



Determinació de constants d'acidesa de substàncies d'interès biològic per electroforesi capil·lar

Joan Marc Cabot Canyelles

ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (www.tdx.cat) i a través del Dipòsit Digital de la UB (diposit.ub.edu) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX ni al Dipòsit Digital de la UB. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX o al Dipòsit Digital de la UB (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (www.tdx.cat) y a través del Repositorio Digital de la UB (diposit.ub.edu) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR o al Repositorio Digital de la UB. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR o al Repositorio Digital de la UB (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (www.tdx.cat) service and by the UB Digital Repository (diposit.ub.edu) has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized nor its spreading and availability from a site foreign to the TDX service or to the UB Digital Repository. Introducing its content in a window or frame foreign to the TDX service or to the UB Digital Repository is not authorized (framing). Those rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author.



DETERMINACIÓ DE CONSTANTS

D'ACIDES A DE SUBSTÀNCIES D'INTERÈS

BIOLÒGIC PER ELECTROFORESI CAPIL·LAR

Joan Marc Cabot Canyelles

Programa de Doctorat
del Departament de Química Analítica de la Universitat de Barcelona
QUÍMICA ANALÍTICA DEL MEDI AMBIENT I DE LA POL·LUCIÓ

**DETERMINACIÓ DE CONSTANTS D'ACIDES A DE
SUBSTÀNCIES D'INTERÈS BIOLÒGIC PER
ELECTROFORESI CAPIL·LAR**

Memòria presentada per

Joan Marc Cabot Canyelles

per optar al grau de

Doctor per la Universitat de Barcelona

Sota la direcció de

Martí Rosés Pascual
Universitat de Barcelona
Departament de Química Analítica

Elisabet Fuguet Jordà
Universitat de Barcelona
Departament de Química Analítica

El Dr. Martí Rosés Pascual, catedràtic del Departament de Química Analítica de la Universitat de Barcelona, i la Dra. Elisabet Fuguet Jordà, professor agregat del mateix Departament,

FAN CONSTAR

Que la present memòria titulada:

“Determinació de constants d'acidesa de substàncies d'interès biològic per electroforesi capil·lar”

ha estat realitzada sota la nostra direcció pel Sr. Joan Marc Cabot Canyelles i que tots els resultats presentats són fruit de les experiències realitzades pel citat doctorant.

I per a que així consti, expedim el present certificat.

Barcelona, Març 2015

Dr. Martí Rosés Pascual

Dra. Elisabet Fuguet Jordà

*Als meus pares,
als meus germans
i a l'Eli*

ÍNDEX

PRÒLEG

i.	RESUM	3
	* <i>ABSTRACT</i>	5
ii.	OBJECTIUS	7
	* <i>AIMS</i>	9

CAPÍTOL I. Introducció 11

1.1. LA CONSTANT D'ACIDESA 13

1.1.1.	LA CONSTANT D'ACIDESA COM A PARÀMETRE D'INTERÈS BIOLÒGIC	13
1.1.1.1.	<i>Ionització i lipofilicitat</i>	14
1.1.1.2.	<i>Ionització i solubilitat</i>	15
1.1.1.3.	<i>Ionització i absorció biològica</i>	16
1.1.2.	EQUILIBRI ÀCID-BASE I LA SEVA CONSTANT	17
1.1.3.	CONSTANTS D'ACIDESA EN PRESENCIA DE COSOLVENTS	20
1.1.4.	LA NECESSITAT DE MÈTODES RÀPIDS PER A LA DETERMINACIÓ DE CONSTANTS D'ACIDESA	21
1.1.5.	MÈTODES PER A LA DETERMINACIÓ DE CONSTANTS D'ACIDESA	22

1.2. ELECTROFORESI CAPIL·LAR 25

1.2.1.	FONAMENT TEÒRIC	25
1.2.2.	PARÀMETRES D'INTERÈS PER A LA DETERMINACIÓ DE CONSTANTS D'ACIDESA	27

1.3. MÈTODES ELECTROFORÈTICS PER A LA DETERMINACIÓ DE CONSTANTS D'ACIDESA 29

1.3.1.	INTRODUCCIÓ	29
1.3.2.	MÈTODE CLÀSSIC	30

1.4. IS-CE, *INTERNAL STANDARD CAPILLARY ELECTROPHORESIS* 33

1.4.1.	PROCEDIMENT	33
1.4.2.	FONAMENT TEÒRIC	34
1.4.2.1.	<i>Àcids monopròtics</i>	34
1.4.2.2.	<i>Bases monopròtiques</i>	35
1.4.2.3.	<i>Utilitzant compostos de diferent naturalesa àcid-base</i>	36
1.4.3.	CONSIDERACIONS	37
1.4.3.1.	<i>Consideracions del BGE</i>	37
1.4.3.2.	<i>Consideracions de l'IS</i>	38
1.4.4.	AVANTATGES	39

CAPÍTOL II. Publicacions 41

Article I. <i>Fast high throughput method for the determination of acidity constants by capillary electrophoresis. II. Acidic internal standards.</i>	43
--	----

Article II. <i>Determination of acidity constants by the capillary electrophoresis internal standard method. IV. Polyprotic compounds.</i>	49
---	----

Article III. <i>Internal Standard Capillary Electrophoresis as a High Throughput Method for pK_a Determination in Drug Discovery and Development.</i>	59
Article IV. <i>High throughput determination $\log P_{o/w}$ / pK_a / $\log D_{o/w}$ of drugs by combination of UHPLC and CE methods.</i>	83
Article V. <i>Determination of acidity constants of sparingly soluble drugs in aqueous solution by the internal standard capillary electrophoresis method.</i>	93
Article VI. <i>Determination of Acidity Constants of Insoluble Drugs in Organic-Water Mixtures by Internal Standard Capillary Electrophoresis Method.</i>	99
Article VII. <i>Novel instrument for automated pK_a determination by Internal Standard Capillary Electrophoresis.</i>	123
 CAPÍTOL III. Resultats i discussió	 147
3.1. ESTABLIMENT D'UNA LLISTA DE PATRONS INTERNS DE REFERÈNCIA	151
3.2. MÈTODE IS-CE PER A COMPOSTOS POLIPRÒTICS	155
3.3. IS-CE COM A MÈTODE <i>HIGH THROUGHPUT</i>	159
3.3.1. ESTUDI D'ESTABILITAT DELS TAMPONS I DELS PATRONS INTERNS	162
3.4. APLICACIÓ DEL MÈTODE IS-CE PER A LA DETERMINACIÓ <i>HIGH THROUGHPUT</i> DE PERFILS DE LIPOFILICITAT	165
3.5. DETERMINACIÓ DE CONSTANTS D'ACIDES A DE COMPOSTOS POC SOLUBLES	169
3.5.1. FÀRMACS AMB SOLUBILITAT LLEUGERAMENT BAIXA	170
3.5.2. FÀRMACS AMB SOLUBILITAT MOLT BAIXA	171
3.5.3. COMPARACIÓ AMB ALTRES MÈTODES	173
3.6. DISSENY D'UN INSTRUMENT ESPECÍFIC PER AL MÈTODE IS-CE	177
3.6.1. DISSENY DE L'EQUIP	177
3.6.2. DETERMINACIÓ AUTOMATITZADA DEL pK_a PEL MÈTODE IS-CE	185
 CONCLUSIONS	 187
*CONCLUSIONS	191
 REFERÈNCIES	 193

**Chapter/section written in English*

PRÒLEG

i. RESUM

Aquesta Tesi es titula *Determinació de constants d'acidesa de substàncies d'interès biològic per electroforesi capil·lar*. La Dissertació es focalitza llavors en l'estudi de la determinació de la constant d'acidesa de fàrmacs utilitzant aquesta tècnica d'electroseparació, l'electroforesi capil·lar. La constant d'acidesa o pK_a és una propietat fisicoquímica útil en el descobriment i desenvolupament de fàrmacs, on el coneixement de l'estat de ionització d'un grup funcional és sovint essencial per a comprendre les propietats farmacocinètiques i farmacodinàmiques de nous fàrmacs potencials. Gràcies a l'ús d'un patró intern (IS), es va desenvolupar una nova metodologia per a la determinació d'aquestes constants per electroforesi capil·lar (CE), el mètode IS-CE. No obstant això, aquest mètode només es va desenvolupar per a substàncies simples monoprotòniques. Així doncs, l'objectiu principal d'aquesta Tesi és establir el mètode IS-CE com a mètode d'alt rendiment per a la determinació de constants d'acidesa de compostos bioactius, així com també dissenyar un prototip específic per aquest mètode per tal d'implementar-lo per a l'anàlisi rutinari de fàrmacs potencials en laboratoris d'anàlisi fisicoquímiques. Aquesta fita ha estat perseguida al llarg d'aquesta Dissertació, cobrint durant el seu desenvolupament cada necessitat que presentava el mètode.

Per començar, en aquesta Tesi s'estableix el pK_a d'un llistat de patrons interns àcids i bàsics de diferent estructura que cobreixi tot l'interval de pH útil en electroforesi capil·lar i s'amplia l'aplicabilitat del mètode a compostos amb diverses constants d'acidesa. Posteriorment s'optimitzen els paràmetres instrumentals per tal de convertir l'IS-CE en un mètode *high throughput* IS-CE. Després s'avalua la capacitat del mètode per a la determinació de compostos de baixa solubilitat en aigua i s'amplia per a la determinació de constants d'acidesa de compostos de molt baixa solubilitat utilitzant mescles metanol-aigua. Arribat aquest punt es dissenya una instrumentació específica adaptable al mètode IS-CE, tot simplificant i agilitzant el procés per a la determinació del pK_a .

En conjunt, aquest treball d'investigació científica ha permès el desenvolupament consolidat d'un mètode ràpid, robust, precís i acurat per a la determinació de constants d'acidesa de compostos bioactius. Així mateix, també ha permès dissenyar un nou prototip per a la implementació d'aquest mètode a l'anàlisi rutinari de fàrmacs

potencials en laboratoris de mesures fisicoquímiques i de *drug discovery and development*.

Aquesta Tesi inclou quatre parts que estan escrites en anglès, el resum, els objectius, les conclusions i també els articles publicats en revistes que formen la base de la feina exposada aquí. Hi ha dues raons per tenir aquestes parts escrites en anglès. En primer lloc, l'anglès és la llengua vehicular de la ciència; i en segon lloc, també cal fer possible que qualsevol persona que no sap català, la llengua en què s'escriu aquesta Dissertació, pugui entendre aquest treball completament. Així doncs, les parts essencials de la Tesi com el resum, els objectius, les conclusions i els articles estan en anglès perquè qualsevol lector de científic de formació científica pugui entendre-la.

i. ABSTRACT

The Thesis is entitled *Determinació de la constant d'acidesa de substàncies d'interès biològic per electroforesi capil·lar*, which could be translated as *Determination of acidity constants of biological compounds by capillary electrophoresis*. The Dissertation is then focused on the study of the acidity constant determination of drugs using this electroseparation technique, capillary electrophoresis. The acidity constant or pK_a is a useful physicochemical property in drug discovery and development, where the knowledge of the ionization state of a functional group is often essential to understand the pharmacodynamic and pharmacokinetic properties of new potential drugs. In the past, a new method to determine acidity constants by capillary electrophoresis (CE) was developed by the use of an internal standard (IS), the IS-CE method. However, this method was only developed for simple monoprotic compounds. Thus, the main objective of this Thesis is to establish the IS-CE method as a high throughput method for the determination of acidity constants of bioactive compounds as well as to design a specific prototype for this method in order to implement it for the routine analysis of potential drugs in physicochemical analysis laboratories. This goal has been pursued throughout this Dissertation in order to meet the need during the development of this method.

First of all, this Thesis establishes the pK_a of a list of acidic and basic internal standards with different structures that covers the entire pH-range useful in capillary electrophoresis, and expands the applicability of this method to compounds with several acidity constants. Afterwards, instrumental parameters and experimental conditions are optimized in order to transform the IS-CE method into a high throughput method. Subsequently, the method is evaluated for sparingly soluble compounds in water and extended for the determination of acidity constants of highly insoluble compounds using methanol-water mixtures. At this point a specific instrumentation is designed and adapted to the IS-CE method, simplifying and accelerating the pK_a determination process.

Overall, the conducted scientific research has allowed the development of a consolidated fast, robust, precise and accurate method for the determination of acidity constants of bioactive compounds. Likewise, a new prototype has been designed for the implementation of this method to the analysis of potential drugs in routine laboratory of physicochemical measurements and drug discovery and development.

This Thesis includes four parts written in English, the abstract, the aim, the conclusions and also the articles published in peer-reviewed journals which form the basis of the work exposed here. There are two reasons for having these parts written in English. First, English is the vehicular language of science; and secondly, it is also necessary that anyone who does not know Catalan, the language in which this Dissertation is written, could understand completely this work. Therefore, the essential parts of the Thesis the abstract, the aim, the conclusions and the papers are in English, because there parts should be enough to allow a scientific English reader to understand it.

ii. OBJECTIUS

Recentment es va desenvolupar una metodologia per a la determinació de constants d'acidesa per electroforesi capil·lar gràcies a l'ús d'un patró intern. Aquest mètode només es va aplicar per a substàncies simples monoprotiques. Per tant, l'objectiu principal d'aquesta Tesi és proporcionar una metodologia robusta, simple i fiable per tal d'avaluar la constant d'acidesa per a qualsevol tipus de substància bioactiva, sense importar la seva complexitat, nombre de constants d'acidesa o solubilitat. Així mateix, la necessitat de les empreses farmacèutiques de mesurar moltes mostres en poc temps requereix l'optimització d'aquest mètode per a convertir-lo en un mètode *high throughput*.

Un altre aspecte a considerar és el desenvolupament d'una instrumentació específica per a l'aplicabilitat d'aquest mètode. Un segon objectiu d'aquesta Tesi és dissenyar un nou prototip automatitzat per tal que pugui ser implementat en laboratoris de mesures fisicoquímiques o de *drug discovery and development*. Així doncs, l'analista podrà determinar de forma simple, ràpida i eficaç la constant d'acidesa d'un fàrmac potencial per a l'estudi de les seves propietats fisicoquímiques i d'absorció en processos biològics.

ii. AIMS

Recently, a method to determine acidity constants by capillary electrophoresis by the use of an internal standard was developed. This method was only applied for simple monoprotic compounds. Thus, the main objective of this Thesis is to provide a robust, simple and reliable methodology to evaluate the acidity constant for any kind of bioactive compound, regardless its complexity, number of acidity constants or solubility. Furthermore, the need of pharmaceutical companies to measure high quantity of samples in short time requires to optimize this method in order to transform it into a high throughput method.

Another aspect to consider is the development of a specific instrumentation for this method. So a second aim of this Thesis is therefore to design a new automated instrumentation to be implemented in physicochemical measurements and drug discovery and development laboratories. Thus, the analyst would be capable of determining in a simple, fast and effective manner the acidity constant of a potential drug in order to study its physicochemical properties and absorption in biological processes.

Capítol I

Introducció

1.1. LA CONSTANT D'ACIDESA

La constant d'acidesa és una propietat fisicoquímica útil per descriure l'abast de la ionització dels grups funcionals d'una molècula respecte al pH. Aquests paràmetres són importants en àrees d'investigació com el descobriment i desenvolupament de fàrmacs, on el coneixement de l'estat de ionització d'un grup funcional és sovint essencial per a comprendre les propietats farmacocinètiques i farmacodinàmiques de nous fàrmacs potencials.

Aquesta primera secció de la introducció es divideix en cinc apartats. El primer explica la importància de la constant d'acidesa de les substàncies bioactives dins del sistema biològic. Seguidament, en el segon i tercer apartat es defineix la constant d'acidesa per a cada tipus d'equilibri àcid-base en medi aquós i en presència de cosolvent. El quart apartat presenta la necessitat de mètodes ràpids per a la determinació de constant d'acidesa, i el cinquè i últim posa de manifest alguns dels mètodes més importants.

1.1.1. La constant d'acidesa com a paràmetre d'interès biològic

La constant d'acidesa, K_a o pK_a (com a $-\log K_a$) és una constant que defineix l'estat de ionització de les substàncies àcid-base en un medi. Aquesta és una propietat fonamental dels àcids i bases febles que afecta a la seva acció biològica, ja que les propietats de les formes ionitzades i neutres són generalment diferents. La forma ionitzada és sovint més soluble en aigua, mentre que la forma neutra és més lipofílica i té major permeabilitat en la membrana [1,2]. Per tant, el grau de ionització és factor indispensable per a l'estudi dels processos d'absorció, distribució, metabolisme, excreció i toxicitat, o altrament anomenat en l'acrònim anglès, processos *ADMET* [3–6]. La Figura 1.1 representa l'impacte de la constant d'acidesa a les propietats fisicoquímiques i les potencials interaccions amb les característiques *ADMET*.

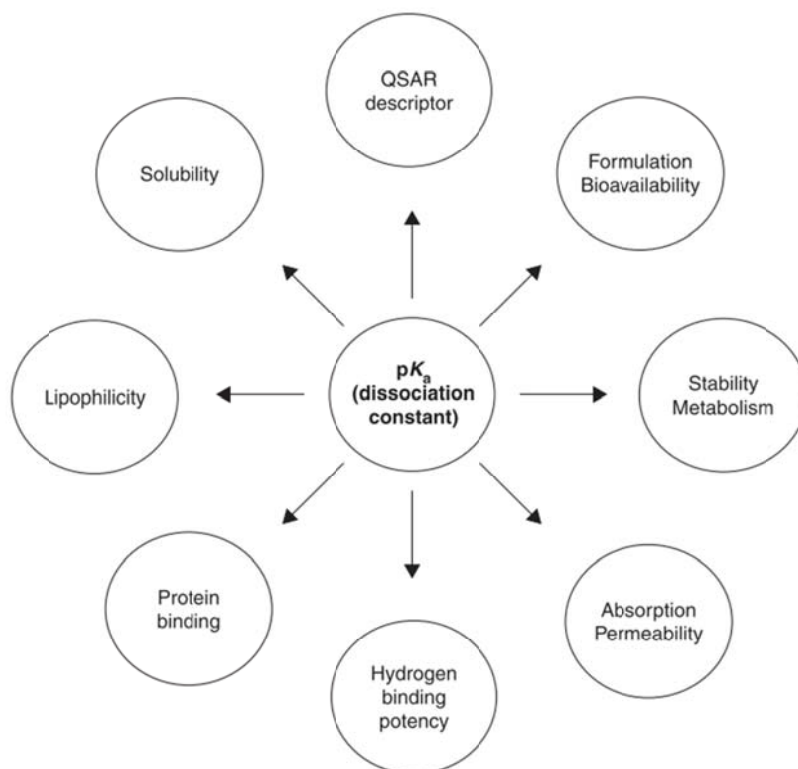


Figura 1.1. Impacte del pK_a a les propietats fisicoquímiques i interaccions amb altres paràmetres [6].

Degut a que molts dels fàrmacs contenen com a mínim un grup funcional àcid-base, el coneixement de la constant d'acidesa esdevé, en molts casos, clau per a conèixer el comportament dels fàrmacs en solució. Al 1988, es va estimar que el 75% dels fàrmacs eren bàsics, el 20% àcids i només el 5% no eren ionitzables [7].

A continuació s'explica la influència que té la ionització en dos paràmetres biològicament tant importants com la lipofilicitat i la solubilitat, i posteriorment la repercussió que té tot això en l'absorció biològica de fàrmacs.

1.1.1.1. Ionització i lipofilicitat

Les molècules són més lipofíliques quan no estan ionitzades, i la seva lipofilicitat disminueix ràpidament quan s'ionitzen. Aquest fet condiciona l'absorció i transport de fàrmacs dins l'organisme com a resultat de la seva permeabilitat per difusió passiva a través de membranes i bicapes lipídiques. La Figura 1.2 mostra la variació de la

lipofilicitat (expressat com a logaritme de la partició octanol / aigua, $\log D$) de tres fàrmacs ionitzables en funció del pH. Tal i com mostra la figura, la lipofilicitat presenta variacions significatives dins del interval de pH i quan la substància és neutre roman constant, aquest valor s'anomena $\log P$.

Hi ha molts factors que poden influir en la conversió de fàrmacs en la seva forma no ionitzada, causant variacions notables en la seva lipofilicitat [8,9]. Per exemple l'exposició a medis de pH diferent com el de la sang i la barrera sang-cervell (7.4), de l'estómac (pot variar de 1 a 7, però normalment es troba entre 1.4-2.1), del tracte gastrointestinal (d'entre 5 i 8) o canvis de pH depenent de si s'està o no en dejú.

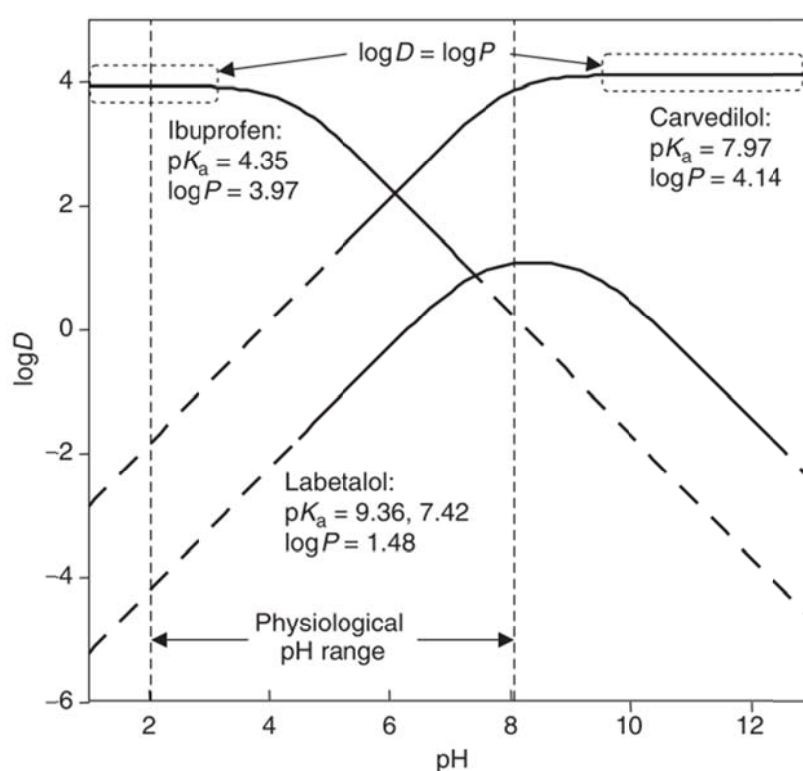


Figura 1.2. Perfils de lipofilicitat en funció del pH de l'ibuprofè (àcid), el carvedilol (base) i el labetalol (zwitterió) [8].

1.1.1.2. Ionització i solubilitat

La solubilitat de les molècules ionitzables és inferior quan es troben en la seva forma neutra, i augmenta ràpidament quan s'ionitzen. Tanmateix, l'espècie neutra en solució va desapareixent a mesura que la iònica augmenta. Si a més, tenim en compte la

lipofilitat explicada anteriorment, només les molècules dissoltes de les espècies neutres passaran a la fase biològica [10]. Així doncs, si un fàrmac no es troba en solució en la seva forma neutra no serà permeable, essent per tant la solubilitat baixa un problema molt important per als laboratoris de descobriment i desenvolupament de fàrmacs, en anglès anomenats *drug discovery and development laboratories*. Els perfils de solubilitat o $\log S$ (logaritme de la solubilitat) en funció del pH de la Figura 1.3 mostren com la solubilitat augmenta quan s'ionitzen molècules. El $\log S_0$ és el logaritme de la solubilitat de la forma neutra de la substància [11].

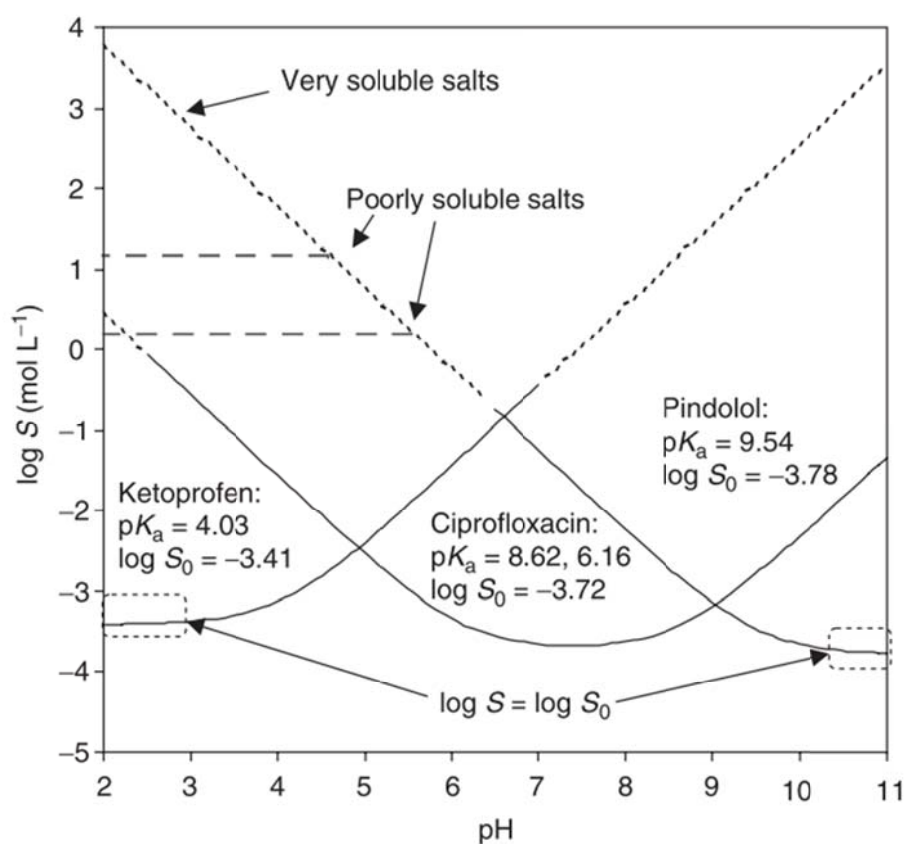


Figura 1.3. Perfils de solubilitat en funció del pH del ketoprofè (àcid), pindolol (base) i ciprofloxacina (zwitterió) [8].

1.1.1.3. Ionització i absorció biològica

Tot i l'existència d'altres mecanismes de transport a través de les membranes lipídiques, com per exemple el transport paracel·lular o el transport actiu [12–15], el principal mètode de transport és la difusió passiva. Com ja s'ha comentat, les

molècules neutres són més permeables per aquesta via mentre que les molècules iòniques romanen en fases aquoses tals com la sang i són excretades a través dels ronyons. Per aquest motiu s'ha de tenir en compte el pH del medi a l'hora de preveure l'absorció de fàrmacs.

Molts fàrmacs bàsics són absorbits en l'intestí, on el pH pot variar entre 5 i 8 depenent de quina part es considera, i si el pacient està alimentat o en dejú (els aliments eleven el pH de l'estómac, però redueixen el de l'intestí). El itraconazol (comercialitzat com Sporanox) és un antifúngic que es pot prendre per via oral. Als pacients se'ls aconsella prendre itraconazole amb els àpats, quan el pH del contingut de l'intestí pot baixar a 5 o menys. La paret de l'intestí està protegida per una capa de mucosa amb un pH al voltant de 6, i no es veu molt afectada pel pH del contingut intestinal [2]. Amb valor de pK_a al voltant de 4 i amb una lipofilitat alta, l'itraconazol és una base que a pH alt no es troba ionitzada i és gairebé insoluble [16]. S'ionitza a un pH baix, i seria bastant més soluble a pH 5 que a pH 6. Gràcies a que els continguts intestinals poden baixar fins a pH 5, algunes molècules s'hi poden dissoldre. D'aquesta manera, l'itraconazol pot acostar-se a la paret i transformar-se en la forma neutra que és de fàcil absorció.

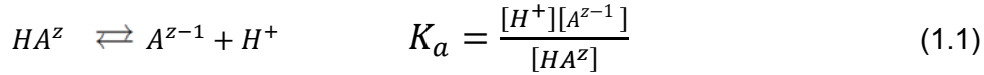
Un altre exemple clar sobre la important relació entre la constant d'acidesa i l'absorció biològica és el sildenafil. Aquest fàrmac és un vasodilatador, i es comercialitza sota el nom de Viagra. És un compost amfòlit, amb un pK_a àcid de 9.12 i un pK_a bàsic de 6.78 [17]. Per tant, el sildenafil es troba carregat a valors de pH alts i baixos, i és neutra un pH entremig d'ambdues constants d'acidesa. Així doncs, al pH intestinal el sildenafil es trobarà en la seva forma més lipofílica, que en altres paraules significa que serà absorbit al tracte gastrointestinal.

Concloent, el coneixement de la constant d'acidesa és de vital importància per a l'estudi del comportament dels fàrmacs dins l'organisme. La ionització de les substàncies altera de forma considerable propietats com la lipofilitat i la solubilitat, fent possible o no l'absorció i la conseqüent funció curativa del fàrmac.

1.1.2. Equilibri àcid-base i la seva constant

L'any 1923, el químic danès Johannes Nicolaus Brønsted i l'anglès Thomas Martin Lowry publicaren de forma independent una teoria sobre el comportament dels àcids i

de les bases que superava la teoria àcid-base vigent d'Arrhenius. La teoria àcid-base de Brøwsted-Lowry defineix que “*un àcid és aquella substància capaç de perdre o donar un protó, mentre que una base és aquella capaç de guanyar o acceptar un protó*”. Així doncs, l'equació general per a una substància monopròtica àcida, HA^z , es pot expressar de la següent manera:



La constant d'acidesa (K_a), és una propietat fonamental dels àcids i bases febles. Per als compostos amb un únic grup ionitzable, es defineix com el pH en el qual un compost s'ionitza al 50%. Aplicant el logaritme a l'expressió (1.1), L.J. Henderson [18] i K.A. Hasselbalch [19] establiren a principis del segle XX l'equació que relaciona el pH (definit com a $-\log [H^+]$) amb el pK_a :

$$pH = pK_a + \log \frac{[base]}{[àcid]} \quad (1.2)$$

No obstant això, quan volem comparar o parlar de constants d'acidesa normalment parlem de constants d'acidesa termodinàmiques (K_a), referida a les activitats, és a dir, a força iònica zero. La constant termodinàmica depèn únicament de la temperatura. L'equació (1.3) mostra la constant per a una àcid i l'equació (1.4) per a una base:

$$K_a = \frac{a_{H^+} a_{A^-}}{a_{HA}} = \frac{a_{H^+} [A^-]}{[HA]} \cdot \frac{\gamma_{A^-}}{\gamma_{HA}} = K'_a \cdot \frac{\gamma_{A^-}}{\gamma_{HA}} \quad (1.3)$$

$$K_a = \frac{a_{H^+} a_B}{a_{BH^+}} = \frac{a_{H^+} [B]}{[BH^+]} \cdot \frac{\gamma_B}{\gamma_{BH^+}} = K'_a \cdot \frac{\gamma_B}{\gamma_{BH^+}} \quad (1.4)$$

Aquí, a_i és l'activitat, i γ_i el coeficient d'activitat de les diferents espècies. La K'_a és la constant a una determinada força iònica.

Considerant que el coeficient d'activitat de les espècies neutres és pràcticament igual a la unitat (γ_{HA} i $\gamma_B \approx 1$), el logaritme de la constant d'acidesa termodinàmica ve donat per:

$$pK_a = pK'_a - \log \gamma_{A^-} \quad (1.5)$$

$$pK_a = pK'_a + \log \gamma_{BH^+} \quad (1.6)$$

on els termes $\log \gamma_{A^-}$ i $\log \gamma_{BH^+}$ s'encarreguen de corregir els efectes de la força iònica del solut ionitzat. Aquests coeficients es poden estimar usant la teoria de Debye-Hückel, equació (1.7):

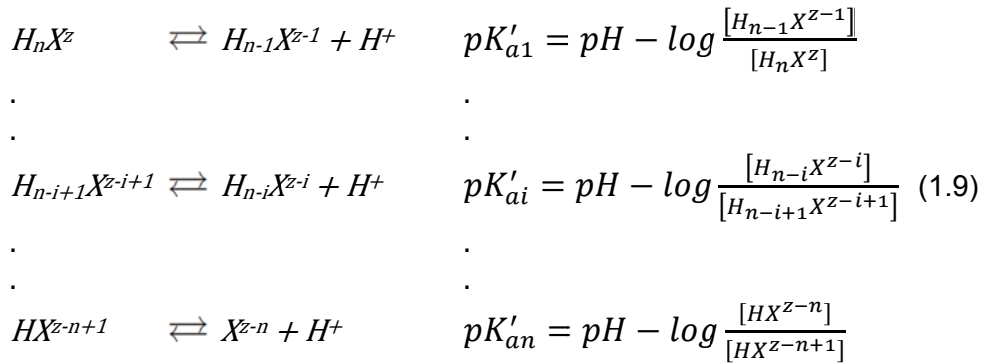
$$-\log \gamma_{A^-} = -\log \gamma_{BH^+} = -\log \gamma = \frac{Az^2\sqrt{I}}{1+Ba_0\sqrt{I}} \quad (1.7)$$

Els paràmetres A i B són dues constants que depenen de la naturalesa del dissolvent i de la temperatura, i la constant a_0 és el radi iònic. Aquestes constants de Debye-Hückel són, per solucions aquoses a 25 °C, 0.5085, 0.3281 i 4.56 Å respectivament. La força iònica de l'equació anterior, I (mol·L⁻¹), és calculable a partir de l'equació (1.8):

$$I = \frac{1}{2} \sum_{i=1}^n c_i z_i^2 \quad (1.8)$$

on z_i és la càrrega i c_i (mol·L⁻¹) és la concentració de cada espècie iònica. Aquesta equació és vàlida per a forces iòniques menors a 0.2 M.

Fins ara s'han tractat equilibris àcid-base per a substàncies monopròtiques. Per a un compost polipròtic totalment protonat, H_nX^z , les equacions en equilibri es representen de la següent manera:



on n és el nombre total de grups ionitzables, z la càrrega de l'espècie totalment protonada, i el pK'_{ai} és la constant d'acidesa a certa força iònica de l'èssim equilibri.

El pK_{ai} termodinàmic es pot calcular a partir dels valors de pK'_{ai} a través dels coeficients d'activitat de les espècies involucrades a l'equilibri ($\gamma_{H_{n-i}X^{z-i}}$ i $\gamma_{H_{n-i+1}X^{z-i+1}}$):

$$pK_{ai} = pK'_{ai} - \log \frac{\gamma_{H_{n-i}X^{z-i}}}{\gamma_{H_{n-i+1}X^{z-i+1}}} \quad (1.10)$$

estimant, de la mateixa manera que per als compostos monopròtics, els coeficients d'activitats a través de l'equació (1.7).

1.1.3. Constants d'acidesa en presència de cosolvents

Les tècniques actuals utilitzades per al desenvolupament de nous fàrmacs a vegades proporcionen molècules poc o gens solubles en aigua. La insolubilitat sovint es converteix en un inconvenient en la caracterització fisicoquímica dels medicaments, ja que molts assajos requereixen que els compost es trobi en solució al llarg del procés experimental [6,20–22]. No obstant això, aquest problema es pot solucionar utilitzant medis mixtes de solvent orgànic i aigua.

S'han utilitzat molts solvent orgànics solubles en aigua com alcohols o acetonitril. Tanmateix, el metanol és el dissolvent més escollit, perquè malgrat que les seves propietats són molt similars a l'aigua, és capaç de dissoldre compostos no solubles en aigua [21–26].

El pK_a en presència de cosolvent s'anomena *constant d'acidesa aparent*, ${}_s pK_a$ (segons la IUPAC), on s significa la mescla de la barreja hidro-orgànica en l'escala de pH de la mateixa.

Per a estimar el pK_a aquós, o altrament dit a composició orgànica zero, existeixen diverses equacions. La més coneguda és la proposada per Yasuda-Shedlovsky, basada en el model electrostàtic de Born [27,28]. El treball experimental implica la determinació del pK_a a diverses mescles hidro-orgàniques i l'aplicació de la següent equació:

$$pK_a + \log[H_2O] = \frac{a_\varepsilon}{\varepsilon} + b_\varepsilon \quad (1.11)$$

que expressa una relació lineal entre el terme $pK_a + \log[H_2O]$ i la inversa de la constant dielèctrica, $1/\varepsilon$. a_ε i b_ε són les constant que depenen de cada substància. El valor de pK_a aquós es troba quan la constant dielèctrica pren el valor corresponent a l'aigua pura, $\varepsilon = 78.3$. L'equació (1.11) és vàlida per a solucions amb valor de $\varepsilon > 50$, Pel cas de mescles metanol/aigua aquest representa un contingut inferior al 66% en volum (o aproximadament al 60% en pes) de metanol. Valors fora d'aquest interval comporten a

una pèrdua de linealitat, atribuïda a què a valors baixos de ϵ , es comença a tenir una presència important de parells iònics. També podria interpretar-se com a una desviació deguda a la solvatació preferencial del solut, el que implicaria que la esfera de solvatació del solut fos de composició diferent, i per tant, tingués un valor de ϵ diferent al del si de la dissolució que s'inclou a la equació (1.11). A proporcions més baixes de metanol el efectes responsables de la variació del pK_a són deguts a efectes electrostàtics [21–23].

1.1.4. La necessitat de mètodes ràpids per a la determinació de constants d'acidesa

El descobriment de fàrmacs ha sofert canvis considerables amb l'avenç de noves tecnologies i estratègies. Aquests avenços han donat lloc a noves oportunitats per reunir i integrar la informació, augmentant l'èxit i l'eficàcia del descobriment de fàrmacs. Tot això fa que les empreses farmacèutiques sintetitzin un gran nombre de fàrmacs potencials i precursors químics en períodes relativament curts [29,30]. Posteriorment, aquells que són els més adequats se seleccionen per a la seva anàlisi posterior i desenvolupament. Els laboratoris que mesuraven menys de deu valors de pK_a al mes fa menys de dos dècades han hagut d'adaptar o desenvolupar nous procediments per gestionar peticions de diversos centenars de mesuraments de pK_a per setmana [31]. Com a conseqüència, es necessita un cribratge d'alt rendiment per a l'avaluació ràpida d'aquests compostos un cop són disponibles després de la seva síntesi [3,4]. La disponibilitat d'informació sobre les propietats fisicoquímiques en les primeres etapes de la selecció d'un compost permet temps de desenvolupament més curts i una reducció en la quantitat de candidats en els programes de recerca de nous fàrmacs [6,20,31]. Així doncs, es requereix la mesura de moltes mostres en poc temps, en altres paraules, es necessiten mètodes d'alt rendiment, o més conegut en anglès com a mètodes *high throughput*.

1.1.5. Mètodes per a la determinació de constants d'acidesa

Per a mesurar valors de pK_a cal exposar el compost a un ambient de canvi de pH, i supervisar una propietat que canvia en funció de l'estat de ionització de la molècula. A més, és imprescindible mantenir la temperatura i la força iònica constant per a totes les mesures. Diverses consideracions afecten la tria del mètode. Per exemple, si la mostra es troba en la seva forma no ionitzada i és poc soluble en aigua, ha de ser mesurada en una solució molt diluïda, o bé ser analitzada en barreges d'aigua-dissolvent. Si se'n disposa d'una quantitat molt petita (per exemple, menys d'1 mg), els únics mètodes pràctics impliquen majoritàriament l'espectroscòpia UV. D'altra banda, com s'ha comentat a la secció 1.1.4, factors com la rapidesa en la determinació *high throughput* d'aquestes constants també són d'importància vital a l'hora d'escollir una metodologia adequada.

Hi ha diverses tècniques per a la determinació de les constants d'acidesa. Els mètodes més utilitzats es basen en valoracions potenciomètriques [21–23,32–35], espectroscòpiques [32,36–39] i mesures electroforètiques [40–55]. També se n'han desenvolupat d'altres menys utilitzades com ara espectroscòpia Raman, RMN, conductivimetria, termometria o la cromatografia de líquids [32,56]. Al llarg dels últims 15 anys s'han publicat molts articles comparant alguns dels avantatges i inconvenients d'aquestes tècniques [57–62]. Tanmateix, en aquest apartat s'explicarà una breu pinzellada dels més importants, i més extensament el mètode per a electroforesi capil·lar en les seccions 1.3 i 1.4.

Les vastament conegudes valoracions potenciomètriques donen resultats molt precisos, però la mostra ha de ser de gran puresa i totalment soluble en tot l'interval de pH. Per tal de detectar un canvi significatiu al punt d'equivalència de la corba, es necessita treballar per sobre de concentracions de l'ordre de $5 \cdot 10^{-4}$ M. Una valoració típica entre pH 3 i 11 requereix al voltant de 45 valors de pH mesurats. A més, la necessitat d'esperar a l'equilibri per a cada mesura de pH fa que no sigui un mètode ràpid, fins i tot per a valoracions automatitzades.

D'altra banda, les valoracions espectrofotomètriques són més simples i requereixen concentracions significativament menors, però també són lentes i només s'apliquen quan el compost d'interès té un grup cromòfor en la seva estructura amb diferents

propietats espectrals entre la forma iònica i la forma neutra. A més, igual que en les valoracions potenciomètriques, la puresa elevada de la mostra també és obligatòria.

Una variació molt atractiva és l'instrument híbrid de pH-metric/UV [8,36,63]. Aquest instrument és eficient i altament automatitzat, on el resultat s'obté a partir dels canvis en l'espectre UV en funció del pH. En alguns casos, les mesures es poden realitzar fins i tot quan apareix precipitació acoblant una sonda anomenada DPAS (*dip probe absorption spectroscopy*) [64].

L'electroforesi capil·lar és una altra tècnica automatitzada i a més, altament utilitzada per a la determinació de les constants d'acidesa. Aquesta tècnica necessita petites quantitats de mostra i reactius. Les impureses no són un problema perquè són separades de l'anàlit en el procés d'electroforesi. Els temps de separació poden ser de l'ordre de pocs minuts. No es requereix informació sobre la concentració ja que només es necessiten les mobilitats electroforètiques i normalment disposa de sistemes per a controlar la temperatura.

També val la pena esmentar aquí, que la comparació dels resultats obtinguts mitjançant diverses tècniques analítiques i dades de la literatura assenyalen la consistència satisfactòria dels resultats i la possibilitat d'utilitzar diferents tècniques indistintament. Entre aquestes tècniques, la CE sembla ser una de les preferides per a mesures ràpides i precises [55]. En altres paraules, l'electroforesi capil·lar s'ha creat un lloc entre tota la col·lecció de mètodes per a la determinació de constants d'acidesa.

1.2. ELECTROFORESI CAPIL·LAR

1.2.1. Fonament teòric

L'electroforesi capil·lar és un mètode de separació que es basa en la diferent velocitat de migració de les espècies carregades en el si d'un medi conductor o electròlit suport (conegut en anglès com a *background electrolyte* o BGE) dins d'un capil·lar de diàmetre intern petit (normalment de 50 μm) a través del qual s'hi aplica un camp elèctric constant.

A valors per sobre de pH 2, la paret interna d'un capil·lar de sílice presenta càrrega negativa degut a la ionització dels grups silanol (Si-OH) de la superfície. Els cations del BGE s'agrupen sobre la superfície negativa del capil·lar de sílice per formar la doble capa elèctrica: d'una banda, es crea una capa interna on els cations queden fixats a la paret del capil·lar; d'altra banda, com aquests cations no són suficients per a neutralitzar tota la càrrega negativa dels grups silanol, una segona capa de cations és atreta cap a la superfície del capil·lar però amb menor força, per la qual cosa aquesta segona capa és mòbil. En aplicar una diferència de potencial, els cations de la capa exterior són atrets cap al càtode o elèctrode negatiu, creant un flux de líquid que es coneix com a flux electroosmòtic (EOF). Degut a que els ions estan solvatats, arrosseguen la resta de la solució al llarg del capil·lar. L'electroosmosi provoca un flux en la dissolució amb un perfil pla, perpendicular al capil·lar (Figura 1.4). [65].

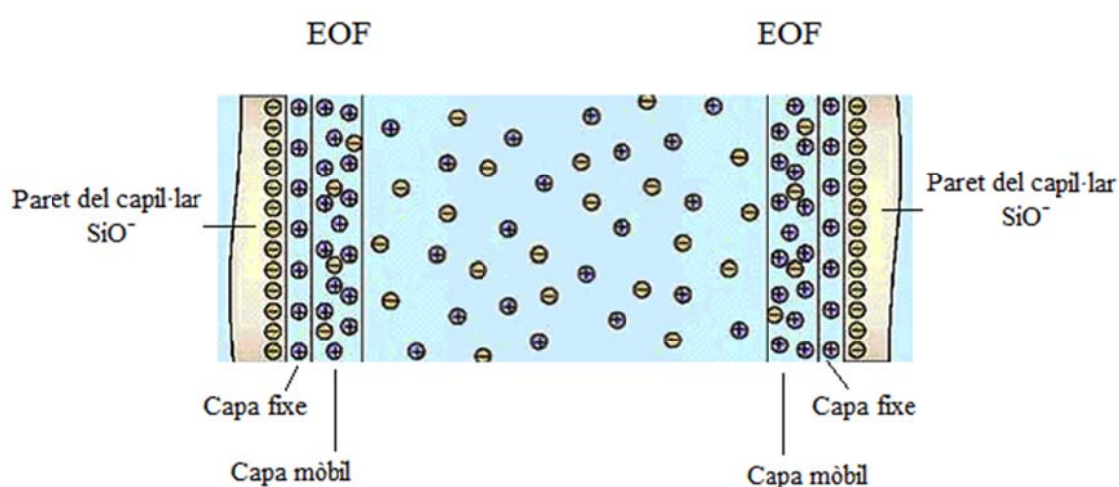


Figura 1.4. Generació del flux electroosmòtic.

