases móviles con mayor contenido en acetonitrilo porque proporcionan una mayor sensibilidad, ya que como ya apuntamos en algún momento, son más fáciles de nebulizar y facilitan la ionización de los analitos. Es importante señalar que las condiciones de separación óptimas, a veces no coinciden con las condiciones de detección óptimas [Art. 3.3-3.4]. Esto se ha de tener especialmente en cuenta en el caso de los crudos de síntesis de hormonas peptídicas en las que algunos de los subproductos e impurezas se encuentran a muy baja concentración con lo que para su detección se requieren unas condiciones determinadas aunque éstas impliquen una menor resolución entre los diferentes componentes de la mezcla. Para los crudos de síntesis analizados en este trabajo [Art. 3.1-3.4], se han obtenido buenos resultados por LC-ESI-MS con fases móviles que contienen desde un 25% a un 33% (v/v) de MeCN y un 0.1% (v/v) de TFA. Paralelamente, la adición post-columna de una pequeña cantidad de dioxano produjo mejoras significativas en los Cromatogramas Totales de Iones (TIC) de los crudos de leuprolide y goserelina. En ambos casos, se emplearon además dos fases móviles con proporciones de MeCN diferentes, con el objeto de caracterizar, en un tiempo razonable, todos los componentes de la mezcla, incluso los que presentan una mayor retención.

Los espectros de masas obtenidos a partir de los TIC correspondientes a cada una de las mezclas peptídicas, nos han permitido determinar las masas moleculares de las sustancias detectadas en las mezclas. La asignación de una estructura a las impurezas presentes en los crudos se ha hecho sobre la base de las diferencias entre su masa molecular y la obtenida para el péptido principal, teniendo en cuenta además el procedimiento de síntesis seguido en cada caso.

De este modo, a continuación se resumen los subproductos de síntesis más frecuentemente encontrados en los crudos estudiados:

En todos los crudos se han encontrado **diastereoisómeros** de la hormona peptídica principal, cuya formación es habitual durante la síntesis, la activación y el acoplamiento de cada nuevo aminoácido. Para minimizar su obtención, se suele utilizar una elevada concentración de reactivos en estas etapas, con lo que se asegura una velocidad de reacción adecuada, además de aditivos como la 4-dimetilaminopiridina (4-DMAP) y el 1-hidroxibenzotriazol.

Dependiendo del esquema de protección seguido en la síntesis de las hormonas peptídicas, es frecuente encontrar **secuencias parcialmente protegidas,** que reflejan una eliminación insuficiente de los grupos protectores. Así, en el caso de los crudos de eledoisina, leuprolide y triptorelina, se han encontrado numerosas secuencias con las cadenas laterales aún *tert*-butiladas. Menos común es encontrar fragmentos del péptido principal que aún conserven el grupo Fmoc, como en los crudos de carbetocina y leuprolide (Figura 6.4).

Fmoc-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-CH_2-CH_3

Figura 6.4 Impureza $I4_2$ del crudo de leuprolide

Una eliminación incompleta del grupo protector, también puede provocar la formación de estructuras idénticas a las del péptido principal pero donde falta alguno de los aminoácidos. Esto es lo que se denomina **secuencias de deleción**. Se han observado en todos los crudos de síntesis, siendo más numerosas en los crudos de eledoisina y goserelina (Figura 6.5).

Figura 6.5 Impureza D del crudo de eledoisina. Ausencia del aminoácido Lys.

El uso de un exceso de reactivos en las etapas de acoplamiento, aunque asegura una eficacia elevada en la reacción, también puede provocar la **inserción adicional de algún aminoácido** en la secuencia del péptido principal. Se han identificado este tipo de subproductos en todos los crudos de síntesis estudiados, particularmente en el de triptorelina, en el que se han identificado hasta nueve impurezas de este tipo (Figura 6.6).

Figura 6.6 Impurezas $I5_1$, I6, I8, I12 e $I13_1$ del crudo de triptorelina. Son diastereoisómeros entre si.

Los aminoácidos de la cadena en crecimiento, pueden sufrir **reacciones en las cadenas laterales** (aminaciones, oxidaciones, reducciones, ...) a lo largo de todo el proceso de síntesis, si no se encuentran convenientemente protegidos. Un caso especialmente llamativo es la conversión de la arginina en ornitina durante la síntesis de la goserelina, lo que provoca la presencia de un grupo amino adicional en el péptido en crecimiento, de forma que la cadena puede crecer por dos lugares diferentes. Esto ha originado la presencia de numerosos subproductos de reacción relacionados con esta reacción lateral en este crudo de síntesis (Figura 6.7) [Art. 3.2,3.4].

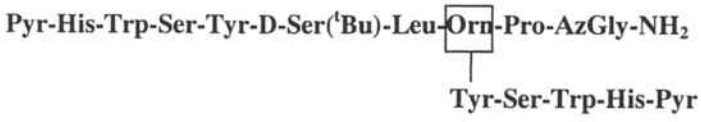

Figura 6.7 Impureza I11 del crudo de goserelina

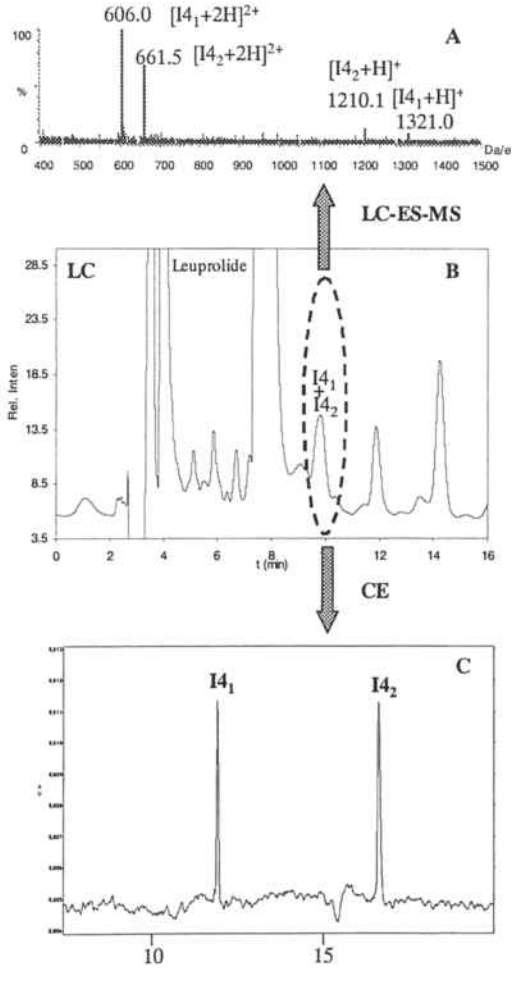

Figura 6.8 A) Espectro de masas del pico cromatográfico I4 obtenido por LC-ESI-MS, B) Análisis por LC del crudo de síntesis del leuprolide, C) Análisis por CE del pico cromatográfico I4 [Art. 3.5].

Aunque la LC-ESI-MS proporciona, en la mayoría de los casos, la suficiente información como para poder identificar y cuantificar sustancias que coeluyen en un mismo pico cromatográfico (Figura 6.8-a), en algunas ocasiones puede resultar interesante combinar dos técnicas de separación complementarias para conseguir un mayor poder de resolución, por ejemplo, si coeluyen dos sustancias con concentraciones muy diferentes, o con masas moleculares idénticas o cuando una de ellas suprime la ionización de la otra. En esta línea, se ha empleado la CE para separar el crudo de síntesis del leuprolide y la complementariedad entre ambas técnicas se ha aprovechado para resolver un mayor número de componentes de la mezcla [Art. 3.5]. Se ha realizado una combinación secuencial de LC y CE, donde las fracciones correspondientes a picos cromatográficos previamente identificados por LC-ESI-MS se han analizado por CE, utilizando unas condiciones de separación que permiten la resolución de sustancias que previamente no se separaban por LC y a la inversa (Figura 6.8-b). Esto nos ha permitido asociar los diferentes picos electroforéticos con las sustancias que previamente habían sido identificadas por LC-ESI-MS. De acuerdo a la estructura propuesta por LC-ESI-MS, se ha calculado la carga de la molécula (q) y se han empleado los modelos semiempíricos, que describen la m_e de las sustancias peptídicas en función de la carga total (q) y la masa molecular (M) o el número de aminoácidos (n), (Ecuaciones 6.3 y 6.4, respectivamente), para confirmar las asignaciones estructurales realizadas a partir de la datos previamente obtenidos por LC-ESI-MS (Figura 6.9-a):

$$m_e = A\frac{q}{M^{\alpha}} \quad \alpha = 1/3,\ 1/2 \text{ o } 2/3 \tag{6.3}$$