Gràcies a aquest factor, el flux electroosmòtic no contribueix de manera significativa a l'eixamplament de banda de la mateixa manera que ho fa el flux laminar conduït per la pressió en HPLC.

Encara que tota la solució de dins el capil·lar és conduïda pel flux electroosmòtic, en injectar una mescla de compostos carregats i elèctricament neutres i aplicar una diferència de potencial entre els extrems del capil·lar, s'observen diferències en el moviment de les substàncies. Aquest fet es deu a que el moviment de les substàncies carregades no està regit únicament per la velocitat de l'EOF, sinó també per la mobilitat electroforètica, μ_e , que varia per a qualsevol molècula carregada segons la seva mida i càrrega. Fent balanç entre les forces d'electromobilitat i fricció, la mobilitat electroforètica, constant per a cada ió determinat, pot expressar-se com:

$$\mu_e = \frac{q}{6 \pi \eta r} \quad (1.12)$$

on q és la càrrega de l'ió, η és la viscositat del BGE i r és el radi de l'ió solvatat. Si tenim una mescla de diferents substàncies, a l'aplicar un camp elèctric les diferents μ_e faran que els anions migrin cap al pol positiu (ànode) i els cations cap al pol negatiu (càtode). A més, els ions més petits i més carregats migraran a major velocitat que els de mida més gran i càrrega menor. En conseqüència, cada molècula carregada té una mobilitat diferent, anomenada mobilitat electroforètica efectiva, μ_{eff} , suma de la mobilitat de l'EOF i la pròpia μ_e . En canvi, les substàncies neutres en no experimentar ni atracció ni repulsió respecte els pols, es mouen totes amb el flux electroosmòtic i per tant, no és possible la separació entre elles (Figura 1.5) [65,66].

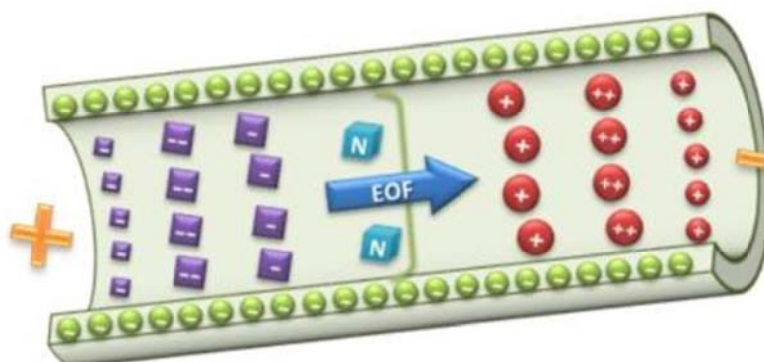


Figura 1.5. Representació de la migració electroforètica de diferents molècules en un capil·lar de sílice fosa. N representa les substàncies neutres.

Els principals paràmetres a tenir en compte en l'optimització d'una separació per CE són la naturalesa i composició del BGE, sovint una solució tampó, és a dir la seva concentració i pH. Altres factors importants que afecten a la separació i que en alguns casos estan interrelacionats són: la quantitat de mostra a injectar; la mida del capil·lar (longitud i diàmetre); la calor generada per efecte Joule; la temperatura de treball; i el voltatge emprat en la separació. El control d'aquests factors és imprescindible per a poder aconseguir separacions ràpides, reproduïbles i d'alta resolució [66].

1.2.2. Paràmetres d'interès per a la determinació de constants d'acidesa

En un equilibri àcid-base com al que es mostra en l'equació (1.1) de la secció 1.1.2, la relació entre $[A^-]$ i $[HA]+[A^-]$ determina el grau de ionització d'una determinada substància. De la mateixa manera, la mobilitat efectiva, μ_{eff} , d'un àcid monopròtic (HA) depèn d'aquest grau de ionització. Quan només existeix A^- aquesta mobilitat és constant i s'anomena mobilitat de l'espècie desprotonada, μ_{A^-} , o mobilitat límit. La relació d'ambdues mobilitats amb la constant d'acidesa ve determinada per la següent equació:

$$\mu_{eff} = \frac{\mu_{A^-}}{1+10^{pK_a-pH}} \quad (1.13)$$

Igual que pels àcids, la μ_{eff} d'una base monopròtica (B) ve determinada pel seu grau de ionització. Quan només existeix BH^+ aquesta mobilitat és constant i s'anomena mobilitat de l'espècie protonada, μ_{BH^+} o mobilitat límit. La relació d'ambdues mobilitats amb la constant d'acidesa ve determinada per la següent equació:

$$\mu_{eff} = \frac{\mu_{BH^+}}{1+10^{pH-pK_a}} \quad (1.14)$$

Extrapolant aquestes bases a compostos polipròtics seguint la nomenclatura de l'equació (1.9) present en de la secció 1.1.2, la mobilitat efectiva es pot expressar en funció del pH i del pK_a de cada equilibri amb l'equació general (1.15):

$$\mu_{eff} = \frac{\mu_{H_n X^z} + \sum_{i=1}^n 10^{ipH - \sum_{j=1}^i pK'_{aj}} \mu_{H_{n-i} X^{z-i}}}{1 + \sum_{i=1}^n 10^{ipH - \sum_{j=1}^i pK'_{aj}}} \quad (1.15)$$

on $\mu_{H_n X^z}$ és la mobilitat de l'espècie més protonada i $\mu_{H_{n-i} X^{z-i}}$ és la mobilitat de les espècies desprotonades successives.

Experimentalment la μ_{eff} ($\text{cm}^2 \text{V}^{-1} \text{min}^{-1}$) es pot calcular directament a través de l'equació (1.16).

$$\mu = \frac{L_T L_D}{V} \left(\frac{1}{t_m} - \frac{1}{t_0} \right) \quad (1.16)$$

Aquesta depèn bàsicament de dos paràmetres: el primer és el temps de migració del compost (t_m); i el segon el temps de migració del marcador de flux electroosmòtic (substància neutra no retinuda que marca la velocitat del flux electroosmòtic, t_0). La longitud total del capil·lar (L_T), la longitud efectiva (distància entre l'extrem del capil·lar més allunyat de la finestra de detecció i la pròpia finestra, L_D) i el voltatge (V) són constants dins d'un sistema electroforètic específic.

1.3. MÈTODES ELECTROFORÈTICS PER A LA DETERMINACIÓ DE CONSTANTS D'ACIDESA

S'han publicat molts articles científics sobre la determinació de constants d'acidesa per electroforesi capil·lar, on la majoria han estat compilats per S.K. Poole et al. [31] i P. Nowak et al [55]. El compendi comprèn una gran diversitat de compostos i metodologies dins l'abast de la tècnica. En aquesta secció es comenten les característiques i tipus de metodologies existents per a la determinació de les constants d'acidesa per electroforesi capil·lar.

1.3.1. Introducció

La majoria de metodologies es basen en el mètode clàssic, consistent en tres passos: (i) preparació de diversos tampons a diferents valors de pH; (ii) mesura de les mobilitats electroforètiques d'anàlits i de marcador de EOF; (iii) i per últim, la determinació del punt d'inflexió de la corba mobilitat versus pH traçada per regressió no lineal. No obstant, s'han introduït diverses modificacions com per exemple: monitoritzar l'absorbància en lloc de la mobilitat electroforètica o utilitzar electròlits que contenen cosolvents orgànics. A la Figura 1.6 es mostra un petit esquema sobre les possibilitats que ofereix la determinació de constants d'acidesa per CE.

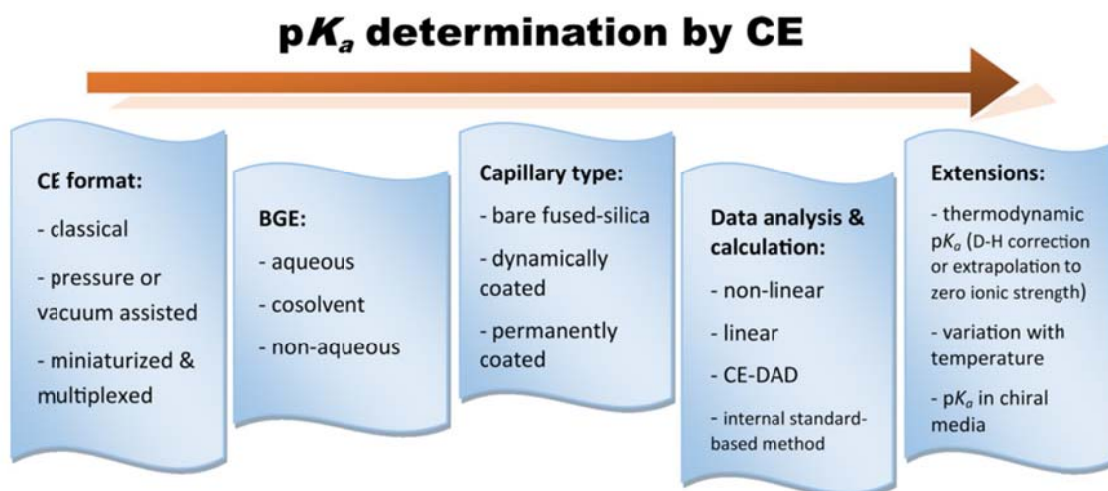


Figura 1.6. Esquema dels diversos passos per a la determinació pK_a per CE. D-H: model de Debye-Hückel. DAD: acrònim angles de *diode array detector* [55].

Cada requadre d'aquesta figura permet una opció i totes les combinacions entre aquests elements particulars estan permeses. Aquest fet representa un ampli espectre de solucions i possibilitats, algunes de les quals fins ara han estat inexplorades.

Aplicar pressió a l'entrada del capil·lar o el buit a la seva sortida, són algunes de les metodologies emprades per tal d'aconseguir separacions més ràpides [44–47,67,68]. Alternativament, alguns autors [69–71] utilitzen recobriments dinàmics o permanents en capil·lars de sílice (il·lustrat com a capil·lars *dynamically or permanently coated* a la Figura 1.6) per eliminar, incrementar o mantenir constant el flux electroosmòtic a qualsevol pH de treball.

Seguidament, s'explicarà més detalladament en què consisteix el mètode clàssic. D'altra banda, cal esmentar que el nostre grup de treball ha desenvolupat un nou mètode electroforètic basat en la utilització d'un patró intern. Degut a que aquesta tesi està enfocada principalment en el desenvolupament i optimització d'aquest mètode, se li ha dedicat una secció única i exclusiva de la introducció, la secció 1.4.

1.3.2. Mètode clàssic

El mètode clàssic es basa en determinar la mobilitat del compost a diversos valors de pH. Per tal d'utilitzar aquest mètode cal tenir varies solucions tampó que cobreixin un interval mínim de pH entre $pK_a \pm 4$ de l'anàlit per tal d'assegurar que el punt d'inflexió de la corba correspon a la constant.

Cada pH és mesurat potenciomètricament i de forma acurada just abans de l'anàlisi. Segons el pH, la substància analitzada presenta diferents graus de ionització, donant temps de migració diferents dins del capil·lar. Aquestes diferències en els temps de migració condueixen a una mobilitat electroforètica o una altra. A partir dels temps de migració es registren i es calculen les mobilitats a partir de l'equació (1.16) de la secció 1.2.2. Sabent que aquesta mobilitat és la mobilitat efectiva de l'anàlit i que el pH ha estat mesurat prèviament via pH-metre, obtenim llavors una sèrie de valors representables en una gràfica, on a l'eix de les abscisses s'hi representa el valor de pH de la substància i al d'ordenades el valor de μ_{eff} . Les dades s'ajusten a l'equació no lineal que relaciona ambdues variables i els valors de pK_a es determinen per interpolació. Aquestes equacions es troben resumides a la secció 1.2.2, essent per a

substàncies monopròtiques àcides l'equació (1.13), per a bases monopròtiques l'equació (1.14), i per a polipròtics l'equació general (1.15).

Un exemple de l'aplicació d'aquest mètode és la quinina, una base feble dipròtica utilitzada anteriorment per combatre la malària i actualment en begudes refrescants. Aquesta substància pot trobar-se en tres estats diferents en funció del pH: H_2X^{2+} , HX^+ , X . Calculant la μ_{eff} a diferents valors de pH i representant-los en un gràfic, trobarem el perfil d'una corba tal i com es mostra en la Figura 1.7, marcats com a ($\circ$). Seguidament, aquest valors s'ajusten (línia contínua de la figura) i els valors de pK_a , marcats com a ($\bullet$), es determinen per l'ajust.

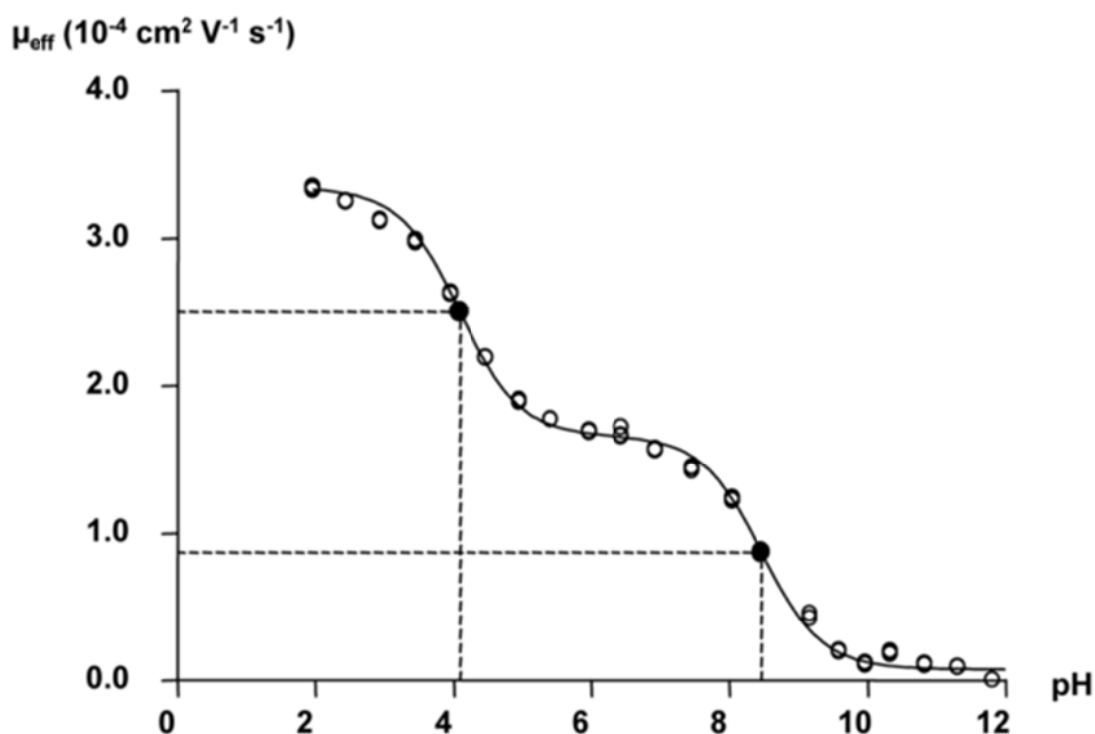


Figura 1.7. Relació entre la μ_{eff} i el pH del tampó per un compost dipròtic bàsic, la quinina. Els punts experimentals estan representats com a ($\circ$) i l'ajust a l'equació general (1.15) com a línia contínua. Els punts ($\bullet$) representen els valors de pK_a extrapolats [69].

Per a una bona determinació de la constant d'acidesa, factors com ara la tria de les solucions tampó i la seva concentració poden resultar claus. Els tampons han de tenir capacitat amortidora suficient, han de minimitzar el problema de l'escalfament per efecte Joule i no han de presentar interaccions específiques amb l'anàlit.

En aquest mètode, el pH de les solucions tampó és crític i cal que sigui mesurat just abans de determinar la mobilitat. Un mal condicionament del capil·lar amb la solució tampó, l'electròlisi d'aquest a causa de la diferencia de potencial i l'absorció de CO₂ de l'atmosfera són problemes freqüents que afecten al valor de pH de les mesures. Tanmateix, alguns d'aquests problemes es poden minimitzar canviant les solucions amortidores per unes de noves cada 5 o 6 anàlisis o de vegades preparant-les de nou.

En termes de rendiment, el mètode clàssic és un mètode lent ja que les mobilitats electroforètiques s'han de determinar en una ampli interval de valors de pH. Tenint en compte que s'han de preparar al voltant de 20 solucions tamponades per a cobrir tot l'interval de pH útil en CE, es necessiten unes hores per a realitzar la determinació d'una constant d'acidesa. No obstant això, en els darrers anys algunes aproximacions i nous dissenys s'han dut a terme per a la determinació *high throughput* d'aquestes constants per CE. Entre elles destaquen: la utilització de sistemes amb 96 capil·lars electroforètics [47–49,72,73]; la miniaturització per microxip [70]; o la determinació simultània de la μ_{eff} d'un conjunt elevat de compostos utilitzant detectors més sensibles i selectius com l'espectrometria de masses [74].

1.4. IS-CE, INTERNAL STANDARD CAPILLARY ELECTROPHORESIS

El grup de treball ha desenvolupat un nou mètode per a la determinació de constants d'acidesa anomenat mètode del patró intern per electroforesi capil·lar o IS-CE (*Internal Standard Capillary Electrophoresis*) [75]. El mètode IS-CE és més ràpid i eficaç que el mètode clàssic. Utilitza l'electroforesi capil·lar, però se centra en l'ús d'un compost de referència, anomenat patró intern o IS (de l'acrònim anglès *Internal Standard*), de naturalesa i pK_a similars als de l'anàlit. Aquest mètode calcula el pK_a de l'anàlit a partir de les diferències entre les mobilitats relatives d'ambdós compostos i la constant d'acidesa de l'IS, suposant que l'IS i l'anàlit es comporten de la mateixa manera en les condicions d'anàlisi. Cal afegir, que el mètode IS-CE ha estat provat per a compostos monopròtics de diferent naturalesa química, obtenint valors de pK_a amb desviacions mitges respecte els valors de la literatura de 0.05 unitats [75,76].

Per a una millora en la lectura i comprensió d'aquest mètode, aquesta secció s'ha dividit en quatre parts: en la primera s'explicarà com funciona el mètode; en la segona els fonaments i equacions per utilitzar-lo; en la tercera es comentaran algunes consideracions a tenir en compte per a la seva utilització; i en la quarta i última part, els seus avantatges respecte als altres mètodes.

1.4.1. Procediment

El mètode IS-CE consisteix en injectar l'anàlit i el patró intern conjuntament a dos o més valors de pH diferents. Un primer pH on l'anàlit i l'IS estiguin parcialment ionitzats, és a dir, a un interval de $pH \pm 1$ unitats respecte al valor de pK_a a fi de tenir un percentatge acceptable tant d'espècie iònica com de neutra dels dos compostos. I un segon pH on ambdós estiguin totalment ionitzats, en altres paraules, a $pH > pK_a + 2$ en el cas dels àcids i $pH < pK_a - 2$ en el cas de les bases.

Un cop obtinguts els dos electroferogrames, a partir dels temps de migració s'obtenen les mobilitats efectives i de les formes totalment ionitzades de l'anàlit i de l'IS. De la relació entre les diferents mobilitats de l'anàlit i del patró intern s'obté la diferència

entre els valors de pK_a de l'anàlit i de l'IS. Per tant, coneixent exactament el pK_a de l'IS pot determinar-se fàcilment el de l'anàlit.

1.4.2. Fonament teòric

En aquesta secció s'expliquen els fonaments i les equacions emprades en el mètode IS-CE per a la determinació de constants d'acidesa d'àcids i bases monopròtics. Atès que la determinació de constants d'acidesa de substàncies polipròtiques forma part de la recerca original d'aquesta tesi, seran considerades a la segona secció del Capítol III d'aquest treball. També es pot trobar a la publicació presentada a l'article II.

1.4.2.1. Àcids monopròtics

Suposant un àcid monopròtic, HA, la seva mobilitat electroforètica (μ_{eff}) es pot relacionar amb la constant d'acidesa (o el logaritme d'aquesta, pK_a) i el pH, tal com es reflecteix a l'equació (1.13) de la secció 1.2.2. Aïllant el pK_a de la equació s'obté:

$$pK'_a = pH + \log \frac{\mu_{A^-} - \mu_{eff}}{\mu_{eff}} = pH + \log Q \quad (1.17)$$

on Q és el coeficient que relaciona la mobilitat límit (μ_{A^-}) i la mobilitat efectiva (μ_{eff}). Aquestes mobilitats ($\text{cm}^2\text{V}^{-1}\text{min}^{-1}$) es poden calcular directament amb el temps de migració del compost (t_m) i el marcador de flux electroosmòtic (t_0) a través de l'equació (1.16) de la secció 1.2.2.

Utilitzant un patró intern (IS), l'equació (1.17) es pot escriure dues vegades: una per a l'anàlit (AN), equació (1.18); i una altra per a l'IS, equació (1.19).

$$pK'_{a(AN)} = pH + \log \frac{\mu_{AN^-} - \mu_{eff(AN)}}{\mu_{eff(AN)}} = pH + \log Q_{AN} \quad (1.18)$$

$$pK'_{a(IS)} = pH + \log \frac{\mu_{IS^-} - \mu_{eff(IS)}}{\mu_{eff(IS)}} = pH + \log Q_{IS} \quad (1.19)$$

Restant ambdues equacions el pH es simplifica i deixa de ser una variable a determinar. Si s'aïlla la constant de l'anàlit s'obté:

$$pK'_{a(AN)} = pK'_{a(IS)} + \log Q_{AN} - \log Q_{IS} \quad (1.20)$$

Si AN i IS són compostos de la mateixa naturalesa àcid-base i càrrega, els coeficients d'activitats se simplifiquen. Així doncs, la constant termodinàmica es pot trobar directament tal i com es mostra a la equació (1.21).

$$pK_{a(AN)} = pK_{a(IS)} + \log Q_{AN} - \log Q_{IS} \quad (1.21)$$

Per tant, injectant els dos compostos en un tampó a un pH on estiguin totalment desprotonats (per determinar μ_{AN^-} i μ_{IS^-}) i en un altre tampó on estiguin parcialment ionitzats (per determinar $\mu_{eff(AN)}$ i $\mu_{eff(IS)}$), podrem calcular el pK_a sense determinar exactament el pH, ja que aquest no es troba implicat a l'equació.

1.4.2.2. Bases monopròtiques

Quan es té una base monopròtica, B, la mobilitat també es podrà relacionar amb la constant d'acidesa i el pH, tal com es reflecteix a l'equació (1.14) de la secció 1.2.2. Si s'aïlla el pK'_a d'aquesta equació s'obté:

$$pK'_a = pH - \log \frac{\mu_{BH^+} - \mu_{eff}}{\mu_{eff}} = pH - \log Q \quad (1.22)$$

on Q torna a ser el coeficient que relaciona la mobilitat límit (μ_{BH^+}), i la mobilitat efectiva (μ_{eff}). De la mateixa manera que per als àcids, amb un patró intern (IS), l'equació (1.22) es pot escriure per al AN, equació (1.23), i per a l'IS, equació (1.24).

$$pK'_{a(AN)} = pH - \log \frac{\mu_{AN^+} - \mu_{eff(AN)}}{\mu_{eff(AN)}} = pH - \log Q_{AN} \quad (1.23)$$

$$pK'_{a(IS)} = pH - \log \frac{\mu_{IS^+} - \mu_{eff(IS)}}{\mu_{eff(IS)}} = pH - \log Q_{IS} \quad (1.24)$$

Restant aquestes dues equacions i aïllant el $pK'_{a(AN)}$:

$$pK'_{a(AN)} = pK'_{a(IS)} - \log Q_{AN} + \log Q_{IS} \quad (1.25)$$

Novament, al restar, el pH se simplifica i deixa de ser una variable a determinar. Si els dos compostos també tenen la mateixa naturalesa àcid-base, els coeficients d'activitat s'eliminen i la constant de l'anàlit s'obté tal com indica l'equació (1.26).

$$pK_{a(AN)} = pK_{a(IS)} - \log Q_{AN} + \log Q_{IS} \quad (1.26)$$

Aquí la determinació del $pK_{a(AN)}$ també requereix la determinació de les mobilitats de les espècies ionitzades, μ_{AN^+} i μ_{IS^+} , les mobilitats de les espècies parcialment ionitzades, $\mu_{eff(AN)}$ i $\mu_{eff(IS)}$, i, el valor del $pK_{a(IS)}$.

1.4.2.3. Utilitzant compostos de diferent naturalesa àcid-base

Sempre que es pugui, es recomana que el patró intern sigui de la mateixa naturalesa àcid-base que la de l'anàlit. No obstant això, un treball publicat pel nostre grup de recerca [77] ha demostrat que es pot utilitzar un IS de naturalesa àcida per a determinar el pK_a d'un anàlit bàsic, i l'inrevés, argumentant que la similitud estructural entre AN i IS no suposa un requeriment essencial en el mètode IS-CE. Per tant, podrem determinar constants d'acidesa utilitzant mesclures monoprotiques mixtes per dues vies.

El primer cas és la determinació d'un AN àcid a partir d'un IS bàsic. Igual que pels àcids i les bases monoprotiques, podem restar les equacions (1.18) i (1.24) i aïllar el $pK'_{a(AN)}$ per donar l'equació següent:

$$pK'_{a(AN)} = pK'_{a(IS)} + \log Q_{AN} + \log Q_{IS} \quad (1.27)$$

El segon cas és la determinació d'un AN bàsic a partir d'un IS àcid. De la mateixa manera, restarem les equacions (1.23) i (1.19) i aïllarem el $pK'_{a(AN)}$, obtenint l'equació següent:

$$pK'_{a(AN)} = pK'_{a(IS)} - \log Q_{AN} - \log Q_{IS} \quad (1.28)$$

Un cop més, aquestes equacions no són pH-dependents. Per calcular la constant d'acidesa de l'anàlit, cal saber la constant del patró intern i les mobilitats iòniques i efectives dels dos compostos. Degut a que en aquests casos la naturalesa àcid-base no és la mateixa, la constant d'acidesa termodinàmica només podrà ser calculada corregint la força iònica a partir de l'equació (1.5) de la secció 1.1.2, si es tracta d'un anàlit àcid, o l'equació (1.6) de la secció 1.1.2, si es tracta d'una base. Llavors, substituït i arreglant termes, l'equació (1.27) es transformarà en la següent:

$$pK_{a(AN)} = pK_{a(IS)} + \log Q_{AN} + \log Q_{IS} - 2\log \gamma \quad (1.29)$$

i l'equació (1.28):

$$pK_{a(AN)} = pK_{a(IS)} - \log Q_{AN} - \log Q_{IS} + 2\log \gamma \quad (1.30)$$

Es pot observar que aquestes dues equacions no depenen pH, però si depenen de l'activitat iònica dels coeficients del AN i de l'IS ($\log \gamma$). Aquest coeficients es poden estimar seguint l'aproximació de Debye-Hückel, equació (1.7) de la secció 1.1.2.

1.4.3. Consideracions

Fins ara en aquesta secció s'han comentat els procediments i els fonaments teòrics per a la determinació de constants d'acidesa per al mètode IS-CE. Tanmateix, el mètode requereix d'unes consideracions per a disposar de tampons de pH i patrons interns adequats. En aquesta secció es discuteix quins són els principals punts a tenir en compte per tal d'utilitzar bons BGE i bons IS.

1.4.3.1. Consideracions del BGE

Com en el mètode clàssic d'electroforesi capil·lar, hi ha varis factor que s'han de controlar. Entre tots ells, tres són els principals a considerar de cara a una bona determinació de les constants d'acidesa.

Primerament, la força iònica (I) ha de ser la mateixa per a totes les determinacions. Per aquesta raó, tots els tampons s'han de preparar al pH desitjat amb una força iònica determinada i constant. Si s'utilitzen solucions d'electròlits diluïts, aquests han de proporcionar una bona capacitat amortidora, suficient com per mantenir un pH fix quan la mostra és injectada. S'ha estimat que per a determinacions electroforètiques la força iònica òptima està entre 10-100 mM [31,78].

El segon factor a controlar és la temperatura. Encara que el capil·lar estigui termostatzat, l'aplicació d'una diferència de potencial i la generació d'un corrent elèctric dins del capil·lar fa augmentar la temperatura (efecte Joule). Només en el cas que el calor es dissipï d'una manera apropiada, la temperatura dins del capil·lar es mantindrà constant. Una forma fàcil de comprovar si les condicions de treball són adequades o no, és mesurar la intensitat generada per una solució tampó a diversos valors de tensió. Si la temperatura no canvia, llavors la relació entre intensitat i voltatge (I/V) romandrà constant, d'acord amb la llei d'Ohm [79]. Aquest efecte es va comprovar al nostre grup de treball [76], i es va demostrar que per a tots els tampons utilitzats a l'estudi i amb capil·lars de 40 cm de longitud efectiva (L_D), tampons amb força iònica de 50 mM i voltatge de 20 kV, la relació I/V seguia la llei d'Ohm.

El tercer i últim factor a tenir en compte és la interacció específica entre els anàlits o els patrons interns i l'electròlit del tampó. En un altre treball [52] es van avaluar aquestes interaccions amb diferents tipus de tampons, utilitzats normalment per a la determinació de constants d'acidesa en sistemes de CE. Es va observar la formació d'alguns parells iònics entre tampons i anàlits, donant una clara desviació de la mobilitat esperada. Com a conclusió es van escollir un conjunt de tampons que no presentaven interaccions amb els anàlits, que són els s'han utilitzat en aquest treball. Aquesta llista es presentarà en la primera secció del capítol III.

1.4.3.2. Consideracions de l'IS

La consideració més important a tenir en compte a l'hora d'escollir un patró intern és que el pK_a sigui similar al de l'anàlit, ja que les mobilitats efectives dels dos han de ser determinades conjuntament en el mateix electroferograma. Si tinguéssim valors de pK_a massa diferents, no es trobaria un pH on ambdós compostos estiguessin parcialment

ionitzats. Això implica tenir a priori un coneixement aproximat del valor de pK_a de l'anàlit.

Avui en dia existeixen programes de càlcul força bons que permeten una primera aproximació al pK_a . Entre ells destaquen l'ACD/Labs [80] i el SPARC [81]. Un cop obtinguda la primera aproximació del pK_a de l'anàlit, s'escull un pH i un patró adequats per a la determinació. Si el pH del tampó està en la zona de $pK_a \pm 1$ del compost a mesurar, aquest es troba significativament ionitzat (10-90%) i s'escull un IS amb un pK_a a la mateixa zona. A vegades la primera estimació de pK_a de l'anàlit no és correcta. Llavors, el pK_a del AN i de l'IS són massa diferents i com a conseqüència el pH on es realitza la mesura de la μ_{eff} no és l'adequat. Per a un compost monopròtic bàsic, per exemple, l'anàlit mostrarà una mobilitat molt pròxima a la de la seva forma protonada (en el cas que es seleccioni un pH massa baix), o una mobilitat propera a zero (en cas que es seleccioni un pH massa alt). Pel cas d'un àcid, passarà exactament el contrari. Si això passa, el mètode no es pot aplicar. Com que el valor de la μ_{eff} estarà sempre comprès entre la seva mobilitat límit i zero, depenent del valor obtingut de μ_{eff} a la primera aproximació, es pot escollir fàcilment un nou pH i IS, si es necessari. Per exemple, si la μ_{eff} d'una base feble és molt similar a la seva forma iònica, μ_{BH^+} , voldrà dir que el pH utilitzat per a la determinació és massa baix. Suposant que tenim un coneixement previ erroni del pK_a de l'anàlit, és bastant probable que la mesura adequada de la μ_{eff} sigui a un pH més alt, per exemple dues unitats de pH per sobre. En cas que el μ_{eff} sigui zero, voldrà dir que el pH és massa alt i la base és en la seva forma neutra. Un cop més, s'haurà de realitzar la mesura de la μ_{eff} dues unitats de pH per sota. Per tant, quan la primera estimació de la pK_a no és prou bona sovint és necessari un altre assaig.

Tot i que probablement no sigui un requisit estricte, és recomanable que els compostos escollits com a patrons interns siguin de naturalesa similar a la dels anàlits.

1.4.4. Avantatges

Com a primer avantatge cal esmentar que el mètode IS-CE utilitza l'electroforesi capil·lar, per la qual cosa aprofita tots els avantatges d'aquesta tècnica ja comentades al penúltim paràgraf de la secció 1.1.4. Alguns exemples són: cal poca mostra i

reactius; és una tècnica de separació; no necessita informació sobre les concentracions dels anàlits i està molt automatitzada.

Apart de tots els beneficis com a tècnica electroforètica, el principal avantatge del mètode IS-CE és que l'ús del patró intern compensa alguns error sistemàtics durant l'experiment. Així doncs amb tant sols dues injeccions en dos valors de pH diferents, es pot determinar la constant d'acidesa d'un nou compost. El factor limitant de la velocitat del mètode clàssic és la necessitat de realitzar moltes anàlisis experimentals de forma separada a diferents valors de pH. Aquest pas, que no aplica als mètodes de valoració potenciomètriques o espectrofotomètriques, tampoc aplica al mètode IS-CE. Mentre que el mètode clàssic necessita aproximadament unes hores per a determinar una constant d'acidesa, l'IS-CE implica un temps mitjà de tant sols 20 minuts.

D'altra banda, un treball realitzat en el mateix grup de recerca [82] ha aplicat aquest mètode per a la determinació de constants a diferents temperatures. En aquest treball es demostra que si l'AN i IS tenen increments d'entalpies estàndards similars, la utilització d'un patró intern compensa possibles fluctuacions incontrolades de temperatura a dintre del capil·lar, com per exemple les degudes a l'efecte Joule.

Capítol II

Publicacions



Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast high-throughput method for the determination of acidity constants by capillary electrophoresis. II. Acidic internal standards

Joan Marc Cabot, Elisabet Fuguet, Clara Ràfols, Martí Rosés*

Departament de Química Analítica, Institut de Biomedicina de la Universitat de Barcelona (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

ARTICLE INFO

Article history:

Received 15 August 2010

Received in revised form 7 October 2010

Accepted 14 October 2010

Available online 23 October 2010

Key words:

Acidity constant

Capillary electrophoresis

 pK_a

Internal standard

High throughput method

ABSTRACT

A fast method for the determination of acidity constants by CZE has been recently developed. This method is based on the use of an internal standard of pK_a similar to that of the analyte. In this paper we establish the reference pK_a values of a set of 24 monoprotic neutral acids of varied structure that we propose as internal standards. These compounds cover the most usual working pH range in CZE and facilitate the selection of adequate internal standards for a given determination. The reference pK_a values of the acids have been established by the own internal standard method, i.e. from the mobility differences between different acids of similar pK_a in the same pH buffers. The determined pK_a values have been contrasted to the literature pK_a values and confirmed by determination of the pK_a values of some acids of the set by the classical CE method. Some systematic deviations of mobilities have been observed in NaOH buffer in reference to the other used buffers, overcoming the use of NaOH in the classical CE method. However, the deviations affect in a similar degree to the test compounds and internal standards allowing thus, the use of NaOH buffer in the internal standard method. This fact demonstrates the better performance of the internal standard method over the classical method to correct mobility deviations, which together with its fastness makes it an interesting method for the routine determination of accurate pK_a values of new pharmaceutical drugs and drug precursors.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The knowledge of the acidity constant K_a (or in logarithmic scale, pK_a) of chemical compounds with acid–base properties is essential in many fields. In fact, the neutral and ionic forms of the chemical substances can have very different physicochemical and biological properties, the acidity constant being sometimes decisive for a given application. This is the case in the pharmaceutical industry, in which the pK_a values have a particular importance [1–3]. Many drugs are weak acids or bases and new applications will depend on their particular physicochemical properties such as acidity, hydrophobicity, and solubility [3,4]. Therefore, pharmaceutical companies must characterize a great number of potential drugs and chemical precursors in a relatively short time to select those which are more suitable for their further test and development. Need of rapid patents of the promising drugs is evident for such companies, which normally compete for similar compounds and applications, thus justifying the increasing demand of fast and reliable physicochemical characterization techniques of potential drugs [1,2].

There are several methodologies for the determination of acidity constants. The ones most used are based on potentiometric [5–7], spectrophotometric [5,8] and electrophoretic measurements [9–15]. Capillary electrophoresis (CE) has been widely used to determine acidity constants. It is a highly automatized technique that uses small amounts of samples and reagents. In addition, impurities are not a problem because they can be easily separated from the analyte during the electrophoretic process [13–17].

Recently, our research group has developed a new method to determine acidity constants by capillary zone electrophoresis (CZE) [18,19], faster and more efficient than the classical one. This method is based on the use of a reference compound or internal standard (IS), with nature and pK_a similar to those of the analyte. The reference compound and the analyte behave in a similar manner in the conditions of the analysis and therefore, the difference between the values of their relative mobilities is directly related to their different acidity. In comparison with other CE methods, the internal standard method requires less electrophoretic measurements and there is no need to measure the pH value of the buffer solution. In fact, for routine pK_a estimation the analyte and the internal standard can be injected at only two different pH values: a first one where the analyte and the IS are partially ionized (at $pH = pK_a \pm 1$) in order to calculate the effective mobility (μ_{eff}), and a second one in which both are completely ionized to calculate the limiting mobility (μ_{A^-} or μ_{BH^+}) for monoprotic compounds. From these mobility

* Corresponding author. Tel.: +34 93 403 92 75; fax: +34 93 402 12 33.

E-mail addresses: marti.roses@ub.edu, marti@apolo.qui.ub.es (M. Rosés).

measurements the pK_a can be directly obtained if the pK_a of the IS is well known. The CE internal standard method is, therefore, an attractive alternative to other methods, which are more expensive because they need higher amounts of pure sample and solvents, and they are more time consuming [5–15]. The main purpose of this paper is to establish the pK_a of a series of acids with different functional groups and structures, covering all the useful pH range in CE, which can potentially be employed as internal standards for the fast and efficient knowledge of the pK_a of new drugs and precursors.

2. Theory

The effective electrophoretic mobility, μ_{eff} , of a monoprotic neutral acid HA can be related to the acidity constant K_a and pH through the following equation [16]:

$$\mu_{\text{eff}} = \frac{\mu_{A^-}}{1 + 10^{pK'_a - \text{pH}}} \quad (1)$$

where μ_{A^-} is the mobility of the completely deprotonated species, and pK'_a is related to the thermodynamic pK_a and the activity coefficient of the anionic species γ_{A^-} , which corrects the effect of the ionic strength (I), as follows:

$$pK_a = pK'_a - \log \gamma_{A^-} \quad (2)$$

The pK_a of the test compound can be easily obtained from Eq. (1):

$$pK'_a = \text{pH} + \log \frac{\mu_{A^-} - \mu_{\text{eff}}}{\mu_{\text{eff}}} = \text{pH} + \log Q \quad (3)$$

For an acid HA, Q is the coefficient of the relative amounts of the neutral and ionic forms, related to μ_{A^-} and μ_{eff} . Such mobilities ($\text{cm}^2 \text{V}^{-1} \text{min}^{-1}$) can be directly calculated from the retention time of the analyte t_m and the electroosmotic flow marker t_0 (min) by means of the expression:

$$\mu = \frac{L_T L_D}{V} \left(\frac{1}{t_m} - \frac{1}{t_0} \right) \quad (4)$$

where L_T and L_D are the total length and the effective length of the capillary, respectively (cm), and V is the applied voltage (V).

In the internal standard method, Eq. (3) is applied to both, the analyte (AN) and the internal standard (IS). The difference between the two equations obtained leads to:

$$pK'_{a,AN} - pK'_{a,IS} = \log Q_{AN} - \log Q_{IS} \quad (5)$$

This equation is independent of pH. Therefore, when injecting both compounds using a given buffer solution in which they are all completely deprotonated (to obtain μ_{A^-}), and a second buffer solution with a different pH where they are partially ionized (to obtain μ_{eff}), $pK'_{a,AN}$ can be calculated despite the pH is not exactly known (since it is not involved in the equation):

$$pK'_{a,AN} = pK'_{a,IS} + \log Q_{AN} - \log Q_{IS} \quad (6)$$

To determine $pK'_{a,AN}$, the value of $pK'_{a,IS}$ is obviously needed, and it should be as accurate as possible.

Notice, that if the ionic forms of the analyte and internal standard have the same charge (such as in all acids studied in this work) then Debye–Hückel equation predicts the same activity coefficient correction for Eq. (2). In this instance, pK'_a can be replaced by pK_a in Eq. (6) for both analyte and internal standard. In other words, the pK_a (or pK'_a) value of the analyte is obtained in the same working conditions (ionic strength) than the pK_a value of the internal standard used to calculate it.

3. Experimental

3.1. Apparatus

Capillary electrophoresis experiments have been performed using a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. The fused-silica capillaries were 50.2 cm length (40 cm to the detector) $\times$ 50 μm ID and were obtained from Composite Metal Services Ltd. (Shipley, West Yorkshire, UK). The temperature of the capillary was $25.0 \pm 0.1^\circ\text{C}$. The samples were injected at a hydrodynamic pressure of 0.5 psi for 3 s, and the applied voltage was 20 kV.

Before the injections, the capillary was conditioned according to the following steps [17,18]: 1.0 M NaOH (20 min), H_2O (10 min), and finally the buffer to be used in the experiment (20 min). Between consecutive injections, the capillary was washed with the same buffer (3 min), and after 4–5 injections, the vial containing the buffer was replaced. When a different buffer was used, the capillary was washed with water (10 min) and afterwards, with the new buffer solution (20 min). At the end of each session, the capillary was washed with 0.1 M NaOH (10 min) and water (15 min).

3.2. Reagents

Benzyl alcohol (analytical-reagent grade), 0.5 M sodium hydroxide, 0.5 M hydrochloric acid, and potassium chloride (>99.5%) were from Merck (Darmstadt, Germany). Anhydrous sodium acetate (>99.6%) was purchased from J.T. Baker (Deventer, The Netherlands). 2-(Cyclohexylamino)ethanesulfonic acid (CHES, >99%), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, >98%) were from Sigma (St. Louis, MO, USA). 2,2-Bis(hydroxymethyl)-2,2'-nitrilotriethanol (BisTris), and sodium formate were from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)amino-methane (Tris, >99.9%) was purchased from Aldrich (Milwaukee, WI, USA). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 M Ω cm.

The test solutes employed were: 2-chlorobenzoic acid, 2,6-dibromo-4-nitrophenol, 4-nitrobenzoic acid, 2,6-dinitrophenol, 3-bromobenzoic acid, 2,4-dinitrophenol, benzoic acid, ibuprofen, nicotinic acid, warfarin, 2,5-dinitrophenol, sulfacetamide, 2,4,6-tribromophenol, 4-nitrophenol, vanillin, 4-hydroxybenzaldehyde, phenobarbital, 3,5-dichlorophenol, methylparaben, 2-chlorophenol, 3-chlorophenol, 4-bromophenol, paracetamol, and phenol. All the compounds were reagent grade or of chromatographic quality and were obtained from Sigma, Fluka, or Carlo Erba (Milan, Italy).

3.3. Preparation of samples and buffers

The buffers employed covered practically all the useful CE pH range (from 3 to 12). They are shown in Table 1, together with the corresponding pH range and their stock solutions. The buffer solutions have been prepared as described in previous work [17]. Briefly, to prepare the buffers at the desired pH and constant ionic strength, 0.5 M HCl, 0.5 M NaOH or 0.5 M KCl was added to 50 mL of the stock solution (0.1 M). Finally, the solution obtained was diluted by adding water up to a volume 100 mL, thus allowing keeping a constant ionic strength of 50 mM.

The internal standard solutions were prepared at a concentration of 1000 $\mu\text{g mL}^{-1}$, in water or in a methanol/water mixture (when they were not soluble in water). Afterwards they were diluted to 100 $\mu\text{g mL}^{-1}$ with water. Benzyl alcohol (100 $\mu\text{g mL}^{-1}$) was then added as the EOF marker to determine the mobilities.

All the samples and buffers were filtered through a nylon mesh 0.45 μm porous size (Whatman, Maidstone, UK) and stored at 4°C until used.

Table 1

Buffer solutions used to determine acidity constants with the internal standard and classical methods by capillary electrophoresis.