$$m_e = A\frac{\ln(1+q)}{n^{0.43}} \tag{6.4}$$

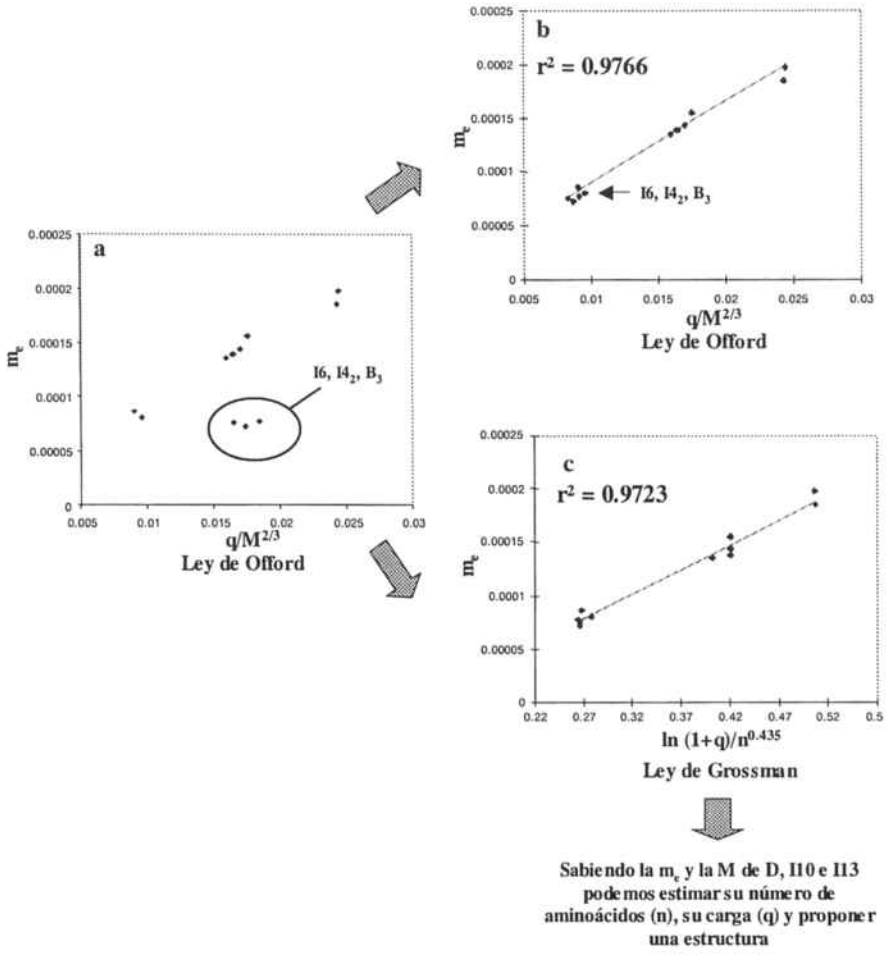

Figura 6.9 Modelos semiempíricos de migración electroforética aplicados a los péptidos del crudo de síntesis de Leuprolide [5]: Ley de Offord a) teniendo en cuenta las M determinadas por LC-ESI-MS y las estructuras originalmente propuestas, b) teniendo en cuenta las nuevas estructuras propuestas para I6, $I4_2$ y B_3 c) Ley de Grossman teniendo en cuentra las nuevas estructuras propuestas para I6, $I4_2$ y B_3.

Como podemos observar en las Figura 6.9-a, la migración electroforética de todas las impurezas se ajusta al modelo semiempírico propuesto, excepto la de las impurezas I6, $I4_2$ y B_3. Como la M ha sido medida experimentalmente por LC-ESI-MS, el problema tiene que deberse a la carga de la molécula, si ésta se ha calculado a partir de una asignación estructural errónea. Se han propuesto nuevas estructuras,

que proporcionan un buen ajuste a los modelos propuestos, para I6, $I4_2$ y B_3 (Figura 6.9-b y 6.9-c). Además, de acuerdo con estos nuevos ajustes, se ha obtenido información estructural (la carga, Figura 6.9-b, y el número de aminoácidos, Figura 6.9-c) de otras impurezas que no habían sido identificadas anteriormente por LC-ESI-MS. Así se ha asignado una estructura coherente con la información obtenida mediante ambas técnicas a las impurezas D e I10 del crudo de síntesis del leuprolide [Art. 3.5].

6.3. SEPARACIÓN Y CARACTERIZACIÓN DE HORMONAS PEPTÍDICAS MEDIANTE CE-ESI-MS.

La metodología que ha sido empleada para identificar los picos que aparecen en el electroferograma del crudo de síntesis del leuprolide es muy interesante cuando se dispone del acoplamiento LC-ESI-MS pero no es posible disponer de un instrumento de CE-ESI-MS, como ocurre en la mayoría de los laboratorios actuales. El análisis mediante CE-ESI-MS del crudo de síntesis peptídico nos hubiera proporcionado la misma información estructural de una manera mucho más rápida y sencilla. Sin embargo, tal y como comentábamos en la introducción inicial, el uso de la CE-ESI-MS no está aún tan extendido como el de la LC-ESI-MS, y dos de las principales causas son su aún elevado coste y su poca robustez, lo que en ambos casos está íntimamente relacionado con un déficit de estudios donde se identifiquen y optimicen sistemáticamente los parámetros que afectan al acoplamiento y a las separaciones.