Buffer	pK _a	pH range	Stock solution (I = 50 mM)
HCOOH/HCOO ⁻	3.75	2.60–4.80	0.1 M HCOONa + 0.5 M HCl
CH ₃ COOH/CH ₃ COO ⁻	4.76	3.70–5.80	0.1 M CH ₃ COONa + 0.5 M HCl
BisTrisH ⁺ /BisTris	6.48	5.50–7.50	0.1 M BisTrisHCl + 0.5 M NaOH
H ₂ PO ₄ ⁻ /HPO ₄ ²⁻ ^a	7.21	5.80–8.20	0.1 M NaH ₂ PO ₄ + 0.1 M Na ₂ HPO ₄
TrisH ⁺ /Tris	8.08	7.00–9.00	0.1 M TrisHCl + 0.5 M NaOH
H ₃ BO ₃ /H ₂ BO ₃ ^{-a}	9.24	8.20–10.40	0.1 M Na ₂ B ₄ O ₇ + 0.5 M HCl
CHES/CHES ⁻	9.50	8.40–10.10	0.1 M CHESNa + 0.5 M HCl
CAPS/CAPS ⁻	10.40	9.40–11.60	0.1 M CAPSNa + 0.5 M HCl
NaOH ^b		11.8–13.00	0.1 M NaOH + 0.5 M KCl

^a Used only in the classical method.^b Not recommended for the classical method (see text).

4. Results and discussion

Table 2 lists the acids selected as internal standards together with their literature pK_a values (pK_{a,lit}) at 25 °C and zero ionic strength [20–24]. They are of diverse chemical nature and cover the pH range most useful for CE. We selected pK_a values from general sources which are compilations of pK_a values [20,21]. Only when the pK_a of the compound was not found in this reference literature, it was taken from specific research articles [22–24]. Since different pK_a values can be found in the literature for a same compound, it was difficult to choose one reference value for each internal standard. To set up a list with reliable pK_a values, we applied an iterative process with our own experimental electrophoretic data. The advantage of using this procedure is that during the refining process, the initial pK_a values, which could be no good enough, converge to the correct final value (consistent with the measured electrophoretic mobilities for the whole set of internal standards).

The procedure to establish the pK_a reference values was performed as follows. The pK_a value for each acid (pK_{a,AN}) was determined from the electrophoretic measurements using a series of appropriate acids from the same set in Table 2 with pK_{a,IS} = pK_{a,AN} ± 1, used as internal standards. 4–7 internal standards were generally used for each compound. In a first approach the literature pK_a values of the internal standard were used as pK_{a,IS} in Eq. (6) for the calculation of pK_{a,AN}. Then the pK_{a,AN} val-

ues obtained with the different internal standards for the same analyte were averaged. This procedure provided a new list of pK_a values for all the studied compounds, slightly different from the initial literature ones. An iterative process was then applied to obtain a new pK_a value for each compound, from the already measured electrophoretic mobilities and the new obtained pK_a values for the internal standards (instead of the literature ones). A new set of refined average pK_a values was thus obtained. The procedure was repeated until achieving pK_a values differing only ≤0.01 units between consecutive refined values.

Table 3 shows the evolution of the pK_a values of some compounds of Table 2 through the consecutive refining steps. For example, the pK_a of 2-chlorobenzoic acid was calculated from the pK_{a,lit} values of 2,6-dibromo-4-nitrophenol, 4-nitrobenzoic acid, 2,6-dinitrophenol, and 3-bromobenzoic acid, used as internal standards (N=4), and the corresponding electrophoretic results. The calculated pK_a values of 2-chlorobenzoic acid from these four internal standards were 2.91, 2.91, 2.86 and 2.77, respectively, with an average value of 2.86 (see the first value in the column of pK_{a,R1} shown in Table 3). At the same time, the pK_a of 2,6-dibromo-4-nitrophenol was calculated from the pK_{a,lit} values of 2-chlorobenzoic acid, 4-nitrobenzoic acid, 2,6-dinitrophenol, 3-bromobenzoic acid, 2,4-dinitrophenol and benzoic acid (N=6) obtaining pK_{a,R1} = 3.32. Similarly, the pK_{a,R1} of 4-nitrobenzoic acid was calculated from the pK_{a,lit} values of five different internal stan-

Table 2Literature (pK_{a,lit}) and experimental reference (pK_{a,ref}) pK_a values at 25 °C and zero ionic strength of the acidic internal standards, together with the mobilities of the completely deprotonated species μ_A⁻. N is the number of internal standards used in the determination.

Compound	N	pK _{a,lit}	Ref.	pK _{a,ref}	μ _A ⁻ × 10 ⁴ (cm ² min ⁻¹ V ⁻¹)
2-Chlorobenzoic acid	4	2.92	[20]	2.84 ± 0.02	-137.1 ± 0.5
2,6-Dibromo-4-nitrophenol	6	3.38	[20]	3.31 ± 0.03	-122.8 ± 1.1
4-Nitrobenzoic acid	5	3.43	[20]	3.37 ± 0.01	-138.1 ± 0.5
2,6-Dinitrophenol	7	3.74	[20]	3.69 ± 0.01	-149.6 ± 0.9
3-Bromobenzoic acid	8	3.83	[20]	3.79 ± 0.02	-134.1 ± 1.0
2,4-Dinitrophenol	7	4.10	[20]	4.12 ± 0.02	-140.8 ± 1.4
Benzoic acid	7	4.19	[20]	4.22 ± 0.03	-146.2 ± 1.9
Ibuprofen	6	4.43	[23]	4.49 ± 0.02	-95.7 ± 0.6
Nicotinic acid	7	4.82	[21]	4.85 ± 0.03	-143.8 ± 1.6
Warfarin	6	5.08	[23]	5.17 ± 0.04	-84.8 ± 2.3
2,5-Dinitrophenol	4	5.22	[20]	5.30 ± 0.05	-144.2 ± 0.8
Sulfacetamide	4	5.27, 5.32	[23,24]	5.42 ± 0.05	-114.7 ± 0.4
2,4,6-Tribromophenol	5	6.10	[20]	6.04 ± 0.08	-116.0 ± 1.3
4-Nitrophenol	5	7.18	[20]	7.09 ± 0.05	-144.1 ± 0.6
Vanillin	6	7.40	[21]	7.36 ± 0.06	-122.8 ± 1.6
4-Hydroxybenzaldehyde	6	7.51	[20]	7.61 ± 0.04	-137.2 ± 1.7
Phenobarbital	6	7.46	[22]	7.53 ± 0.04	-113.0 ± 1.1
3,5-Dichlorophenol	7	8.18	[20]	8.18 ± 0.04	-130.1 ± 1.8
Methylparaben	7	8.37	[20]	8.35 ± 0.03	-122.1 ± 1.7
2-Chlorophenol	7	8.51	[20]	8.50 ± 0.04	-145.0 ± 1.1
3-Chlorophenol	6	9.02	[20]	9.04 ± 0.01	-144.6 ± 1.3
4-Bromophenol	5	9.36	[19]	9.28 ± 0.01	-136.4 ± 1.2
Paracetamol	4	9.45, 9.75	[21,23]	9.58 ± 0.02	-110.7 ± 0.8
Phenol	3	9.99	[20]	9.89 ± 0.01	-150.7 ± 0.3

Table 3Procedure for establishment of the reference pK_a values ($pK_{a,ref}$) from the literature initial pK_a values ($pK_{a,lit}$) after several pK_a refinements ($pK_{a,Ri}$).

Compound	N	$pK_{a,lit}$	$pK_{a,R1}$	$pK_{a,R2}$	$pK_{a,R3}$	$pK_{a,R4}$	$pK_{a,ref}$
2-Chlorobenzoic acid	4	2.92	2.86	2.85	2.85	2.84	2.84 ± 0.02
2,6-Dibromo-4-nitrophenol	6	3.38	3.32	3.32	3.31	3.31	3.31 ± 0.03
4-Nitrobenzoic acid	5	3.43	3.40	3.38	3.38	3.37	3.37 ± 0.01
2,6-Dinitrophenol	7	3.74	3.69	3.69	3.69	3.69	3.69 ± 0.01
3-Bromobenzoic acid	8	3.83	3.80	3.79	3.79	3.79	3.79 ± 0.02
2,4-Dinitrophenol	7	4.10	4.12	4.12	4.12	4.12	4.12 ± 0.02
Benzoic acid	7	4.19	4.20	4.20	4.21	4.21	4.22 ± 0.03
Ibuprofen	6	4.43	4.47	4.48	4.48	4.49	4.49 ± 0.02
Nicotinic acid	7	4.82	4.80	4.84	4.84	4.85	4.85 ± 0.03
Warfarin	6	5.08	5.13	5.15	5.15	5.16	5.17 ± 0.04
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮

dards, etc. Once the column of $pK_{a,R1}$ values was completed, the procedure was repeated for each compound with the same electrophoretic data and internal standards, but using the new $pK_{a,R1}$ values instead of the literature pK_a values. A new series of $pK_{a,R2}$ values is thus obtained. The refining process was stopped after calculating the $pK_{a,R5}$ series, which was almost equal to $pK_{a,R4}$ (difference ≤ 0.01). The last column shows the $pK_{a,R5}$ values, which have been considered the reference ones ($pK_{a,ref}$). The standard error obtained for the use of N internal standards is also indicated.

For each compound μ_{eff} and μ_{A-} were calculated in buffers with an appropriate pH from the experimental migration times by means of Eq. (4), and the corresponding $pK_{a,AN}$ was obtained from Eq. (6). The reference μ_{A-} value was determined as the average of all the limiting mobilities obtained using the N internal standards. Table 2 shows all the limiting mobilities and the experimental pK_a values ($pK_{a,exp}$) with the corresponding standard deviation.

As shown in Table 2, the experimental results obtained in this paper agree well with the literature ones; in general they differ in less than 0.1 units. Table 2 provides, therefore, a series of monoprotic acidic internal standards with a well established pK_a value, useful to determine pK_a of test compounds through the internal standard method.

To further validate the method, the pK_a values of some of the studied compounds were also determined by the classical CE method. In the classical method, the effective mobility of the analyte is measured at pH values close to its pK_a , at some pH values much higher than pK_a and usually, although not strictly needed for a neutral acid, at some pH values much lower than pK_a . The plot of the mobility vs. pH gives a sigmoidal curve with an inflection point at $pH = pK_a$. In our case, the mobility was determined at pH increments of about 0.2 pH units in the middle of the curve, and at pH increments of about 0.5 pH units at the beginning and at the end of the curve. Fitting the corresponding effective mobility to pH through Eq. (1), the pK_a and μ_{A-} parameters are obtained.

In particular, the pK_a values of paracetamol (with two quite different literature pK_a values) and phenol (with a 0.1 pK_a difference between literature and experimental values) were checked. When determining the pK_a values by the classical method, an abnormal behaviour was found at the end of the curve (see Fig. 1). The mobilities determined using NaOH as buffer showed a shift to more negative values than expected. It is difficult to discard NaOH because it is apparently innocuous and specific interactions with analytes have not been reported. For this reason CHES/CHES⁻ and CAPS/CAPS⁻ buffers (see Table 1) were supposed to be the cause of the deviation in the plots of Fig. 1. They are buffers with a more complex structure and could exhibit a selective capacity of interaction greater than that of the NaOH buffer. However, it is generally accepted that the zwitterionic buffers such as CHES and CAPS do not present interactions with acids of this type [17]. In addition, both have a similar chemical nature and it is not easy to ascertain from Fig. 1 that they promote deviations.

In order to test the effect of NaOH as buffer on the mobility–pH curves, we selected three other acids from Table 2, of different nature and lower acidity constants. This would allow drawing a baseline in the high pH region, where the buffers under study may present problems. These compounds are 3-bromobenzoic acid, 2,5-dinitrophenol, and 4-nitrophenol, with pK_a values of 3.79, 5.30, and 7.09, respectively.

Fig. 2 depicts the mobility–pH curves for such three compounds. It is clearly observed that when $H_2PO_4^-/HPO_4^{2-}$ and $H_3BO_3/H_2BO_3^-$ are used as buffers, their experimental points follow the same trend as the ones of CHES/CHES⁻ and CAPS/CAPS⁻ buffers. No deviation is observed in this case. In addition, a negative deviation in the mobility is clearly observed when NaOH buffer is employed, very similar to the negative deviation observed for paracetamol and phenol. These results allow conclusion that NaOH buffers are not adequate to determine pK_a values with the classical method. We do not know the exact reason of this behaviour, although, we presume it may be caused by attack of

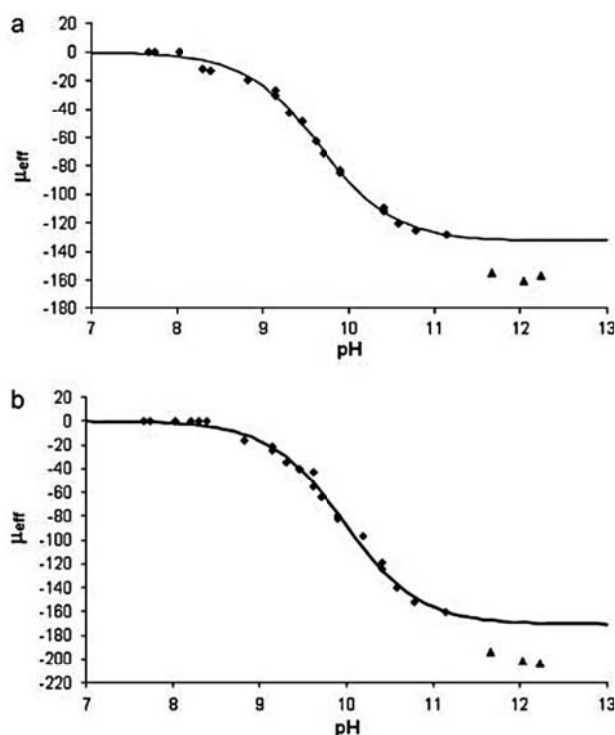


Fig. 1. Mobility ($cm^2 \text{ min}^{-1} V^{-1} \times 10^4$) vs. pH plots for (a) paracetamol and (b) phenol. The employed buffers are: (●) TrisH⁺/Tris, CHES/CHES⁻, and CAPS/CAPS⁻, and (▲) NaOH. Solid lines correspond to the fitting to Eq. (1).

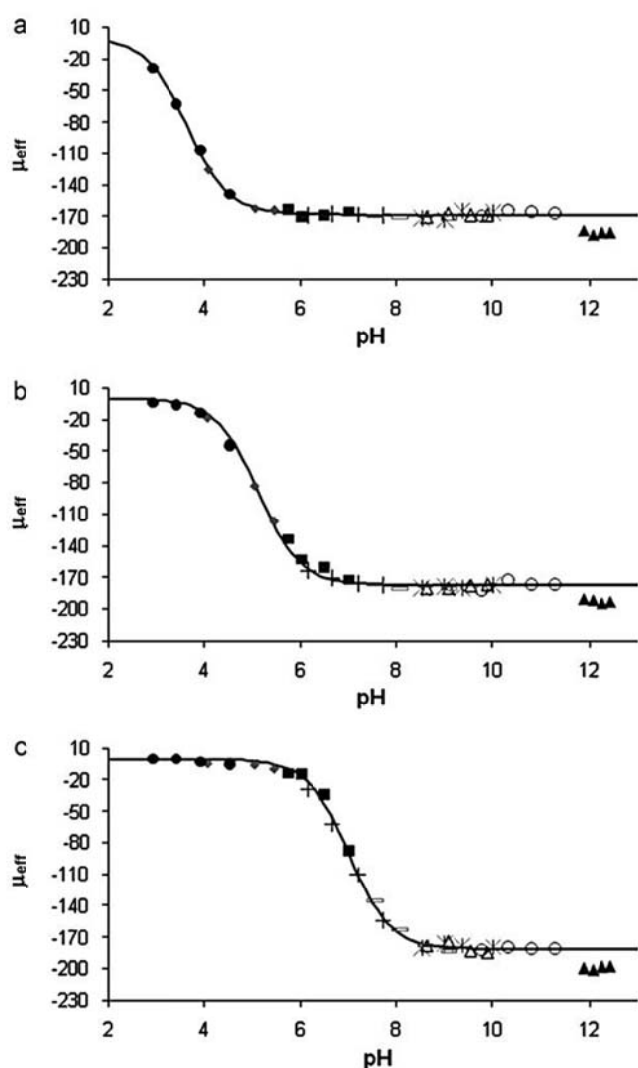


Fig. 2. Mobility ($\text{cm}^2 \text{min}^{-1} \text{V}^{-1} \times 10^4$) vs. pH plots for (a) 3-bromobenzoic acid, (b) 2,5-dinitrophenol, and (c) 4-nitrophenol. The employed buffers are: (●) $\text{HCOOH}/\text{HCOO}^-$, (◆) $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$, (■) $\text{BisTrisH}^+/\text{BisTris}$, (+) $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$, (–) $\text{TrisH}^+/\text{Tris}$, (*) $\text{CHES}/\text{CHES}^-$, (Δ) $\text{H}_3\text{BO}_3/\text{H}_2\text{BO}_3^-$, (○) $\text{CAPS}/\text{CAPS}^-$, (▲) NaOH . Solid lines correspond to the fitting to Eq. (1).

the NaOH buffer to the silica capillary wall that modifies solute mobilities.

The pK_a values of the compounds of Figs. 1 and 2 were then calculated by fitting the experimental data to Eq. (1) but excluding the points obtained with NaOH buffer. The curve obtained from the fit is plotted as a solid line in Figs. 1 and 2, and the obtained pK_a values are listed in Table 4, where they are compared to those

obtained using the internal standards method. It can be seen that they are practically equal.

In order to see if NaOH buffer had any effect in the measurements performed in the IS method, those analysis performed at high pH values with NaOH as buffer were repeated using CAPS/CAPS $^-$. It was observed that although the limiting mobility (μ_{A^-}) was shifted to more negative values for NaOH than for CAPS, the same pK_a values were obtained independently of the buffer used. The reason is that although the analyte shows mobilities in NaOH more negative than those observed in CAPS, the same happens with the internal standard, so the effect is balanced and the same final pK_a value is obtained. Therefore, NaOH can be used to determine pK_a values using internal standard method proving the better performance of this method over the classical one to deal with mobility shifts caused by unexpected effects and interactions that affect in a similar degree to the analyte and the internal standard.

5. Conclusions

A reference set of monoprotic neutral acids with different functional groups and structures, and reference pK_a values that cover all the useful pH range in CE, has been established. These compounds can be systematically employed as internal standards for routine and accurate determination of pK_a values in a fast way. They can be especially interesting for the pK_a determination of new drugs and drug precursors in the pharmaceutical industry, which may need to deal with a lot of compounds in a short time.

For routine analysis of neutral acidic compounds an internal standard with a pK_a similar to the one of the analyte can be selected from the set presented in Table 2. Then, both compounds are injected together in a buffer with a pH where they are partially ionized and in a buffer with a pH where they are totally ionized. The electrophoretic mobilities in these conditions are calculated from the migration times, and finally the pK_a of the analyte is calculated through Eq. (6). This procedure provides a pK_a value for the analyte as accurate as the one of the internal standard. Higher precision and accuracy can be obtained selecting several adequate internal standards from the list, and averaging the results obtained.

It has been also observed, that the internal standard method compensate some systematic deviations of mobility, which is a clear advantage over the classical CE method. In fact, it has been proved that mobilities determined using NaOH as buffer show a shift to more negative values than expected from other buffers. The NaOH buffer is, therefore, not suitable for accurate determinations when the classical method is used, whereas these problems are overcome with the internal standard method.

Acknowledgements

We thank the Ministerio de Educación y Ciencia of the Spanish Government and the Fondo Europeo de Desarrollo Regional of the European Union (Project CTQ2007-61608/BQU) for financial support.

References

- [1] R.F. Cookson, Chem. Rev. 74 (1974) 5.
- [2] H. Wan, J. Ulander, Expert Opin. Drug Metabol. Toxicol. 2 (2006) 139.
- [3] K. Valkó, in: K. Valkó (Ed.), Separation Methods in Drug Synthesis and Purification, Elsevier, Amsterdam, 2000.
- [4] E.H. Kerns, J. Pharm. Sci. 90 (2001) 1838.
- [5] A. Albert, E.P. Serjeant, The Determination of Ionization Constant, Chapman & Hall, London, 1971.
- [6] A. Avdeef, J.E.A. Comer, S.J. Thomson, Anal. Chem. 65 (1993) 42.
- [7] A. Avdeef, K.J. Box, J.E.A. Comer, M. Gilges, M. Hadley, C. Hibbert, W. Patterson, K.Y. Tam, J. Pharm. Biomed. Anal. 20 (1999) 631.
- [8] K.Y. Tam, K. Takács-Novák, Anal. Chim. Acta 434 (2001) 157.

Table 4

Comparison of experimental pK_a values at 25 °C and zero ionic strength, obtained through the internal standard method (Table 2) and through the classical method (statistics of the fitting to Eq. (1) are also shown).

Compound	IS method	Classical method			
	$\text{pK}_a \pm s$	$\text{pK}_a \pm s$	r^2	s	F
Paracetamol	9.58 ± 0.02	9.57 ± 0.02	0.996	2.85	4509.0
Phenol	9.89 ± 0.01	9.89 ± 0.03	0.994	4.22	3377.5
3-Bromobenzoic acid	3.79 ± 0.02	3.73 ± 0.01	0.995	2.29	6133.9
2,5-Dinitrophenol	5.30 ± 0.05	5.22 ± 0.02	0.996	3.86	8075.7
4-Nitrophenol	7.09 ± 0.05	7.09 ± 0.02	0.997	4.15	11458

- [9] I. Ishihama, Y. Oda, N. Asakawa, J. Pharm. Sci. 83 (1994) 1500.
- [10] J.A.J. Cleveland, M.H. Benkö, S.J. Gluck, Y.M. Walbroehl, J. Chromatogr. A 652 (1993) 301.
- [11] S.J. Gluck, J.S.J. Cleveland, J. Chromatogr. A 680 (1994) 43.
- [12] S.J. Gluck, J.A.J. Cleveland, J. Chromatogr. A 680 (1994) 49.
- [13] S.J. Gluck, K.P. Steele, M.H. Benkoe, J. Chromatogr. A 745 (1996) 117.
- [14] A. Šlampová, L. Krivánková, P. Gebauer, P. Boček, J. Chromatogr. A 1213 (2008) 25.
- [15] S.K. Poole, S. Patel, K. Dehring, H. Workman, C.F. Poole, J. Chromatogr. A 1037 (2004) 445.
- [16] J.M. Herrero-Martínez, M. Sanmartín, M. Rosés, E. Bosch, C. Ràfols, Electrophoresis 26 (2005) 1886.
- [17] E. Fuguet, M. Reta, C. Gibert, M. Rosés, E. Bosch, C. Ràfols, Electrophoresis 29 (2008) 2841.
- [18] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, J. Chromatogr. A 1216 (2009) 3646.
- [19] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, Chem. Biodiver. 6 (2009) 1822.
- [20] F. Rived, M. Rosés, E. Bosch, Anal. Chim. Acta 374 (1998) 309.
- [21] G. Kortüm, W. Vogel, K. Andrussov, Dissociation Constants of Organic Acids in Aqueous Solution, Butterworths, London, 1962.
- [22] L. Geiser, Y. Henchoz, A. Galland, P.-A. Carrupt, J.-L. Veuthey, J. Sep. Sci. 28 (2005) 2374.
- [23] H. Wan, A.G. Holmén, Y. Wang, W. Lindberg, M. Englund, M.B. Nágård, R.A. Thompson, Rapid Commun. Mass Spectrom. 17 (2003) 2639.
- [24] M. Shalaeva, J. Kenseth, F. Lombardo, A. Bastin, J. Pharm. Sci. 97 (2008) 7.



Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of acidity constants by the capillary electrophoresis internal standard method. IV. Polyprotic compounds

Joan Marc Cabot, Elisabet Fuguet, Clara Ràfols, Martí Rosés*

Departament de Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

ARTICLE INFO

Article history:

Received 12 November 2012

Received in revised form

21 December 2012

Accepted 3 January 2013

Available online 10 January 2013

Keywords:

 pK_a

Acidity constant

Capillary electrophoresis

Internal standard

High throughput method

Polyprotic compounds

ABSTRACT

The IS-CE method is developed for pK_a determination of polyprotic compounds. In this method, the internal standard (IS) and the polyprotic test compound are injected into the capillary electrophoresis (CE) system in buffers with appropriate pH. The pH of the buffers is not externally measured, but determined inside the capillary from the mobilities of the internal standards. Then the pK_a values of the polyprotic compounds are obtained by fitting its mobilities to the in situ pH values. The method is faster than the classical CE method (a diprotic compound can be done in less than 15 min), and also than other methods like potentiometric and spectrophotometric titrations. The method has been successfully applied to 20 polyprotic test compounds of different chemical nature, including compounds with extreme or very close pK_a values.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Acidity determination is important in many fields of research, specifically in drug development because the ionization state of a compound governs its physicochemical properties such as solubility, lipophilicity and protein binding. Many drug compounds contain at least one acidic and/or basic functional group and it has been estimated that 95% of the commercial drugs are ionizable [1,2].

A need for rapid filing of patents for promising drugs compels pharmaceutical companies to characterize a great number of potential drugs and chemical precursors in a relatively short time to select the ones which are more suitable for their further test and development. This fact justifies the increasing demand of fast and reliable physicochemical characterization techniques [1,2]. In addition, there is a significant need of measuring the dissociation constants of environmentally relevant drugs and pollutants to determine their occurrence, fate and effects [3].

Our research group has developed the internal standard (IS) method, based on capillary electrophoresis (CE) for the determination of acidity constants [4–7]. The IS-CE method is fast and efficient, and offers an attractive alternative to the classical method

[8–14] and to other common methods, such as potentiometric [15–17] and spectroscopic titrations [15,18]. It is easily automatable and requires very small amounts of compounds that may only be available in very limited quantities (e.g. from combinatorial syntheses). Using this method, the acidity constant of a monoprotic compound can be easily determined in few minutes in a standard CE instrument.

The method is based on the use of an internal standard with pK_a similar to that of the analyte. The analyte and the internal standard are injected through sequential injection into the capillary and run sequentially in two different buffers: a buffer in which the analyte and the IS are partially ionized (pH in the range $pK_a \pm 1$), and a second buffer in which both are completely ionized ($pH \gg pK_a$ for a neutral acid or $pH \ll pK_a$ for a neutral base). The difference between the pK_a of the analyte and the one of the IS can be determined from the mobility ratio of the analyte and the IS. Consequently, when the pK_a of the IS is well known, the pK_a of the analyte can be accurately determined [4–7]. This procedure provides a measure of the pK_a of the test compound as accurate as the pK_a of the IS. In any case, the precision can be improved by using more internal standards and/or buffer solutions.

At the moment, the IS-CE method has been only developed for the pK_a determination of monoprotic weak acids and bases [4–7]. However, many drugs contain more than one acidic and/or basic group and thus the method needs to be extended to polyprotic compounds. The present paper extends the application of the method to compounds with several acidity constants, and emphasizes those

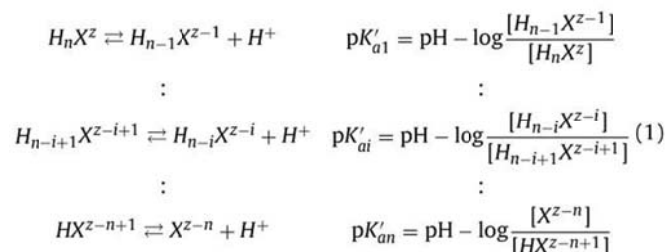
* Corresponding author at: Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain.
Tel.: +34 93 403 92 75; fax: +34 93 402 12 33.

E-mail address: marti.roses@ub.edu (M. Rosés).

particularly troublesome cases, like compounds with extreme or very close pK_a values.

2. Theory

The general acid–base equilibria for a fully protonated species, H_nX^z , can be expressed by:



where n is the total number of ionizable groups, z the charge of the fully protonated species, and pK'_{ai} the dissociation equilibrium constant of the i th dissociation step (at constant ionic strength).

As the ionization degree is related to the acidity constants, the effective electrophoretic mobility (μ_{eff}) of a polyprotic compound can be expressed as a function of the pK_a values of each species and the pH of the background electrolyte through the following general equation [9,11,19–22]:

$$\mu_{eff} = \frac{\mu_{H_nX^z} + \sum_{i=1}^n 10^{ipH - \sum_{j=1}^i pK'_{aj}} \mu_{H_{n-i}X^{z-i}}}{1 + \sum_{i=1}^n 10^{ipH - \sum_{j=1}^i pK'_{aj}}} \quad (2)$$

where $\mu_{H_{n-i}X^{z-i}}$ is the limiting mobility of the subscript species. The term corresponding to the uncharged species has $\mu = 0$ and it is removed from expression (2).

Such mobilities can be directly calculated from the migration time of the test solute t_m and the electroosmotic flow marker t_0 (min) by means of the expression:

$$\mu = \frac{L_T L_D}{V} \left(\frac{1}{t_m} - \frac{1}{t_0} \right) \quad (3)$$

where L_T and L_D are the total length and the effective length of the capillary, respectively (cm), and V is the applied voltage (V).

In order to obtain each pK'_{ai} of a studied compound, data pairs $pH - \mu_{eff(AN)}$ can be fitted to Eq. (2) using nonlinear regression analysis. Later, the thermodynamic pK_{ai} values can be easily calculated from the working pK'_{ai} values by using Eq. (4):

$$pK_{ai} = pK'_{ai} - \log \frac{\gamma_{H_{n-i}X^{z-i}}}{\gamma_{H_{n-i+1}X^{z-i+1}}} \quad (4)$$

where $\gamma_{H_{n-i}X^{z-i}}$ and $\gamma_{H_{n-i+1}X^{z-i+1}}$ are the activity coefficients of the involved species. Activity coefficients are usually estimated by Debye–Hückel equation, which depends on the ionic strength (I) of the solution:

$$-\log \gamma = \frac{Az^2\sqrt{I}}{1 + Ba\sqrt{I}} \quad (5)$$

where A and B depend on the solvent dielectric constant and temperature (their values are 0.509 and 0.33, respectively, in water at 25 °C), z is the charge of the ion, and a is the hydrated radius of the ion. The value of a depends on the hydrated ion, although a value of 4.5 Å (value for hydrogen ion) is commonly taken for most ions. This equation is valid for ionic strength values lower than 0.2 M. As usual, activity coefficients of neutral species ($z = 0$) are assumed to be unity.

In the classical CE method, the pH of the buffer solution is measured by potentiometry and the mobility of the analyte in this

buffered solution by capillary electrophoresis. In the IS–CE method, both parameters can be obtained at the same time in the same CE experiment. The test compound and the IS are injected together into the capillary. Since the pK_a of the IS is known, once μ_{eff} and the limiting mobility of the IS have been measured in an appropriate buffer, the pH of the buffer solution inside the capillary can be easily calculated from Eq. (2) avoiding pH variations between potentiometric pH and electrophoretic mobility measurements.

The calculation of the pH inside the capillary is particularly simple if we chose monoprotic internal standards, such as the ones we proposed in earlier work [6,7]. In this instance, Eq. (2) can be transformed into Eq. (6) when the IS is a monoprotic neutral acid ($n = 1$, $z = 0$), or Eq. (7) when it is a monoprotic neutral base ($n = 1$, $z = 1$) [4].

$$pH = pK'_{a(IS)} - \log \frac{\mu_{IS^-} - \mu_{eff(IS)}}{\mu_{eff(IS)}} \quad (6)$$

$$pH = pK'_{a(IS)} + \log \frac{\mu_{IS^+} - \mu_{eff(IS)}}{\mu_{eff(IS)}} \quad (7)$$

In these equations the pK'_a is related to the thermodynamic pK_a and the activity coefficient using Eq. (4), μ_{IS^-} is the mobility of the fully deprotonated neutral acid IS (measured at $pH \gg pK'_{a(IS)}$) and μ_{IS^+} is the mobility of the fully protonated neutral base IS (measured at $pH \ll pK'_{a(IS)}$).

μ_{eff} of the analyte is also measured together with μ_{eff} of the IS. Fitting all pairs $pH - \mu_{eff(AN)}$ to Eq. (2) and using nonlinear regression analysis, all pK'_{ai} and limiting mobilities of the test compound not directly measured can be calculated.

3. Experimental

3.1. Apparatus

Experiments have been performed using an Agilent Technologies (Santa Clara, CA, USA) Capillary Electrophoresis system equipped with a diode-array spectrophotometric detector. Capillary was of fused silica 50 μ m I.D., 375 μ m O.D., and 33.5 cm of total length (25 cm to the detector). The temperature of the capillary was 25.0 °C $\pm$ 0.1 °C. Samples were injected hydrodynamically at a pressure of 50 mbar for 2 s and the applied voltage was 20 kV. Sequential injection was performed. A pressure of 25 mbar is applied during the electrophoretic runs. With these conditions each run took around 1.5 min. The UV detector was set at 214, 254, and 280 nm.

The capillary conditioning previously reported [6,7,22] was time optimized. The very first time the capillary was conditioned by rinsing successively with 1.0 M NaOH (20 min), H₂O (10 min), and the buffer solution (10 min). Before every other use, the capillary was conditioned with 1.0 M NaOH (2 min), H₂O (0.5 min), and finally the buffer to be used in the experiment (2 min). When the pH of the buffer used was changed, the capillary was cleaned with the new buffer solution (10 s). At the end of each session, the capillary was washed with 0.1 M NaOH (2 min) and water (2 min).

3.2. Reagents

Dimethyl sulfoxide (DMSO, >99.9%), 0.5 M sodium hydroxide, 0.5 M hydrochloric acid, and sodium dihydrogenphosphate monohydrate (>99%) were from Merck (Darmstadt, Germany). Anhydrous sodium acetate (>99.6%) was purchased from J.T. Baker (Deventer, The Netherlands). 2-(Cyclohexylamino)ethanesulfonic acid (CHES, >99%), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, >98%) were from Sigma (St. Louis, MO, USA). 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (BisTris, >99.9%), and sodium formate were from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)amino-methane (Tris, >99.9%) was purchased

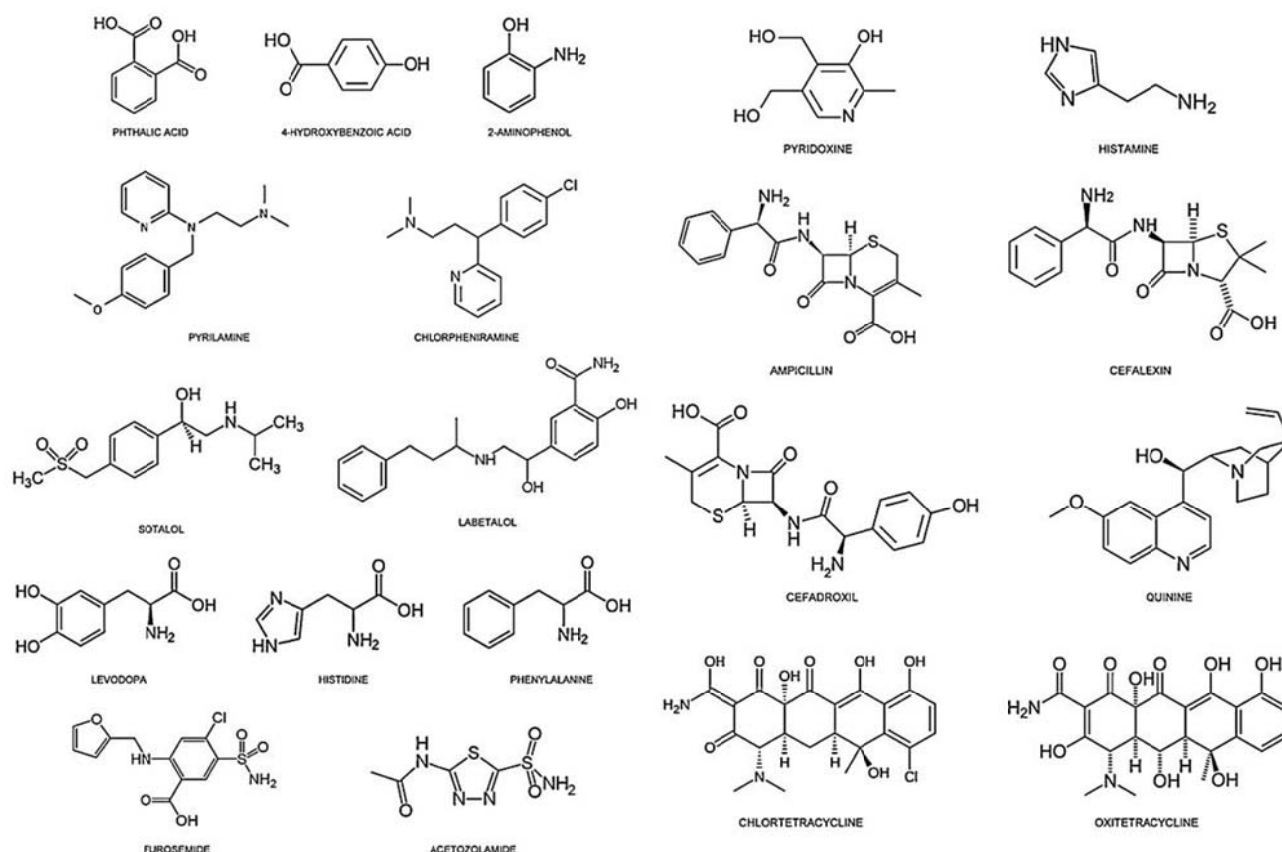


Fig. 1. Structures of the studied test compounds.

from Aldrich (Milwaukee, WI, USA). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 M Ω cm.

The acidic internal standards used were: 2-chlorobenzoic acid, 2,6-dibromo-4-nitrophenol, 2,4-dinitrophenol, 2,5-dinitrophenol, sulfacetamide, 4-nitrophenol, vanillin, phenobarbital, 3-chlorophenol, 4-bromophenol, paracetamol, phenol. The basic internal standards used were: aniline, quinoline, pyridine, 4-*tert*-butylpyridine, trazodone, 2,4,6-trimethylpyridine, bupivacaine, 1-phenylpiperazine, *N,N*-dimethyl-*N*-benzylamine, procainamine, propranolol, nortriptyline. The polyprotic test solutes employed were: furosemide, labetalol, phthalic acid, 2-aminophenol, 4-hydroxybenzoic acid, histamine, pyrilamine, pyridoxine, phenylalanine, chlorpheniramine, acetazolamide, histidine, quinine, chlortetracycline, levodopa, cefalexin, cefadroxil, ampicillin, sotalol and oxitetracycline. All the compounds were reagent grade or of chromatographic quality and obtained from Sigma, Fluka, Aldrich, or Carlo Erba (Milan, Italy). Fig. 1 shows the structures of the studied compounds.

3.3. Preparation of samples and buffers

Buffer solutions covering practically all the useful CE pH range (from 2 to 12) were prepared as described in previous work [22]. Table 1 shows the different electrophoretic solutions together with the corresponding pH range and the stock solutions used for their preparation. To prepare the buffers at the desired pH and 50 mM ionic strength the corresponding amounts of 0.5 M HCl or 0.5 M NaOH were added to 50 mL of the stock solution (0.1 M). Finally, the solution obtained was diluted by adding water up to a volume of 100 mL.

Stock solutions of test compounds and ISs were prepared at a concentration of 100 mg L⁻¹ and 0.4% of DMSO was added as electroosmotic flow marker. They were solubilized in water or in a methanol/water mixture (when they were not soluble in pure water).

All the samples and buffers were filtered through a nylon mesh 0.45 μ m pore size (Whatman, Maidstone, UK) and stored at 4 °C until used.

3.4. Calculations

Fits of Eq. (2) to the experimental data have been performed using TableCurve 2D v2.01, a 2D linear and non-linear curve fitting software from Systat Software Inc. (SSI) (San Jose, CA, USA).

4. Results and discussion

Several polyprotic compounds going from simple compounds, such as aminophenols, to more complex ones, such as cephalosporins or tetracycline derivatives (with three acidity constants) or compounds with very close pK_a values were selected as test analytes. They were chosen considering their acid–base characteristics and pharmacologic importance, as well as the difficulty in the determination of their acidity constants. A total of twenty compounds of diverse chemical nature were selected for this study (Fig. 1), which can be classified according to the nature of their acid–base moieties: acidic compounds (all acid–base groups are acidic), basic compounds (all are basic) and amphiprotic compounds (they have acidic and basic groups).

The IS–CE method requires choosing buffers and internal standards with pH and pK_a values close to those of the test compound.

Table 1
Buffer solutions used to determine the acidity constants by the IS-CE method.

Buffer	pK_a	pH range	Stock solution	pH modifier
H ₃ PO ₄ /H ₂ PO ₄ ⁻	2.15	2.10–3.10	0.1 M NaH ₂ PO ₄	0.5 M HCl
HCOOH/HCOO ⁻	3.75	2.60–4.80	0.1 M HCOONa	0.5 M HCl
CH ₃ COOH/CH ₃ COO ⁻	4.76	3.70–5.80	0.1 M CH ₃ COONa	0.5 M HCl
BisTrisH ⁺ /BisTris	6.48	5.50–7.50	0.1 M BisTrisHCl	0.5 M NaOH
TrisH ⁺ /Tris	8.08	7.00–9.00	0.1 M TrisHCl	0.5 M NaOH
CHES/CHES ⁻	9.50	8.40–10.10	0.1 M CHESNa	0.5 M HCl
CAPS/CAPS ⁻	10.40	9.40–11.60	0.1 M CAPSNa	0.5 M HCl

Table 2
Internal standards set [6,7].

IS	pK_a	IS	pK_a
2-Chlorobenzoic acid	2.84	4-Nitrophenol	7.09
2,6-Dibromo-4-nitrophenol	3.31	Vanillin	7.36
4-Nitrobenzoic acid	3.37	2,4,6-Trimethylpyridine	7.51
2,6-Dinitrophenol	3.69	Phenobarbital	7.53
3-Bromobenzoic acid	3.79	4-Hydroxybenzaldehyde	7.61
2,4-Dinitrophenol	4.12	Lidocaine	7.93
Benzoic acid	4.22	Clonidine	8.10
Ibuprofen	4.49	Bupivacaine	8.19
Aniline	4.63	3,5-Dichlorophenol	8.18
Nicotinic acid	4.85	Methylparaben	8.35
Quinoline	4.93	2-Chlorophenol	8.50
4-tert-Butylaniline	4.93	1-Phenylpiperazine	8.75
Warfarin	5.17	N,N-Dimethyl-N-benzylamine	8.95
N,N-Dimethyl-N-phenylamine	5.17	3-Chlorophenol	9.04
Pyridine	5.28	Diphenhydramine	9.08
2,5-Dinitrophenol	5.30	Procainamide	9.26
Sulfacetamide	5.42	4-Bromophenol	9.28
Acridine	5.55	Imipramine	9.37
2,4,6-Tribromophenol	6.04	Propranolol	9.47
4-tert-Butylpyridine	6.03	1-Aminoethylbenzene	9.52
Papaverine	6.41	Paracetamol	9.58
2,4-Lutidine	6.81	Ephedrine	9.72
Trazodone	6.84	Phenol	9.89
Pilocarpine	7.08	Nortriptyline	10.08

Thus, the pK_a values of the test compounds were estimated by the SPARC program ($pK_{a(pred)}$) [23] and pH buffers close to these $pK_{a(pred)}$ values were prepared from the solutions listed in Table 1. In the same way, we selected appropriate internal standards of pK_a value close to the one expected for the test, and as far as possible with the same acid–base nature (monoprotic acids as ISs for acidic groups and monoprotic bases as ISs for basic groups), from the list of Table 2. This table presents the internal standards of well known pK_a values as established in earlier work [6,7]. Sometimes the initial estimation of a pK_a is not correct, so wrong buffer solutions and internal standard are selected. These cases are easily detectable because then the effective mobility of the test compound becomes

almost zero or very close to the limiting mobility, since the pH in which experiments are performed is much lower or higher than the true pK_a value. In any case, effective mobility would not change when changing the pH of the buffer solution. Then, new buffers and IS must be selected as reported elsewhere [4].

Once the appropriate IS was chosen, IS and analyte were injected together into the capillary, and the μ_{eff} and limiting mobilities were determined in the buffers of appropriate pH. These mobilities were calculated from the experimental migration times by means of Eq. (3). The exact pH (pH_{det}) of the buffer solution where IS and analyte were partially ionized was then obtained from Eq. (6) and/or Eq. (7), through the pK'_a and the effective and limiting mobilities of the internal standard (the latter measured in the buffer where IS was completely ionized). Then pH_{det} and $\mu_{eff(AN)}$ data were fitted to Eq. (2), so $pK'_{a(AN)}$ was obtained. A maximum of 4 runs were performed for each pK_a (three at pH close to the pK_a and one in a buffer appropriate to measure the corresponding limiting mobility). Since each run took around 1.5 min, about 6 min were needed for each pK_a .

Several examples of increasing complexity are discussed for illustration.