En esta línea, se ha estudiado sistemáticamente el efecto de diferentes parámetros sobre la detección y la separación por CE-ESI-MS de la mezcla de hormonas peptídicas que había sido analizada previamente por CE-UV, empleando una interfase con líquido auxiliar coaxial y un espectrómetro de masas con analizador TOF (Figura 6.10) [Art. 4.1].

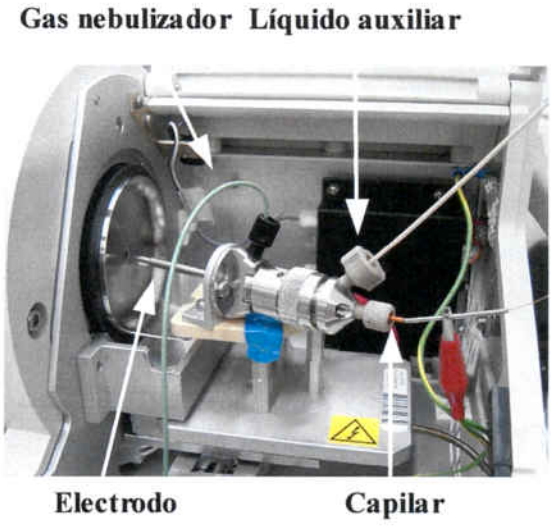

Figura 6.10 Interfase de líquido auxiliar coaxial (*Agilent Technologies*) empleada para acoplar una CE (HP 3DCE, *Agilent Technologies*) y un espectrómetro de masas (Mariner TOF-MS, *Perseptive Biosystems*).

Inicialmente se ha considerado una serie de factores para asegurar un correcto funcionamiento del acoplamiento de ambos instrumentos tal y como se muestra en la Figura 6.11: se ha situado la entrada y la salida del capilar de separación a la misma altura, para evitar un efecto sifón no deseado (Figura 6.11-a); se ha comprobado la importancia de cortar limpiamente el extremo de salida del capilar de separación para conseguir una nebulización eficaz, estable y reproducible (Figura 6.11-b); se ha optimizado la distancia entre los extremos del capilar y el electrodo para que se produzca adecuadamente la mezcla (en nuestro caso fue 0.1 mm) (Figura 6.11-c); y se ha empleado para suministrar el líquido auxiliar una bomba de LC seguida de un divisor de flujo, para evitar las oscilaciones en la línea de base que provoca el uso de una bomba de infusión a velocidades de flujo bajas (Figura 6.11-d).

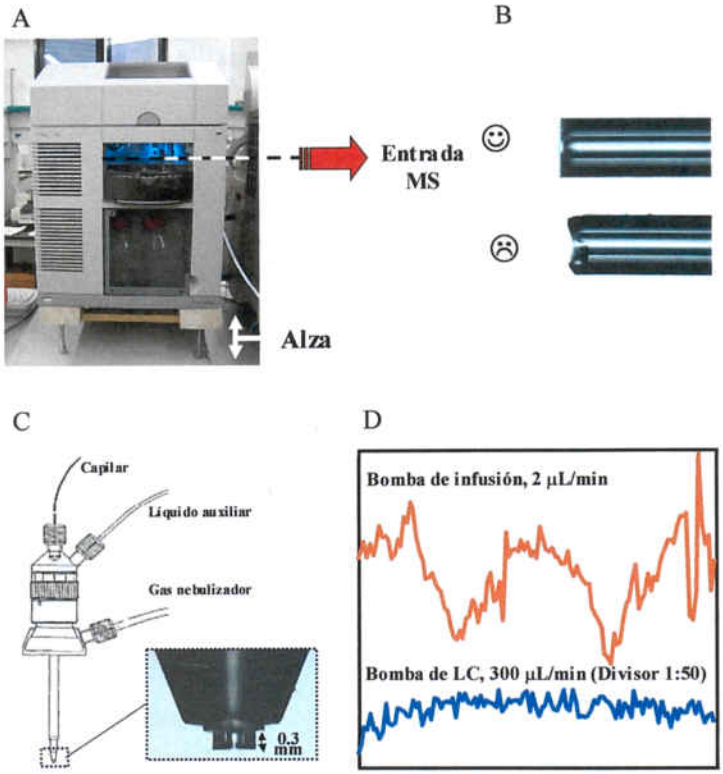

Figura 6.11 Parámetros a considerar en la puesta a punto del acoplamiento CE-ESI-MS empleando una interfase con líquido auxiliar coaxial, a) Efecto sifón por diferencia de alturas entre la entrada del capilar y la salida, b) Extremo de salida del capilar de separación, c) Distancia entre los extremos del capilar y el electrodo (se ha situado a 0.3 mm para facilitar la observación, en nuestro caso la distancia óptima es 0.1 mm) y d) Efecto del tipo de bomba.