4.1. Polyprotic compounds with separate intermediate pK_a values

The simplest case is a diprotic compound with one acidic group and one basic group, and separate pK_a values, like pyridoxine. Predicted pK_a values for pyridoxine were 5.29 and 9.47, so pyridine and paracetamol, with pK_a values of 5.28 and 9.58, respectively, were used as internal standards. Analyte and each IS were injected together in three pH solutions where they were partially ionized (nominal pH 5.0, 5.5 and 6.0 for the first pK_a , and 8.5, 9.0 and 9.5 for the second pK_a). Also, they were injected at nominal pH 3.0 to obtain the limiting mobilities of pyridine and pyridoxine ($\mu_{H_2X^+}$) and at nominal pH 11.5 to determine the limiting mobilities of paracetamol and pyridoxine (μ_{X^-}). Eqs. (6) and (7) were used to calculate pH. pH_{det} values were then related to $\mu_{eff(AN)}$ values through Eq. (2). In this case, experimentally measured $\mu_{H_2X^+}$ and μ_{X^-} were directly used in the fitting equation. Table 3 shows the data corresponding to the pK_a determination of pyridoxine and Fig. 2 the corresponding fit. pK_{a1} and pK_{a2} obtained for pyridoxine in this way were 4.89 ± 0.01 and 9.01 ± 0.02 , respectively (about 0.4 pK_a

Table 3
Data for pyridoxine pK_a determination.

IS	$pK_{a(IS)}$	pH_{nom}	IS		Analyte ($\mu \times 10^4$) ^a
			$\mu \times 10^4$ ^a	pH_{det}	
Pyridine	5.28	3.0	240.5	–	137.2
		5.0	163.0	5.04	63.7
		5.5	106.6	5.47	33.3
		6.0	34.3	6.15	8.7
Paracetamol	9.58	11.5	–122.2	–	–111.0
		9.5	–62.0	9.61	–94.4
		9.0	–34.3	9.19	–71.4
		8.5	–11.4	8.61	–35.0

^a Mobility in $cm^2 V^{-1} min^{-1}$.

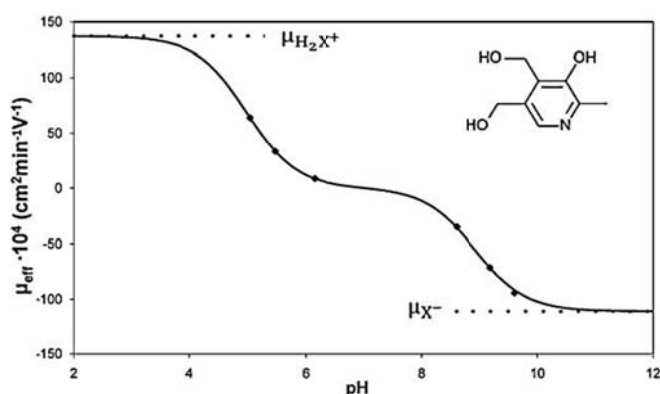


Fig. 2. Mobility vs. pH plot for pyridoxine. (■) Experimental points. Solid line is the fit to Eq. (2). Dashed lines show the directly measured limiting mobilities.

units lower than SPARC predictions). Histidine and 2-aminophenol behave in the same way as pyridoxine.

In case of compounds with pK_a values separated more than 3 units, like pyridoxine, histidine and 2-aminophenol, it is also possible to determine each pK_a separately, applying the IS-CE method for monoprotic compounds [4–7]. For example, the pK_a of pyridoxine determined in this way obtained practically identical results ($pK_{a1} = 4.89 \pm 0.01$ and $pK_{a2} = 9.01 \pm 0.02$). Moreover, as the charge of the ionized form of pyridoxine and the IS are the same (–1 for the acidic group and +1 for the basic one) there is no need of ionic strength correction.

4-Hydroxybenzoic acid is presented as example of a compound with two groups of the same nature and separate pK_a values. Estimation through SPARC program provides 4.17 and 8.85 as possible pK_a values, so according to Table 2, 2,4-dinitrophenol and 3-chlorophenol were chosen as acidic internal standards (their pK_a values are 4.12 and 9.04, respectively). 4-Hydroxybenzoic acid was injected together with the corresponding IS in three pH solutions for each pK_a where they were partially ionized to calculate $\mu_{eff(AN)}$ and $\mu_{eff(IS)}$. In this case, the solutions were of nominal pH 4.0, 4.5 and 5.0 when 2,4-dinitrophenol was used as IS for the first pK_a , and 8.5, 9.0 and 9.5 for 3-chlorophenol as IS for the second pK_a . 2,4-Dinitrophenol was also injected at nominal pH 7 to get its limiting mobility and the mixture of 3-chlorophenol and analyte was injected at 11.5 to determine their limiting mobilities. Obtained data is shown in Table 4. Effective and limiting mobilities of the internal standard were used to determine the exact pH (pH_{det}) of the buffer solutions using Eq. (6). As the limiting mobility of the species X^{2-} was directly measured in the buffer at pH_{nom} 11.5, its value was introduced into Eq. (2). Although it could be measured in a restricted pH range because the pK_a values are separated enough, the limiting mobility of the intermediate species (μ_{HX^-}) was not measured, but obtained through the fit. Therefore, the fit

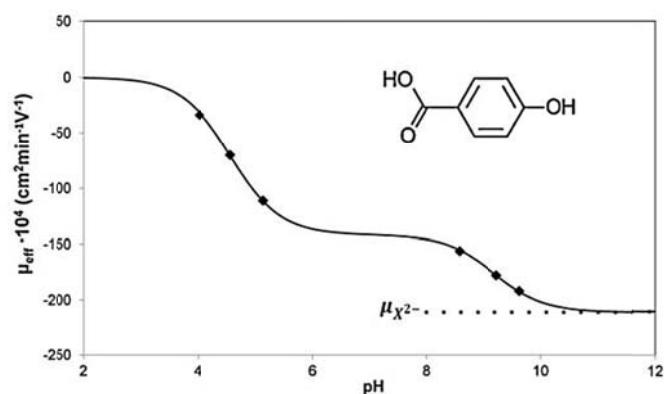


Fig. 3. Mobility vs. pH plot for 4-hydroxybenzoic acid. Symbols as in Fig. 2.

of the experimental values (pH_{det} and $\mu_{eff(AN)}$) to Eq. (2) allowed determination of pK_{a1} , pK_{a2} (4.65 ± 0.02 and 9.45 ± 0.04 , respectively 0.5–0.6 pK_a units higher than expected by SPARC) and μ_{HX^-} (-141.7 ± 2.2) for 4-hydroxybenzoic acid. Fig. 3 shows the experimental points and the curve corresponding to the fit. Furosemide has the same behavior and it was determined in the same way.

As pK_a values are quite separated, the determination can be done individually, like a monoprotic compound. However, in this case ionic strength correction is needed for the second pK_a because the charge of the IS (–1) is not the same as the one of 4-hydroxybenzoic acid (–2). When the determination is done separately, very similar results are obtained ($pK_{a1} = 4.63 \pm 0.02$ and $pK_{a2} = 9.42 \pm 0.02$).

Other compounds of Fig. 1 (pyrilamine, histamine, chlorpheniramine and quinine) are diprotic bases with relatively separated pK_a values. Although the procedure followed to determine the pK_a s is analogous to the one of 4-hydroxybenzoic acid, there are some differences. For example, the limiting mobility (corresponding to BH^{2+} species) must be determined at acidic pH values, and ideally, monoprotic basic internal standards should be used. In this case, ionic strength correction also must be done, but for the first acidity constant, because of the charge difference between the IS (+1) and the one of the most acidic form of the compounds (+2).

4.2. Polyprotic compounds with extreme pK_a values

An interesting case to check the method is the one of compounds with very low or high pK_a values. Compared to previous cases, a difficulty appears because it is not possible to measure directly the limiting mobilities. In these cases, an IS of different nature must be selected, i.e., an acidic IS for a basic test pK_a or a basic IS for an acidic test pK_a , because μ_{lim} of IS cannot be measured at highly acidic or basic pH regions. For instance, phenylalanine has a predicted pK_{a1} value of 2.21. The mobility of the most protonated form ($\mu_{H_2X^+}$) could not be directly determined because

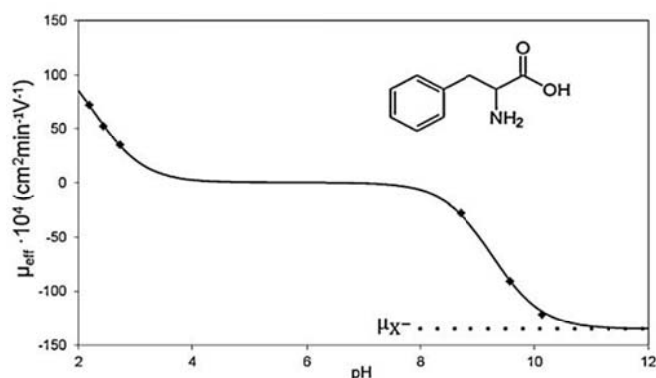
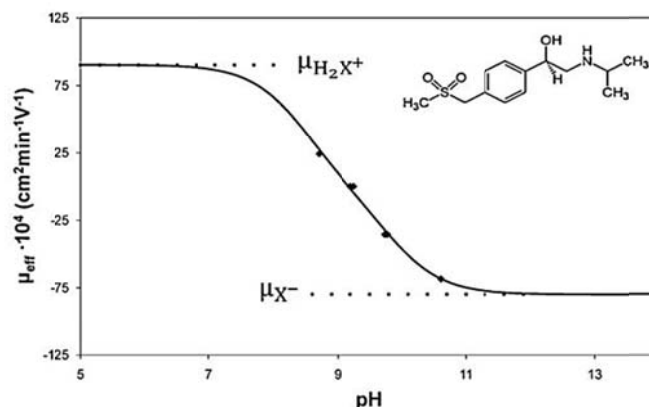
Table 4
Data for 4-hydroxybenzoic acid pK_a determination.

IS	$pK_{a(IS)}$	pH_{nom}	IS		Analyte ($\mu \times 10^4$) ^a
			$\mu \times 10^4$ ^a	pH_{det}	
2,4-Dinitrophenol	4.12	7.0	–153.9	–	–
		5.0	–142.6	5.14	–111.2
		4.5	–119.2	4.57	–70.2
		4.0	–76.6	4.03	–33.9
3-Chlorophenol	9.04	11.5	–144.3	–	–211.0
		9.5	–119.0	9.63	–192.0
		9.0	–94.0	9.23	–177.9
		8.5	–42.7	8.58	–156.3

^a Mobility in $cm^2 V^{-1} min^{-1}$.

Table 5
Data for phenylalanine pK_a determination.

IS	$pK_{a(IS)}$	pH_{nom}	IS		Analyte ($\mu \times 10^4$ ^a)
			$\mu \times 10^4$ ^a	pH_{det}	
2-Chlorobenzoic acid	2.84	6.0	–164.4	–	0.0
		2.8	–79.2	2.73	34.6
		2.5	–52.9	2.44	52.3
		2.3	–34.7	2.19	71.8
4-Bromophenol	9.28	11.5	–146.0	–	–135.0
		10.0	–131.8	10.16	–121.8
		9.5	–105.3	9.60	–90.7
		9.0	–37.4	8.73	–27.7

^a Mobility in $cm^2 V^{-1} min^{-1}$.**Fig. 4.** Mobility vs. pH plots for phenylalanine. Symbols as in Fig. 2.**Fig. 5.** Mobility vs. pH plots for sotalol. Symbols as in Fig. 2.

the compound should be injected at very low pH value, nor an IS of the same nature as the analyte (i.e. a basic one) could be used to determine the first pK_a because its limiting mobility must be also measured at a very low pH value. Thus, 2-chlorobenzoic acid (with pK_a similar to the analyte) was used as internal standard for the first pK_a of phenylalanine. Both analyte and IS were injected at the most acidic nominal pH values possible (2.3, 2.5 and 2.8) using $H_3PO_4/H_2PO_4^-$ buffer (Table 1). The limiting mobility of the IS was measured in a buffer of nominal pH 6.0, as pointed out in Table 5. Of course, ionic strength correction was needed to obtain the thermodynamic pK_a since the charge of the first pK_a of phenylalanine (+1) is different from the one of the IS (–1). The second pK_a of phenylalanine ($pK_{a(pred)} = 9.23$), corresponding to an acidic group, was determined with 4-bromophenol as IS (Table 5) following the same procedure as in previous sections. The mobility of the protonated form of phenylalanine, $\mu_{H_2X^+}$, was calculated through extrapolation of the mobility vs. pH curve (i.e. from the fitting to Eq. (2)) and pK_a values were determined from the fit as well (Fig. 4). Therefore, pK_a values (2.20 ± 0.09 and 9.35 ± 0.02) and $\mu_{H_2X^+}$ (129.3 ± 13.9) were obtained, close to the SPARC predicted ones. The pK_a and

the extrapolated limiting mobility obtained have a standard deviation somewhat higher than those cases where mobilities could be directly determined instead of extrapolated. Compounds such as levodopa, ampicillin, cefalexine, cefadroxil, chlortetracycline and oxitetracycline have also extreme pK_a values and therefore their parameters were determined in the same way.

4.3. Polyprotic compounds with close pK_a values

The IS–CE method is also applicable to compounds with several close pK_a values. In these cases pK_a determination cannot be done separately, like individual monoprotic compounds. For instance, sotalol has two close pK_a values (one as neutral base and the other as neutral acid). According to SPARC, they are 9.16 and 10.35, respectively, therefore *N,N*-dimethyl-*N*-benzylamine ($pK_a = 8.95$) and phenol ($pK_a = 9.89$) were selected as adequate ISs. The following procedure is the same as in previous examples. However, as the two pK_a values are very close, some selected pH values to determine μ_{eff} can be the same for both IS, as can be observed in Fig. 5

Table 6
Data for sotalol pK_a determination.

IS	$pK_{a(IS)}$	pH_{nom}	IS		Analyte ($\mu \times 10^4$ ^a)
			$\mu \times 10^4$ ^a	pH_{det}	
<i>N,N</i> -Dimethyl- <i>N</i> -benzylamine	8.95	6.0	144.7	–	90.3
		9.0	98.3	8.71	24.3
		9.5	55.0	9.25	0.0
		10.0	22.1	9.78	–35.5
Phenol	9.89	11.5	–122.5	–	–79.8
		10.5	–106.0	10.61	–68.4
		10.0	–56.9	9.74	–35.5
		9.5	–24.3	9.20	0.0

^a Mobility in $cm^2 V^{-1} min^{-1}$.

Table 7
Data for phthalic acid pK_a determination.

IS	$pK_{a(IS)}$	pH_{nom}	IS		Analyte ($\mu \times 10^4$ ^a)
			$\mu \times 10^4$ ^a	pH_{det}	
2-Chlorobenzoic acid	2.84	6.0	–157.0	–	–
		4.0	–153.5	4.40	–140.5
		3.5	–127.8	3.40	–99.3
		3.0	–78.6	2.77	–50.9
2,5-Dinitrophenol	5.30	7.5	–163.1	–	–252.3
		6.5	–151.4	6.48	–244.6
		6.0	–129.9	5.96	–233.9
		5.5	–83.4	5.39	–208.7

^a Mobility in $cm^2 V^{-1} min^{-1}$.

and Table 6. In this case, buffers at nominal pH 9.5 and 10 have been used for the experimental determination of both pK_a s. As a result of that, fewer electrophoretic runs are needed so that both ISs and the test compound are injected together in the same run to determine simultaneously two fitting points. Table 6 presents the electrophoretic data of sotalol pK_a determination. Obtained pK_a values are 8.36 ± 0.05 and 9.92 ± 0.05 . It can be observed that their standard deviations are low. The fact of obtaining experimental data for the calculation of two different pK_a values through the same electrophoretic run has been performed in the pK_a determination of other compounds with close pK_a values, such acetazolamide, labetalol, cefadroxil, chlortetracycline and oxytetracycline. This last compound, for example, has three acidity constants (pK_a s of 3.28, 7.48, and 8.88) and two of them are very close (less than 1.5 pK_a units between the second and the third one).

Phthalic acid is also worth discussing. This compound is a diprotic neutral acid (H_2A), with close pK_a values. According to SPARC estimation pK_a s are 3.05 and 5.57, so 2-chlorobenzoic acid ($pK_a = 2.84$) and 2,5-dinitrophenol ($pK_a = 5.30$) were selected as ISs. Compared to sotalol, phthalic acid presents the difficulty that the mobility of the amphiprotic species (HA^-) is not zero, and it cannot be experimentally determined because there is no plateau region between the two jumps. Then, μ_{HX^-} must be estimated in the fit of experimental points to Eq. (2), like the two pK_a values. Table 7 shows the experimental data for phthalic acid pK_a determination, and Fig. 6 the fit of experimental points to Eq. (2). Obtained results are $pK_{a1} = 3.02 \pm 0.05$, $pK_{a2} = 5.38 \pm 0.05$, and $\mu_{HX^-} = -127.4 \times 10^4 cm^2 V^{-1} min^{-1}$. pK_a values show very good agreement with most of the values of literature.

4.4. General discussion

The results obtained by the application of the IS-CE method to the different studied polyprotic acids are presented in Table 8.

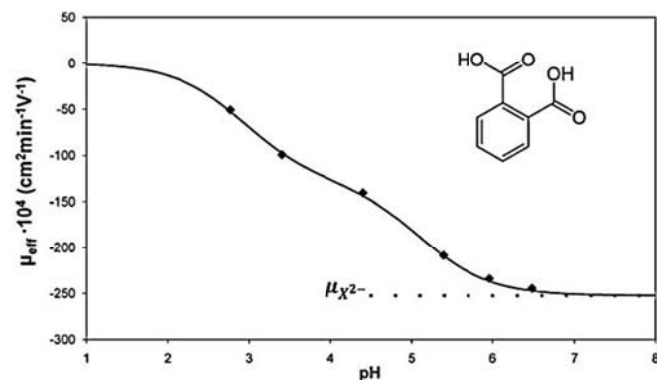


Fig. 6. Mobility vs. pH plots for phthalic acid. Symbols as in Fig. 2.

This table shows, for each test compound, the pK_a estimated by SPARC, the pK'_a and limiting mobilities of the different species at the working ionic strength, the $pK_{a(exp)}$ at 25 °C after ionic strength correction, and statistics of the fit. When possible, limiting mobilities of the test compounds were experimentally determined, and their value introduced into Eq. (2). However, in those cases where the pK_a of the compounds were in extreme regions of the pH scale, or the compound had very close pK_a values, limiting mobilities could not be experimentally measured and were obtained through the fit. Mobilities of the neutral species are, obviously, zero. In general, the obtained standard deviations are lower than 0.1 pK_a units, being for most pK_a values 0.05 or less.

Literature pK_a values ($pK_{a(lit)}$) for all tested compounds at 25 °C and zero ionic strength are also shown in Table 8. These values were collected mainly from general compilation sources [3,8,9,18,24–44] in order to compare the results obtained through the IS-CE method to the results obtained by other methods. When the experimentally determined pK_a values ($pK_{a(exp)}$) of polyprotic compounds are compared to literature values, very good agreement is observed (<0.1 pK_a units) for most pK_a s and compounds. For compounds such as chlorpheniramine, labetalol, sotalol, ampicillin, cefadroxil, chlortetracycline or levodopa there is a 0.2–0.3 variations difference for some of their pK_a values. However, bibliographic information is scarce and disperse regarding the acidity constants of these compounds, so a real estimation of the accuracy of the obtained pK_a values is not possible. Fig. 7, that shows the correlation between $pK_{a(lit)}$ and $pK_{a(exp)}$, proves the good general accuracy of the IS-CE method comparing our results with those reported in the literature.

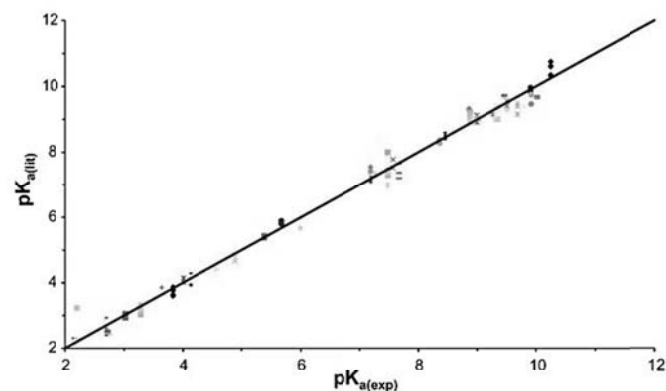


Fig. 7. Literature pK_a vs. experimental pK_a plot. Test compounds are: (◆) furosemide, (■) phthalic acid, (▲) 4-hydroxybenzoic acid, (♦) acetazolamide, (■) pyrilamine, (●) histamine, (□) chlorpheniramine, (▲) quinine, (■) levodopa, (■) histidine, (◆) phenylalanine, (▲) 2-aminophenol, (●) pyridoxine, (■) labetalol, (○) sotalol, (■) ampicillin, (◆) cefalexine, (●) cefadroxil, (♦) chlortetracycline, (■) oxytetracycline.

Table 8
pK_a values, limiting mobilities and statistics of the pK_a calculation for the compounds studied in this work.

Compound	pK _{a(pred)}	pK _{a(exp)}	pK _{a(lit)}	s	μ _{lim} × 10 ⁴ ^a	z ^b	R	s	F
Furosemide	3.18	3.75	3.78 [18]; 3.87 [18]; 3.64 [24]; 3.76 [25]; 3.60 [26]	0.05	-106.5	-1	0.9977	3.85	1749
Phthalic acid	10.07	9.99	10.62 [18]; 10.75 [18]; 10.6 [24]; 10.32 [25]; 10.35 [26]	0.07	-187.3	-2	0.9990	2.84	1449
4-Hydroxybenzoic acid	3.05	2.93	3.06 [8]; 2.73 [9]; 2.94 [42]; 2.95 [43]	0.05	-127.4	-2	0.9997	1.45	4682
Acetazolamide	5.57	5.11	5.38 [8]; 4.78 [9]; 5.43 [42]; 5.41 [43]	0.05	-252.3	-2	0.9996	1.61	2881
Pyrimidine	4.17	4.57	4.45 [25]; 4.57 [42]	0.02	-141.7	-1	0.9973	6.07	1479
Histamine	8.85	9.19	9.32 [25]; 9.46 [42]	0.04	-211.0	-2	0.9990	2.13	2630
Chlorpheniramine	6.51	7.46	7.52 [28]; 7.78 [29]	0.03	-161.8	-1	0.9995	1.78	15,857
Quinine	8.85	9.01	9.41 [28]; 9.57 [29]	0.04	-265.5	-2	0.9997	1.91	4339
Levodopa	4.64	4.27	4.14 [26]; 4.02 [31]	0.03	201.6	+2	0.9999	1.06	34,186
Histidine	8.57	9.07	9.12 [26]; 8.92 [31]	0.02	122.4	+1	0.9996	1.59	3978
Phenylalanine	6.28	5.92	5.80 [26]; 5.89 [40]	0.04	349.5	+2	0.9977	2.86	647
2-Aminophenol	9.55	10.00	9.96 [26]; 9.85 [40]	0.04	187.6	+1	0.9999	1.01	11,309
Pyridoxine	3.95	3.90	3.86 [41]	0.04	214.9	+2	0.9999	0.56	29,707
Labetalol	9.53	9.36	9.13 [30]; 9.18 [41]	0.03	118.4	+1	0.9999	0.62	21,892
Sotalol	4.72	4.37	3.95 [18]; 3.92 [18]; 3.95 [24]; 4.29 [26]; 4.14 [30]	0.07	205.1	+2	0.9995	1.63	2534
Ampicillin	9.10	8.52	8.46 [18]; 8.43 [18]; 8.60 [24]; 8.53 [26]; 8.39 [30]	0.04	111.4	+1	0.9997	2.92	906
Cefalexine	2.33	2.24	2.31 [31]; 2.30 [44]	0.18	134.6	+1	0.9999	1.39	9769
Cefadroxil	8.75	9.38	9.74 [31]; 9.70 [44]	0.04	-193.7	-1	0.9995	1.63	2534
Chlortetracycline	6.47	6.08	5.96 [32]; 5.94 [32]; 5.67 [33]	0.01	167.9	+1	0.9982	2.92	906
Oxitetracycline	8.74	9.23	9.27 [32]; 9.31 [32]; 8.99 [33]	0.02	-145.5	-1	0.9996	1.59	3978
	2.21	2.28	2.16 [26]; 2.23 [34]	0.09	129.3	+1	0.9999	0.56	29,707
	9.23	9.27	9.01 [26]; 9.32 [34]	0.02	-135.0	-1	0.9999	0.62	21,892
	5.22	4.80	4.63 [35]; 4.78 [42]; 4.72 [43]	0.01	184.4	+1	0.9995	1.63	2534
	9.87	9.71	9.42 [35]; 9.97 [42]; 9.71 [43]	0.01	-166.7	-1	0.9999	1.06	34,186
	5.29	4.98	4.67 [24]; 4.87 [34]; 4.77 [36]; 4.87 [39]	0.01	137.2	+1	0.9995	1.63	2534
	9.47	8.93	9.02 [24]; 9.11 [34]; 9.04 [36]; 8.99 [39]	0.02	-111.0	-1	0.9995	2.92	906
	8.88	7.36	7.28 [18]; 7.36 [18]; 7.35 [26]	0.03	85.5	-1	0.9996	1.59	3978
	10.01	9.60	9.49 [18]; 9.40 [18]; 9.16 [26]	0.04	-72.5	-1	0.9977	2.86	647
	9.16	8.46	8.38 [26]; 8.28 [34]	0.05	90.3	+1	0.9999	1.01	11,309
	10.35	9.83	9.47 [26]; 9.75 [34]	0.05	-79.8	-1	0.9999	0.56	29,707
	3.46	2.84	2.50 [24]; 2.41 [24]; 2.42 [25]	0.07	82.7	+1	0.9999	0.56	29,707
	6.71	7.39	7.04 [24]; 6.94 [24]; 7.30 [25]	0.02	-106.5	-1	0.9999	0.56	29,707
	3.59	2.76	2.40 [25]; 2.61 [26]; 2.53 [37]; 2.93 [37]	0.13	91.1	+1	0.9999	0.56	29,707
	6.67	7.09	7.27 [25]; 7.08 [26]; 7.13 [37]; 7.18 [37]	0.03	-93.0	-1	0.9999	0.56	29,707
	3.59	2.81	2.55 [26]; 2.52 [37]; 2.48 [37]	0.04	90.4	+1	0.9999	0.56	29,707
	6.92	7.58	7.21 [26]; 7.65 [37]; 7.37 [37]	0.02	-104.5	-1	0.9999	0.56	29,707
	9.66	9.78	9.71 [26]; 9.64 [37]	0.04	-168.5	-2	0.9999	0.56	29,707
	1.57	3.37	3.33 [3]; 3.30 [27]; 3.3 [38]	0.02	92.8	+1	0.9999	0.56	29,707
	5.67	7.10	7.55 [3]; 7.44 [27]; 7.4 [38]	0.04	-53.6	-1	0.9999	0.56	29,707
	9.12	8.60	9.33 [3]; 9.27 [27]; 9.3 [38]	0.04	-97.6	-2	0.9999	0.56	29,707
	1.15	3.37	3.22 [3]; 3.10 [18]; 3.04 [24]; 3.27 [27]; 3.3 [38]	0.02	90.3	+1	0.9999	0.56	29,707
	5.42	7.39	7.46 [3]; 7.35 [18]; 8.00 [24]; 7.32 [27]; 7.3 [38]	0.05	-59.0	-1	0.9999	0.56	29,707
	8.92	8.62	8.94 [3]; 9.20 [18]; 9.11 [27]; 9.1 [38]	0.06	-104.1	-2	0.9999	0.56	29,707

^a μ_{lim}: limiting mobility, expressed in cm² V⁻¹ min⁻¹.^b z is the charge of the limiting mobility.

5. Conclusions

The IS-CE is a fast and reliable method for the determination of the pK_a values of polyprotic compounds. It is not only applicable to the simplest cases where the pK_a values are quite separated, but also to compounds with extreme or very close pK_a values, with very good results in terms of precision and accuracy of the obtained values. The method is fast (the determination can be done in less than 15 min for a diprotic compound), easily automatable, requires very small amounts of compounds, there is no need of previous measurements of the exact pH of the buffer solutions, and possible experimental errors during the determination are corrected by the use of an internal standard. Therefore, we provide a very attractive alternative for the acidity constant determination in physicochemical screening processes, such as in drug-discovery.

References

- [1] R. Mannhold, H. Kubinyi, G. Folkers (Eds.), *Methods and Principles in Medicinal Chemistry*, vol. 18, Drug Bioavailability, Wiley-VCH, Weinheim, 2003.
- [2] H. Wan, J. Ulander, *Expert Opin. Drug Metabol. Toxicol.* 2 (2006) 139.
- [3] Z. Qiang, C. Adams, *Water Res.* 38 (2004) 2874.
- [4] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, *J. Chromatogr. A* 1216 (2009) 3646.
- [5] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, *Chem. Biodivers.* 6 (2009) 1822.
- [6] J.M. Cabot, E. Fuguet, C. Ràfols, M. Rosés, *J. Chromatogr. A* 1217 (2010) 8340.
- [7] E. Fuguet, C. Ràfols, M. Rosés, *J. Chromatogr. A* 1218 (2011) 3928.
- [8] S.K. Poole, S. Patel, K. Dehring, H. Workman, C.F. Poole, *J. Chromatogr. A* 1037 (2004) 445.
- [9] S.J. Gluck, K.P. Steele, M.H. Benkoe, *J. Chromatogr. A* 745 (1996) 117.
- [10] A. Šlampová, L. Křivánková, P. Gebauer, P. Boček, *J. Chromatogr. A* 1213 (2008) 25.
- [11] I. Ishihama, Y. Oda, N. Asakawa, *J. Pharm. Sci.* 83 (1994) 1500.
- [12] J.A.J. Cleveland, M.H. Benkoe, S.J. Gluck, Y.M. Walbroehl, *J. Chromatogr. A* 652 (1993) 301.
- [13] S.J. Gluck, J.S.J. Cleveland, *J. Chromatogr. A* 680 (1994) 43.
- [14] S.J. Gluck, J.A.J. Cleveland, *J. Chromatogr. A* 680 (1994) 49.
- [15] A. Albert, E.P. Serjeant, *The Determination of Ionization Constant*, Chapman & Hall, London, 1971.
- [16] A. Avdeef, J.E.A. Comer, S.J. Thomson, *Anal. Chem.* 65 (1993) 42.
- [17] A. Avdeef, K.J. Box, J.E.A. Comer, M. Gilges, M. Hadley, C. Hibbert, W. Patterson, K.Y. Tam, *J. Pharm. Biomed. Anal.* 20 (1999) 631.
- [18] K.Y. Tam, K. Takács-Novák, *Anal. Chim. Acta* 434 (2001) 157.
- [19] J.M. Herrero-Martínez, M. Sanmartín, M. Rosés, E. Bosch, C. Ràfols, *Electrophoresis* 26 (2005) 1886.
- [20] L. Geiser, Y. Henchoz, A. Galland, P.-A. Carrupt, J.-L. Veuthey, *J. Sep. Sci.* 28 (2005) 2374.
- [21] V. Sanz-Neboit, F. Benavente, I. Toro, J. Barbosa, *Electrophoresis* 22 (2001) 4333.
- [22] E. Fuguet, M. Reta, C. Gibert, M. Rosés, E. Bosch, C. Ràfols, *Electrophoresis* 29 (2008) 2841.
- [23] SPARC: <http://archemcalc.com/sparc> (October 2011 release w4.6.1691-s4.6.1687).
- [24] H. Wan, A.G. Holmén, Y. Wang, W. Lindberg, M. Englund, M.B. Nágård, R.A. Thompson, *Rapid Commun. Mass Spectrom.* 17 (2003) 2639.
- [25] G. Völgyi, R. Ruiz, K. Box, J. Comer, E. Bosch, K. Takács-Novák, *Anal. Chim. Acta* 583 (2007) 418.
- [26] M. Shalaeva, J. Kenseth, F. Lombardo, A. Bastin, *J. Pharm. Sci.* 97 (2008) 7.
- [27] C.R. Stephens, K. Murai, K.J. Brunings, R.B. Woodward, *J. Am. Chem. Soc.* 78 (1956) 4155.
- [28] S. Ferrer, J. Borrás, E. Garciaespana, *J. Inorg. Biochem.* 39 (1990) 297.
- [29] E. Chufán, F. Suvire, R. Enriz, J. Pedregosa, *Talanta* 49 (1999) 859.
- [30] H. Wan, A. Holmén, M. Nágård, W. Lindberg, *J. Chromatogr. A* 979 (2002) 369.
- [31] N.G. Lordi, J.E. Christian, *J. Am. Pharm. Assoc.* 45 (1956) 300.
- [32] Y. Henchoz, J. Schappler, L. Geiser, J. Prat, P.-A. Carrupt, J.-L. Veuthey, *Anal. Bioanal. Chem.* 389 (2007) 1869.
- [33] L. Yang, Z. Yuan, *Electrophoresis* 20 (1999) 2887.
- [34] A. Avdeef, *Sirius Technical Application Notes*, vol. 1, Sirius Analytical Instruments Ltd., UK, 1994.
- [35] C.-E. Lin, Y.-T. Chen, *J. Chromatogr. A* 871 (2000) 357.
- [36] K. Takács-Novák, K.Y. Tam, *J. Pharm. Biomed. Anal.* 21 (2000) 1171.
- [37] M. Andrási, P. Buglyo, L. Zekany, G. Attila, *J. Pharm. Biomed. Anal.* 44 (2007) 1040.
- [38] P.K. Martindale, *The Complete Drug Reference*, 32nd ed., Pharmaceutical Press, London, 1999.
- [39] V.R. Williams, J.B. Neilands, *Arch. Biochem. Biophys.* 53 (1954) 56.
- [40] I.F. Holmes, F. Jones, *J. Chem. Soc.* (1960) 2398.
- [41] P. Wiczling, M. Waszczuk-Jankowska, M.J. Markuszewski, R. Kaliszán, *J. Chromatogr. A* 1214 (2008) 109.
- [42] A. Albert, E.P. Serjeant, *Determination of Ionization Constants*, Chapman & Hall Ltd., London, 1971.
- [43] A. Albert, E.P. Serjeant, *Ionization Constants of Acids and Bases*, Butler & Tanner Ltd., London, 1962.
- [44] J.E. Gorton, R.F. Jameson, *J. Chem. Soc. A* (11) (1968) 2615.

Internal Standard Capillary Electrophoresis as a High-Throughput Method for pK_a Determination in Drug Discovery and Development

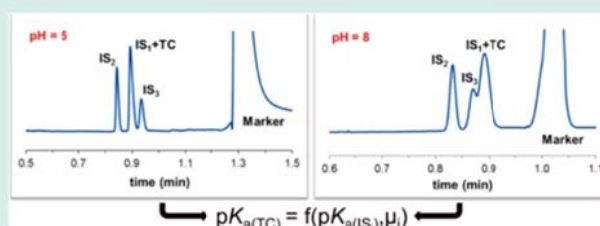
Joan M. Cabot, Elisabet Fuguet, and Martí Rosés*

Departament de Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

S Supporting Information

ABSTRACT: A novel high-throughput method for determining acidity constants (pK_a) by capillary electrophoresis (CE) is developed. The method, based on the use of an internal standard (IS-CE), is implemented as a routine method for accurate experimental pK_a determination of drugs undergoing physicochemical measurements in drug discovery laboratories. Just two electropherograms at 2 different pH values are needed to calculate an acidity constant. Several ISs can be used in the same buffer and run to enhance precision. With 3 ISs, for example, the pK_a of the test compound (TC) can be obtained in triplicate in less than 3 min of electrophoresis. It has been demonstrated that the IS-CE method eliminates some systematic errors, maintaining, or even increasing the precision of the results compared with other methods. Furthermore, pH buffer instability during electrophoretic runs is not a problem in the IS-CE method. It is also proved that after 16 h of electrophoresis using the same buffer vial, pH may change by around one unit; but the pK_a calculated by the IS-CE method remains constant. Thus, IS-CE is a powerful high-throughput method for pK_a determination in drug discovery and development.

KEYWORDS: acidity constant, IS-CE, high-throughput method, pK_a determination, internal standard, capillary electrophoresis



$$pK_{a(TC)} = f(pK_{a(IS_i)}, \mu_i)$$

INTRODUCTION

Drug discovery has undergone considerable changes with the advent of novel technologies and strategies that have given rise to new opportunities for gathering and integrating information and that can increase the success and efficiency of drug discovery. Pharmaceutical companies synthesize a large number of potential drugs and chemical precursors in relatively short periods.^{1,2} Those that are those most suitable are then selected for further testing and development. As a consequence, high-throughput screening for rapid evaluation of compounds is needed as soon as they are available from synthesis.^{3–5}

Physicochemical parameters are frequently used as predictors of ADME properties (absorption, distribution, metabolism and excretion) of drugs. One important physicochemical property that affects the pharmaceutical potential of a compound is its acid–base dissociation, defined by its acidity constant (or pK_a on a logarithmic scale). Many potential drugs are weak acids or bases, and their performance in advanced studies depends strongly on their pK_a , since it determines the degree of ionization of the compound under given conditions of pH. Therefore, in drug discovery there is a major need for fast pK_a determination of a large number of compounds.^{5,6}

Capillary zone electrophoresis (CZE) has been widely used as a technique for aqueous pK_a determination.^{7–15} It is a highly automated technique which requires only small amounts of the sample and reagents. In addition, CZE does not require substances with a high degree of purity because it allows for the separation of impurities and decomposition products from the

main compound. Thus, CZE is an attractive alternative compared to the most common techniques for pK_a determination, such as potentiometric or spectrophotometric titrations, which have some limitations based on the sample quantity, solubility, or purity, among other factors.^{13–15}

The classic capillary electrophoresis (CE) method for acidity constant determination involves measuring the mobility of the substance of interest at several pH values which are set by the preparation of suitable buffers at constant ionic strength in different pH ranges.^{16,17} However, the classic CZE method is quite slow and not very useful for high-throughput screening. It also has other limitations: interactions between the compound being studied and the different buffers must be avoided; and it requires strict control of temperature to minimize the Joule effect. If such undesired effects are not avoided, deviations in the mobility vs pH curve can result, which affect the final pK_a determination. A faster method to determine acidity constants by CE based on the use of internal standards (ISs) was recently developed by our research group.¹⁸ The IS-CE method is based on the use of an IS with a similar pK_a value to that of the test compound (TC). The IS and the TC are supposed to behave in the same way under the same measurement conditions. Therefore, if they are injected together, the differences in the mobility values of the compounds can be directly related to

Received: April 11, 2014

Revised: August 29, 2014

Published: August 29, 2014

differences in their acidity. Provided that the pK_a of the IS is well-known, the pK_a of the drug can be calculated easily. So the IS-CE method benefits from all the advantages of CE as a method for pK_a determination, but in the IS-CE method far fewer measuring points are required. Furthermore, an external accurate measurement of the pH of the buffer solution is not needed because the IS allows the pH to be determined in situ inside the capillary.^{18,19}

IS-CE has been applied to determine the acidity constants of simple compounds, such as monoprotic and polyprotic ions with separated pK_a values, as well as more complex ones, ranging from compounds with several close pK_a values to others with acidity constants at the limits of the pH range that is useful in CE.^{20–22} The procedure requires a minimum of only 2 CE runs for each pK_a value, which is determined through the ratio between the mobility of the TC and that of the IS. It has been demonstrated that the IS-CE method minimizes the effect of interactions between the TC and the buffer components,²⁰ and the use of an adequate IS corrects possible systematic experimental errors, such as temperature or pH variations during the determination.²³ In previous work, a set of suitable ISs (acidic and basic monoprotic compounds) with different chemical structure and pK_a values that cover the entire pH range that is useful for CE was established.^{20,21} Table 1 lists this proposed set, which is used and tested in the current work.

Table 1. Internal Standard Set^a

IS	pK_a	IS	pK_a
2-chlorobenzoic acid	2.84	4-nitrophenol	7.09
2,6-dibromo-4-nitrophenol	3.31	vanillin	7.36
4-nitrobenzoic acid	3.37	2,4,6-trimethylpyridine	7.51
2,6-dinitrophenol	3.69	phenobarbital	7.53
3-bromobenzoic acid	3.79	4-hydroxybenzaldehyde	7.61
2,4-dinitrophenol	4.12	lidocaine	7.93
benzoic acid	4.22	clonidine	8.10
ibuprofen	4.49	bupivacaine	8.19
aniline	4.63	3,5-dichlorophenol	8.18
nicotinic acid	4.85	methylparaben	8.35
quinoline	4.93	2-chlorophenol	8.50
4-tert-butylaniline	4.93	1-phenylpiperazine	8.75
warfarin	5.17	N,N-dimethyl-N-benzylamine	8.95
N,N-dimethyl-N-phenylamine	5.17	3-chlorophenol	9.04
pyridine	5.28	diphenhydramine	9.08
2,5-dinitrophenol	5.30	procainamide	9.26
sulfacetamide	5.42	4-bromophenol	9.28
acridine	5.55	imipramine	9.37
2,4,6-tribromophenol	6.04	propranolol	9.47
4-tert-butylpyridine	6.03	1-aminoethylbenzene	9.52
papaverine	6.41	paracetamol	9.58
2,4-lutidine	6.81	ephedrine	9.72
trazodone	6.84	phenol	9.89
pilocarpine	7.08	nortriptyline	10.08

^aThermodynamic reference pK_a values at 25°C.^{20,21}

Although IS-CE is a fast method, it cannot be considered a genuine high-throughput method as several parameters have not yet been optimized. Capillary conditioning processes prior to analysis take some time, and electrophoretic determination could be improved to become faster, particularly at low pH values where the electroosmotic flow is very slow. Con-

sequently, here we sequentially test and optimize several instrumental parameters and different experimental conditions to reduce run time and both the pre- and postconditioning steps as much as possible without losing precision. Furthermore, other important aspects of the implementation of the method for accurate experimental pK_a determination in day-to-day analysis in physicochemical measurement laboratories are checked and improved where possible; for instance, the use of consecutive injections or the stability of the ISs and separation buffers.

THEORY

The apparent mobility of a compound (μ_{ap}) is the sum of the electrophoretic (μ_{ep}) and electroosmotic flow (μ_{EOF}) mobilities and can be expressed by means of eq 1

$$\mu_{ap} = \mu_{ep} + \mu_{EOF} \quad (1)$$

Any of these mobilities can easily be calculated from the capillary length to the detector (L_D , usually in cm), the electric field applied (E , in $V \cdot cm^{-1}$), the observed migration time of the compound (t_m), and an electroosmotic flow marker (t_0). Thus

$$\mu_{ap} = \frac{L_D}{E \cdot t_m} \quad (2)$$

$$\mu_{ep} = \frac{L_D}{E} \left(\frac{1}{t_m} - \frac{1}{t_0} \right) \quad (3)$$

where E is calculated from the applied potential (V) and the total capillary length (L_T) thus

$$E = \frac{V}{L_T} \quad (4)$$

For an ionizable acid–base compound, the electrophoretic mobility depends on the degree (or degrees) of ionization of the compound. Thus, the general acid–base equilibria for a monoprotic species, HX^z , can be expressed as

$$HX^z \rightleftharpoons X^{z-1} + H^+ \quad pK'_a = pH - \log \frac{[X^{z-1}]}{[HX^z]} \quad (5)$$

where z is the charge of the protonated species and pK'_a the logarithmic form of the acidity constant (at a given ionic strength).

The μ_{ep} , also called the effective mobility (μ_{eff}), of a monoprotic compound can be expressed as a function of the pK'_a of the species and the pH of the background electrolyte through the following general equation⁸

$$\mu_{eff} = \frac{\mu_{HX^z} + (\mu_{X^{z-1}})10^{pH-pK'_a}}{1 + 10^{pH-pK'_a}} \quad (6)$$

where μ_{HX^z} and $\mu_{X^{z-1}}$ are the limiting electrophoretic mobilities of the subscripted species.

In the classic CE method, the pH of the buffer solution is measured by potentiometry and related to the mobility of the compound in the same buffered solution measured by CE. In the IS-CE method, both parameters are obtained at the same time in the same CE experiment. The use of an IS allows direct calculation of the pH of the buffered solution inside the capillary. This value is then used to calculate pK'_a of the compound being studied.