El electrolito de separación, la proporción de disolvente orgánico y la concentración del aditivo en el líquido auxiliar, así como la velocidad de flujo a la que se bombea, son parámetros fundamentales para obtener una buena sensibilidad en CE-ESI-MS y se han de optimizar para cada aplicación concreta. El electrolito de separación previamente optimizado para separar la serie de hormonas peptídicas estudiada por CE [Art. 2.3-2.4], 50 mM ácido acético: 50 mM ácido fórmico ajustado a pH 2.85 con amoníaco, es volátil y compatible con CE-ESI-MS. De entre todos los líquidos auxiliares estudiados, el que contiene un 60% v/v de 2-propanol y

un 0.05% (v/v) de ácido fórmico, es el que proporciona los picos electroforéticos más intensos cuando se bombea a 2 μL/min. La velocidad de flujo del gas de nebulización no afecta significativamente a la intensidad de la señal, pero sí de forma importante a las separaciones, tal y como se ha puesto de manifiesto en los resultados obtenidos, con lo que también debe ser optimizada. En nuestro caso, esto nos ha obligado a reajustar su valor hasta 0.5 L/min, cuando se deseaba separar la mezcla de siete hormonas peptídicas [Art. 4.1]. En las condiciones de separación y detección óptimas, el método de análisis desarrollado para estas hormonas peptídicas presenta una buena reproducibilidad y repetitividad en tiempos de migración y áreas de picos, con valores de RSD de alrededor de un 2% y un 10%, respectivamente, buenas linealidades en intervalos amplios de concentración y límites de detección en el intervalo comprendido entre 1 y 4 μg/mL, para cada una de las hormonas peptídicas. Se ha comprobado que el espectrómetro de masas con analizador TOF, además de proporcionar una excelente sensibilidad, permite obtener espectros de elevada resolución, en un amplio intervalo de relaciones carga/masa, lo que puede ser de extraordinaria ayuda para la caracterización de moléculas complejas con pequeñas diferencias estructurales, tales como las glicoformas de una glicoproteína como la EPO.

6.4. PRECONCENTRACIÓN, SEPARACIÓN Y CARACTERIZACIÓN DE HORMONAS PEPTÍDICAS MEDIANTE SPE-CE Y SPE-CE-ESI-MS.

A pesar de los bajos límites de detección obtenidos para cada una de las hormonas peptídicas anteriores cuando se analizan por CE-ESI-MS [Art. 4.1], estamos lejos de los límites de detección a los que algunos péptidos y proteínas, como la EPO, se encuentran en ciertas muestras, tales como matrices biológicas [10]. Este es el motivo por el cual se han explorado las posibilidades de la SPE-CE como alternativa para la preconcentración y la separación de hormonas peptídicas en

muestras diluidas [Art. 4.2]. Empleando rellenos no selectivos de tipo C18, se ha descrito sistemáticamente el procedimiento a seguir para la construcción de los cartuchos de extracción en las columnas capilares de separación de forma rápida y sencilla. En la Figura 6.12 se puede observar una imagen ampliada de uno de estos cartuchos de extracción visto a través de un microscopio. Es importante señalar que se ha utilizado el diseño que ha resultado más adecuado para el desarrollo de nuestros experimentos, pero existen otras posibilidades, que ya hemos apuntado o que aún no han sido estudiadas, que pueden resultar adecuadas para la construcción de cartuchos de extracción en línea con la CE.

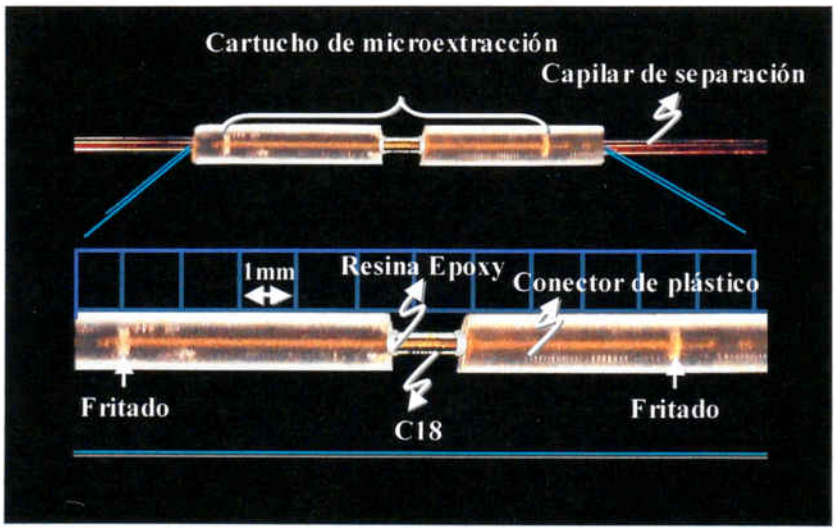

Figura 6.12 Cartucho de extracción en fase sólida utilizado para realizar SPE-CE.

Por los resultados obtenidos, queda demostrado que tan importante como diseñar un buen cartucho de extracción, es optimizar sistemáticamente las diferentes etapas involucradas en el proceso de preconcentración. El tipo de muestra, su concentración, el número de analitos en la muestra, la velocidad de flujo y el tiempo de introducción, son parámetros importantes a la hora de seleccionar las condiciones

a las que se introduce la muestra para conseguir los mejores factores de preconcentración y llegar a desarrollar metodologías de SPE-CE cuantitativas. Las disoluciones que se utilizan para lavar la fase estacionaria tras la introducción de la muestra son importantes porque sólo deben eliminar los contaminantes, las impurezas y las moléculas de analito que no interaccionaron específicamente. En esta línea, hemos demostrado que se ha de tener especialmente en cuenta la composición y pH del electrolito de separación empleado en CE, ya que éste también puede eluir moléculas de analito adsorbidas específicamente. Esto ha ocurrido con la Angiotensina II, al emplear un electrolito de separación que contenía 20 mM de CHES ajustado a pH 9.5 con amoníaco, lo que nos ha impedido detectar disoluciones de concentración inferior a 10 μg/mL. En cambio, empleando un electrolito de separación que contiene 20 mM de ACES ajustado a pH 7.4 con amoníaco, es posible detectar disoluciones de Angiotensina II de hasta 100 ng/mL, lo que supone una mejora de tres órdenes de magnitud en los límites de detección de esta sustancia respecto a los valores obtenidos por CE-UV. La disolución empleada para eluir los analitos cuantitativamente también debe ser optimizada para cada aplicación concreta. En el caso del uso de C18 como fase estacionaria y del análisis de las hormonas peptídicas seleccionadas, se utiliza una disolución 80:20 (v/v) MeCN (HFor 25 mM):H_2O inyectada hidrodinámicamente a 50 mbar de presión durante 5 s. Los resultados obtenidos para la preconcentración de mezclas de Angiotensina II y Leu-encefalina, parecen indicar que la presencia de Leu-encefalina, favorece la preconcentración de la Angiotensina II, obteniéndose mejores resultados con la mezcla que introduciendo una disolución de Angiotensina II por separado. Los tiempos de análisis y las separaciones de esta misma mezcla en SPE-CE también difieren de los obtenidos en CE para una mezcla de similares características, por lo que ha sido necesario reoptimizarlas, en este caso, empleando capilares de mayor longitud. Por otro lado, el uso de un electrolito de separación y de un eluyente de composición compatible con la detección por ESI-MS, nos ha

permitido el traslado de las condiciones de preconcentración y separación establecidas en SPE-CE a SPE-CE-ESI-MS. En este caso, se ha utilizado un espectrómetro de masas con analizador de cuadrupolo, que no permite conseguir límites de detección tan bajos, ni resoluciones tan elevadas, como el analizador TOF utilizado anteriormente. Sin embargo, mediante la preconcentración en línea se ha conseguido disminuir los límites de detección tres órdenes de magnitud, hasta detectar 100 ng/mL de Angiotensina II.

6.5. Separación y caracterización de rHuEPO mediante CE y MS

En general, cuando se analizan sustancias peptídicas de bajo peso molecular mediante CE en capilares de sílice fundida, la adsorción irreversible de los analitos sobre las paredes internas del capilar no es un fenómeno muy importante. Este proceso comienza a tener especial relevancia a medida que aumenta el peso molecular de la sustancia peptídica analizada, como en el caso de la EPO, lo que nos obliga a desarrollar alguna estrategia para minimizarlo. La separación de las glicoformas de la EPO natural o de sus formas recombinantes, tiene la dificultad añadida de que estas glicoformas presentan mínimas diferencias estructurales entre ellas por lo que son muy difíciles de separar. El método de CE incluido en la Farmacopea Europea del año 2002, para analizar disoluciones relativamente concentradas de rHuEPO, emplea un electrolito de separación que contiene putrescina, para recubrir dinámicamente el capilar y una elevada concentración de urea [11]. Aunque de este modo se obtiene la resolución de hasta 8 bandas correspondientes a glicoformas, nuestros experimentos han puesto de manifiesto la falta de repetitividad y reproducibilidad del método, la influencia negativa sobre las separaciones de las especies de bajo peso molecular que es frecuente encontrar en los preparados farmacéuticos y la incompatibilidad de las condiciones de separación