For example, for a neutral acid used as the IS, where z is 0, the pH value can be given, by rearranging eq 6, by

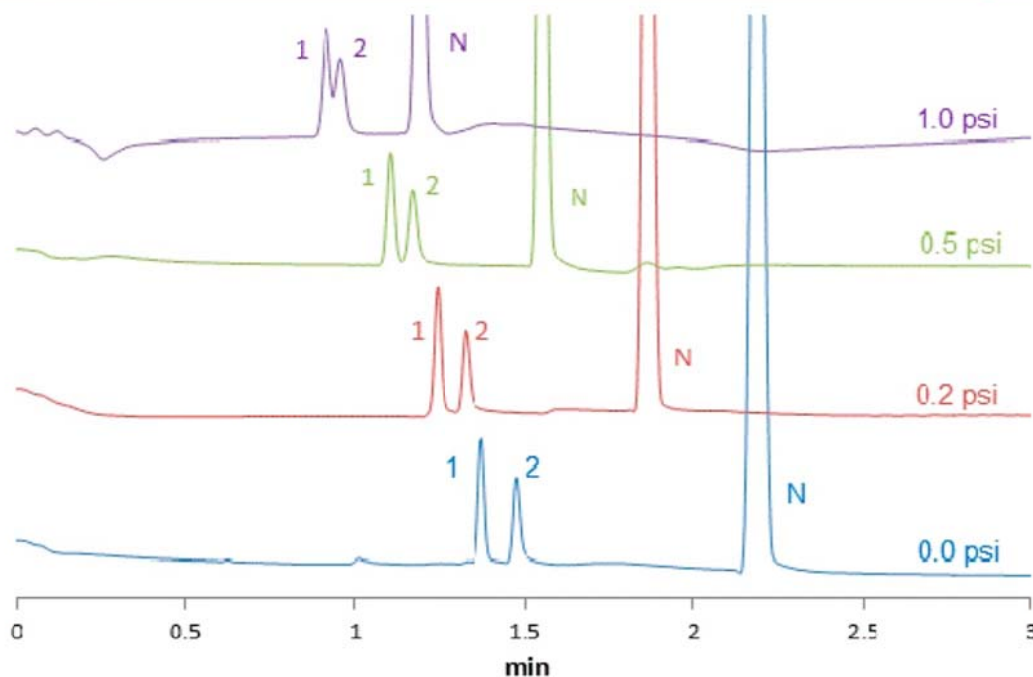


Figure 1. Effect of pressure during the separation on the migration time and resolution. Buffer: $\text{HCOOH}/\text{HCOO}^-$, $I = 50 \text{ mM}$, $\text{pH} = 3.5$; compounds, clonidine (1) and imipramine (2) at 100 ppm ; neutral marker DMSO (N).

$$\text{pH} = \text{p}K'_{\text{a}(\text{IS})} - \log \frac{\mu_{\text{IS}^-} - \mu_{\text{eff}(\text{IS})}}{\mu_{\text{eff}(\text{IS})}} \quad (7)$$

where $\mu_{\text{eff}(\text{IS})}$ is the effective mobility and μ_{IS^-} the limiting mobility (mobility of the fully charged negative species) of the IS. The term corresponding to the uncharged species, μ_{HX} , has zero mobility and is removed from eq 6. Since $\text{p}K'_{\text{a}(\text{IS})}$ of the IS is well-known, only two runs at two different pH values are needed to calculate the pH: the first where the pH is in the range $\text{p}K_{\text{a}} \pm 1$ to calculate the effective mobility; and a second in which it is completely ionized to calculate the limiting mobility.

Once the pH inside the capillary is known, the acidity constant of the TC can be calculated by rearranging eq 6 again:

$$\text{p}K'_{\text{a}(\text{TC})} = \text{pH} - \log \frac{\mu_{\text{HX}^z} - \mu_{\text{eff}(\text{TC})}}{\mu_{\text{eff}(\text{TC})} - \mu_{\text{X}^{z-1}}} \quad (8)$$

where μ_{HX^z} and $\mu_{\text{X}^{z-1}}$ refer to the limiting mobilities of the fully charged z and $z - 1$ species of the TC.

In these equations, $\text{p}K'_{\text{a}(\text{TC})}$ is related to the thermodynamic $\text{p}K_{\text{a}}$ by the activity coefficients, calculated by means of the Debye–Hückel equation.²²

EXPERIMENTAL SECTION

Apparatus. CE experiments were performed with a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. The capillary was made of fused silica, $50 \mu\text{m}$ I.D., $375 \mu\text{m}$ O.D., 35.2 cm length (25 cm to the detector) and was obtained from Composite Metal Services Ltd. (Shipley, West Yorkshire, UK). The temperature of the capillary was set to $25.0 \text{ }^\circ\text{C} \pm 0.1 \text{ }^\circ\text{C}$. The TCs and ISs were injected sequentially (one-by-one before separation) at a hydrodynamic pressure of 0.5 psi for 3 s ($1 \text{ psi} = 6897.76 \text{ Pa}$), and the applied voltage during separation was 20 kV . An additional 0.5 psi of hydrodynamic flow was applied

during separation. The UV detector was set at 214 , 254 , and 280 nm .

Chemicals. Dimethyl sulfoxide $>99.9\%$ (DMSO), 0.5 M sodium hydroxide trititol, 0.5 M hydrochloric acid trititol, and sodium dihydrogen phosphate monohydrate $>99\%$ were obtained from Merck (Darmstadt, Germany). Anhydrous sodium acetate $>99.6\%$ was purchased from J.T. Baker (Deventer, Netherlands). 2-(Cyclohexylamino)ethanesulfonic acid $>99\%$ (CHES) and 3-(cyclohexylamino)-1-propanesulfonic acid $>98\%$ (CAPS) were purchased from Sigma (St. Louis, MO, USA). 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol $>99.9\%$ (BisTris) and sodium formate were bought from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)amino-methane $>99.9\%$ (Tris) was purchased from Aldrich (Milwaukee, WI, USA). Water was purified using a Milli-Q plus system from Millipore (Bedford, MA, USA), to a resistivity of $18.2 \text{ M}\Omega\text{cm}$.

The ISs and TCs used were reagent grade or of chromatographic quality and obtained from Sigma, Fluka, Aldrich, or Carlo Erba (Milan, Italy).

Sample and buffer preparations are detailed in the Supporting Information, section 1.

RESULTS AND DISCUSSION

To consider IS-CE a high-throughput method for accurate experimental $\text{p}K_{\text{a}}$ determination in day-to-day analysis, instrumental parameters and experimental conditions need to be optimized to reduce the run time, conditioning steps and sample preparation as much as possible. IS and buffer solution stability must also be studied to determine how many times they can be used before requiring replacement. The precision and accuracy of the method should also be established.

Instrumental Conditions. In accordance with ideal requirements for the IS-CE method, a CE P/ACE MDQ Beckman instrument was selected as the most adequate for the method. The samples are placed in a different tray from the

buffers and they can be removed easily from their position for storage. Moreover, the samples can be stored directly at 4 °C inside the instrument, and the instrument allows for the application of pressure during separation as well as sequential injecting.

Modifications were made to the experimental conditions and methodology reported in previous work^{18–21,23} to minimize the analysis time. The capillary was cut to the minimum length the instrument configuration allows: 25.0 cm to the detector and 35.2 cm total length. To avoid the Joule effect, a voltage of 20 kV was selected as the maximum to maintain linearity between the voltage applied and the current generated (according to Ohm's law) for all the buffers used. Under these capillary and voltage conditions, migration times were reduced compared to those in previous work, down to average analysis times of 3 min for anionic compounds, which are the last compounds eluted.

To avoid the necessity to prepare solutions of specific mixtures of the TC and the appropriate ISs prior to the analysis, sequential injection was used. It must be taken into account that the sample plug length (TC plus IS plugs) should be less than 1% or 2% of the total length of the capillary to avoid overloading. Consequently, the lowest pressure that the instrument allowed (0.5 psi) was applied for the 3 s of injection time.

To speed up the migration of the compounds, pressure was applied during separation: the higher the pressure, the shorter the migration times. However, high pressure may result in overlapping peaks. In order to optimize the pressure, the migration time and separation of several compounds with different structures and limiting mobilities were compared at different pressures (0.0, 0.2, 0.5, and 1.0 psi). The example shown in Figure 1 illustrates as when pressure increases, run time decreases, as does the resolution between peaks. 0.5 psi was chosen as the best compromise between high resolution and a short run time.

Pre- and postconditioning methods (at the beginning of the session; between injections with the same buffer; when the buffer is changed; and at the end of the working session) were optimized to reduce the rinsing time and, as a consequence, total analysis time. Table 2 shows the conditioning sequences after the optimization processes. The total rinsing time was drastically reduced just to obtain reproducible results.

Table 2. Capillary Conditioning Optimization

rinse type	CE conditioning (at 20 psi)
beginning of the session	2.0 min NaOH 1.0 M
	0.5 min H ₂ O
	2.0 min buffer
replicates	no rinse
change pH	0.2 min new buffer
end of the session	2.0 min NaOH 0.1 M
	2.0 min H ₂ O

A more detailed description of these processes is available in the Supporting Information (section 2 Instrumental conditions).

Electroosmotic Flow Marker. Although many organic solvents can be used as EOF markers, the common use of DMSO in the syntheses of new drugs together with the fact that it is the most commonly used solvent for preparing compound libraries in the pharmaceutical industry,^{24–26} make it appropriate for our purpose. Many potential samples may

already have this EOF marker incorporated, prior to the analysis.^{27,28}

To test the performance of the marker, several compounds were totally dissolved in DMSO at a concentration of 1000 $\mu\text{g mL}^{-1}$. Solutions were then prepared for injection at a concentration of 100 $\mu\text{g mL}^{-1}$ by dilution with water. When the samples were injected, significant peak tailing appeared due to the high concentration of DMSO (Figure 2a). For cationic

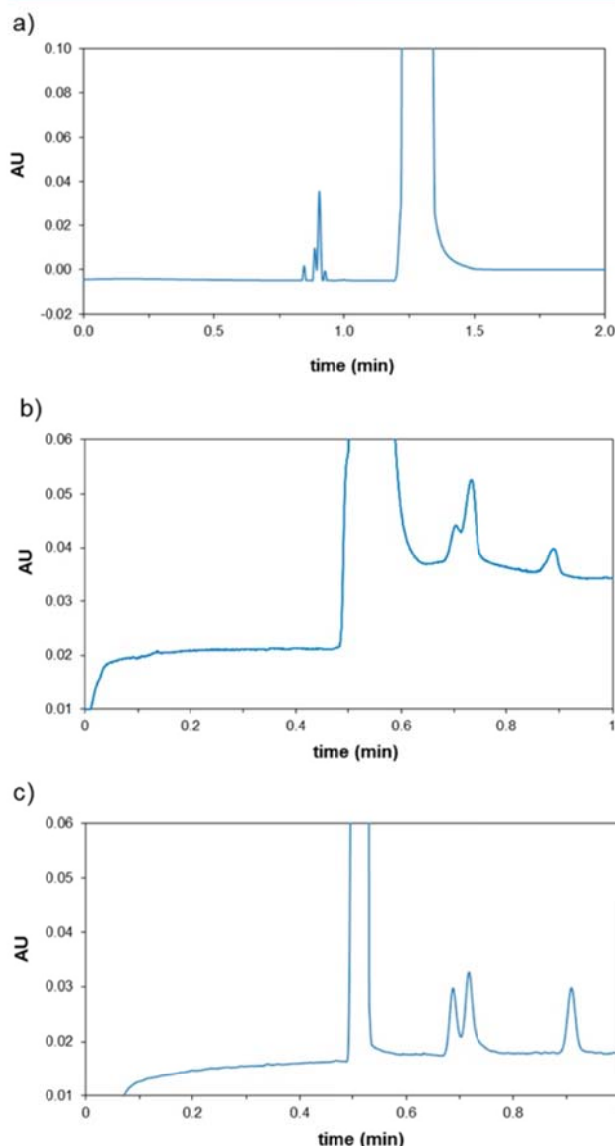


Figure 2. Electropherograms of (a) cationic compounds (metoprolol, diphenidramine, propranolol, ephedrine) at pH = 5.0 and 10% DMSO; (b) anionic compounds (ibuprofen, nicotinic acid, warfarin) at pH = 8.5 and 10% DMSO; (c) anionic compounds (ibuprofen, nicotinic acid, warfarin) at pH = 8.5 and 0.4% DMSO.

compounds no problems were observed because they elute before the EOF marker; however, the anionic compounds were hardly detectable since they were diffused in the tail of the DMSO peak (Figure 2b). Consequently, the quantity of DMSO in the samples was reduced 20-fold. So that 4% DMSO was added to the stock solutions and the final volume was made up with water. After 1/10 dilution, the content of DMSO in the

injection vial was about 0.4%. In these conditions, the large peak tail disappeared, as shown in Figure 2c, thereby increasing the peak efficiency.

Stability of the Buffers. The buffers must satisfy several requirements for meaningful experimental results to be obtained. For use in CE, a buffer must have a good buffering capacity in the pH range of use and a low detection signal.¹⁶ The buffers should also be inert so that they do not interact with the analyte or the walls of the capillary. Moreover, the whole CE system must be filled with the same buffer to keep parameters, such as conductivity, electric field, temperature and pH, as constant as possible.^{10,16}

The precise determination of a pK_a by CE requires the measurement of the effective mobility in several electrolytes with a low and constant ionic strength. This ionic strength must be low enough to minimize activity corrections, temperature gradients and viscosity differences; but there must be enough buffer capacity to maintain a fixed pH, even after introduction of the sample.^{16,23} Thus, an ionic strength of 0.05 M is a reasonable compromise between the need to minimize Joule heat production inside the capillary and the buffering capacity required. Several running solutions covering the pH range that is useful in CE system were prepared at this ionic strength. Supporting Information Table S1 shows the buffers used, their pK_a value and the stock solution used in their preparation. These buffers have an effective range of approximately two pH units, centered on their pK_a value.

The pH of the buffer can be affected by several factors, such as volatilization (e.g., ammonia buffers), electrolysis when a voltage is applied (almost all buffers), or even CO_2 dissolution (NaOH and highly basic buffers), which cause changes in the pH and migration velocity of the compounds over time.^{17,29} Even though with the IS-CE method the pH of the buffer solution inside the capillary can be easily calculated for every run, it is important to know for how many runs a buffer can be used before its properties stray from an acceptable range. Consequently, the recommended buffers were monitored over time to ensure that they kept their initial properties. One way to monitor stability is through the pH value. This is easy to monitor because the pH inside the capillary can be calculated at any time just from knowing the pK_a value and mobilities (limiting and effective) of any compound injected (in fact, of an IS) in a capillary filled with the buffer under study.

The pH values of all the buffers shown in Supporting Information Table S1 were monitored over time (every 5 min for a total of 16 h of electroseparation) using three different ISs of appropriate pK_a from Table 1. Figure 3 shows the average pH obtained with the three ISs against the electrophoretic run time. As can be seen, in all the cases the pH decreases over the run time. This can easily be explained by the electrolysis processes that occur in the separation vials: reactions involving the generation (inlet buffer) and consumption (outlet buffer) of hydrogen ions occur at the electrodes.³⁰ As explained above, in the IS-CE method alterations in the conditions of analysis can be minimized by the use of an IS that undergoes the same alteration as the TC. This means that these pH variations should not affect the pK_a determination of any compound provided that the TC and IS remain adequately ionized.

Therefore, at the same time as monitoring the pH inside the capillary, the pK_a of two compounds, 2,6-dibromo-4-nitrophenol and acridine, was determined with the same frequency. For 2,6-dibromo-4-nitrophenol, 2-chlorobenzoic acid was used as the IS, and the experiments were performed with an acetic/

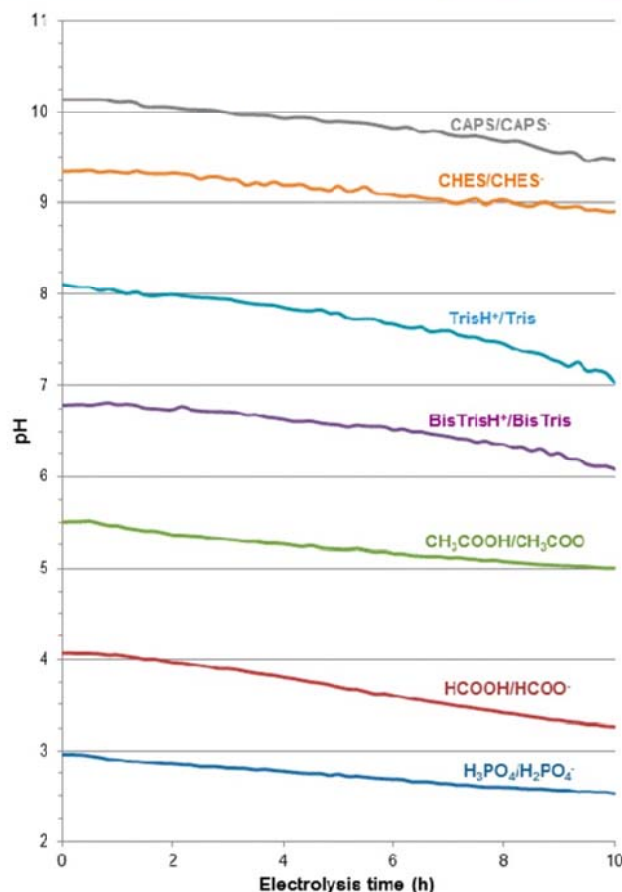


Figure 3. pH of the background electrolytes as a function of electrophoretic run time.

acetate buffer at pH 5.5 and 50 mM ionic strength. In the case of acridine, pyridine was used as the IS and $H_3PO_4/H_2PO_4^-$ as the separation buffer at pH 3.0 ($I = 50$ mM). Figure 4 shows the pK_a of the compounds (solid line) and the pH of the buffers (dashed line) over the electroseparation time. It can clearly be seen that while the pH decreases during voltage application, the calculated pK_a remains constant all through the 16 h of the study (around 500 injections). This demonstrates that the IS-CE method does not require the buffer vials to be replaced frequently; whereas in other work,^{11,17,29} where the classic CE method is used for pK_a determination, pH variation is minimized by replacement of the buffer solutions approximately every 1–2 h of electrodriven separation.

Nevertheless, after several hours of separation, the pH of the buffer may become drastically reduced. For example, Figure 3 shows that the pH of TRIS or CAPS decreases nearly one unit after 10 h of separation. The buffering capacity is then clearly diminished and, what is more important, the IS and TC selected may be in different ionization states from the initial one. In order to prevent this, we recommend buffer replacement after 10 h of use; considering a mean run time of 2 min, this corresponds to 300 injections.

Stability of the Internal Standards. Table 1 shows the pK_a values of the 48 monoprotic acidic²⁰ and basic²¹ ISs established in previous works. The list comprises a reference set of compounds with well-determined acidity constants that facilitates the process of selecting an appropriate IS for a given pK_a determination. Thus, when the pK_a of a TC is to be

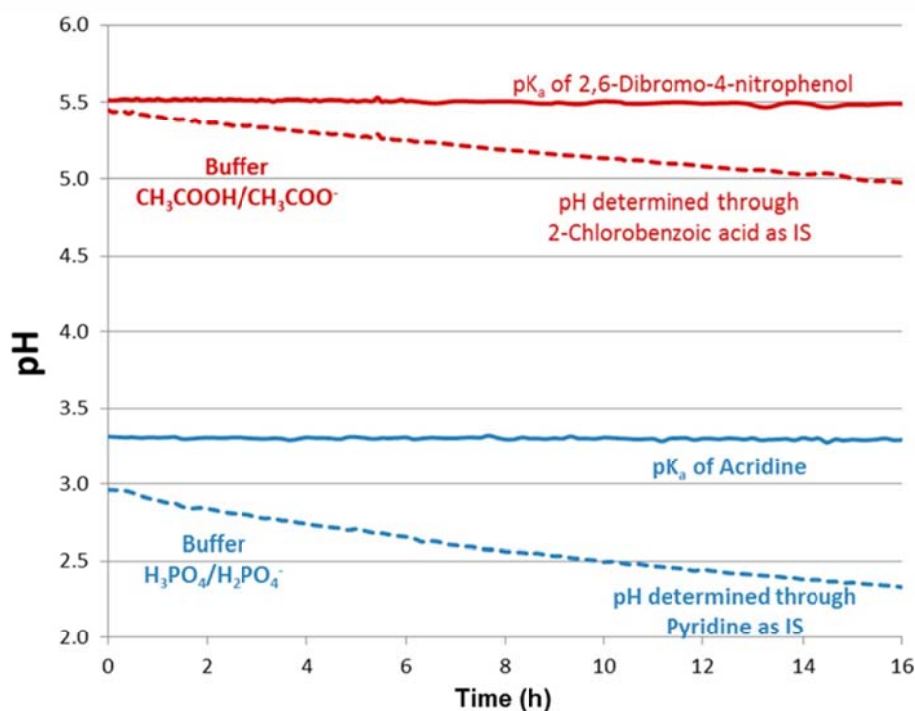


Figure 4. pH of the buffer solution vs electrolysis time. Solid lines correspond to the pK_a of test compounds. Dashed lines are the pH of the buffer calculated using the ISs. Buffers used: pH = 3.0, $H_3PO_4/H_2PO_4^-$; pH = 5.5, CH_3COOH/CH_3COO^- .

determined, one or several appropriate ISs from the sample tray are sequentially injected with the TC. However, the replacement time of the solutions must be known because of possible degradation.

To evaluate the stability of the ISs, each compound was injected once per week at a pH at which it was totally ionized. The peak area and the mobility of the compounds were monitored over this time. Nicotinic acid, *N,N*-dimethyl-*N*-phenylamine, pyridine, and 4-*tert*-butylpyridine showed a decrease of 10% in their area in a few days; so they must be replaced daily. In the case of vanillin, aniline, quinolone, 4-*tert*-butylaniline, 2,4-lutidine, trazodone, pilocarpine, 2,4,6-trimethylpyridine, bupivacaine, imipramine, and propranolol, weekly replacement is required. Warfarin, 2,4,6-tribromophenol, acridine, papaverine, and procainamine require only monthly replacement. For the rest of ISs, the peak area was still 90% or more after a four-month period.

Precision and Accuracy. The precision of the pK_a values obtained (standard deviation, sd) can be evaluated in several ways. Effective mobilities can be determined using several buffers with pH values close to the pK_a of the TC. For example, the pK_a of mepivacaine was determined using bupivacaine as the IS ($pK_{a(IS)} = 8.19$) at three pH values near to their pK_a values. Considering the μ_{eff} of the TC and IS at pH values of 7.5, 8.0, and 8.5, the following results were obtained: 7.90, 7.95, and 7.93, respectively. So, final pK_a value obtained for mepivacaine is 7.93 ± 0.03 . In this case, four electropherograms are required to determine the acidity constant in triplicate. Alternatively, and much faster, the precision can be improved if more than one IS is used for any pK_a determination. Several ISs can be sequentially injected together with the TC in the same run. For example, the pK_a of mepivacaine was determined using three different ISs (lidocaine, clonidine and bupivacaine) in the same pH buffer and run, obtaining an average pK_a for the three

ISs of 7.92 ± 0.02 . In this way, just two runs of 1.1 and 1.6 min are needed to obtain the pK_a value as determined in triplicate, since the four compounds (mepivacaine and the three ISs) are injected together. Figure 5 shows both electropherograms.

To date, we have used the IS-CE method to determine 129 pK_a values for 93 different drugs ($pK_{a(IS-CE)}$). These compounds range from simple structures (with just one acidity constant) to more complex ones with up to four constants. To estimate the accuracy of the experimental values, we correlated them with compiled values obtained from the literature ($pK_{a(lit)}$) at 25 °C and zero ionic strength^{20–22,31,32} or measured in our laboratory by other methods. The overall data is presented in Table 2S in the Supporting Information. Figure 6 shows $pK_{a(IS-CE)}$ as a function of $pK_{a(lit)}$ and the fitted line. The equation obtained is

$$pK_{a(IS-CE)} = (0.995 \pm 0.006)pK_{a(lit)} + (0.05 \pm 0.05)$$

$$SD = 0.17 \quad F = 24783 \quad (9)$$

The slope and intercept of the correlation are not significantly different from 1 and 0, respectively, for a 95% confidence level. These fitting results demonstrate the accuracy of the IS-CE method compared with reference literature values.

CONCLUSION

The proposed IS-CE method is a fast and attractive alternative to longer time-consuming methods for the determination of acidity constants, such as potentiometric and spectrophotometric titrations, or the classic CZE method. Several instrumental parameters and different experimental conditions were studied and optimized to reduce the time of pK_a determination. With a short capillary of just 25 cm to the detector, reduced capillary conditioning times, 0.4% DMSO marker, hydrodynamic sequential injection at 0.5 psi for 3s, and the application of 20 kV and 0.5 psi during separation, the time

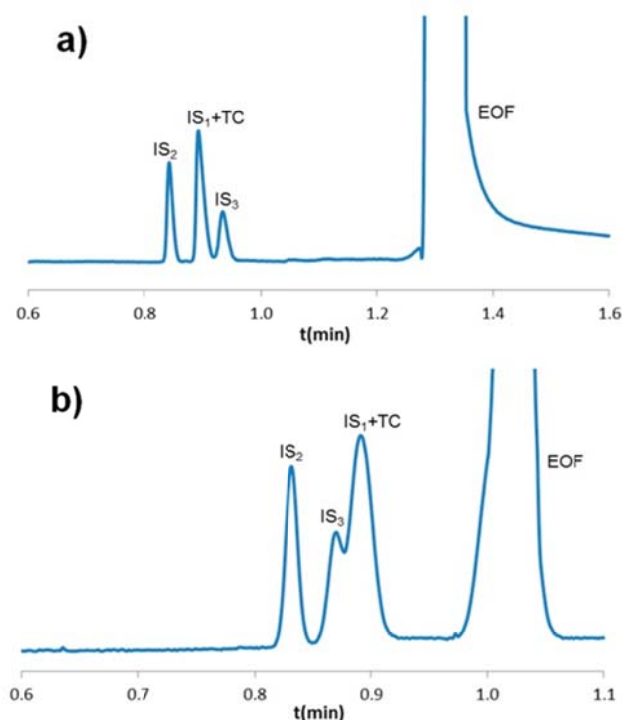


Figure 5. Electropherograms of mepivacaine (TC) and 3 internal standards (IS₁, lidocaine; IS₂, clonidine; IS₃, bupivacaine): (a) pH = 5, limiting mobilities and (b) pH = 8, effective mobilities.

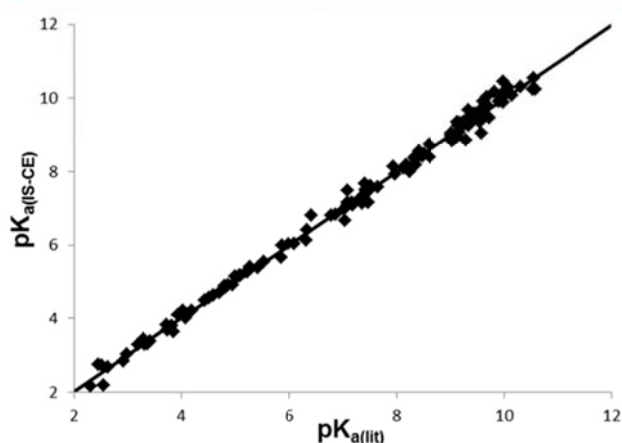


Figure 6. Measured pK_a ($pK_{a(IS-CE)}$) vs literature pK_a ($pK_{a(lit)}$). Fitted line is shown as the solid line.

required for the electropherograms can be reduced in such a way that any pK_a can be obtained in less than 5 min using the proposed buffers and ISs. 129 pK_a values from 93 drugs, ranging from simple structures to more complex ones with up to four constants, were determined by this IS-CE method and satisfactorily correlated with reference values from the literature.

In conclusion, IS-CE is a powerful high-throughput method suitable for routine pK_a determination in drug discovery and development laboratories.

■ ASSOCIATED CONTENT

■ Supporting Information

Detailed experimental samples and buffers preparation, instrumental conditions, and precision and accuracy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: (+34) 93 403 92 75. Fax (+34) 93 402 12 33. E-mail: marti.roses@ub.es.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the Spanish *Ministerio de Ciencia e Innovación* and the Fondo Europeo de Desarrollo Regional (FEDER) of the European Union (Project CTQ2010-19217/BQU) for financial support. Furthermore, J.M. Cabot thanks the *Secretaria d'Universitats i Recerca* of the Department d'Economia i Coneixement of the Catalan Government for a supporting scholarship (2013FI_B1 00209).

■ REFERENCES

- (1) Comer, W. T. Perspectives in Drug Discovery. *Ref. Module Chem., Mol. Sci. Chem. Eng.* **2013**, DOI: 10.1016/B978-0-12-409547-2.02699-8.
- (2) Ganellin, R.; Roberts, S.; Jefferis, R.; Stocks, M. The Small Molecule Drug Discovery Process—From Target Selection to Candidate Selection. *Introd. Biol. Small Mol. Drug Res. Dev.* **2013**, 81–126.
- (3) Vachal, P.; Tata, J.; Athanasopoulos, J. High-Throughput Purification Platform in Support of Drug Discovery. *ACS Comb. Sci.* **2012**, 14, 51–59.
- (4) Li, S.; Julien, L.; Tidswell, P.; Goetzinger, W. Enhanced Performance Test Mix for High-Throughput LC/MS Analysis of Pharmaceutical Compounds. *J. Comb. Chem.* **2006**, 8, 820–828.
- (5) Wan, H.; Ulander, J. High-Throughput pK_a Screening and Prediction Amenable for ADME Profiling. *Expert Opin. Drug Metab. Toxicol.* **2006**, 2, 139–155.
- (6) Reijenga, J.; van Arno, H.; van Antonie, L.; Teunissen, B. Development of Methods for the Determination of pK_a Values. *Anal. Chem. Insights* **2013**, 8, 53–71.
- (7) Cleveland, J. A., Jr.; Benko, M. H.; Gluck, S. J.; Walbroehl, Y. M. Automated pK_a Determination at Low Solute Concentrations by Capillary Electrophoresis. *J. Chromatogr. A* **1993**, 652, 301–308.
- (8) Gluck, S. J.; Steele, K. P.; Benkö, M. H. Determination of Acidity Constants of Monoprotic and Diprotic Acids by Capillary Electrophoresis. *J. Chromatogr. A* **1996**, 745, 117–125.
- (9) Jiménez-Lozano, E.; Marqués, I.; Barrón, D.; Beltrán, J. L.; Barbosa, J. Determination of pK_a Values of Quinolones from Mobility and Spectroscopic Data Obtained by Capillary Electrophoresis and a Diode Array Detector. *Anal. Chim. Acta* **2002**, 464, 37–45.
- (10) Poole, S. K.; Patel, S.; Dehring, K.; Workman, H.; Poole, C. F. Determination of Acid Dissociation Constants by Capillary Electrophoresis. *J. Chromatogr. A* **2004**, 1037, 445–454.
- (11) Herrero-Martínez, J. M.; Sanmartín, M.; Roses, M.; Bosch, E.; Rafols, C. Determination of Dissociation Constants of Flavonoids by Capillary Electrophoresis. *Electrophoresis* **2005**, 26, 1886–1895.
- (12) Šlampová, A.; Krivánková, L.; Gebauer, P.; Boček, P. Standard Systems for Measurement of pK_s and Ionic Mobilities: 1. Univalent Weak Acids. *J. Chromatogr. A* **2008**, 1213, 25–30.
- (13) Babić, S.; Horvat, A. J. M.; Mutavdžić Pavlović, D.; Kaštelan-Macan, M. Determination of pK_a Values of Active Pharmaceutical Ingredients. *Trends Anal. Chem.* **2007**, 26, 1043–1061.

- (14) Andrási, M.; Buglyó, P.; Zekany, L.; Gaspar, A. A Comparative Study of Capillary Zone Electrophoresis and pH-Potentiometry for Determination of Dissociation Constants. *J. Pharm. Biomed. Anal.* **2007**, *44*, 1040–1047.
- (15) Barbosa, J.; Barrón, D.; Jiménez-Lozano, E.; Sanz-Nebot, V. Comparison between Capillary Electrophoresis, Liquid Chromatography, Potentiometric and Spectrophotometric Techniques for Evaluation of pKa Values of Zwitterionic Drugs in Acetonitrile–Water Mixtures. *Anal. Chim. Acta* **2001**, *437*, 309–321.
- (16) Beckers, J. L.; Bocek, P. The Preparation of Background Electrolytes in Capillary Zone Electrophoresis: Golden Rules and Pitfalls. *Electrophoresis* **2003**, *24*, 518–535.
- (17) Fuguet, E.; Reta, M.; Gibert, C.; Rosés, M.; Bosch, E.; Ràfols, C. Critical Evaluation of Buffering Solutions for pKa Determination by Capillary Electrophoresis. *Electrophoresis* **2008**, *29*, 2841–2851.
- (18) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. A Fast Method for pKa Determination by Capillary Electrophoresis. *Chem. Biodiversity* **2009**, *6*, 1822–1827.
- (19) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. Fast High-Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis: I. Monoprotic Weak Acids and Bases. *J. Chromatogr. A* **2009**, *1216*, 3646–3651.
- (20) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. Fast High-Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. II. Acidic Internal Standards. *J. Chromatogr. A* **2010**, *1217*, 8340–8345.
- (21) Fuguet, E.; Ràfols, C.; Rosés, M. A Fast High Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. 3. Basic Internal Standards. *J. Chromatogr. A* **2011**, *1218*, 3928–3934.
- (22) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. Determination of Acidity Constants by the Capillary Electrophoresis Internal Standard Method. IV. Polyprotic Compounds. *J. Chromatogr. A* **2013**, *1279*, 108–116.
- (23) Gravador, R. S.; Fuguet, E.; Ràfols, C.; Rosés, M. Temperature Variation Effects on the Determination of Acidity Constants through the Internal Standard-Capillary Electrophoresis Method. *Electrophoresis* **2013**, *34*, 1203–1211.
- (24) Avdeef, A. Physicochemical Profiling (Solubility, Permeability and Charge State). *Curr. Trends Med. Chem.* **2001**, *1*, 277–351.
- (25) Lipinski, C. A. Solubility in Water and DMSO: Issues and Potential Solutions. *Biotechnol.: Pharm. Aspects* **2004**, *1*, 93–125.
- (26) Lipinski, C. Drug Solubility in Water and Dimethylsulfoxide. *Methods Princ. Med. Chem.* **2008**, *37*, 257–282.
- (27) Wan, H.; Holmén, A.; Näägård, M.; Lindberg, W. Rapid Screening of pKa Values of Pharmaceuticals by Pressure-Assisted Capillary Electrophoresis Combined with Short-End Injection. *J. Chromatogr. A* **2002**, *979*, 369–377.
- (28) Lipka, E.; Folly-Klan, M.; Charton, J.; Vaccher, M.-P.; Bonte, J.-P.; Vaccher, C. Determination of pKa Values of Benzimidazole Derivatives from Mobility Obtained by Capillary Electrophoresis. *J. Pharm. Biomed. Anal.* **2010**, *53*, 1267–1271.
- (29) Foulon, C.; Duhal, N.; Lacroix-Callens, B.; Vaccher, C.; Bonte, J. P.; Goossens, J. F. Determination of pKa Values of Benzoxa-, Benzothia-, and Benzoselena-Zolinone Derivatives by Capillary Electrophoresis. Comparison with Potentiometric Titration and Spectrometric Data. *Eur. J. Pharm. Sci.* **2007**, *31*, 165–171.
- (30) Zhu, T.; Sun, Y.; Zhang, C.; Ling, D.; Sun, Z. Variation of the pH of the Background Electrolyte as a Result of Electrolysis in Capillary Electrophoresis. *J. High Resolut. Chromatogr.* **1994**, *17*, 563–564.
- (31) Box, K.; Bevan, C.; Comer, J.; Hill, A.; Allen, R.; Reynolds, D. High-Throughput Measurement of pKa Values in a Mixed-Buffer Linear pH Gradient System. *Anal. Chem.* **2003**, *75*, 883–892.
- (32) Box, K. J.; Comer, J. E. A. Using Measured pKa, LogP and Solubility to Investigate Supersaturation and Predict BCS Class. *Curr. Drug Metab.* **2008**, *9*, 869–878.

Supporting information

Internal Standard Capillary Electrophoresis as a High Throughput Method for pK_a Determination in Drug Discovery and Development.

*Joan M. Cabot, Elisabet Fuguet and Martí Rosés**

Departament de Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Corresponding Author. E-mail: marti.roses@ub.es

Table of contents

1. Preparation of samples and buffers.....	S2
2. Instrumental conditions.....	S3
3. Precision and accuracy.....	S7
FIGURES.....	S9
TABLES	S10
REFERENCES	S15

1. Preparation of samples and buffers

Buffer solutions covering practically all the useful pH range (from 2 to 12 separated at intervals of 0.5 pH units) were prepared at the approximate pH and 50 mM ionic strength as described elsewhere.^{1S} Table 1S shows the employed buffers, its pK_a value and the stock solution used in their preparation. To prepare the buffers at the desired pH and 50 mM ionic strength, caculated amounts of 0.5 M HCl or 0.5 M NaOH were added to 50 mL of the stock solution (0.1 M), according to Table 1S. Finally, the solution obtained was diluted by adding water up to a volume of 100 mL. Buffers were stored at 4°C until used.

Stock solutions of test compounds and ISs were prepared at a concentration of 1 mg mL⁻¹ and 4% of DMSO was added as electroosmotic flow (EOF) marker. They were diluted in water or in a methanol/water mixture (when they were not soluble in water itself). Afterwards, a 1/10 dilution of the stock solution in water was prepared for injection (100 mg L⁻¹, 0.4% DMSO). Internal standards and test compounds were stored at 4°C until used.

All the samples and buffers were filtered through a nylon mesh 0.45 µm porous size (Whatman, Maidstone, UK) and vials were filled up to 1.6 mL. When the instrument was not in use, sample tray and inlet and outlet buffer trays were removed and stored in a refrigerator at 4°C.

2. Instrumental conditions

2.1. Instrument selection

To implement IS-CE method for high-throughput analysis the commercial instrument has to accomplish a series of requirements: it must have a high number of positions in the sample and buffer trays to accommodate all standards and buffers; it is convenient for the sample and buffer trays to be in separate positions for easy replacement of samples; trays must be easily removable to store them in the refrigerator at 4°C when they are not used; sample trays should be thermostated while the instrument is working to avoid decomposition of ISs; the instrument must be able to apply pressure during the separation and capable of sequential injection to speed up the process.

According to these requirements, capillary electrophoresis P/ACE MDQ Beckman instrument was selected, among others, as the most adequate for developing the method. As illustrated by Figure 1S, samples are placed in a different tray than buffers and they can be removed easily from their position for storage. Moreover, samples can be directly stored at 4°C inside the instrument, and this instrument can apply pressure during separation as well as injecting sequentially.

2.2. Optimization of instrumental parameters

Modifications to experimental conditions and methodology reported in previous works^{2S-6S} have been performed in order to minimize the analysis time. As explained before (Theory Section), the mobility of a compound, μ_{ap} , can be expressed by means of eq 2. When migration time is isolated, eq 2 becomes eq 1S.

$$t_m = \frac{L_D}{E \cdot \mu_{ap}} \quad (1S)$$

This equation demonstrates that if low migration times are obtained, minimum capillary length and maximum electrical field must be used. Consequently, the capillary was cut as short as allowed by the instrument configuration, 25.0 cm until detector and 35.2 cm of total length. According to eq 4, voltage should be as high as possible to obtain the maximum electrical field. Migration time decreases when high voltages are applied, but on the contrary, efficiency is reduced because of the Joule heat generated.^{7S,8S} So, even though the rate of analysis becomes faster as the voltage is increased, the use of very high voltages can be a disadvantage because efficiency of separations as well as robustness of the method can be dramatically affected.^{7S-10S} It has been demonstrated that in pK_a determination through the IS-CE method some mobility fluctuations of the test compound can be corrected by an appropriate IS (if temperature variations affect equally both compounds).^{6S} Anyway, the linearity between the applied voltage and the generated current (according to Ohm's law) was tested for all the employed buffers in order to avoid the Joule effect. The voltage of 20 kV was selected as the maximum value for keeping linearity. In these conditions of capillary length and voltage, migration times were reduced in reference to previous works, obtaining average analysis times of 3 minutes for anionic compounds, which are the last eluted compounds.

2.3.Sequential injection and pressure-assisted separation

The IS-CE method implies the injection of the test compound and one or more internal standards (depending on the desired accuracy and precision) at the same time for the mobility measurement. The usual procedure would imply the preparation of solutions of specific mixtures of the TC and the appropriate ISs prior to the analysis, which enlarges the sample preparation step. However, this handicap can be overcome by sequential injection. In this way only the TC solu-

tion must be prepared (together with the EOF marker) prior to the test analysis. Then, the test compound is injected followed by all the required ISs, which are already in the sample tray of the CE instrument. Once ISs and TC are mixed into the capillary, voltage is applied to start the separation. This procedure drastically reduces the amount of mixtures TC-IS to be prepared

A pressure of 0.5 psi was applied during separation. Some authors^{11S} point out that the use of pressure introduces an additional hydrodynamic flow which generates a non-linear EOF. Therefore, it is interesting to know whether the applied pressure influences the effective mobility measurement, especially for short migration times. When electrophoretic mobilities were calculated (by eq 3), a slight decrease was observed (for basic compounds) or increase (for acidic compounds) of mobility values. Such deviations reach a relative variation around 8-10% when a pressure of 1 psi is applied. However, as in the IS-CE method any variation on the EOF affects equally the TC and the IS, the effect is compensated and the final determination of pK_a remains unaffected. This advantage enables the IS-CE method to obtain more accurate results than other CE methods.

2.4. Capillary conditioning

Pre- and post-conditioning methodologies (at the beginning of the session; between injections with the same buffer; when buffer is changed; and at the end of the working session) have been optimized to reduce the rinsing time, and as a consequence, the total analysis time. In order to assess this reduction, mobility of a compound (at pH where it was half ionized) was monitored as the rinsing time gradually decreased. A maximum variation of 3% was accepted, choosing as mobility reference value the one obtained in the much longer CE conditioning step used in previous works.^{4S,5S} The lowest rinsing time that did not exceed this error was selected.

Table 2 summarizes the conditioning sequences after the optimization processes. At the beginning of the session, capillary is intensively rinsed with sodium hydroxide, water and buffer. The rinsing time was drastically reduced from 50 to 4.5 minutes, which was enough to obtain reproducible results. When the buffer is changed, attention must be paid to eliminate completely the old buffer and replenish the capillary with the new one, otherwise ionization state of the injected compounds could not be the desired one. 5 s were needed to fill the capillary until detector and reach the final absorbance. For security, the rinsing time was set at 0.2 min (12 s) to assure the whole replacement of the solution inside the capillary and thus keeping an acceptable relative error. It was observed that there was no need at all to rinse the capillary between injections using the same buffer solution. As pressure was applied during the separation, buffer solution was constantly entering during all the separation time so at the end of the analysis the capillary was full of fresh buffer and ready for a new separation. Postconditioning of the capillary was also reduced from 25 to 4 minutes without loss of performance. Capillary conditioning time has been reduced nearly 12 times overall.

3. *Precision and accuracy*

The 129 pK_a values of 93 different drugs determined by the IS-CE method are presented in Table 2S.. This table also shows the average of pK_a values published in the literature^{4S,5S,12S-14S} as well as its differences with the IS-CE method determined ones. We include the range of literature values to illustrate the variability of results of some drugs.

The overall average difference between the pK_a values measured in this work and the average literature pK_a values was found to be -0.01 units. However, for some compounds there is a 0.2-0.4 variations difference. These deviations are mainly observed for compounds that have close

and/or extreme pK_a values, compounds with complex structures, or compounds that are unstable under basic conditions. The pK_a of these compounds are difficult to measure by any technique. For them, bibliographic information is scarce and dispersed. Some other compounds are poorly soluble in water and their pK_a values were determined in hydro-organic mixtures and extrapolated to the 0% co-solvent, which always implies higher error than direct determination in aqueous buffers. The good performance of the IS-CE method (low sample concentration and short analysis time requirements) allows direct determination of the pK_a of these compounds in water media.

FIGURES

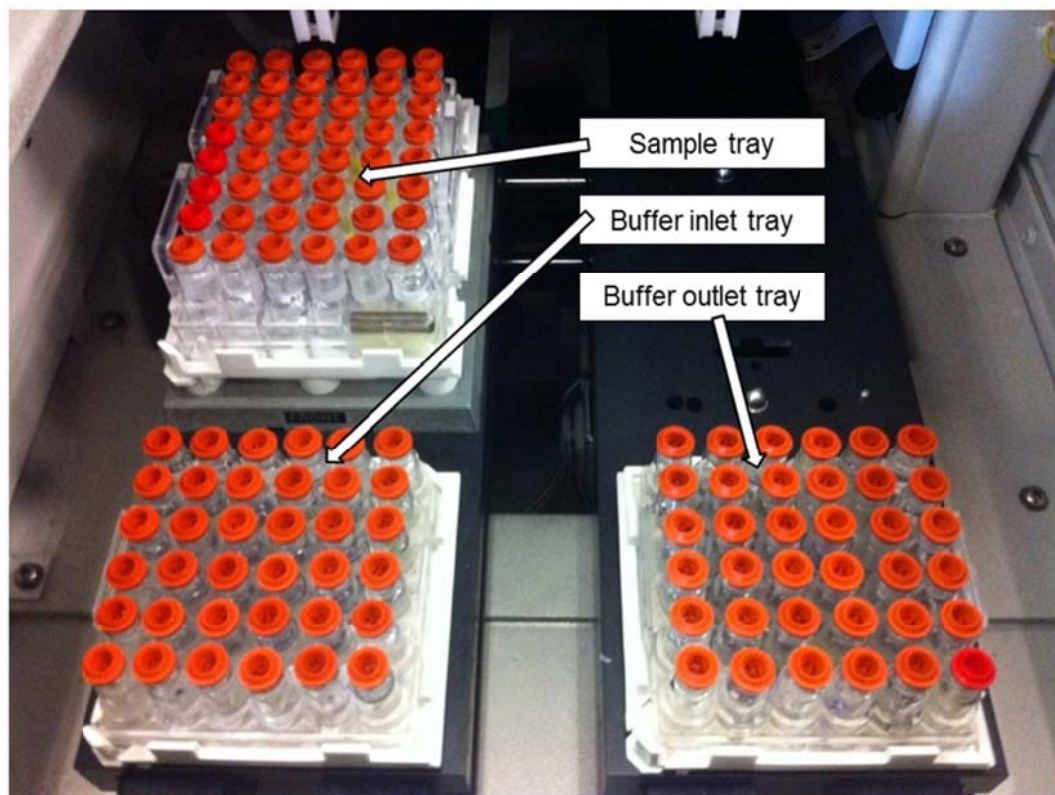


Figure 1S. Sample and buffer trays of Capillary Electrophoresis P/ACE MDQ instrument from Beckman.