con la detección en línea empleando ESI-MS [Art. 5.1]. Con el objeto de solventar estos inconvenientes, se ha estudiado en este trabajo el uso de capilares de sílice fundida, además de otros tipos de capilares recubiertos, con diferentes electrolitos de separación volátiles, de manera que sea posible poner a punto una metodología alternativa compatible con la CE-ESI-MS [Art. 5.1]. Así, capilares recubiertos de forma permanente con polybrene dieron los mejores resultados y se optimizaron sistemáticamente los parámetros involucrados en la separación, empleando electrolitos de separación volátiles, que contenían HAc o HFor principalmente, ajustados a diferentes pH con amoníaco.

Las mejores separaciones de las glicoformas de rHuEPO se obtuvieron con un electrolito de separación que contiene 400 mM de HAc ajustado a pH 4.75 con amoníaco. En estas condiciones, potencialmente compatibles con la detección en línea empleando ESI-MS, se ha conseguido la resolución parcial de hasta 5 bandas correspondientes a glicoformas. Las separaciones son rápidas y reproducibles y es posible diferenciar entre diferentes tipos de rHuEPO.

La ESI-MS y la MALDI-TOF se han empleado, sin separación previa, para caracterizar las glicoformas de la rHuEPO. El espectro de masas obtenido mediante ESI-MS es excesivamente complejo y no ha sido posible deconvolucionarlo para obtener información sobre la masa molecular de las glicoformas. Sin embargo, a partir del espectro de masas de MALDI-TOF se puede obtener un valor para la masa molecular media de la molécula (28443 ± 183 Da), aunque se ha puesto de manifiesto que este valor depende de la energía del láser empleada para ionizarla, lo que sugiere cierto grado de fragmentación de la molécula durante la medida. Esto se ha de tener en cuenta para poder comparar valores de masas moleculares obtenidas con diferentes instrumentos.

Los trabajos realizados al final de esta tesis doctoral y que se han recopilado en los capítulos 4 y 5, marcan la línea principal de las investigaciones que nos han ocupado y nos ocuparán durante los próximos años, cuyo objetivo concreto es llegar

a establecer una metodología analítica que nos permita determinar de forma inequívoca, rápida y sencilla la presencia de rHuEPO o sus análogos en fluidos biológicos. De acuerdo con nuestros trabajos, la SPE-CE-ESI-MS, empleando soportes de afinidad, puede constituir una buena alternativa para determinar y caracterizar estructuralmente una sustancia en disoluciones diluidas. Los recientes resultados obtenidos en esta línea, mediante CE-ESI-MS y SPE-CE, se han presentado en forma de comunicación oral en dos congresos internacionales [12-13]

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Capítulo 7. Conclusiones

7. CONCLUSIONES

A partir de los resultados obtenidos en las investigaciones que se recopilan en esta memoria de tesis doctoral se pueden extraer las siguientes conclusiones generales:

I. Se han desarrollado dos modelos generales que describen el comportamiento cromatográfico y electroforético, respectivamente, de los analitos ionizables en función del pH de la fase móvil, los pK_a de los analitos y los coeficientes de actividad.

II. Se han utilizado estos modelos generales para predecir el comportamiento cromatográfico y electroforético de péptidos y hormonas peptídicas en mezclas complejas, a partir de un reducido número de medidas experimentales, y de esta forma poder seleccionar el valor de pH óptimo para las separaciones.

III. Se han utilizado los modelos cromatográfico y electroforético desarrollados, para determinar los pK_a de péptidos y hormonas peptídicas, tanto en medio acuoso, como en mezclas hidroorgánicas.

IV. Se ha constatado la validez del método LSER para predecir, en función de la polaridad de la fase móvil, el comportamiento cromatográfico de péptidos en mezclas complejas, como los crudos que se obtienen tras la síntesis en fase sólida del leuprolide, la goserelina, la carbetocina, la triptorelina y la eledoisina.

V. Se ha utilizado el método LSER para predecir la retención cromatográfica de las sustancias presentes en crudos que se obtienen en la síntesis en fase sólida, a partir de un reducido número de medidas experimentales y de esta forma poder seleccionar la proporción óptima de disolvente orgánico en la fase móvil para las separaciones previas, tanto para la purificación de los

péptidos sintetizados a escala preparativa, como para su posterior caracterización, junto a las impurezas, empleando LC-ESI-MS.

VI. En los crudos de síntesis de las hormonas peptídicas analizadas mediante LC-ESI-MS, se han identificado mayoritariamente: diastereoisómeros, secuencias parcialmente protegidas, secuencias de deleción, productos que resultan de la inserción de algún aminoácido adicional y moléculas en las que las cadenas laterales del péptido principal han experimentado algún tipo de reacción (oxidaciones, reducciones, amidaciones, conversión de arginina en ornitina, ...).

VII. Se ha comprobado la complementariedad de LC y CE para la separación de mezclas peptídicas complejas. La combinación de ambas técnicas se ha aprovechado para resolver un mayor número de componentes en un crudo de síntesis de leuprolide.

VIII. Se ha demostrado que los modelos semiempíricos que explican la migración electroforética de las sustancias peptídicas en función del la carga global y la masa molecular o el número de aminoácidos, son de gran ayuda para corroborar las asignaciones estructurales realizadas mediante LC-ESI-MS, que se basan principalmente en la masa molecular obtenida experimentalmente.

IX. Se ha realizado un estudio sistemático de las variables que afectan a la sensibilidad y selectividad de las separaciones de una serie de hormonas peptídicas en CE-ESI-MS, empleando una interfase de líquido auxiliar coaxial y un analizador TOF. Entre estas variables, las que tienen una mayor influencia en los resultados son: la composición del electrolito de separación, la composición y el flujo del líquido auxiliar y el flujo de gas nebulizador.

X. Para la separación y caracterización de una serie de hormonas peptídicas mediante CE-ESI-MS se ha seleccionado un electrolito de separación volátil

que contiene 50 mM ácido acético: 50 mM ácido fórmico ajustado a pH 2.85 con amoníaco. Los mejores resultados se obtuvieron empleando un líquido auxiliar que contiene un 60% de 2-propanol y un 0.05% de ácido fórmico a 2 μL/min. El flujo de gas nebulizador afecta ligeramente a la sensibilidad, pero enormemente a la selectividad de las separaciones y se ha de optimizar adecuadamente. Se han determinado los parámetros de calidad en las condiciones de análisis óptimas: se obtienen límites de detección para cada una de las hormonas peptídicas en el intervalo entre 1.2 y 3.65 μg/mL, y buenas repetitividades y reproducibilidades.

XI. Se ha realizado un estudio sistemático de las variables que afectan a la preconcentración de los analitos peptídicos utilizando SPE-CE y SPE-CE-ESI-MS. Se ha descrito detalladamente el diseño y construcción de los cartuchos de extracción y las columnas de separación modificadas. Se ha comprobado que la concentración de la muestra , el número de analitos que contiene, y la velocidad de flujo a la que se introduce determinan el tiempo necesario para operar en condiciones que no impliquen la saturación de la fase estacionaria. La composición y el pH del electrolito de separación pueden provocar la elución de sustancias adsorbidas específicamente. Las condiciones de separación en SPE-CE pueden requerir un reajuste respecto a las condiciones originalmente optimizadas en CE.

XII. Se ha comprobado que empleando una fase estacionaria no selectiva de tipo C18 en SPE-CE y SPE-CE-ESI-MS, se pueden conseguir mejoras de alrededor de tres órdenes de magnitud en los límites de detección obtenidos en el análisis de hormonas peptídicas por CE y CE-ESI-MS, cuando se utiliza una disolución de 80:20 (v/v) MeCN (HFor 25 mM):H_2O para eluir y un electrolito de separación que contiene 20 mM ACES ajustado a pH 7.4 con amoníaco.

XIII. Se ha propuesto un nuevo método para separar mediante CE las glicoformas de la rHuEPO, como alternativa al actualmente propuesto por la Farmacopea Europea. De esta forma se pueden separar hasta 5 picos correspondientes a glicoformas de rHuEPO. Las ventajas de este nuevo método son que las separaciones son más rápidas y reproducibles, no es necesario ningún pretratamiento de la muestra y las condiciones de separación son potencialmente compatibles con la operación en CE-ESI-MS.

XIV. Se ha comprobado la imposibilidad de obtener información sobre las masas moleculares de las glicoformas de rHuEPO si se analiza la glicoproteína directamente mediante ESI-MS.

XV. Se ha obtenido la masa molecular media de 28443 Da para la rHuEPO empleando MALDI-TOF. Se ha constatado la gran influencia de la energía del láser empleada sobre la masa molecular determinada, debido a la gran labilidad de la molécula.