TABLES

Table 1S. Buffer solutions used to determine the acidity constants by the IS-CE.

Buffer	pK _a	pH prepared	Stock solution	pH modifier
H ₃ PO ₄ /H ₂ PO ₄ ⁻	2.15	2.0; 2.5; 3.0	0.1 M NaH ₂ PO ₄	0.5 M HCl
HCOOH/HCOO ⁻	3.75	3.5; 4.0	0.1 M HCOONa	0.5 M HCl
CH ₃ COOH/CH ₃ COO ⁻	4.76	4.5; 5.0; 5.5	0.1 M CH ₃ COONa	0.5 M HCl
BisTrisH ⁺ /BisTris	6.48	6.0; 6.5; 7.0	0.1 M BisTrisHCl	0.5 M NaOH
TrisH ⁺ /Tris	8.08	7.5; 8.0; 8.5	0.1 M TrisHCl	0.5 M NaOH
CHES/CHES ⁻	9.50	9.0; 9.5	0.1 M CHESNa	0.5 M HCl
CAPS/CAPS ⁻	10.40	10.0; 10.5, 11.0	0.1 M CAPSNa	0.5 M HCl
NaOH	-	11.5; 12.0; 12.5	0.1 M NaOH + 0.5M KCl	-

Table 2S. IS-CE measured and literature pK_a values at 25°C and zero ionic strength.

Compound	IS-CE measured pK_a	Reference literature pK_a^a	Literature pK_a range	ΔpK_a^b	Ref.
2-Chlorobenzoic acid	2.84	2.92	2.92	0.08	4S
2,6-Dibromo-4-nitrophenol	3.31	3.38	3.38	0.07	4S
4-Nitrobenzoic acid	3.37	3.43	3.43	0.06	4S
2,6-Dinitrophenol	3.69	3.74	3.74	0.05	4S
3-Bromobenzoic acid	3.79	3.83	3.83	0.04	4S
2,4-Dinitrophenol	4.12	4.10	4.10	-0.02	4S
Benzoic acid	4.22	4.19	4.19	-0.03	4S
Ibuprofen	4.49	4.43	4.43	-0.06	4S
Aniline	4.63	4.60	4.60	-0.03	5S
Nicotinic acid	4.85	4.82	4.82	-0.03	4S
Quinoline	4.93	4.87	4.87	-0.06	5S
4- <i>tert</i> -butylaniline	4.93	4.95	4.95	0.02	5S
Warfarin	5.17	5.08	5.08	-0.09	4S
<i>N,N</i> -dimethyl- <i>N</i> -phenylamine	5.17	5.07	5.07	-0.10	5S
Pyridine	5.28	5.22	5.22	-0.06	5S
2,5-Dinitrophenol	5.30	5.22	5.22	-0.08	4S
Sulfacetamide	5.42	5.30	5.27-5.32	-0.12	4S
Acridine	5.55	5.52	5.52	-0.03	5S
2,4,6-Tribromophenol	6.04	6.10	6.10	0.06	4S
4- <i>tert</i> -butylpyridine	6.03	5.99	5.99	-0.04	5S
Papaverine	6.41	6.34	6.34	-0.07	5S
2,4-Lutidine	6.81	6.79	6.79	-0.02	5S
Trazodone	6.84	6.87	6.87	0.03	5S
Pilocarpine	7.08	7.08	7.08	0.00	5S
4-Nitrophenol	7.09	7.18	7.18	0.09	4S
Vanillin	7.36	7.40	7.40	0.04	4S
2,4,6-Trimethylpyridine	7.51	7.43	7.43	-0.08	5S
Phenobarbital	7.53	7.46	7.46	-0.07	4S
4-Hydroxybenzaldehyde	7.61	7.51	7.51	-0.10	4S
Lidocaine	7.93	7.96	7.96	0.03	5S
Clonidine	8.10	8.12	8.12	0.02	5S
Bupivacaine	8.19	8.35	8.35	0.16	5S
3,5-Dichlorophenol	8.18	8.18	8.18	0.00	4S
Methylparaben	8.35	8.37	8.37	0.02	4S
Quinine	8.45	8.48	8.48	0.03	5S
2-Chlorophenol	8.50	8.51	8.51	0.01	4S
1-Phenylpiperazine	8.75	8.60	8.60	-0.15	5S
<i>N,N</i> -dimethyl- <i>N</i> -benzylamine	8.95	9.14	9.14	0.19	5S

3-Chlorophenol	9.04	9.02	9.02	-0.02	4S
Diphenhydramine	9.08	9.17	9.17	0.09	5S
Procainamide	9.26	9.35	9.35	0.09	5S
4-Bromophenol	9.28	9.36	9.36	0.08	4S
Imipramine	9.37	9.30	9.30	-0.07	5S
Propranolol	9.47	9.48	9.48	0.01	5S
1-Aminoethylbenzene	9.52	9.49	9.49	-0.03	5S
Paracetamol	9.58	9.60	9.45-9.75	-0.13	4S
Ephedrine	9.72	9.66	9.66	-0.06	5S
Phenol	9.89	9.99	9.99	0.10	4S
Nortryptiline	10.08	10.14	10.14	0.06	5S
Furosemide	3.83	3.73	3.60-3.87	-0.10	12S
	10.25	10.53	10.32-10.75	0.28	12S
Phthalic acid	3.02	2.98	2.73-3.06	-0.04	12S
	5.38	5.41	4.78-5.43	0.03	12S
4-Hydroxybenzoic acid	4.57	4.51	4.45-4.57	-0.06	12S
	9.51	9.39	9.32-9.46	-0.12	12S
Acetazolamide	7.57	7.65	7.52-7.78	0.08	12S
	9.51	9.49	9.41-9.57	-0.02	12S
Pyrilamine	4.01	4.08	4.02-4.14	0.07	12S
	8.99	9.02	8.92-9.12	0.03	12S
Histamine	5.67	5.85	5.80-5.89	0.18	12S
	9.91	9.91	9.85-9.96	0.00	12S
Chlorpheniramine	3.64	3.86	3.86	0.22	12S
	9.27	9.16	9.13-9.18	-0.11	12S
Quinine	4.11	3.94	3.92-4.29	-0.17	12S
	8.43	8.48	8.39-8.60	0.05	12S
Levodopa	2.16	2.31	2.30-2.31	0.15	12S
	9.47	9.72	9.70-9.74	0.25	12S
Histidine	5.99	5.86	5.67-5.96	-0.13	12S
	9.32	9.19	8.99-9.31	-0.13	12S
Phenylalanine	2.20	2.20	2.16-2.23	0.00	12S
	9.35	9.24	9.01-9.32	-0.11	12S
2-Aminophenol	4.71	4.71	4.63-4.78	0.00	12S
	9.80	9.70	9.42-9.97	-0.10	12S
Pyridoxine	4.89	4.80	4.67-4.87	-0.09	12S
	9.02	9.04	8.99-9.11	0.02	12S
Labetalol	7.27	7.33	7.28-7.36	0.06	12S
	9.69	9.35	9.16-9.49	-0.34	12S
Sotalol	8.36	8.33	8.28-8.38	-0.03	12S
	9.92	9.61	9.47-9.75	-0.31	12S

Ampicillin	2.76	2.44	2.41-2.50	-0.32	12S
	7.48	7.09	6.94-7.30	-0.39	12S
Cefalexin	2.68	2.62	2.40-2.93	-0.06	12S
	7.17	7.17	7.08-7.27	0.00	12S
Cefadroxil	2.72	2.52	2.48-2.55	-0.20	12S
	7.67	7.41	7.21-7.65	-0.26	12S
	10.02	9.68	9.64-9.71	-0.34	12S
Chlortetracycline	3.28	3.31	3.30-3.33	0.03	12S
	7.19	7.46	7.40-7.55	0.27	12S
	8.86	9.30	9.27-9.33	0.44	12S
Oxitetracycline	3.28	3.19	3.04-3.30	-0.09	12S
	7.48	7.49	7.30-8.00	0.01	12S
	8.88	9.09	8.94-9.20	0.21	12S
Atenolol	9.67	9.55	9.54-9.56	-0.12	13S,14S
Procaine	9.08	9.10	9.04-9.15	0.02	13S,14S
Alprenolol	9.37	9.52	9.48-9.55	0.15	13S,14S
Diltiazem	7.99	8.13	8.02-8.24	0.14	13S,14S
Fluoxetine	10.08	10.05	10.00-10.09	-0.05	13S,14S
Flurbiprofen	4.19	4.01	4.01	-0.18	14S
Maprotiline	10.23	10.57	10.57	0.34	14S
Chlorpromazine	9.26	9.24	9.24	-0.02	14S
Diclofenac	4.22	4.03	4.03	-0.19	14S
Haloperidol	8.56	8.42	8.42	-0.14	14S
Miconazole	6.14	6.32	6.32	0.18	14S
Acebutolol	9.36	9.55	9.55	0.19	13S
Metoprolol	9.53	9.58	9.58	0.05	13S
Morin	5.15	4.99	4.99	-0.16	- ^c
	8.15	7.93	7.93	-0.22	
	10.46	9.99	9.99	-0.47	
Quercetin	7.15	7.09	7.09	-0.06	- ^c
	8.84	9.04	9.04	0.20	
	10.31	10.30	10.30	-0.01	
Kaemferol	7.16	7.47	7.47	0.31	- ^c
	9.05	9.58	9.58	0.53	
	10.54	10.54	10.54	0.00	
Fisetin	7.14	7.36	7.36	0.22	- ^c
	8.39	8.34	8.34	-0.05	
	10.28	10.08	10.08	-0.20	
Galangin	7.25	7.37	7.37	0.12	- ^c
	9.54	9.39	9.39	-0.15	
Naringenin	6.68	7.04	7.04	0.36	- ^c

	10.16	9.82	9.82	-0.34	
Hesperetin	6.97	7.02	7.02	0.05	- ^c
	10.13	9.96	9.96	-0.17	
Apigenin	7.60	7.52	7.52	-0.08	- ^c
	9.35	9.14	9.14	-0.21	
Chrysin	7.47	7.44	7.44	-0.03	- ^c
Ketoconazole	3.45	3.29	3.29	-0.16	13S
	6.81	6.42	6.42	-0.39	13S
Theophylline	8.39	8.63	8.63	0.24	13S

^a: pK_a average of all literature values.

^b: $\Delta pK_a = pK_a(\text{literature}) - pK_a(\text{IS-CE})$.

^c: Determined potentiometrically at our laboratory.

REFERENCES

- (1S) Fuguet, E.; Reta, M.; Gibert, C.; Rosés, M.; Bosch, E.; Ràfols, C. Critical Evaluation of Buffering Solutions for pKa Determination by Capillary Electrophoresis. *Electrophoresis* **2008**, *29*, 2841–2851.
- (2S) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. A Fast Method for pKa Determination by Capillary Electrophoresis. *Chemistry & Biodiversity* **2009**, *6*, 1822–1827.
- (3S) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. Fast High-Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis: I. Monoprotic Weak Acids and Bases. *Journal of Chromatography A* **2009**, *1216*, 3646–3651.
- (4S) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. Fast High-Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. II. Acidic Internal Standards. *Journal of Chromatography A* **2010**, *1217*, 8340–8345.
- (5S) Fuguet, E.; Ràfols, C.; Rosés, M. A Fast High Throughput Method for the Determination of Acidity Constants by Capillary Electrophoresis. 3. Basic Internal Standards. *Journal of Chromatography A* **2011**, *1218*, 3928–3934.
- (6S) Gravador, R. S.; Fuguet, E.; Ràfols, C.; Rosés, M. Temperature Variation Effects on the Determination of Acidity Constants through the Internal Standard-Capillary Electrophoresis Method. *Electrophoresis* **2013**, *34*, 1203–1211.
- (7S) Lacey, M. E.; Webb, A. G.; Sweedler, J. V. On-Line Temperature Monitoring in a Capillary Electrochromatography Frit Using Microcoil NMR. *Analytical Chemistry* **2002**, *74*, 4583–4587.
- (8S) Evenhuis, C. J.; Guijt, R. M.; Macka, M.; Marriott, P. J.; Haddad, P. R. Temperature Profiles and Heat Dissipation in Capillary Electrophoresis. *Analytical Chemistry* **2006**, *78*, 2684–2693.
- (9S) Rathore, A. S. Joule Heating and Determination of Temperature in Capillary Electrophoresis and Capillary Electrochromatography Columns. *Journal of Chromatography A* **2004**, *1037*, 431–443.
- (10S) Taylor, D. R. Capillary Electrophoresis. Techniques in Analytical Chemistry Series by R. Baker. *Chromatographia* **1996**, *43*, 94.
- (11S) Wan, H.; Holmén, A.; Någård, M.; Lindberg, W. Rapid Screening of pKa Values of Pharmaceuticals by Pressure-Assisted Capillary Electrophoresis Combined with Short-End Injection. *Journal of Chromatography A* **2002**, *979*, 369–377.

- (12S) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. Determination of Acidity Constants by the Capillary Electrophoresis Internal Standard Method. IV. Polyprotic Compounds. *Journal of Chromatography A* **2013**, 1279, 108–116.
- (13S) Box, K.; Bevan, C.; Comer, J.; Hill, A.; Allen, R.; Reynolds, D. High-Throughput Measurement of P K a Values in a Mixed-Buffer Linear pH Gradient System. *Analytical Chemistry* **2003**, 75, 883–892.
- (14S) Box, K. J.; Comer, J. E. A. Using Measured pKa, LogP and Solubility to Investigate Supersaturation and Predict BCS Class. *Current Drug Metabolism* **2008**, 9, 869–878.

Original scientific paper

High throughput determination $\log P_{o/w}$ / pK_a / $\log D_{o/w}$ of drugs by combination of UHPLC and CE methods

Joan Marc Cabot, Xavier Subirats, Elisabet Fuguet and Martí Rosés*

Departament de Química Analítica – Institut de Biomedicina, Universitat de Barcelona, Martí i Franquès 1-11, 08028 Barcelona

*Corresponding Author: E-mail: marti.roses@ub.edu; Tel.: +34-934039275; Fax: +34-934021233

Received: July 03, 2014; Revised: July 11, 2014; Published: July 18, 2014

Abstract

In 1997 Valkó et al. developed a generic fast gradient HPLC method, based on the calculation of the Chromatographic Hydrophobicity Index (CHI) from the gradient retention times, in order to measure lipophilicity. We have employed the correlations between CHI and $\log P_{o/w}$ and adapted the rapid gradient HPLC method to UHPLC obtaining excellent resolution and repeatability in a short analysis time (< 4min). $\log P_{o/w}$ values can be easily obtained from these CHI measurements but, unfortunately, these correlations are only valid for non-ionized compounds. Consequently, in order to determine the effective $\log P_{o/w}$ value at a particular pH, a fast high-throughput method for pK_a determination was required. The IS-CE method, based on the use of internal standards (IS) and capillary electrophoresis (CE), is a fast and attractive alternative to other methods for pK_a determination, since it offers multiple advantages compared to them: low amounts of test compounds and reagents are needed, high purity is not required, specific interactions between test compounds and buffers are corrected, etc. In addition, it allows the determination of a pK_a value in less than 5 minutes. Both CHI and IS-CE have been combined in order to describe a high throughput alternative in the determination of the lipophilicity profiles of bioactive compounds.

Keywords: lipophilicity, drug discovery, acidity constants, chromatographic hydrophobicity index, CHI, internal standard

Introduction

The drug discovery and development process requires the high-throughput determination of physicochemical parameters of drug candidates. Parameters measuring lipophilicity, acidity, solubility or protein interaction must be determined in a fast way for a high number of compounds of potential interest to select the most promising compounds for further development.

One of the most important physical properties affecting the biological activity of substances is lipophilicity, traditionally expressed as the logarithm of the octanol-water partition coefficient ($\log P_{o/w}$). In the common case of drug candidates with acid-base properties, the effective partition rate at a particular pH is noted as $\log D_{o/w}$. Although being these the most widely used lipophilicity indexes, experimental reference procedures are usually time consuming and require a high purity and a relatively high amount of sample. In order to decrease the analysis time and overcome these limitations, instrumental analytical

methods have been developed to determine lipophilicity, and the values therefrom obtained in a particular lipophilicity scale have been correlated to $\log P_{o/w}$ or $\log D_{o/w}$ scale.

About twenty year ago Klára Valkó and Péter Slégel [1] proposed a new hydrophobicity index measured by a reversed-phase HPLC method, named φ_0 , defined as the concentration of organic modifier in the mobile phase (methanol or acetonitrile) which is required to obtain a retention time (t_R) double than the dead time (t_0). In these conditions, the logarithm of the retention factor equals to zero ($\log k' = \log ((t_R - t_0)/t_0) = 0$) and, therefore, the compound is equally distributed between the mobile and the stationary phases. The higher the value, the more hydrophobic is the compound. This index is characteristic of a compound and independent of the column dimensions, the mobile phase composition and flow-rate. It only depends on the stationary phase, the particular organic modifier employed, the temperature and, for acidic or basic compounds, the pH. However, φ_0 values were experimentally determined in isocratic mode using several mobile phases containing low fractions of methanol or acetonitrile, which involved long run times for hydrophobic substances. A few years later, Valkó and co-workers [2] proposed a new high-throughput Chromatographic Hydrophobicity Index (CHI), based on φ_0 but derived from retention times observed in a fast gradient reversed-phase HPLC method. Compounds of previously determined CHI values can be used as calibration set, and the retention times of new compounds can be correlated to their corresponding CHI values. In that work the C_{18} column employed was an ODS2-IK5 Inertsil with the dimensions of 150×4.6 mm, and the gradient program lasted for about 15 min. In a paper published in 2001 in collaboration with Michael H. Abraham [3] a much shorter column was used, a 50×4.6 mm Phenomenex Luna C-18(2), and therefore gradient times were reduced to 5 minutes. In addition, correlations between CHI and $\log P_{o/w}$ were improved by means of hydrogen-bond acidity descriptors, since it was found that the major difference between these scales is their sensitivity towards the hydrogen bond acidity of the compounds. However, in the case of compounds with acid/base properties, poor correlations between CHI and $\log D_{o/w}$ have been obtained so far. In 2002 Stephen F. Donovan and Mark C. Pescatore [4] used a 20×4.2 mm octadecyl–poly(vinyl alcohol) cartridge and a fast gradient methanolic elution in order to estimate $\log P_{o/w}$ from chromatographic data. Due to the pH stability of this polymeric column, the pH of the mobile phase could be adjusted to the desired value to ensure the ionizable analyte to be in its neutral form. Nowadays, UHPLC technology presents also an excellent stability of C_{18} columns over a wide range pH, besides higher resolution and sensitivity, and shorter equilibration times within consecutive runs in gradient elution.

Since mostly of the potential drug candidates are acids or bases, $\log D_{o/w}$ is a fundamental parameter to be determined in the drug discovery process in order to estimate the pharmacokinetics of a compound of interest. Especially relevant is the $\log D_{o/w}$ at pH 7.4, the physiological value, which indicates the lipophilicity of a drug in the blood plasma. As long as this effective partition rate at any particular pH can be estimated from the measured $\log P_{o/w}$ for the neutral substance and the pK_a of the compound, we propose a high-throughput methodology based on the combination of UHPLC for CHI measurements and CE for pK_a determination in order to define the $\log D_{o/w}$ profile of drug candidates.

In a series of previous works [5-7], we have proposed a high-throughput method to determine acidity constants of weak acids and bases by capillary zone electrophoresis. This method is based on the use of an internal standard (IS) of similar nature and acidity constant as the test compound, which is injected into the capillary just after the sample compound and analyzed in the same electrophoretic run. This method requires only two electrophoretic runs: a first one at a pH where both the test compound and the IS are totally ionized, and a second one at another pH at which both are partially ionized. Thus, from the mobilities of the compounds at these two pH values and the pK_a value of the IS, the pK_a of the analyte can

be easily calculated [5,8]. Besides its rapidness, the main advantages of this method are the compensation of systematic errors due to simultaneous analysis of analyte and IS, and the unnecessary of an accurate pH measurement.

Experimental

Apparatus

For UHPLC measurements, a Shimadzu (Kyoto, Japan) Nexera UHPLC system was used. The system was equipped with two LC-30AD high-pressure pumps, a DGU-20A₅ online degasser, a CTO-10ASvp oven thermostated at 25°C, a SIL-30AC autosampler, a SPD-M20A diode array detector and a CBM-20Alite controller. Retention data were obtained from a Waters (Milford, MA, USA) Acquity BEH C₁₈ column, 50 mm × 2.1 mm, which allowed the study of basic drugs because of their extended working pH range (up to 12). Injected samples were prepared in DMSO at a concentration of 0.5 mg/mL and thus, due to the melting point of DMSO (19°C), the autosampler temperature was set at 20°C. Aqueous buffers were, in all cases, 50 mM ammonium acetate at the desired pH value. Acidic buffers were prepared from glacial acetic acid and the pH was adjusted with small volumes of concentrated ammonia (25%), and basic buffers were prepared inversely. Medium acidic and basic buffers were obtained by solving the salt and adjusting the pH with concentrated ammonia or glacial acetic acid.

Capillary electrophoresis experiments were performed using a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode array detector. Capillary was made of fused silica 50 µm I.D., 375 µm O.D., 35.2 cm length (25 cm to the detector) and purchased from Composite Metal Services Ltd (Shipley, West Yorkshire, UK). The temperature of the capillary was 25.0±0.1°C and the applied voltage during separation was 20 kV. IS and analyte samples were sequentially injected at 0.5 psi for 3 s and analyzed in the same run. Additional 0.5 psi of hydrodynamic flow during separation was applied. The capillary was initially conditioned with 1 M NaOH (2.0 min), water (0.5 min), and running buffer (2.0 min). Between replicates with the same buffer the capillary was not rinsed. 20 psi of pressure was applied during rinsing processes. Running buffers were prepared at different pH values and 50 mM ionic strength as described elsewhere [8]. Injected samples were prepared in methanol/water mixtures at a concentration of 0.1 mg/mL, containing DMSO as neutral marker. Appropriate IS were selected from the set proposed in previous works [8]. All samples and buffers were filtered through a nylon mesh 0.45 µm porous size (Whatman, Maidstone, UK) before use.

pH measurements were performed with a combined Crison (Alella, Spain) 5014 electrode in a Crison GLP22 pH meter. The electrode system was standardized with ordinary aqueous buffers of pH 4.01, 7.00 and 9.21.

Chemicals

Acetonitrile HPLC gradient grade was purchased from VWR (West Chester, PA, USA). Water was purified by the Milli-Q® plus system from Millipore (Billerica, MA, USA) with a resistivity of 18.2 MΩ cm. The chemicals used for buffer preparation were anhydrous sodium acetate (>99.6%) and glacial acetic acid from J.T. Baker (Deventer, The Netherlands), 2-(Cyclohexylamino)ethanesulfonic acid (CHES, >99%), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, >98%) from Sigma (St. Louis, MO, USA), 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (BisTris, >99.9%) from Fluka (Buchs, Switzerland), sodium hydroxide, hydrochloric acid and ammonia from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO, >99.9%) was purchased from J.T. Baker. The injected compounds were purchased from Merck, Sigma-Aldrich and Fluka, or obtained from Almirall (Barcelona, Spain), all of them in high purity grade.

Results and Discussion

Measurement of CHI_{MeCN} values by UHPLC

The HPLC fast gradient method developed by Valkó and co-workers [3] was transferred to our UHPLC system taking into consideration the column and particle sizes, the flow rate, the injection volume, and the dwell time of the UHPLC instrument. The final UHPLC conditions, together with the original HPLC ones, are shown in Table 1. DMSO was used as sample solvent because its ability to solve hydrophobic compounds sparingly soluble in MeCN/water mixtures. In the present work MeCN was used as organic modifier as long as this solvent allows better correlations between $\log P_{o/w}$ and CHI than methanol.

Several substances covering a wide range of CHI_{MeCN} and $\log P_{o/w}$ values were tested as calibration standards. Finally, the representative compounds shown in Table 2 were selected. They are mainly unionizable substances that can be used at any pH value (acetanilide and phenones), but 4-hydroxybenzyl alcohol is a phenol that should be used in its neutral form below pH 8. When using aqueous buffers above this pH value, caffeine was used instead of 4-hydroxybenzyl alcohol. A representative chromatogram of the calibration set is shown in Figure 1. After plotting the CHI_{MeCN} values against the retention times in the fast gradient elution, a quadratic calibration curve is obtained with a typical coefficient of determination (r^2) higher than 0.998 and a root mean square error (RMSE) lower than 1.5.

Table 1. Comparison between HPLC and UHPLC conditions to measure CHI values

	HPLC [3]	UHPLC (present work)
Column	C18, 50 x 4.6 mm, 5 μ m	BEH C18, 50 x 2.1 mm, 1.7 μ m
Flow rate	2.0 mL/min	0.5 mL/min
Temperature	25°C	25°C
Fast gradient program	% MeCN	% MeCN
	0.0 min -> 0%	0.0 min -> 0%
	0.5 min -> 0%	0.4 min -> 0%
	3.0 min -> 100%	2.5 min -> 100%
	3.5 min -> 100%	2.9 min -> 100%
	3.7 min -> 0%	3.1 min -> 0%
	4.5 min -> 0%	3.8 min -> 0%
Injection volume and solvent	3 μ L - MeCN/aqueous buffer	0.2 μ L - DMSO

Similarly to $\log D_{o/w}$ scale, CHI values of acidic or basic compounds depend on the ionization degree. The lower the ionization, the higher the CHI value [9,10]. Therefore, acidic substances present the highest CHI when aqueous buffers of low pH are used, and consequently basic compounds exhibit the reversed trend. On the other hand, non-ionizable analytes show nearly the same CHI values independently of the pH. In the present work all studied analytes were injected at three different pH values (3.0, 7.4 and 11.0), and a representative collection supporting the above statements is presented in Table 3.

Table 2. Compounds used as calibration standards.

Compound	CHI_{MeCN} [11]	$\log P_{o/w}$ [12]	pK_a [12]
4-hydroxybenzyl alcohol ^a	18.91	0.25	9.83
Caffeine ^b	24.45	-0.07	0.60
Acetanilide	42.38	1.16	-
Acetophenone	63.26	1.58	-
Propiophenone	76.93	2.19	-
Butyrophenone	87.14	2.66	-
Valerophenone	96.53	3.28	-
Hexanophenone	105.13	3.79	-
Heptanophenone	112.79	4.32	-

^a)Used below pH 8. ^b)Used above pH 8.

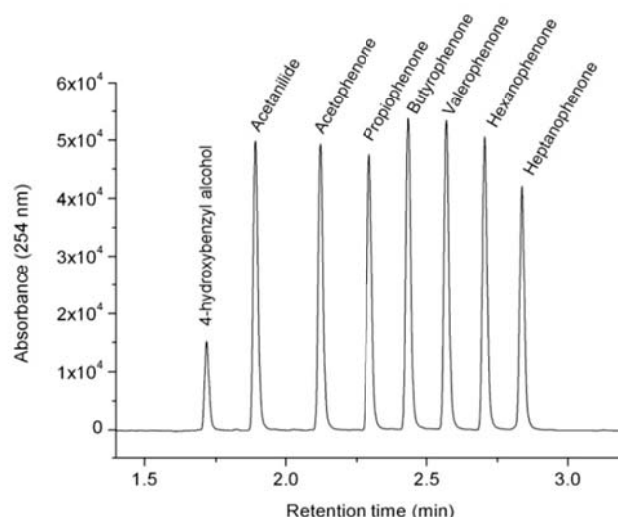


Figure 1. Chromatogram of the calibration set after subtraction of the DMSO blank. UHPLC conditions according to Table 1, 50 mM ammonium acetate pH 7.4 as aqueous buffer.

Table 3. CHI_{MeCN} values obtained at pH 3.0, 7.4 and 11.0.

Compounds	CHI_{MeCN}			Analyte behavior
	pH 3.0	pH 7.4	pH 11.0	
Paracetamol	21.2	20.7	-137.2	Acidic
Theophylline	21.4	20.2	-55.9	Acidic
Atenolol	19.1	18.6	32.1	Basic
Colchicine	43.9	43.4	43.8	-
Salicylic acid	46.0	21.1	-12.5	Acidic
Benzoic acid	48.0	-64.4	-143.2	Acidic
Hydrocortisone	50.2	50.0	50.3	-
Metoprolol	38.1	35.9	61.5	Basic
Procaine	24.9	30.1	62.9	Basic
Propranolol	54.2	51.0	80.9	Basic
Warfarin	82.9	41.8	25.9	Acidic
Ketoconazole	70.8	83.4	83.9	Basic
Lidocaine	35.4	77.9	86.4	Basic
Haloperidol	70.1	66.4	88.9	Basic
Thymol	90.0	89.9	89.5	Acidic
Indomethacin	90.7	54.5	37.7	Acidic
Napthalene	97.6	97.6	97.5	-
Phenothiazine	98.4	98.4	98.2	-
Reserpine	82.7	95.6	98.7	Basic
Anthracene	111.5	111.7	112.3	-

Determination of $\log P_{o/w}$ / pK_a / $\log D_{o/w}$

As mentioned in the introduction, Valkó and co-workers proposed an equation to correlate CHI_{MeCN} and $\log P_{o/w}$ indexes, which involved the Abraham's solute hydrogen-bond acidity descriptor (A , also noted in the original paper as $\Sigma\alpha_2^H$) [3]:

$$\log P_{o/w} = 0.054 \cdot CHI_{MeCN} + 1.319 \cdot A - 1.877 \quad (n = 86, R = 0.970, s = 0.29, F = 655) \quad (1)$$

Table 4. Validation set used for the determination of log $P_{o/w}$ and log $D_{o/w}$.

Behavior	Ref.	Compound	CHI_{MeCN}	A	log $P_{o/w}$ (CHI)	pK_a (IS-CE)	log $D_{7.4}$ (CHI+IS-CE)	log $D_{7.4}$ (reference)
Non-ionizable	1	Acetanilide	40.70	0.41	0.86	-	0.86	1.19 ^a
	2	Acetophenone	62.50	0.00	1.50	-	1.50	1.58 ^a
	3	Anthracene	112.30	0.00	4.19	-	4.19	4.49 ^a
	4	Butyrophenone	87.90	0.00	2.87	-	2.87	2.65 ^a
	5	Caffeine	25.90	0.00	-0.48	-	-0.48	-0.10 ^a
	6	Colchicine	43.90	0.26	0.83	-	0.83	1.09 ^a
	7	Heptanophenone	112.10	0.00	4.18	-	4.18	4.46 ^a
	8	Hexanophenone	105.20	0.00	3.80	-	3.80	3.69 ^a
	9	Hydrocortisone	50.30	0.73	1.80	-	1.80	1.58 ^a
	10	Naphthalene	97.50	0.00	3.39	-	3.39	3.19 ^a
	11	Phenothiazine	98.40	0.13	3.61	-	3.61	4.11 ^a
	12	Propiophenone	77.20	0.00	2.29	-	2.29	2.24 ^a
	13	Valerophenone	97.00	0.00	3.36	-	3.36	3.40 ^a
Basic	14	Papaverine	66.93	0.00	1.74	6.41	1.69	-
	15	Reserpine	98.70	0.31	3.86	6.64	3.79	3.89 ^a
	16	2,4-Lutidine	49.35	0.00	0.79	6.81	0.69	-
	17	Ketoconazole	83.90	0.00	2.65	6.81	2.55	3.42 ^a
	18	Trazodone	76.34	0.00	2.25	6.84	2.14	2.54 ^b
	19	Pilocarpine	20.86	0.00	-0.75	7.08	-0.92	-
	20	2,4,6-Trimethylpyridine	57.17	0.00	1.21	7.51	0.85	-
	21	Lidocaine	86.40	0.26	3.13	7.89	2.52	1.61 ^a
	22	Clonidine	51.30	0.42	1.44	8.10	0.67	0.62 ^b
	23	Bupivacaine	101.75	0.26	3.96	8.19	3.11	-
	24	Quinine	66.42	0.23	2.01	8.45	0.93	-
	25	Haloperidol	88.90	0.31	3.34	8.56	2.15	2.77 ^a
	26	Procaine	62.90	0.23	1.82	9.08	0.14	0.24 ^a
	27	Propranolol	80.90	0.29	2.88	9.48	0.83	1.23 ^a
	28	Metoprolol	61.50	0.29	1.83	9.53	-0.26	-0.25 ^a
	29	Atenolol	32.10	0.78	0.89	9.67	-1.33	-1.81 ^a
Acidic	30	Paracetamol	21.20	0.91	0.47	9.58	0.46	0.35 ^a
	31	2-Chlorophenol	63.64	0.33	2.00	8.50	1.96	-
	32	Theophylline	21.40	0.35	-0.27	8.39	-0.31	-0.07 ^a
	33	Methylparaben	52.33	0.66	1.82	8.35	1.78	-
	34	3,5-Dinitrophenol	85.97	0.83	3.86	8.18	3.80	-
	35	4-Hydroxybenzaldehyde	38.36	0.66	1.07	7.61	0.86	-
	36	Phenobarbital	51.84	0.52	1.61	7.53	1.37	1.51 ^b
	37	Vanillin	41.37	0.44	0.94	7.36	0.61	-
	38	4-Nitrophenol	55.31	0.67	1.99	7.09	1.51	-
	39	Warfarin	82.90	0.31	3.01	5.17	0.83	0.89 ^a
	40	Benzoic acid	48.00	0.57	1.47	4.22	-1.40	-1.27 ^a
	41	Indomethacin	90.70	0.57	3.77	4.16	0.88	-

^a) Shake-flask method, ref. [13]. ^b) Experimental value, ref. [14].

With the aim of validating the UHPLC method proposed, CHI_{MeCN} were measured for several non-ionizable, acidic and basic compounds in the log $P_{o/w}$ range between -0.07 and 4.45. All analytes were injected using aqueous mobile phases of pH 3.0, 7.4, and 11.0, and the highest value was considered for log $P_{o/w}$ determination. These 41 compounds are listed in Table 4, together with the CHI_{MeCN} values of neutral species determined in the present work, the A solute descriptors obtained from the ACD/Percepta platform [15], and the log $P_{o/w}$ determined from CHI_{MeCN} and A according to Eq. (1). There is a good correlation between literature log $P_{o/w}$ values (*Mlog P* values compiled in Bio-Loom database [12]) and the ones measured in the present work (slope: 0.99(±0.06), intercept: -0.15(±0.15), r: 0.940, RMSE: 0.45). Basic

solutes correlate slightly worse than non-ionizable and acidic compounds. Nevertheless, from the 16 studied bases only 6 presented deviations from literature values higher than 0.7 log $P_{o/w}$ units. In four of those cases the determined log $P_{o/w}$ values were lower than the bibliographic ones (compounds 14, 16, 17, and 19 in Table 4), and in two cases (solutes 21 and 29) the reverse trend was shown.

As previously commented, poor correlations are obtained between log $D_{o/w}$ and CHI_{MeCN} values for ionized compounds. However, the effective distribution rate of a drug at a particular pH value can be estimated from its acidity constant and the partition coefficients of the neutral and fully ionized species [16]. The current state of the art does not allow the direct measurement of the partition coefficient of ions but it can be assumed that a typical value is 3.15 units lower than that of the neutral species, according to the work published by Donovan and Pescatore [4]. Thus, log $D_{o/w}$ values of monoprotic compounds can be calculated from the following expressions at any desired pH value:

$$\text{Acidic compound: } \log D_{o/w} = \log \left(P_{o/w} + (P_{o/w} - 3.15) \cdot 10^{\text{pH} - \text{pK}_a} \right) - \log \left(1 + 10^{\text{pH} - \text{pK}_a} \right) \quad (2)$$

$$\text{Basic compound: } \log D_{o/w} = \log \left(P_{o/w} + (P_{o/w} - 3.15) \cdot 10^{\text{pK}_a - \text{pH}} \right) - \log \left(1 + 10^{\text{pK}_a - \text{pH}} \right) \quad (3)$$

Consequently, the measurement of aqueous acidity constants was required in order to estimate log $D_{o/w}$ values and the IS-CE fast method was presented as a very appropriate option. This method allows an accurate determination of acidity constants, which normally present an excellent match with reference values found in literature. In the present work, the correlation between the measured pK_a values for some of the studied acidic and basic compounds (Table 4) and the ones found in literature was remarkable (slope: $1.01(\pm 0.01)$, intercept: $-0.07(\pm 0.09)$, r : 0.998, RMSE: 0.11).

Table 4 shows the log $D_{o/w}$ values at pH 7.4 resulting from log $P_{o/w}$ determinations and pK_a measurements (Eqs. (2-3)) and those obtained from reference methods [13,14]. As shown in Figure 2, there is a good agreement between both sets of values, with the only exception of lidocaine, which presented yet one of the worst matches in log $P_{o/w}$ determination.

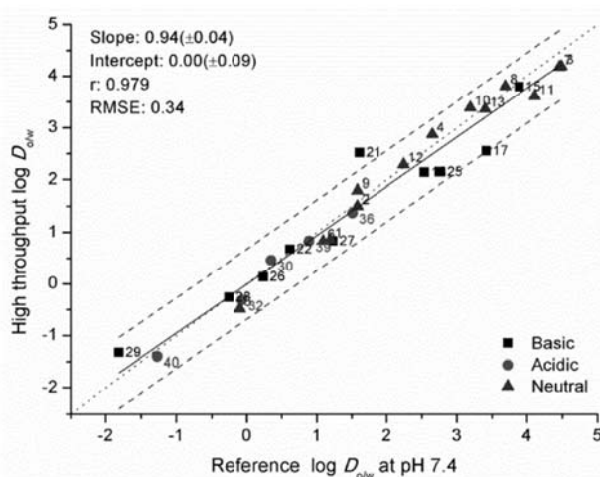


Figure 2. Correlation between reference log $D_{o/w}$ values at pH 7.4 (Table 4) and those obtained from log $P_{o/w}$ and pK_a measurements (Eqs. (2-3)). Linear regression (continuous lines) and the statistics are also shown, together with the lines corresponding to $\pm 2 \cdot \text{RMSE}$ (dashed lines).

Figure 3 shows the $\log D_{o/w}$ profile obtained in the present work for vanillin and clonidine, which present acidic and basic nature, respectively. For the sake of comparison, the curve calculated by ACD/Labs software is also presented, together with their corresponding calculated and measured pK_a values. In the case of vanillin, the ACD/Labs calculated partition ratio (1.32) is moderately higher than the measured one (0.94), whereas both pK_a values, estimated and experimental, match very well (7.30 and 7.36, respectively). Thus, the ACD/Labs estimated curve shifts up from the experimental one. For clonidine, the ACD/Labs calculated $\log P_{o/w}$ (2.05) is higher than the CHI_{MeCN} measured one (1.44) by 0.6 units, whereas the pK_a (7.90) is lower than the IS-CE determined one (8.10). Therefore, the ACD/Labs estimated curve moves up and left from the measured one, being the values determined in the present work more accurate according to literature (8.05 and 1.43, for pK_a and $\log P_{o/w}$, respectively [12]).

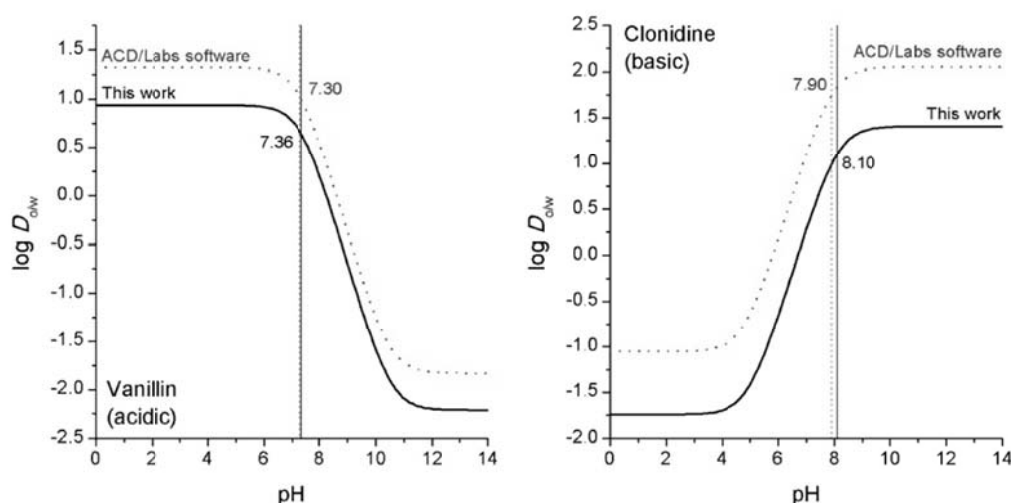


Figure 3. $\log D_{o/w}$ profile for vanillin and clonidine according to Eqs. (2-3) (continuous line) and calculated profile using ACD/Labs software (dotted lines), together with their corresponding measured and calculated pK_a values.

Conclusions

In the present work the combination of two high-throughput methods have been proposed in order to estimate the $\log D_{o/w}$ of an ionizable compound at any desired pH. On the one hand, a fast gradient reversed-phase methodology was successfully transferred into UHPLC in order to determine $\log P_{o/w}$ from chromatographic retention times, based on the formerly developed CHI lipophilicity index. After calibration of the system, a single fast-gradient run performed within 4 minutes is enough to determine the CHI_{MeCN} value of a compound, and then the value can be transformed into $\log P_{o/w}$ lipophilicity scale by means of the established equation. In the case of analytes with acid/base properties, runs performed at 3 different pH values (3.0, 7.4 and 11) are recommended, and the highest CHI_{MeCN} is selected. On the other hand, the IS-CE allows the determination of a pK_a value from only two runs, lasting less than 5 minutes (about 2 min each). Finally, from both the $\log P_{o/w}$ of the neutral species (UHPLC- CHI procedure) and the pK_a value (IS-CE method), the effective distribution rate $\log D_{o/w}$ can be estimated at any particular pH value.

Acknowledgements: Financial support from the Ministerio de Ciencia e Innovación of the Spanish Government and the Fondo Europeo de Desarrollo Regional (FEDER) of the European Union (Project CTQ2010-19217/BQU) is acknowledged. JMC also thanks the Secretaria d'Universitats i Recerca of the Departament d'Economia i Coneixement of the Catalan Government for a supporting scholarship (2013FI_B1 00209).

References

- [1] K. Valkó, P. Slégel, *J. Chromatogr. A* **631** (1993) 49-61.
- [2] K. Valkó, C. Bevan, D. Reynolds, *Anal. Chem.* **69** (1997) 2022-2029.
- [3] K. Valkó, C.M. Du, C. Bevan, D.P. Reynolds, M.H. Abraham, *Curr. Med. Chem.* **8** (2001) 1137-1146.
- [4] S.F. Donovan, M.C. Pescatore, *J. Chromatogr. A* **952** (2002) 47-61.
- [5] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, *J. Chromatogr. A* **1216** (2009) 3646-3651.
- [6] J.M. Cabot, E. Fuguet, C. Ràfols, M. Rosés, *J. Chromatogr. A* **1217** (2010) 8340-8345.
- [7] E. Fuguet, C. Ràfols, M. Rosés, *J. Chromatogr. A* **1218** (2011) 3928-3934.
- [8] J.M. Cabot, E. Fuguet, C. Ràfols, M. Rosés, *J. Chromatogr. A* **1279** (2013) 108-116.
- [9] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, *J. Chromatogr. A* **1173** (2007) 110-119.
- [10] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, *J. Chromatogr. A* **1216** (2009) 7798-7805.
- [11] K. Valkó, C.M. Du, C.D. Bevan, D.P. Reynolds, M.H. Abraham, *J. Pharm. Sci.* **89** (2000) 1085-1096.
- [12] Bio-Loom, BioByte Corp. (Claremont, CA, USA), Version 1.5.
- [13] A. Andrés, M. Rosés, C. Ràfols, E. Bosch, S. Espinosa, V. Segarra, J.M. Huerta, *Manuscript in preparation*.
- [14] A. Avdeef, *Absorption and drug development: solubility, permeability, and charge state*, Wiley, Hoboken, NJ, USA, 2003.
- [15] ACD/Labs, Advanced Chemistry Development, Inc. (Toronto, Canada), build 2203 (29-Jan-2013).
- [16] C. Horváth, W. Melander, I. Molnár, *Anal. Chem.* **49** (1977) 142-154.

Joan Marc Cabot
Elisabet Fuguet
Martí Rosés

Departament de Química
Analítica and Institut de
Biomedicina de la Universitat de
Barcelona, Universitat de
Barcelona, Barcelona, Spain

Received July 21, 2014
Revised September 1, 2014
Accepted September 1, 2014

Research Article

Determination of acidity constants of sparingly soluble drugs in aqueous solution by the internal standard capillary electrophoresis method

A set of 33 drugs with different solubilities, ranging from soluble to very insoluble, has been chosen in order to evaluate the performance of the internal standard CE method to determine acidity constants of compounds with limited solubility. The set of drugs tested in this work has been chosen as a function of their intrinsic solubility. For the most insoluble compounds, several analytical conditions to overcome the insolubility in aqueous buffers have been tested. This paper assesses the compound solubility limits for the IS-CE method in aqueous pK_a determinations, and also compares the determined pK_a s with the results from the literature data obtained by other methods. It is proved that IS-CE method determines acidity constants of sparingly soluble drugs in aqueous media (compounds with $\log S$ down to around -6), whereas other reference methods require the use of aqueous–organic solvent buffers and extrapolation procedures to obtain the aqueous pK_a for the same compounds.

Keywords:

Acidity constant / Capillary electrophoresis / Internal standard / pK_a determination / Solubility
DOI 10.1002/elps.201400353

1 Introduction

Drug discovery has undergone considerable changes with the introduction of new technologies and strategies, adding new opportunities for gathering and integrating information, which can increase drug discovery success and efficiency. Pharmaceutical companies synthesize a large number of potential drugs and chemical precursors in a relatively short time. This fact requires selecting those drugs that are most suitable for further test and development. As a consequence, high-throughput screening for rapid evaluation of compounds is needed as soon as they are available after synthesis [1, 2].

Physicochemical parameters are frequently used as predictors of ADME (absorption, distribution, metabolism, and excretion) properties of drugs. One main physicochemical property that affects the pharmaceutical potential of a compound is its acid–base dissociation, which is defined by its acidity constant (or pK_a in logarithmic scale). Many potential drugs are weak acids or bases, and their use in further studies strongly depends on their pK_a , since it determines

the ionization degree of the compound in the environment pH [2, 3]. Modern techniques of drug discovery often produce molecules that are highly lipophilic and sparingly soluble in water. Poor aqueous solubility often becomes a drawback in the physicochemical characterization of drugs, because many assays require the drugs to be in solution during measurement. Therefore, the determination of the acidity constant of sparingly soluble drugs can be a serious problem in drug discovery [2, 4–6].

Potentiometric titrations are commonly used as a reference method for pK_a determination due to their accuracy and high precision. But, by contrast, potentiometry is a relatively slow method (it takes normally 20–40 min per compound), and solutions of at least 5×10^{-4} M are required in order to detect a significant change in the shape of the titration curve [7].

An alternative to potentiometric titrations are the UV-Vis spectrophotometric titrations. They require a lower concentration, and thus can handle compounds with lower solubility that show favorable molar absorption coefficient. However, the classical spectrophotometric method is not fully automated and hence becomes a time-consuming method. A very attractive variation is the hybrid pH-metric/UV method [6, 8, 9]. This method is also efficient and highly automated approach, where the result is obtained from changes in multiwavelength UV spectra when the pH changes. In some instances, measurements can be done even when precipitation occurs if a probe to detect precipitation (dip probe absorption spectroscopy, DPAS) is present [10]. The main drawbacks of spectrophotometric methods are that the drug must contain a

Correspondence: Professor Martí Rosés, Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1–11, E-08028-Barcelona, Spain
Fax: +34-93-402-12-33
E-mail: marti.roses@ub.es

Abbreviations: IS, internal standard; IS-CE, internal standard capillary electrophoresis

UV-active chromophore close enough to the site of the acid–base function in the molecule, and the requirement of high purity.

CE is another widely used technique to determine acidity constants. One of the main advantages of CE is that less amount of reagents and samples is needed, and in addition, high purity is not required because it is a separation technique. Also in case of decomposition of the compound at some pH values, in most instances the main peak can be monitored to perform the pK_a determination. Few years ago, a high-throughput multiplexed CE method based on parallel capillaries with UV detection was presented [11, 12]. With such a 96-capillary array, the throughput capacity was significantly increased as compared to a single-capillary instrument, but this method requires specific complex instrumentation. In the classic CE method [13–17], electrophoretic mobilities of the drugs must be determined in solutions whose pH has been accurately measured, keeping temperature constant and being aware of possible specific interactions between the buffers and drugs. Based on the CE classic method, CE-MS has also been successfully developed as an advanced technique [18] for pK_a determination. It provides high selectivity as well as high sensitivity, so that lower concentration of drugs can be used. Furthermore, the CE-MS method has a distinct advantage; throughput can be significantly increased by pooling a number of compounds into a single sample. However, in addition to the above-mentioned problems of the CE classic method, if the number of compounds is not high, pK_a determination becomes time-consuming and expensive.

When the pK_a of sparingly soluble drugs cannot be determined in aqueous media, alternative methods based on the determination of the pK_a at different proportions of aqueous–organic solvent mixtures are used. Aqueous pK_a values at zero organic solvent composition are then estimated by extrapolation (e.g. the Yasuda–Shedlovsky method). The mixed-solvent procedures mainly using alcohols or ACN–aqueous mixtures provide a good alternative for sparingly soluble compounds [7, 11, 13, 14, 19, 20]. However, a final extrapolation to 0% organic solvent is always required—fact that may worsen the accuracy of the determination compared to the direct measurement in aqueous media.

Recently, a high-throughput method to determine acidity constants based on CE and the use of internal standards (IS-CE, internal standard CE) has been developed [21–27]. This method does not need an accurate external measurement of the pH of the buffer because it measures the pH inside the capillary from the mobilities of the IS. In addition, the method may correct the interactions of the test compound with the buffers, and possible experimental and systematic errors—such as temperature or buffer pH variations due to electrolysis processes during run analysis—using an adequate IS.

Unlike other methods, the IS-CE presents a big advantage for the sparingly soluble drugs. The method requires only two CE runs at two different pH values to determine a pK_a value: the first one where compounds (drug and IS) are totally ionized (limiting mobility measurement) and the second one where they are partially ionized (effective mobil-

ity measurement). As solubility increases with the ionization degree, limiting mobility is usually easy to measure. In case of effective mobilities, if precipitation of the drug is detected, a new pH in which the compound is ionized in a higher but still measurable degree can be selected in some cases for the determination.

In the present work, we report and discuss the determination of pK_a values of drugs in aqueous solution by the IS-CE method. The aim of this study is to evaluate the ability of the method for the determination of acidity constants of sparingly soluble compounds, compared to other methods for pK_a determination.

2 Materials and methods

2.1 Apparatus

CE experiments have been performed with a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. Capillary was made of fused silica (50 μm id, 375 μm od, 35.2 cm length (25 cm to the detector)) and was obtained from Composite Metal Services (Shipley, West Yorkshire, UK). The temperature of the capillary was set to $25.0 \pm 0.1^\circ\text{C}$. Test compounds and ISs were injected sequentially (one-by-one before separation) at a hydrodynamic pressure of 0.5 psi for 3 s (1 psi = 6897.76 Pa), and the applied voltage during separation was 20 kV. Additional 0.5 psi of hydrodynamic flow during separation was applied. The UV detector was set at 214, 254, and 280 nm. Capillary conditioning has been described recently [27].

2.2 Chemicals

DMSO >99.9%, 0.5 M sodium hydroxide trititol, 0.5 M hydrochloric acid trititol, and sodium dihydrogenphosphate monohydrate >99% were from Merck (Darmstadt, Germany). Anhydrous sodium acetate >99.6%, CHES >99%, and CAPS >98% were from Sigma (St. Louis, MO, USA). 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol >99.9% (Bis-Tris) and sodium formate were from Fluka (Buchs, Switzerland). Tris >99.9% was purchased from Aldrich (Milwaukee, WI, USA). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 M Ω cm. All studied drugs (described in Table 1) and ISs were of reagent grade or better, and were purchased from Sigma, Aldrich, Fluka, Merck, and Carlo Erba (Milan, Italy).

2.3 Buffer preparation

Buffer solutions covering practically all the useful pH range (from 2 to 12 separated within intervals of 0.5 pH units) were prepared at the desired pH, and 50 mM ionic strength as described elsewhere [28]. The employed buffers, its pK_a value, and the stock solution used in their preparation have been summarized previously [25]. All buffers were filtered

Table 1. Test compounds with their log*S* from literature and the determined IS-CE *pK_a* values

Compound	log <i>S</i> _(lit)	<i>pK_a</i> _(IS-CE)	% Ionization at the working pHs	<i>μ</i> _{ionic} ^{a)}
<i>Sample prepared in water</i>				
Theophylline	−1.10 ^{b)}	8.39	73	−162.9
Atenolol	−1.29 ^{c)}	9.67	60	84.6
Procaine	−1.72 ^{c)}	9.08	65	98.9
Alprenolol	−2.63 ^{c)}	9.37	57	92.1
Diltiazem	−2.94 ^{c)}	7.99	45	78.9
Fisetin	−3.00 ^{b)}	7.14	46; 70; 88	−51.9
		8.39	129; 154; 167	−142.6
		10.28	216; 230; 270	−184.4
Morin	−3.02 ^{b)}	5.15	19; 42; 69	−93.1
		8.15	118; 142; 168	−172.6
		10.46	217; 240; 258	−209.2
Kaempferol	−3.37 ^{b)}	7.16	48; 69; 89	−84.7
		9.05	128; 137; 169	−146.3
		10.54	216; 239; 268	−220.0
Quercetin	−3.40 ^{b)}	7.15	21; 47; 70	−77.9
		8.84	119; 139; 148	−102.2
		10.31	225; 254; 273	−164.3
Apigenin	−3.47 ^{b)}	7.60	25; 48; 70	−129.9
		9.35	156; 172; 189	−171.1
Naringenin	−3.48 ^{b)}	6.68	53; 77; 82	−95.6
		10.16	144; 165; 183	−185.0
Hesperetin	−3.50 ^{b)}	6.97	54; 78; 83	−88.6
		10.13	144; 162; 182	−166.3
Oxyphencyclimine	−3.58 ^{b)}	11.01	73	78.3
Dibucaine	−3.68 ^{b)}	8.57	21	122.3
Fluoxetine	−3.92 ^{c)}	10.08	60	91.2
Galangin	−3.93 ^{b)}	7.25	17; 42; 65	−88.9
		9.54	138; 159; 181	−169.0
Diflunisal	−4.20 ^{b)}	2.97	80	−118.6
Flurbiprofen	−4.30 ^{c, d)}	4.19	38	−105.9
Ketoconazole	−4.40 ^{b)}	3.45	21; 50; 71	64.9
		6.81	150; 165; 181	131.3
Maprotiline	−4.69 ^{c)}	10.23	58	131.7
Cyproheptadine	−4.94 ^{b)}	8.90	37	107.2
Reserpine	−5.05 ^{b)}	6.64	70	63.8
Fenbufen	−5.19 ^{e)}	4.73	65	−108.3
Chlorpromazine	−5.29 ^{c, d)}	9.26	61	95.0
<i>Sample prepared in 1 mM acid or base</i>				
Diclofenac	−5.45 ^{c, d)}	4.22	66	−103.0
Haloperidol	−5.47 ^{c)}	8.56	72	80.0
Miconazole	−5.62 ^{c)}	6.14	86	87.1
Mifepristone	−5.75 ^{c, e)}	5.23	78	75.6
Indomethacin ^{g)}	−5.77 ^{d, e)}	4.16	84	−128.8
Niclosamide	−6.15 ^{f)}	7.25	82	−93.4
<i>Unable to be determined in aqueous media</i>				
Mefenamic acid	−6.34 ^{c)}	—	—	−104.0
Loperamide	−7.13 ^{c)}	—	—	62.0
Amiodarone	−8.17 ^{c)}	—	—	—

a) Mobility of the ionic species in $\times 10^4 \text{ cm}^2 \text{ V}^{-1} \text{ min}^{-1}$.

b) ACD/Percepta Platform v2012 Software, ACD/Labs 2012 [29].

c) From [8].

d) From [31].

e) From [32].

f) From [33].

g) Degradation was observed when prepared in 1 mM acid or base.

through a nylon mesh with 0.45 μm pore size (Whatman, Maidstone, UK).

2.4 Sample preparation

Stock solutions of test compounds and ISs were prepared at a concentration of 1 mg/mL and 4% of DMSO was added as EOF marker. They were diluted in water or in a methanol/water mixture (when they were not soluble in water itself). Afterwards, a 1/10 dilution of the stock solution in water was prepared for injection (100 mg/L). Test compounds and ISs were filtered through a nylon mesh with 0.45 μm pore size (Whatman).

2.5 *pK_a* determination

The determination of acidity constant by IS-CE has been recently reported [22–25]. Briefly, the method is based on the use of an IS with *pK_a* similar to that of test compound ($\Delta pK_a < 1$) choosing as first approximation for the *pK_a* of the test compound the one predicted by ACD/Labs software [29], although any other *pK_a* prediction software can be used. Then, mobilities of the IS and test compound were calculated in two different buffers: a buffer in which the analyte and IS were completely ionized ($\text{pH} \gg pK_a$ for an acid or $\text{pH} \ll pK_a$ for a base); and a second buffer in which both were partially ionized (pH in the range $pK_a \pm 1$). For polyprotic compounds with close acidity constants, one IS was selected for each constant and effective mobilities were determined in triplicate at three different pH values close to the *pK_a*. From these mobility measurements, the *pK_a* of the test compound can be directly obtained if the *pK_a* of the IS is well known. ISs and their reference *pK_a* values were chosen from a set of suitable acidic and basic monoprotic compounds [23, 24]. Finally, acidity constant at the working ionic strength is related to the thermodynamic *pK_a* by the activity coefficients, calculated through Debye–Hückel equation [30], if it is required.

3 Results and discussion

The set of test compounds compiled for the evaluation of the performance of the IS-CE method to determine the *pK_a* of compounds with limited solubility includes a total of 33 substances (46 *pK_a* values) with most of them being commercially available drugs. This set of compounds was selected as a function of their intrinsic solubility. Table 1 shows the compounds together with the logarithmic form of their intrinsic solubility ($\log S_{\text{lit}}$), solubility in mol/L, the acidity constant determined through the IS-CE method (*pK_a*_(IS-CE)), as well as the mobility of the ionic species. Compounds are listed in decreasing order of solubility values, going from very soluble such as theophylline with $\log S$ of −1.10 to other much more insoluble such as amiodarone with $\log S$ of −8.17. These solubility values were collected from several sources [8, 31–33], or predicted through ACD/Percepta software [29] when experimental data were not available in the literature. Average

of logS is provided for the compounds having more than one literature value.

Commonly, solubility increases with the ionization degree and, therefore, precipitation can be avoided in most cases if ionization is increased. For this reason, when effective mobilities of the partially ionized test compounds were determined, ionization degree was monitored and is summarized in Table 1 for the particular experiments realized.

In a CE instrument, precipitation is identifiable by a significant broadening of the analyte peaks or the appearance of a plateau instead of the typical Gaussian peak profile obtained for compounds in solution. Another indication of precipitation is an abrupt disappearance of the analyte peak at a particular pH value.

3.1 Determination of the acidity constant of soluble drugs

For a drug with high enough solubility, pK_a was determined by the usual IS-CE method without special problems. As solubility goes down, pK_a of the drug becomes more difficult to be determined. Diclofenac, with logS of -5.45 units, was the least insoluble compound that precipitated from its stock solution (1 mg/mL) when it was prepared at 100 ppm in water. It can be said, therefore, that precipitation is not observed in the whole process of pK_a determination through the IS-CE method for compounds with logS values higher than around -5 .

Maprotiline and chlorpromazine are the two compounds located in the boundary whose pK_a value has been widely determined by different techniques. Consequently, it is convenient to contrast the IS-CE method with others presented in the literature. Multiplexed CE method [11] allowed the determination of the pK_a of maprotiline using aqueous buffers, whereas chlorpromazine was found to precipitate and its pK_a was not determined without employing methanol as cosolvent. Nevertheless, it was possible to measure directly the aqueous pK_a of chlorpromazine through CE-MS [18]. The aqueous pK_a of maprotiline could be directly determined through spectrophotometric titrations, but chlorpromazine chromophores were not close enough to give appropriate changes in multiwavelength spectra, so the use of the potentiometric method in the presence of cosolvent was needed to determine pK_a [7, 8, 34]. The IS-CE method allowed the determination of the pK_a of both compounds in aqueous buffers.

3.2 Determination of the acidity constant of sparingly soluble drugs

Compounds with logS lower than that of chlorpromazine, such as diclofenac, haloperidol, miconazole, mifepristone, indomethacin, niclosamide, mefenamic acid, loperamide, and amiodarone, precipitated when the stock solution was diluted in water. However, several strategies were adopted to overcome this problem. The first option was to add the neces-

sary amount of HCl or NaOH in order to ionize the compound in the sample vial and, consequently, increase its solubility. Since the amount of compound injected into the capillary is very small, many times precipitation can be avoided if the compound is previously ionized and solubilized, even when pH changes inside the capillary. However, if precipitation in capillary was detected, the measurement of the effective mobility could be done at a new pH closer to the pH where ionic form predominates, which implies a higher ionization degree of the drug. The third strategy was to further dilute drugs approximately to 5–10 ppm. At these concentrations, detection can be performed correctly. The explained strategies, alone or combined, allowed the determination of pK_a s directly in aqueous solution of drugs that presented logS values down to approximately -6 . In fact, niclosamide was the last drug determined in this way. As Table 1 shows, the percentage of ionization was around 70% or more for these compounds. This degree of ionization was high enough to keep the drug in solution, but low enough to allow pK_a calculation.

Table 2 shows a comparison of methodologies for the pK_a determination of these sparingly soluble drugs. For the majority of the compounds, multiple literature values ($pK_{a(lit)}$) have been reported, determined by a variety of methods and experimental conditions. All pK_a reported values are at zero ionic strength. The acidity constants of mifepristone and niclosamide were not found in any reliable source, so comparison is not provided.

Similar pK_a values are observed when comparing the results of the IS-CE method to the ones obtained with other methods. When data from literature are analyzed, it is observed that at these solubility levels, potentiometric titrations require the use of cosolvents and extrapolation methods to obtain aqueous pK_a values. As regards spectrophotometric titrations, except for diclofenac, all measurements were also performed in hydroorganic measurements. In addition, it has been reported [9] that miconazole and indomethacin presented precipitation during the spectrophotometric titration. It should be noted that this method, combined with a probe to detect precipitation (spectrophotometric titrations with DPAS), allows the determination of acidity constants even if precipitation takes place, just avoiding spectra in the pH range where presence of precipitate is observed, to perform calculations [10]. The classic CE method is in the boundary between the use or not of cosolvents. For example, the acidity constant of indomethacin has been determined in aqueous media by multiplexed CE, whereas the one of miconazole requires the use of methanol–aqueous mixtures and extrapolation through Yasuda–Shedlovsky equation. Finally, CE-MS allows the direct determination in aqueous buffers, probably due to high sensitivity of the method, which allows working at very low concentration.

3.3 Determination of the acidity constant of highly insoluble drugs

As regards compounds with very low solubility such as mefenamic acid, loperamide, and amiodarone (logS < -6.34), they

Table 2. Comparison of pK_a determination by the IS-CE and by literature methods, for low-solubility compounds

Compound	$\log S_{(lit)}$	$pK_{a(IS-CE)}$	$pK_{a(lit)}$	Method
<i>Sample prepared in 1 mM acid or base</i>				
Diclofenac	−5.45 ^{a, b)}	4.22	4.16	Potentiometry cosolvent [35]
			4.09	Potentiometry cosolvent [36]
			4.03	GLpK _a spectrophotometry aqueous media [8]
			4.21	CE-MS aqueous media [18]
Haloperidol	−5.47 ^{a)}	8.56	8.65	Potentiometry cosolvent [8]
			8.55	Potentiometry cosolvent [34]
			8.42	GLpK _a potentiometry cosolvent [8]
			8.96	CE-MS aqueous media [18]
Miconazole	−5.62 ^{a)}	6.14	6.32	GLpK _a spectrophotometry cosolvent [8]
			6.38	Multiplexed CE cosolvent [11]
Mifepristone	−5.75 ^{a, c)}	5.23	—	—
Indomethacin ^{e)}	−5.77 ^{b, c)}	4.16	4.53	Classic CE cosolvent [13]
			4.15	CE-MS aqueous media [18]
			4.18	Multiplexed CE aqueous media [11]
Nicosamide	−6.15 ^{d)}	7.25	—	—
<i>Unable to be determined in aqueous media</i>				
Mefenamic acid	−6.34 ^{a)}	—	4.22	GLpK _a spectrophotometry cosolvent [8]
Loperamide	−7.13 ^{a)}	—	8.90	GLpK _a spectrophotometry cosolvent [8]
Amiodarone	−8.17 ^{a)}	—	8.80	Potentiometry cosolvent [36]
			8.73	GLpK _a spectrophotometry cosolvent [8]
			8.85	Classic CE cosolvent [14]
			8.71	Multiplexed CE cosolvent [11]

a) From [8].

b) From [31].

c) From [32].

d) From [33].

e) Degradation was observed when prepared in 1 mM acid or base.

precipitated during aqueous measurements. In case of mefenamic acid and loperamide, there were no problems when mobilities of the ionic species were determined. However, precipitation appeared in the measurement of effective mobilities, when the percentage of ionization was lower than 100%, despite they were diluted to approximately 5–10 ppm. Amiodarone mobility could not be determined in aqueous buffers even when the compound was fully in its ionic form. In the same way, experimental literature values of Table 2 show that such insoluble drugs cannot be determined in aqueous media, and in all cases the use of cosolvent is strictly necessary.

4 Concluding remarks

The ability of the fast high-throughput IS-CE method to determine pK_a of sparingly soluble compounds has been evaluated. It has been stated that the limit of application of the method is around an intrinsic solubility value of 10^{-6} M. However, from compounds with S values around 10^{-5} M, different simple strategies should be adopted to avoid precipitation of the compound during the pK_a determination process. When the drug has a solubility with a $\log S$ value lower than −6, the use of cosolvents and extrapolation procedures is strictly necessary.

Compared to other methods, potentiometry and spectrophotometric titrations require the use of cosolvents for compounds with higher solubilities. As regards other CE-

based methods, the classic CE method even in multiplexed CE systems shows a drug solubility limit similar to that of the IS-CE method. However, in some cases, the use of cosolvents is needed in the classical CE method, whereas in the IS-CE method it is still not needed. Finally, the CE-MS method allows the use of a lower drug concentration, so pK_a values of more insoluble drugs can be directly determined in aqueous buffers. However, the latter method is time-consuming and expensive unless many drugs are pooled for a simultaneous determination.

The authors thank the Ministerio de Ciencia e Innovación of the Spanish Government and the Fondo Europeo de Desarrollo Regional (FEDER) of the European Union (Project CTQ2010-19217/BQU) for financial support. Furthermore, J. M. Cabot thanks the Secretaria d'Universitats i Recerca of the Departament d'Economia i Coneixement of the Catalan Government for a supporting scholarship (2013FI_B1 00209).

The authors have declared no conflict of interest.

5 References

- [1] Babić, S., Horvat, A. J. M., Mutavdžić Pavlović, D., Kaštelan-Macan, M., *Trends Anal. Chem.* 2007, 26, 1043–1061.

- [2] Wan, H., Ulander, J., *Expert Opin. Drug Metab. Toxicol.* 2006, 2, 139–155.
- [3] Reijenga, J., van Arno, H., van Antonie, L., Teunissen, B., *Anal. Chem. Insights* 2013, 8, 53–71.
- [4] Alsenz, J., Kansy, M., *Adv. Drug Deliver. Rev.* 2007, 59, 546–567.
- [5] Kerns, E., *Drug Discov. Today* 2003, 8, 316–323.
- [6] Taylor, J. B., Trigg, D. J., Comer, J. E. A., *Comprehensive Medicinal Chemistry II*, Elsevier Ltd., Amsterdam, The Netherlands 2007, pp. 357–397.
- [7] Takács-Novák, K., Box, K. J., Avdeef, A., *Int. J. Pharm.* 1997, 151, 235–248.
- [8] Box, K. J., Comer, J. E. A., *Curr. Drug Metab.* 2008, 9, 869–878.
- [9] Box, K., Bevan, C., Comer, J., Hill, A., Allen, R., Reynolds, D., *Anal. Chem.* 2003, 75, 883–892.
- [10] D-PAS Instruction Manual. Revision 3.0. Sirius Analytical Instruments Ltd, Riverside, UK 2006.
- [11] Shalaeva, M., Kenseth, J., Lombardo, F., Bastin, A., *J. Pharm. Sci.* 2008, 97, 2581–2606.
- [12] Pang, H., Kenseth, J., Coldiron, S., *Drug Discov. Today* 2004, 9, 1072–1080.
- [13] Bellini, S., Uhrova, M., Deyl, Z., *J. Chromatogr. A* 1997, 772, 91–101.
- [14] de Nogales, V., Ruiz, R., Rosés, M., Ràfols, C., Bosch, E., *J. Chromatogr. A* 2006, 1123, 113–120.
- [15] Gluck, S. J., Steele, K. P., Benkő, M. H., *J. Chromatogr. A* 1996, 745, 117–125.
- [16] Šlampová, A., Křivánková, L., Gebauer, P., Boček, P., *J. Chromatogr. A* 2008, 1213, 25–30.
- [17] Jia, Z., Ramstad, T., Zhong, M., *Electrophoresis* 2001, 22, 1112–1118.
- [18] Wan, H., Holmén, A. G., Wang, Y., Lindberg, W., Englund, M., Nånberg, M. B., Thompson, R. A., *Rapid Commun. Mass Spectrom.* 2003, 17, 2639–2648.
- [19] Avdeef, A., Box, K. J., Comer, J. E., Gilges, M., Hadley, M., Hibbert, C., Patterson, W., Tam, K. Y., *J. Pharm. Biomed. Anal.* 1999, 20, 631–641.
- [20] Erdemgil, F. Z., Sanli, S., Sanli, N., Ozkan, G., Barbosa, J., Guiteras, J., Beltrán, J. L., *Talanta* 2007, 72, 489–496.
- [21] Fuguet, E., Ràfols, C., Bosch, E., Rosés, M., *Chem. Biodiv.* 2009, 6, 1822–1827.
- [22] Fuguet, E., Ràfols, C., Bosch, E., Rosés, M., *J. Chromatogr. A* 2009, 1216, 3646–3651.
- [23] Cabot, J. M., Fuguet, E., Ràfols, C., Rosés, M., *J. Chromatogr. A* 2010, 1217, 8340–8345.
- [24] Fuguet, E., Ràfols, C., Rosés, M., *J. Chromatogr. A* 2011, 1218, 3928–3934.
- [25] Cabot, J. M., Fuguet, E., Ràfols, C., Rosés, M., *J. Chromatogr. A* 2013, 1279, 108–116.
- [26] Gravador, R. S., Fuguet, E., Ràfols, C., Rosés, M., *Electrophoresis* 2013, 34, 1203–1211.
- [27] Cabot, J. M., Fuguet, E., Rosés, M., *ACS Comb. Sci.*, DOI: 10.1021/co500059p.
- [28] Fuguet, E., Reta, M., Gibert, C., Rosés, M., Bosch, E., Ràfols, C., *Electrophoresis* 2008, 29, 2841–2851.
- [29] ACD/Percepta Platform v2012 Software, ACD/Labs, Toronto, Canada 2012.
- [30] Poole, S. K., Patel, S., Dehring, K., Workman, H., Poole, C.F., *J. Chromatogr. A* 2004, 1037, 445–454.
- [31] Bard, B., Martel, S., Carrupt, P. A., *Eur. J. Pharm. Sci.* 2008, 33, 230–240.
- [32] Wassvik, C. M., Holmén, A. G., Bergström, C. a S., Zamora, I., Artursson, P., *Eur. J. Pharm. Sci.* 2006, 29, 294–305.
- [33] Yang, W., de Villiers, M. M., *AAPS J.* 2005, 7, E241–E248.
- [34] Völgyi, G., Ruiz, R., Box, K., Comer, J., Bosch, E., Takács-Novák, K., *Anal. Chim. Acta* 2007, 583, 418–428.
- [35] Ràfols, C., Rosés, M., Bosch, E., *Anal. Chim. Acta* 1997, 338, 127–134.
- [36] Box, K. J., Völgyi, G., Ruiz, R., Comer, J.E., Takács-Novák, K., Bosch, E., Ràfols, C., Rosés, M., *Helv. Chim. Acta* 2007, 90, 1538–1553.

Manuscript submitted to Journal of Chromatography A

Determination of Acidity Constants of Sparingly Soluble Drugs in Methanol-Water Mixtures by the Internal Standard Capillary Electrophoresis Method

Joan M. Cabot, Elisabet Fuguet and Martí Rosés*

Departament de Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Corresponding author: Prof. Martí Rosés

Departament de Química Analítica, Universitat de Barcelona

Martí i Franquès 1-11

E-08028-Barcelona, Spain

Tel. 34 93 403 92 75; Fax 34 93 402 12 33

e-mail: marti.roses@ub.es

HIGHLIGHTS

Lower methanol percentages are needed compared to other pK_a determination methods

The aqueous pK_a is obtained in < 30 min from methanol-water measurements

pK_a values of internal standards between 0-40% methanol are determined

ABSTRACT

A fast determination of acidity constants (pK_a) of sparingly soluble drugs has become a necessity in drug discovery because it often produces molecules that are highly lipophilic and sparingly or not soluble in water. In this paper we have adapted the high throughput internal standard capillary electrophoresis (IS-CE) method for the determination of pK_a of water insoluble compounds by measurement in methanol/water buffer mixtures. For this purpose, we have established reference pK_a values for a set of 48 acids and bases of varied structure (internal standards) at several methanol-water mixtures covering a range up to 40% of methanol. In contrast with other pK_a determination methods which require higher amounts of co-solvent, 40% of methanol has been enough to determine the aqueous pK_a of all tested drugs because of the lower requirement of solubility of the internal standard method. Obtained pK_a values (around 200 values determined) have been contrasted with literature data obtaining a good agreement and, therefore consolidating the reference acidity constants of the internal standard set.

The IS-CE method has been successfully applied to 8 test drugs of different chemical nature with intrinsic solubilities lower than 10^{-6} M. The obtained results have been successfully compared to literature ones obtained by other methods. It is concluded that the IS-CE method allows the determination of aqueous pK_a values using lower proportions of co-solvent, becoming then more accurate in the extrapolation procedure than other reference methods.

KEYWORDS

Acidity constant, IS-CE, pK_a determination, internal standard, low solubility, hydro-organic media.

1. INTRODUCTION

The new technologies and strategies for drug discovery and development have increased considerably in the last decades, adding new opportunities for gathering and integrating information to increase drug discovery success and efficiency. Consequently, pharmaceutical companies synthesize a great number of potential drugs and chemical precursors in a relative short time. To select those which are the most suitable for further test and development, there is a need of high throughput screening for rapid evaluation of potential drug candidates as soon as they are available from synthesis [1,2].

ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) and DMPK (Drug Metabolism and Pharmacokinetic) studies frequently use physicochemical parameters for the understanding and simulation of drug properties and processes. One of main properties that affects the pharmaceutical potential of a compound is the dissociation constant (or pK_a in logarithmic scale) since it determines the ionization degree of the compound in the pH of the target. In fact, the neutral and ionic forms of a compound can have very different physicochemical and biological properties, being the pK_a sometimes decisive for a given application [2–4].

Modern techniques of drug discovery often produce molecules that are highly lipophilic and sparingly or not soluble in water. Poor aqueous solubility often becomes a drawback in the physicochemical characterization of drugs, because many assays require the drugs to be in aqueous solution during measurement. Therefore, the determination of the acidity constant of insoluble drugs can be a serious problem in drug discovery [2,4–6].

In order to meet the need for pK_a determination of insoluble compounds, aqueous-organic solvent mixtures can be used. Then, acidity constants are determined at different ratios of hydro-organic mixtures, and aqueous pK_a values at zero organic solvent composition are estimated by extrapolation (e.g., through the Yasuda-Shedlovsky equation). Many water miscible organic solvents have been used such as alcohols or acetonitrile. Nevertheless, methanol is the solvent of choice because it is very similar in properties to water, and it is able to solubilize compounds insoluble in water. So far, it is thought to be the least error-prone of the common co-solvents since its general effect on pK_a values has been studied so extensively [7–12].

Capillary electrophoresis is a particular convenient technique for pK_a determinations because it enables automated and accurate measurements even for minute amounts of sample, does not require the information about concentration, and handle both the impure and complex samples [13]. Recently, a high throughput method to determine acidity constants based on CE and the use of internal standards (IS-CE) has been developed [14], as well as tested for all kinds of compounds [15]. The pK_a of an analyte can be determined in 5 minutes. Furthermore, this method does not need an accurate measure of the pH of the buffer, and it may correct the interactions of test compounds with buffers and possible experimental systematic errors -like temperature or pH buffer variations due to electrolysis processes during run analysis- by the use of an adequate internal standard. Moreover, the IS-CE method has been evaluated for determination of sparingly soluble compounds reaching solubility limits of $10^{-6} \text{ mol} \cdot \text{L}^{-1}$. However, the use of co-solvents and extrapolation procedures to pure water is strictly necessary for those compounds with lower solubility [16].

Working with hydro-organic mixtures implies establishing new reference pK_a values for the internal standards at each methanol volume fraction, as it was previously done in aqueous media [17,18]. In the present work we measure and establish the pK_a of 48 acids and bases (internal standards) at several methanol volume fractions in order to use them as reference values. Furthermore, the validity of the methodology to determine acidity constants of sparingly soluble drugs is proved by comparison of the results to the ones obtained by other methods.

2. MATERIAL AND METHODS

2.1. Apparatus

Capillary electrophoresis experiments have been performed with a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode-array spectrophotometric detector. Capillary was made of fused silica 50 μm I.D., 375 μm O.D., 35.2 cm length (25 cm to the detector) and was obtained from Composite Metal Services Ltd (Shipley, West Yorkshire, UK). The temperature of the capillary was set to 25.0 ± 0.1 °C. Test compounds and internal standards were injected sequentially (one-by-one before separation) at a hydrodynamic pressure of 0.5 psi for 3 s (1 psi = 6894.76 Pa), and the applied voltage during separation was 20 kV. In order to speed up analysis, an additional hydrodynamic pressure of 1.0 psi during

separation was applied. The UV detector was set at 214, 254, and 280 nm. Optimized capillary conditionings were described recently[14]. Briefly, at the beginning of the session capillary was conditioned with NaOH 1.0 M (2.0 min), H₂O (0.5 min) and buffer (2 min); 0.2 min with new buffer when pH was changed; and at the end of the session NaOH 0.1 M (2.0 min) and H₂O (2.0 min). Capillary was not rinsed for replicates.

2.2. Chemicals

Dimethyl sulfoxide >99.9% (DMSO), methanol HPLC grade, 0.5 M sodium hydroxide trititol, 0.5 M hydrochloric acid titritol, and sodium dihydrogenphosphate monohydrate >99% were from Merck (Darmstadt, Germany). Anhydrous sodium acetate >99.6%, 2-(cyclohexylamino)ethanesulfonic acid >99% (CHES), and 3-(cyclohexylamino)-1-propanesulfonic acid >98% (CAPS) were from Sigma (St. Louis, MO, USA). 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol >99.9% (BisTris) and sodium formate were from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)amino-methane >99.9% (Tris) was purchased from Aldrich (Milwaukee, WI, USA). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩcm.

All studied drugs and internal standards [14] were reagent grade or purer, and were purchased from Sigma, Aldrich, Fluka, Merck, or Carlo Erba (Milan, Italy).

2.3. Preparation of samples and buffers

Stock solutions of sodium dihydrogenphosphate, sodium formate, sodium acetate, BisTrisH⁺, TrisH⁺, CHES⁻, CAPS⁻ or NaOH were prepared in aqueous media at 0.25 mol·L⁻¹. In order to obtain BGE solutions at the desired pH, ionic strength, and methanol/water fractions, the appropriated amount of 0.5 M HCl, 0.5 M NaOH or 0.5 M KCl was added into 5 mL of stock buffer solution. Subsequently, pure methanol was added to have the desired mixture (v/v), and afterwards the content of the volumetric flask was diluted with a methanol-water mixture of the same v/v proportion up to 25 mL. Using this procedure the ionic strength was kept constant at 0.05 M.

Buffer solutions covering practically all the useful pH range (from 2 to 12.5 separated within intervals of 0.5 pH units) were prepared at 10, 20, 30 and 40 % (v/v) of methanol-water mixtures. Since the pH is calculated by the IS *in-situ* into the capillary, pH-meter measurements are not required.

Stock solutions of test compounds and ISs were prepared at a concentration of 1 mg mL⁻¹ and 4% of DMSO was added as electroosmotic flow (EOF) marker. They were diluted in water or in a methanol/water mixture (when they were not soluble in water itself). Afterwards, a 1/10 dilution in water of the stock solution was prepared for injection (100 mg L⁻¹; 0.4% DMSO).

All buffers and compound solutions were filtered through a nylon mesh 0.45 µm porous size (Whatman, Maidstone, UK) and stored at 4°C until their use.

2.4. *pK_a* determination

The optimized procedure used for acidity constants determination by the IS-CE method has been recently reported [14]. Briefly, the method is based on the use of an IS with *pK_a* similar to that of test compound ($\Delta pK_a < 1$), choosing as first approximation for the *pK_a* of the test compound the prediction by an appropriate software (ACD/Labs in our case) [19]. Then, mobilities of the IS and the test compound are determined in at least two different buffers for a monoprotic acid-base compound: a buffer in which the analyte and the IS are completely ionized ($pH \gg pK_a$ for an acid or $pH \ll pK_a$ for a base); and a second buffer (or more buffers) in which both compounds are partially ionized (pH in the range $pK_a \pm 1$). From these mobility measurements the *pK_a* of the test compound can be directly obtained if the *pK_a* of the IS is well known [14].

For the determination of the aqueous *pK_a* of sparingly soluble compounds, acidity constants were determined in buffers made of a given electrolyte solved in methanol-water mixtures at different compositions (0.10, 0.20, 0.30, 0.40 v/v). Then, the aqueous *pK_a* was obtained by extrapolation to 0% methanol through the Yasuda-Shedlovsky equation, according to the relationship between *pK_a* values and methanol/water mixtures dielectric constant (equation 1).

$$\text{pK}_a + \log[\text{H}_2\text{O}] = a \frac{100}{\varepsilon} + b \quad (1)$$

In this equation, $\log[\text{H}_2\text{O}]$ is the logarithm of the water molar concentration in a given solvent mixture, and ε is the dielectric constant of the solvent mixture. From the plot of the pK_a vs. $100/\varepsilon$ a linear relationship should be obtained, and extrapolation to pure water would provide the aqueous pK_a of the compound [7].

3. RESULTS AND DISCUSSION

3.1. Establishment of the pK_a of the reference set of internal standards

Working with hydro-organic mixtures implies establishing reference pK_a values for the set of ISs in the whole range of methanol-water working compositions (0-0.4 v/v). The procedure followed to establish these pK_a values is based in the IS-CE methodology and has been used already in previous works to determine the aqueous pK_a of the reference set of compounds [17,18]. Briefly, it consists in an iterative process in which the compounds are used indistinctly as test compounds or as internal standards in the following way: to determine the pK_a of a given compound of the set, the neighboring compounds (with pK_a differing less than 1 pK_a units from the expected one) are used as internal standards. This procedure is done for each compound in the set, using the corresponding neighbors as internal standards. The procedure has been applied separately for acidic ISs and basic ISs, and in all cases the pK_a values have been determined at four different methanol-water compositions, 0.10, 0.20, 0.30 and 0.40 (v/v). In order to start the iteration, empirical equations fitting available experimental pK_a values in methanol-water to the solvent percentage have been used to estimate the initial pK_a values of the ISs. Different equations are used depending on the functional groups of the compounds [20,21]. Table 1 lists the pK_a as well as the standard deviation at each volume fraction for all the ISs at 25°C and zero ionic strength.

In order to know the reference pK_a value at any methanol-water composition between 0 and 0.4, the obtained values together with the aqueous ones established in previous works [17,18] have been fitted to polynomial equations, summarized in Table 2. These quadratic polynomial equations allow the calculation of the acidity constant at any particular volume fraction for any internal standard of the list in a range between 0.00 and 0.40.

Literature pK_a ($pK_{a(lit)}$) values for the set of ISs have been compared to the values obtained by interpolation from the equations in Table 2. Literature values have been obtained from several sources [12,22–42], although most of the values have been summarized in the excellent compilation of Palm et al. [43]. Particularly, 101 pK_a values in methanol-water compositions ranging from 0.11 to 0.45 have been correlated with the literature ones for the established ISs. The overall data is presented in Table 3 as well as the pK_a variation between literature and interpolated acidity constants (ΔpK_a) at 25 °C and zero ionic strength. Figure 1 shows $pK_{a(IS-CE)}$ as a function of $pK_{a(lit)}$ and the corresponding theoretical line.

As bibliographic information is sometimes scarce and disperse regarding the acidity constants of these compounds, a real estimation of the accuracy of the obtained pK_a values is not possible. However, absolute ΔpK_a values do not surpass 0.25 units and in most cases it is less than 0.1. Besides, there are some internal standards for which reliable literature values were not found. Nevertheless, the reliability of their pK_a values is supported by the good agreement of the values obtained from the different neighbor ISs used on their determination.

3.2. Determination of acidity constant of sparingly soluble compounds

The test compounds chosen for the evaluation of the performance of the IS-CE method in hydro-organic media include 8 substances, most of them commercially available drugs (Figure 2). These compounds (5 bases and 3 acids) were selected as their insolubility in water made it impossible to perform an aqueous IS-CE determination [16]. Table 4 shows the compounds together with the logarithmic form of their intrinsic solubility ($\log S_{(lit)}$, in mol L^{-1}). These solubility values were collected from several sources [44,45], or predicted through ACD/Percepta software [19] when experimental data was not available in literature. Compounds are listed in decreasing order of solubility, from mefenamic acid with $\log S$ of -6.34 to tamoxifen with $\log S$ of -8.49.

The pK_a values of all studied drugs were determined at four percentages of methanol as co-solvent (40, 30, 20 and 10% v/v), and extrapolation to 0% co-solvent yields the aqueous pK_a using the Yasuda-Shedlovsky equation, as explained in section 2.4. Table 4 shows the determined pK_a and the fitting parameters to the Yasuda-Shedlovsky equation as well as the extrapolated aqueous acidity constant for each drug. Yasuda-Shedlovsky equation has been chosen to obtain the aqueous pK_a values instead of polynomial fitting because of two reasons:

on one hand, it is a linear equation that requires less input data than polynomial equations, and on the other hand, it is the common equation used for aqueous pK_a extrapolation regardless of the experimental technique used [7,12,30,42,46].

One of the advantages of the IS-CE method when hydro-organic media is required is that the pH of the buffer solution is calculated inside the capillary in each determination by means of the IS, obtaining directly the s_pH (if the pK_a of the IS is in the s_pH scale). In other methods like potentiometry, spectrophotometry and the classical CE, pH is measured with a glass electrode generally calibrated with aqueous buffers, while sample measurements are done in solvent/water mixtures. Therefore, a correction of the pH scale is needed to transform the obtained w_pH into the desired s_pH . These pH scales corrections can be avoided in the IS-CE method.

Table 5 shows a comparison of the pK_a values obtained by the IS-CE method to the values obtained by other methods for the selected drugs. For most of the compounds, multiple literature values ($pK_{a(lit)}$) have been reported, determined by a variety of methods and experimental conditions. The pK_a of sertaconazole was not found in any reliable source, so comparison is not provided. Table 5 also includes information regarding the co-solvent and the composition range (v/v) used in each determination. There are some references which do not specify the range of co-solvent, or even the co-solvent used (n.s. in the Table).

Similarity between extrapolated pK_a values are observed when comparing the results of the IS-CE method to other methods. Analyzing literature data, it is observed that at these solubility levels, all methods (potentiometry, spectrophotometry and CE) require the use of co-solvent and extrapolation methods to obtain the pK_a in water except CE-MS, which allows the direct determination of highly diluted solutions in aqueous buffers due to the high sensitivity of the technique.

In terms of the co-solvent used, it is demonstrated that methanol is the preferred co-solvent. Methanol general effect on pK_a values has been studied so extensively, its properties are very similar to water, and it is able to solubilize many compounds insoluble in water.

Regarding the range of solvent compositions (v/v), almost all the reference methods (potentiometric titrations, spectrophotometric titrations, and classic CE method) require the use of higher volume fractions of methanol than the IS-CE method. The use of MDM (equal volumes of MeOH, 1,4-dioxane and ACN) instead of methanol allows to decrease the content

of organic solvent in the mixtures, as observed for amiodarone. In terms of aqueous pK_a determination, using the Yasuda-Shedlovsky extrapolation, the lower solvent percentage used, the more accurate the aqueous pK_a results should be obtained. Tables 4 and 5 demonstrates that IS-CE method allows the determination of pK_a values from 10% whereas other methods require the use of higher volumes of co-solvent, around 40%-50% of methanol, making the reliability of the IS-CE results higher.

4. CONCLUSIONS

The IS-CE method is a fast and reliable alternative for acidity constants determination of any kind compound, including sparingly soluble ones. The pK_a values in methanol-water mixtures ranging from 0 to 40% (v/v) of a set of 48 acidic and basic internal standards have been successfully established through polynomial equations that relate the pK_a with the composition of the mixture. Literature values from several sources have been satisfactorily correlated to the reference pK_a of the internal standards established in this work. This set can be systematically employed as internal standards for routine and accurate measurements of pK_a by the IS-CE method in methanol-water media.

The method has been tested for a set of commercial drugs with aqueous solubility under 10^{-6} M, which cannot be directly determined in water by the IS-CE method, nor with other techniques. pK_a values of these compounds have been determined at 10, 20, 30 and 40% of methanol, and aqueous acidity constant extrapolated using Y-S equation. Compared to other methods, potentiometry and spectrophotometry titrations as well as other CE-based methods require the use of higher methanol percentages in the determinations, being the lowest 40% of methanol, fact that increases the extrapolation error. The CE-MS method allows the direct determination in aqueous solutions due to the high sensitivity of the technique. However, this CE-MS method is time-consuming and expensive unless many drugs are pooled for a simultaneous determination. In conclusion, the high throughput IS-CE method can be used to determine the acidity constants of water insoluble compounds with a lower co-solvent proportion, increasing the accuracy of the obtained results, compared to other current methods. In addition, the high-throughput performance of the method allows the determination of an aqueous pK_a in several hydro-organic media in less than 30 minutes.

ACKNOWLEDGMENTS

The authors thank the Ministerio de Ciencia e Innovación of the Spanish Government and the Fondo Europeo de Desarrollo Regional (FEDER) of the European Union (Project CTQ2010-19217/BQU) for financial support. Furthermore, J.M. Cabot thanks the Secretaria d'Universitats i Recerca of the Departament d'Economia i Coneixement of the Catalan Government for a supporting scholarship (2013FI_B1 00209).

REFERENCES

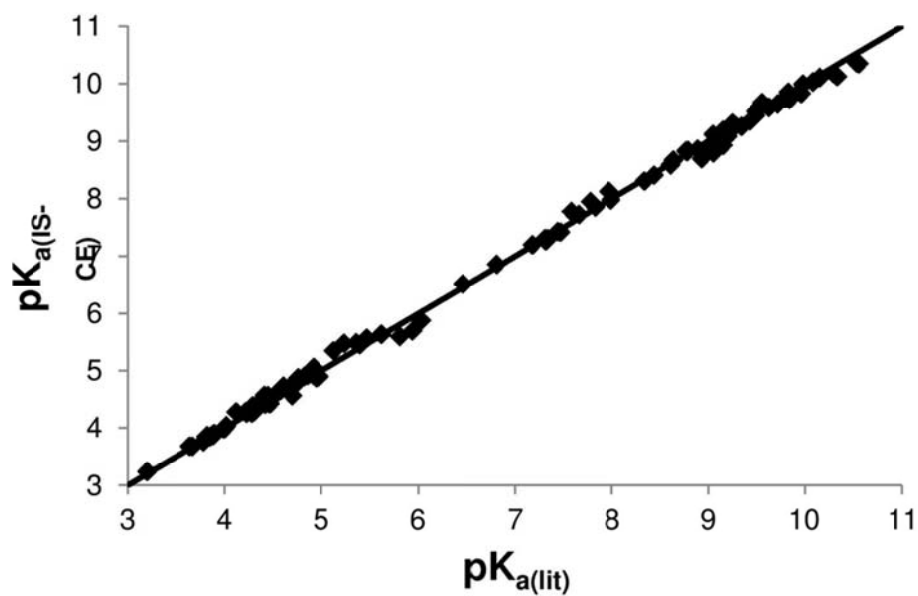
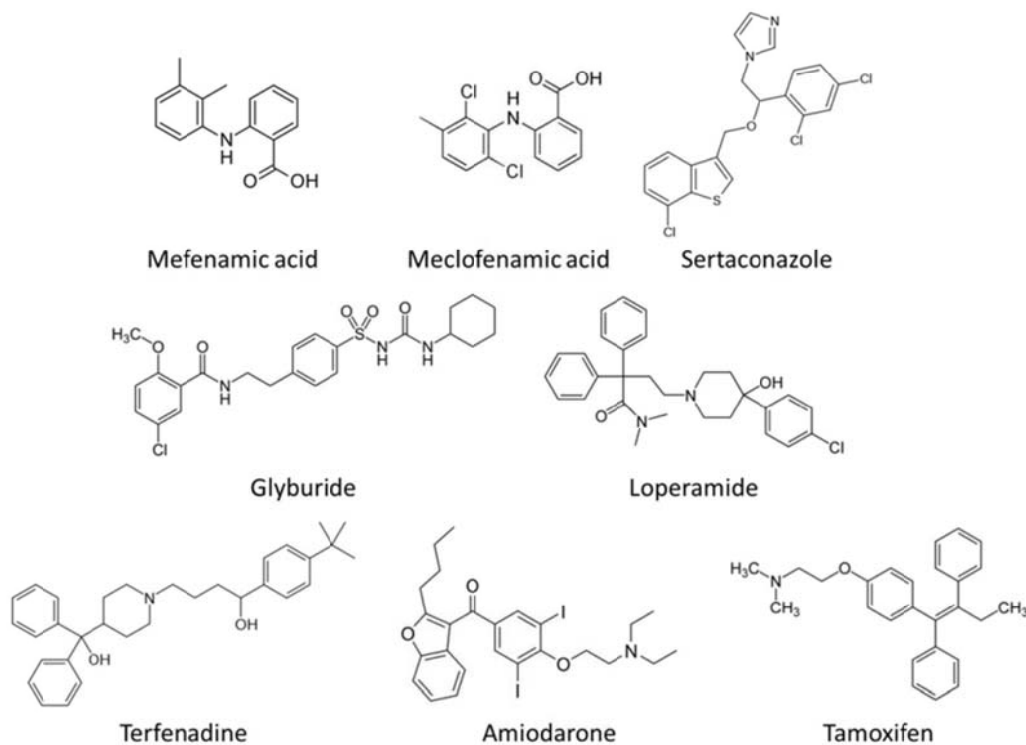
- [1] S. Babić, A.J.M. Horvat, D. Mutavdžić Pavlović, M. Kaštelan-Macan, Determination of pK_a values of active pharmaceutical ingredients, *Trends Anal. Chem.* 26 (2007) 1043–1061.
- [2] H. Wan, J. Ulander, High-throughput pK_a screening and prediction amenable for ADME profiling, *Expert Opinion Drug Metab. Toxicol.* 2 (2006) 139–155.
- [3] J. Reijenga, H. van Arno, L. van Antonie, B. Teunissen, Development of methods for the determination of pK_a values, *Anal. Chem. Insights* 8 (2013) 53–71.
- [4] E. Kerns, Pharmaceutical profiling in drug discovery, *Drug Discov. Today* 8 (2003) 316–323.
- [5] J. Alsenz, M. Kansy, High throughput solubility measurement in drug discovery and development, *Adv. Drug Deliv. Rev.* 59 (2007) 546–567.
- [6] J.B. Taylor, D.J. Triggle, J.E.A. Comer, Ionization Constants and Ionization Profiles, in: *Compr. Med. Chem. II*, 2007: pp. 357–397.
- [7] A. Avdeef, K.J. Box, J.E. Comer, M. Gilges, M. Hadley, C. Hibbert, et al., PH-metric log P 11. pK_a determination of water-insoluble drugs in organic solvent-water mixtures, *J. Pharm. Biomed. Anal.* 20 (1999) 631–641.
- [8] S. Bellini, M. Uhrova, Z. Deyl, Determination of the thermodynamic equilibrium constants of water-insoluble (sparingly soluble) compounds by capillary electrophoresis, *J. Chromatogr. A* 772 (1997) 91–101.
- [9] V. de Nogales, R. Ruiz, M. Rosés, C. Ràfols, E. Bosch, Background electrolytes in 50% methanol/water for the determination of acidity constants of basic drugs by capillary zone electrophoresis, *J. Chromatogr. A* 1123 (2006) 113–120.
- [10] F.Z. Erdemgil, S. Sanli, N. Sanli, G. Ozkan, J. Barbosa, J. Guiteras, et al., Determination of pK(a) values of some hydroxylated benzoic acids in methanol-water binary mixtures by LC methodology and potentiometry, *Talanta*. 72 (2007) 489–496.

- [11] M. Shalaeva, J. Kenseth, F. Lombardo, A. Bastin, Measurement of dissociation constants (pK_a values) of organic compounds by multiplexed capillary electrophoresis using aqueous and cosolvent buffers, *J. Pharm. Sci.* 97 (2008) 2581–2606.
- [12] K. Takács-Novák, K.J. Box, A. Avdeef, Potentiometric pK_a determination of water-insoluble compounds: validation study in methanol/water mixtures, *Int. J. Pharm.* 151 (1997) 235–248.
- [13] P. Nowak, M. Woźniakiewicz, P. Kościelniak, Application of capillary electrophoresis in determination of acid dissociation constant values., *J. Chromatogr. A* 1377 (2015) 1–12.
- [14] J.M. Cabot, E. Fuguet, M. Rosés, Internal Standard Capillary Electrophoresis as a High-Throughput Method for pK_a Determination in Drug Discovery and Development, *ACS Comb. Sci.* 16 (2014) 518–525.
- [15] J.M. Cabot, E. Fuguet, C. Ràfols, M. Rosés, Determination of acidity constants by the capillary electrophoresis internal standard method. IV. Polyprotic compounds, *J. Chromatogr. A* 1279 (2013) 108–116.
- [16] J.M. Cabot, E. Fuguet, M. Rosés, Determination of acidity constants of sparingly soluble drugs in aqueous solution by the internal standard capillary electrophoresis method., *Electrophoresis* 35 (2014) 3564–3569.
- [17] J.M. Cabot, E. Fuguet, C. Ràfols, M. Rosés, Fast high-throughput method for the determination of acidity constants by capillary electrophoresis. II. Acidic internal standards, *J. Chromatogr. A* 1217 (2010) 8340–8345.
- [18] E. Fuguet, C. Ràfols, M. Rosés, A fast high throughput method for the determination of acidity constants by capillary electrophoresis. 3. Basic internal standards, *J. Chromatogr. A* 1218 (2011) 3928–3934.
- [19] ACD/Percepta Platform v2012 Software, ACD/Labs 2012.
- [20] M. Rosés, F. Rived, E. Bosch, Dissociation constants of phenols in methanol--water mixtures., *J. Chromatogr. A* 867 (2000) 45–56.
- [21] F. Rived, I. Canals, E. Bosch, M. Rosés, Acidity in methanol–water, *Anal. Chim. Acta* 439 (2001) 315–333.
- [22] W.L. Bright, H.T. Briscoe, The acidity of organic acids in methanol and ethyl alcohol., *J. Phys. Chem.* 37 (1933) 787–796.
- [23] M. Mizutani, The dissociation of weak electrolytes in aqueous alcoholic solutions. IV. The dissociation of weak electrolytes in methanol., *Z. Physik. Chem.* 118 (1925) 327–341.
- [24] H.M. Dawson, W.R. Bulcraig, G.F. Smith, Elimination of bromide ion from aqueous solutions of phenylbromoacetic acid., *J. Chem. Soc.* (1944) 90–94.

- [25] H. Hartman, The first dissociation constant of ethyl- and phenyl- substituted succinic acids., *Recl Trav. Chim. Pays-Bas Belg.* 57 (1938) 697–702.
- [26] G. Kortuem, K.W. Koch, Conductometric and photometric determination of the dissociation constant of 2,6-dinitrophenol in water-methanol mixtures., *Ber. Bunsenges.* 69 (1965) 677–683.
- [27] R. Gaboriaud, Thermodynamic study of some aqueous organic media., *Ann. Chim. (Paris, France)* 2 (1967) 210–220.
- [28] Z. Voznakova, F. Strafelda, Dissociation constants of some acids in water-methanol mixtures., *Collect. Czechoslov. Chem. Commun.* 36 (1971) 2993–3003.
- [29] J. Juillard, O. Mathe, Dissociation and ionization of various nitrophenols in H₂O/CH₃OH mixtures., *C.R. Seances Acad. Sci., Serie C: Sci. Chim.* 263 (1966) 5–6.
- [30] R. Ruiz, M. Rosés, C. Ràfols, E. Bosch, Critical validation of a new simpler approach to estimate aqueous pK_a of drugs sparingly soluble in water, *Anal. Chim. Acta* 550 (2005) 210–221.
- [31] G.H. Parsons, C.H. Rochester, Acid ionization constants of 4-substituted phenols in methanol-water mixtures., *J. Chem. Soc., Faraday Trans. 1: Phys. Chem. Condens. Phases* 71 (1975) 1058–1068.
- [32] H. Le Moal, Synthesis and physico-chemical study of several α,α -disubstituted succinic and glutaric acids., *Ann. Chim. (Paris)* 8 (1953) 841–899.
- [33] C.H. Rochester, D.N. Wilson, Ionization constants of phenols in methanol + water mixtures., *J. Chem. Soc., Faraday Trans. 1: Phys. Chem. Condens. Phases* 72 (1976) 2930–2938.
- [34] R.A. Robinson, R.H. Stokes, *Electrolyte Solutions*, Second ed., Dover Publications Inc., New York, 2002.
- [35] A.L. Bacarella, E. Grunwald, H.P. Marshall, E.L. Purlee, The potentiometric measurement of acid dissociation constants and pH in the system methanol-water. pK_a values for carboxylic acids and anilinium ions, *J. Org. Chem.* 20 (1955) 747–762.
- [36] J.F.J. Dippy, R.H. Lewis, Chemical constitution and the dissociation constants of monocarboxylic acids. V. Substituted benzoic and phenylacetic acids., *J. Chem. Soc.* (1936) 644–649.
- [37] C. Tissier, M. Tissier, Effect of the substituent on the dissociation of para-substituted pyridinium ions in water-methanol mixtures., *C.R. Seances Acad. Sci., Serie C: Sci. Chim.* 281 (1975) 749–751.
- [38] C. Tissier, M. Tissier, Acidity constants and enthalpies of dissociation of para-substituted pyridinium ions in water-methanol mixtures., *J. Chim. Phys. Phys.-Chim. Biol.* 73 (1976) 149–155.

- [39] R. Gaboriaud, Behavior of uncharged acids in water methyl alcohol mediums., C.R. Seances Acad. Sci., Serie C: Sci. Chim. 263 (1966) 911–914.
- [40] D.H. McDaniel, M. Ozcan, Solvent effects on the basicity of sterically hindered pyridines., J. Org. Chem. 33 (1968) 1922–1923.
- [41] S.M.C. Buckenmaier, D. V McCalley, M.R. Euerby, Determination of ionization constants of organic bases in aqueous methanol solutions using capillary electrophoresis., J. Chromatogr. A 1026 (2004) 251–259.
- [42] R. Ruiz, C. Ràfols, M. Rosés, E. Bosch, A potentially simpler approach to measure aqueous pK_a of insoluble basic drugs containing amino groups, J. Pharm. Sci. 92 (2003) 1473–1481.
- [43] V. Palm, Tables of Rate and Equilibrium Constants of Heterolytic Organic Reactions, VINITI, 1975.
- [44] K.J. Box, J.E.A. Comer, Using measured pK_a , LogP and solubility to investigate supersaturation and predict BCS class, Curr. Drug Metab. 9 (2008) 869–878.
- [45] C.M. Wassvik, A.G. Holmén, C. a S. Bergström, I. Zamora, P. Artursson, Contribution of solid-state properties to the aqueous solubility of drugs, Eur. J. Pharm. Sci. 29 (2006) 294–305.
- [46] A. Avdeef, J.E.A. Comer, S.J. Thomson, pH-Metric log P. 3. Glass electrode calibration in methanol-water, applied to pK_a determination of water-insoluble substances., Anal. Chem. 65 (1993) 42–49.
- [47] H. Wan, A.G. Holmén, Y. Wang, W. Lindberg, M. Englund, M.B. Någård, et al., High-throughput screening of pK_a values of pharmaceuticals by pressure-assisted capillary electrophoresis and mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 2639–2648.
- [48] A. Avdeef, High-throughput measurements of solubility profiles, in: B. Testa, H. Van de Waterbeemd, G. Folkers, R. Guy (Eds.), Pharmacokinet. Optim. Drug Res., Verl. Helv. Chim. Acta, Weinheim (2001) 305–325.
- [49] K.J. Box, G. Völgyi, R. Ruiz, J.E. Comer, K. Takács-Novák, E. Bosch, et al., Physicochemical properties of a new multicomponent cosolvent system for the pK_a determination of poorly soluble pharmaceutical compounds, Helv. Chim. Acta. 90 (2007) 1538–1553.
- [50] A. Avdeef, Absorption and drug development: Solubility, permeability and charge state, Second Ed., Hoboken, John Wiley & Sons, New Jersey, 2012.

FIGURES

Figure 1. IS-CE pK_a ($pK_{a(IS-CE)}$) vs literature pK_a ($pK_{a(lit)}$).**Figure 2.** Studied drugs.

TABLES

Table 1. pK_a values of the internal standards at several methanol-water compositions (v/v), at 25°C and 0 ionic strength.

Methanol (v/v)	0.00*	0.10	0.20	0.30	0.40
Acidic internal standards					
2-Chlorobenzoic acid	2.84 ± 0.02	2.98 ± 0.01	3.29 ± 0.01	3.47 ± 0.01	3.76 ± 0.02
2,6-dibromo-4-nitrophenol	3.31 ± 0.03	3.43 ± 0.01	3.63 ± 0.02	3.79 ± 0.03	3.98 ± 0.01
4-nitrobenzoic acid	3.37 ± 0.01	3.49 ± 0.02	3.65 ± 0.03	3.82 ± 0.03	4.00 ± 0.04
2,6-dinitrophenol	3.69 ± 0.01	3.78 ± 0.01	3.86 ± 0.01	3.97 ± 0.02	4.04 ± 0.02
3-bromobenzoic acid	3.79 ± 0.02	3.95 ± 0.01	4.08 ± 0.02	4.34 ± 0.02	4.55 ± 0.03
2,4-dinitrophenol	4.12 ± 0.02	4.17 ± 0.03	4.26 ± 0.03	4.35 ± 0.02	4.43 ± 0.04
Benzoic acid	4.22 ± 0.03	4.38 ± 0.03	4.56 ± 0.03	4.82 ± 0.04	5.05 ± 0.04
Ibuprofen	4.49 ± 0.02	4.73 ± 0.04	4.92 ± 0.02	5.23 ± 0.04	5.46 ± 0.03
Nicotinic acid	4.85 ± 0.03	4.82 ± 0.03	4.76 ± 0.02	4.72 ± 0.04	4.61 ± 0.04
Warfarin	5.17 ± 0.04	5.36 ± 0.03	5.40 ± 0.02	5.58 ± 0.02	5.70 ± 0.04
2,5-dinitrophenol	5.30 ± 0.05	5.39 ± 0.04	5.45 ± 0.03	5.53 ± 0.03	5.60 ± 0.04
Sulfacetamide	5.42 ± 0.05	5.62 ± 0.02	5.80 ± 0.02	6.11 ± 0.06	6.31 ± 0.04
2,4,6-tribromophenol	6.04 ± 0.08	6.22 ± 0.05	6.44 ± 0.05	6.59 ± 0.06	6.74 ± 0.03
4-nitrophenol	7.09 ± 0.05	7.18 ± 0.05	7.27 ± 0.04	7.37 ± 0.05	7.49 ± 0.03
Vanillin	7.36 ± 0.06	7.49 ± 0.02	7.65 ± 0.04	7.77 ± 0.01	7.92 ± 0.03
Phenobarbital	7.61 ± 0.04	7.69 ± 0.03	7.75 ± 0.05	7.97 ± 0.02	8.13 ± 0.01
4-hydroxybenzaldehyde	7.53 ± 0.04	7.68 ± 0.05	7.84 ± 0.03	7.92 ± 0.02	8.03 ± 0.03
3,5-dichlorophenol	8.18 ± 0.04	8.31 ± 0.04	8.40 ± 0.02	8.53 ± 0.03	8.63 ± 0.03
Methylparaben	8.35 ± 0.03	8.48 ± 0.03	8.60 ± 0.04	8.75 ± 0.02	8.85 ± 0.03
2-chlorophenol	8.50 ± 0.04	8.68 ± 0.02	8.82 ± 0.03	9.00 ± 0.02	9.20 ± 0.04
3-chlorophenol	9.04 ± 0.01	9.18 ± 0.03	9.32 ± 0.03	9.44 ± 0.03	9.57 ± 0.02
4-bromophenol	9.28 ± 0.01	9.43 ± 0.02	9.55 ± 0.02	9.65 ± 0.02	9.81 ± 0.02
Paracetamol	9.58 ± 0.02	9.72 ± 0.01	9.88 ± 0.01	9.98 ± 0.01	10.15 ± 0.01
Phenol	9.89 ± 0.01	10.01 ± 0.02	10.14 ± 0.02	10.25 ± 0.04	10.45 ± 0.01

Basic internal standards

Aniline	4.63 ± 0.02	4.52 ± 0.01	4.43 ± 0.02	4.38 ± 0.02	4.27 ± 0.04
Quinoline	4.93 ± 0.01	4.75 ± 0.01	4.56 ± 0.02	4.32 ± 0.04	4.12 ± 0.04
4- <i>tert</i> -butylaniline	4.93 ± 0.01	4.81 ± 0.02	4.72 ± 0.01	4.61 ± 0.02	4.48 ± 0.04
<i>N,N</i> -dimethyl- <i>N</i> -phenylamine	5.17 ± 0.02	5.02 ± 0.01	4.88 ± 0.02	4.73 ± 0.03	4.57 ± 0.05
Pyridine	5.28 ± 0.01	5.05 ± 0.01	4.89 ± 0.02	4.79 ± 0.01	4.70 ± 0.06
Acridine	5.55 ± 0.06	5.27 ± 0.03	5.10 ± 0.02	4.86 ± 0.03	4.60 ± 0.05
4- <i>tert</i> -butylpyridine	6.03 ± 0.03	5.82 ± 0.04	5.51 ± 0.02	5.35 ± 0.02	5.03 ± 0.04
Papaverine	6.41 ± 0.07	6.13 ± 0.01	5.87 ± 0.02	5.70 ± 0.04	5.60 ± 0.05
2,4-Lutidine	6.81 ± 0.05	6.55 ± 0.04	6.22 ± 0.02	6.04 ± 0.04	5.84 ± 0.04
Trazodone	6.84 ± 0.05	6.60 ± 0.03	6.43 ± 0.04	6.23 ± 0.02	6.08 ± 0.03
Pilocarpine	7.08 ± 0.02	6.76 ± 0.03	6.55 ± 0.03	6.26 ± 0.02	5.91 ± 0.03
2,4,6-Trimethylpyridine	7.51 ± 0.03	7.21 ± 0.06	6.82 ± 0.06	6.61 ± 0.05	6.44 ± 0.02
Lidocaine	7.93 ± 0.01	7.78 ± 0.06	7.70 ± 0.04	7.63 ± 0.03	7.46 ± 0.06
Clonidine	8.10 ± 0.04	8.03 ± 0.06	8.00 ± 0.06	8.01 ± 0.03	7.98 ± 0.06
Bupivacaine	8.19 ± 0.03	8.13 ± 0.04	7.98 ± 0.06	7.97 ± 0.03	7.79 ± 0.06
1-Phenylpiperazine	8.75 ± 0.02	8.56 ± 0.05	8.45 ± 0.04	8.46 ± 0.05	8.37 ± 0.04
<i>N,N</i> -dimethyl- <i>N</i> -benzylamine	8.95 ± 0.04	8.83 ± 0.04	8.74 ± 0.06	8.65 ± 0.04	8.50 ± 0.03
Diphenhydramine	9.08 ± 0.02	8.98 ± 0.04	8.92 ± 0.05	8.86 ± 0.03	8.81 ± 0.05
Procainamide	9.26 ± 0.03	9.07 ± 0.03	8.95 ± 0.03	8.80 ± 0.07	8.70 ± 0.06
Imipramine	9.37 ± 0.02	9.29 ± 0.02	9.20 ± 0.03	9.07 ± 0.06	8.88 ± 0.05
Propranolol	9.47 ± 0.00	9.29 ± 0.02	9.13 ± 0.02	9.04 ± 0.07	8.89 ± 0.03
1-Aminoethylbenzene	9.52 ± 0.01	9.39 ± 0.01	9.29 ± 0.02	9.20 ± 0.04	9.10 ± 0.02
Ephedrine	9.72 ± 0.02	9.59 ± 0.01	9.42 ± 0.03	9.35 ± 0.06	9.26 ± 0.03
Nortriptyline	10.08 ± 0.01	9.94 ± 0.01	9.82 ± 0.02	9.72 ± 0.04	9.64 ± 0.04

*Data from [17,18].

Table 2. Equations and statistics of the fit of pK_a to methanol volume fraction for the set of internal standards. Equations provide the acidity constant at any methanol volume fraction (v) between 0.00 and 0.40, at 25°C and 0 ionic strength.

Compound	Fit equation	R^2	SD	F
Acidic internal standards				
2-Chlorobenzoic acid	$1.188 \cdot v^2 + 1.848 \cdot v + 2.827$	0.9926	0.045	135
2,6-dibromo-4-nitrophenol	$0.677 \cdot v^2 + 1.428 \cdot v + 3.303$	0.9977	0.018	426
4-nitrobenzoic acid	$0.909 \cdot v^2 + 1.223 \cdot v + 3.368$	0.9997	0.006	3878
2,6-dinitrophenol	$-0.090 \cdot v^2 + 0.910 \cdot v + 3.690$	0.9970	0.011	335
3-bromobenzoic acid	$1.644 \cdot v^2 + 1.250 \cdot v + 3.792$	0.9947	0.031	188
2,4-dinitrophenol	$0.362 \cdot v^2 + 0.658 \cdot v + 4.115$	0.9962	0.011	262
Benzoic acid	$1.670 \cdot v^2 + 1.426 \cdot v + 4.218$	0.9986	0.018	695
Ibuprofen	$0.636 \cdot v^2 + 2.177 \cdot v + 4.492$	0.9960	0.034	249
Nicotinic acid	$-0.924 \cdot v^2 - 0.202 \cdot v + 4.849$	0.9829	0.017	57
Warfarin	$-0.078 \cdot v^2 + 1.305 \cdot v + 5.185$	0.9755	0.045	40
2,5-dinitrophenol	$-0.114 \cdot v^2 + 0.792 \cdot v + 5.305$	0.9953	0.011	212
Sulfacetamide	$0.898 \cdot v^2 + 1.901 \cdot v + 5.416$	0.9936	0.040	156
2,4,6-tribromophenol	$-0.790 \cdot v^2 + 2.088 \cdot v + 6.035$	0.9985	0.015	685
4-nitrophenol	$0.466 \cdot v^2 + 0.790 \cdot v + 7.093$	0.9990	0.007	1033
Vanillin	$0.123 \cdot v^2 + 1.354 \cdot v + 7.359$	0.9989	0.010	899
Phenobarbital	$1.228 \cdot v^2 + 0.990 \cdot v + 7.541$	0.9862	0.039	72
4-hydroxybenzaldehyde	$0.028 \cdot v^2 + 1.064 \cdot v + 7.601$	0.9896	0.025	95
3,5-dichlorophenol	$-0.210 \cdot v^2 + 1.206 \cdot v + 8.181$	0.9984	0.010	627
Methylparaben	$-0.078 \cdot v^2 + 1.310 \cdot v + 8.347$	0.9981	0.013	514
2-chlorophenol	$0.540 \cdot v^2 + 1.508 \cdot v + 8.506$	0.9987	0.014	748
3-chlorophenol	$-0.338 \cdot v^2 + 1.462 \cdot v + 9.040$	0.9998	0.004	4867
4-bromophenol	$-0.049 \cdot v^2 + 1.293 \cdot v + 9.288$	0.9958	0.019	237
Paracetamol	$-0.016 \cdot v^2 + 1.407 \cdot v + 9.582$	0.9973	0.016	366
Phenol	$0.994 \cdot v^2 + 0.968 \cdot v + 9.896$	0.9961	0.019	253

Basic internal standards

Aniline	$0.165 \cdot v^2 - 0.932 \cdot v + 4.623$	0.9896	0.020	95
Quinoline	$-0.805 \cdot v^2 - 1.743 \cdot v + 4.934$	0.9986	0.017	721
4- <i>tert</i> -butylaniline	$-0.163 \cdot v^2 - 1.024 \cdot v + 4.925$	0.9981	0.011	532
<i>N,N</i> -dimethyl- <i>N</i> -phenylamine	$-0.158 \cdot v^2 - 1.422 \cdot v + 5.167$	0.9996	0.007	2276
Pyridine	$2.371 \cdot v^2 - 2.380 \cdot v + 5.275$	0.9978	0.015	449
Acridine	$-0.138 \cdot v^2 - 2.251 \cdot v + 5.535$	0.9964	0.031	274
4- <i>tert</i> -butylpyridine	$-0.384 \cdot v^2 - 2.308 \cdot v + 6.033$	0.9914	0.051	115
Papaverine	$3.244 \cdot v^2 - 3.342 \cdot v + 6.415$	0.9994	0.011	1731
2,4-Lutidine	$1.849 \cdot v^2 - 3.200 \cdot v + 6.820$	0.9967	0.032	307
Trazodone	$0.987 \cdot v^2 - 2.286 \cdot v + 6.834$	0.9984	0.017	621
Pilocarpine	$-1.016 \cdot v^2 - 2.431 \cdot v + 7.061$	0.9961	0.040	256
2,4,6-Trimethylpyridine	$3.176 \cdot v^2 - 4.015 \cdot v + 7.530$	0.9945	0.046	180
Lidocaine	$-0.283 \cdot v^2 - 0.981 \cdot v + 7.913$	0.9742	0.040	38
Clonidine	$0.837 \cdot v^2 - 0.601 \cdot v + 8.093$	0.9351	0.017	14
Bupivacaine	$-0.706 \cdot v^2 - 0.676 \cdot v + 8.191$	0.9593	0.044	24
1-Phenylpiperazine	$2.295 \cdot v^2 - 1.776 \cdot v + 8.737$	0.9539	0.044	21
<i>N,N</i> -dimethyl- <i>N</i> -benzylamine	$-0.458 \cdot v^2 - 0.901 \cdot v + 8.941$	0.9909	0.023	109
Diphenhydramine	$0.799 \cdot v^2 - 0.973 \cdot v + 9.077$	0.9986	0.005	739
Procainamide	$1.107 \cdot v^2 - 1.841 \cdot v + 9.257$	0.9983	0.013	575
Imipramine	$-1.850 \cdot v^2 - 0.452 \cdot v + 9.365$	0.9986	0.010	691
Propranolol	$0.905 \cdot v^2 - 1.769 \cdot v + 9.466$	0.9957	0.021	231
1-Aminoethylbenzene	$0.488 \cdot v^2 - 1.222 \cdot v + 9.515$	0.9973	0.012	372
Ephedrine	$1.349 \cdot v^2 - 1.697 \cdot v + 9.725$	0.9929	0.022	139
Nortriptyline	$0.926 \cdot v^2 - 1.437 \cdot v + 10.077$	0.9972	0.013	360

Table 3. Comparison of pK_a at 25°C and 0 ionic strength for the set of internal standards.

Compound	v^*	pK_a		ΔpK_a^{**}
		IS-CE	Literature	
Acidic internal standards				
2-Chlorobenzoic acid	0.20	3.24	3.20 [22]	-0.04
	0.40	3.76	3.78 [23]	0.02
2,6-dibromo-4-nitrophenol	0.24	3.68	3.67 [24]	-0.01
	0.24	3.68	3.64 [25]	-0.04
	0.24	3.68	3.67 [25]	-0.01
4-nitrobenzoic acid	-	-	-	-
2,6-dinitrophenol	0.18	3.85	3.85 [26]	0.00
	0.18	3.85	3.81 [26]	-0.04
	0.20	3.87	3.82 [26]	-0.05
	0.34	3.99	3.99 [26]	0.01
	0.40	4.04	4.02 [26]	-0.03
	0.20	3.87	3.88 [27]	0.01
	0.35	3.99	4.00 [27]	0.01
	0.24	3.90	3.89 [24]	-0.01
	0.24	3.90	3.90 [25]	0.00
3-bromobenzoic acid	-	-	-	-
2,4-dinitrophenol	0.20	4.26	4.23 [27]	-0.03
	0.35	4.39	4.29 [27]	-0.10
	0.24	4.29	4.22 [24]	-0.07
	0.24	4.29	4.22 [25]	-0.07
Benzoic acid	0.12	4.42	4.42 [28]	0.00
	0.24	4.66	4.58 [28]	-0.07
	0.35	4.92	4.86 [28]	-0.06
	0.20	4.57	4.51 [29]	-0.06
	0.20	4.57	4.41 [22]	-0.16
	0.40	5.06	4.92 [23]	-0.14
Ibuprofen	0.36	5.35	5.13 [30]	-0.21
	0.40	5.48	5.24 [30]	-0.24
Nicotinic acid	-	-	-	-
Warfarin	-	-	-	-
2,5-dinitrophenol	0.20	5.46	5.40 [27]	-0.06
	0.35	5.57	5.47 [27]	-0.10
	0.24	5.49	5.36 [24]	-0.13
	0.24	5.49	5.36 [25]	-0.13
	0.45	5.64	5.62 [29]	-0.02
Sulfacetamide	-	-	-	-
2,4,6-tribromophenol	-	-	-	-
4-nitrophenol	0.12	7.20	7.18 [31]	-0.02
	0.24	7.31	7.31 [31]	0.00

	0.35	7.43	7.44 [31]	0.01
	0.20	7.27	7.32 [27]	0.05
	0.35	7.42	7.47 [27]	0.05
	0.24	7.31	7.34 [32]	0.03
Vanillin	-	-	-	-
Phenobarbital	0.20	7.78	7.58 [12]	-0.20
	0.30	7.95	7.78 [12]	-0.17
	0.40	8.13	7.97 [12]	-0.16
4-hydroxybenzaldehyde	0.12	7.73	7.66 [31]	-0.07
	0.24	7.86	7.83 [31]	-0.03
	0.35	7.98	7.99 [31]	0.01
3,5-dichlorophenol	0.11	8.31	8.34 [33]	0.03
	0.20	8.41	8.44 [33]	0.03
	0.36	8.59	8.61 [33]	0.02
Methylparaben	-	-	-	-
2-chlorophenol	0.11	8.67	8.64 [33]	-0.03
	0.20	8.83	8.77 [33]	-0.06
	0.36	9.12	9.05 [33]	-0.07
3-chlorophenol	0.11	9.19	9.15 [33]	-0.04
	0.20	9.32	9.25 [33]	-0.07
	0.36	9.52	9.49 [33]	-0.03
4-bromophenol	0.24	9.59	9.63 [31]	0.04
	0.35	9.74	9.80 [31]	0.06
Paracetamol	0.19	9.85	9.83 [12]	-0.02
	0.29	9.99	9.98 [12]	-0.01
	0.38	10.11	10.15 [12]	0.04
Phenol	0.12	10.03	10.08 [31]	0.05
	0.24	10.18	10.29 [31]	0.11
	0.35	10.36	10.53 [31]	0.17
	0.20	10.13	10.33 [27]	0.20
	0.35	10.35	10.55 [27]	0.20
Basic internal standards				
Aniline	0.20	4.44	4.45 [34]	0.01
	0.40	4.28	4.30 [34]	0.02
	0.20	4.44	4.36 [35]	-0.08
	0.40	4.28	4.12 [35]	-0.15
	0.21	4.43	4.47 [36]	0.04
	0.42	4.26	4.28 [36]	0.02
Quinoline	-	-	-	-
4-tert-butylaniline	-	-	-	-
N,N-dimethyl-N-phenylamine	0.20	4.88	4.95 [34]	0.07
	0.40	4.57	4.70 [34]	0.13
	0.20	4.88	4.76 [35]	-0.11
	0.40	4.57	4.45 [35]	-0.13
Pyridine	0.18	4.92	4.92 [37,38]	0.00

	0.35	4.73	4.61 [37,38]	-0.12
	0.20	4.90	4.82 [39]	-0.08
	0.35	4.73	4.70 [39]	-0.03
	0.20	4.90	4.98 [27]	0.08
	0.35	4.73	4.71 [27]	-0.02
	0.40	4.70	4.66 [40]	-0.04
Acridine	-	-	-	-
4-tert-butylpyridine	-	-	-	-
Papaverine	0.20	5.88	6.03 [12]	0.15
	0.30	5.70	5.94 [12]	0.24
	0.40	5.60	5.81 [12]	0.21
2,4-Lutidine	-	-	-	-
Trazodone	-	-	-	-
Pilocarpine	-	-	-	-
2,4,6-Trimethylpyridine	0.20	6.86	6.81 [39]	-0.05
	0.35	6.52	6.46 [39]	-0.06
Lidocaine	-	-	-	-
Clonidine	-	-	-	-
Bupivacaine	-	-	-	-
1-Phenylpiperazine	-	-	-	-
N,N-dimethyl-N-benzylamine	-	-	-	-
Diphenhydramine	0.20	8.91	9.00 [41]	0.09
	0.30	8.86	8.89 [41]	0.03
	0.40	8.82	8.78 [41]	-0.04
Procainamide	0.20	8.93	9.15 [41]	0.22
	0.30	8.80	9.05 [41]	0.25
	0.40	8.70	8.93 [41]	0.23
Imipramine	0.42	8.84	8.79 [42]	-0.05
Propranolol	0.12	9.27	9.28 [46]	0.01
	0.24	9.09	9.20 [46]	0.11
	0.40	8.90	9.00 [46]	0.10
1-Aminoethylbenzene	-	-	-	-
Ephedrine	0.19	9.45	9.49 [12]	0.04
	0.29	9.35	9.43 [12]	0.08
	0.40	9.26	9.34 [12]	0.07
Nortriptyline	0.37	9.67	9.56 [42]	-0.11
	0.20	9.83	9.96 [41]	0.13
	0.30	9.73	9.84 [41]	0.11
	0.40	9.65	9.72 [41]	0.07

*v: methanol volume fraction

** $\Delta pK_a = pK_a(\text{literature}) - pK_a(\text{IS-CE})$

Table 4. Intrinsic solubility (M) ($\log S_{(iii)}$), pK_a values obtained in methanol-water media, parameters of the Yasuda-Shedlovsky equation ($pK_a + \log[H_2O] = a \cdot 100/\varepsilon + b$) and aqueous pK_a values for the set of test compounds, at 25°C and 0 ionic strength.

Methanol (v/v)	LogS _(iii)	pK _a				a	b	r ²	^w pK _{a(extr)}
		0.10	0.20	0.30	0.40				
1/ε		1.333	1.401	1.482	1.578				0.00
Mefenamic acid	-6.34 [44]	4.39	4.66	4.92	5.33	3.075	1.984	0.9962	4.17 ± 0.02
Meclofenamic acid	-6.86 [44]	4.31	4.53	4.82	5.20	2.917	2.103	0.9959	4.09 ± 0.02
Sertaconazole	-6.99 [19]	6.01	5.98	5.91	5.80	-1.581	9.830	0.9927	6.07 ± 0.01
Glyburide	-7.05 [45]	5.57	5.75	5.93	6.15	1.647	5.078	0.9988	5.44 ± 0.01
Loperamide	-7.13 [44]	8.78	8.68	8.65	8.58	-1.472	12.416	0.9845	8.79 ± 0.02
Terfenadine	-7.74 [44]	9.18	9.11	9.08	9.02	-1.340	12.648	0.9915	9.19 ± 0.01
Amiodarone	-8.17 [44]	8.49	8.30	8.12	7.92	-3.040	14.220	0.9971	8.59 ± 0.02
Tamoxifen	-8.49 [45]	8.37	8.39	8.35	8.32	-0.983	11.394	0.9797	8.40 ± 0.01

Table 5. Comparison of aqueous pK_a determined by the IS-CE method to the values determined by other methods. $pK_{a(IS-CE)}$ were determined by Yasuda-Sheldolovsky extrapolation using methanol as a co-solvent within the range 0.10-0.40 v/v. All acidity constants are at 25°C and 0 ionic strength.

Compound	$pK_{a(IS-CE)}$	$pK_{a(lit)}$	Method	Cosolvent	(v/v) range
Mefenamic acid	4.17	4.22	GLp K_a spectrophotometry [44]	MeOH	n.s.
Meclofenamic acid	4.09	4.10	GLp K_a n.s. [44]	n.s.	n.s.
Sertaconazole	6.07	-	-	-	-
Glyburide	5.44	5.22	CE-MS [47]	None	0.00
Loperamide	8.79	8.90	GLp K_a spectrophotometry [44]	MeOH	n.s.
Terfenadine	9.19	9.25	GLp K_a potentiometry [44]	MeOH	0.50-0.70
		9.74	Potentiometry [48]	n.s.	n.s.
		9.31	Multiplexed CE [11]	MeOH	0.40-0.60
		9.21	CE-MS [47]	None	0.00
Amiodarone	8.59	8.85	Potentiometry [9]	MeOH	0.46-0.60
		8.80	Potentiometry [49]	MDM*	0.36-0.53
		8.73	Potentiometry [49]	MeOH	n.s.
		8.94	Potentiometry [50]	n.s.	n.s.
		8.73	GLp K_a spectrophotometry [44]	MeOH	0.40-0.50
		8.85	Classic CE [9]	MeOH	0.50
		8.62	Multiplexed CE [11]	MeOH	0.50-0.60
Tamoxifen	8.40	8.36	Potentiometry [50]	n.s.	n.s.
		8.51	Multiplexed CE [11]	MeOH	0.40-0.60
		8.71	CE-MS [47]	None	0.00

n.s.: not specified in the reference.

*MDM: mixture consists of equal volumes of MeOH, 1,4-dioxane and ACN.

Manuscript submitted to Analytical Chemistry

Novel instrument for automated pK_a determination by Internal Standard Capillary Electrophoresis

Joan M. Cabot^{†,‡}, Elisabet Fuguet[†], Martí Rosés[†], Pter Smejkal[‡], Michael C. Breadmore^{‡}*

[†] Departament de Química Analítica and Institut de Biomedicina (IBUB), Universitat de
Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

[‡] Australian Centre for Research on Separation Science, School of Chemistry, Faculty of
Science, Engineering and Technology, University of Tasmania, Private Bag 75, Hobart,
Tasmania 7001, Australia

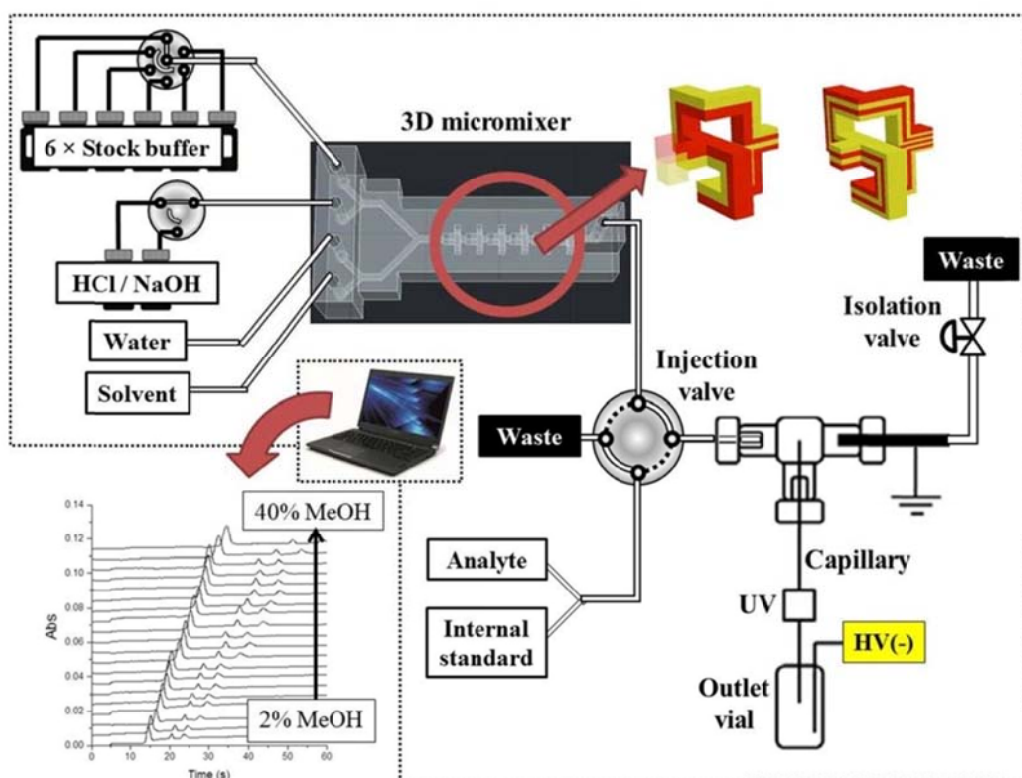
KEYWORDS

Automated CE, buffer mixer, on-line buffer generation system, 3D printing, acidity constant, pK_a determination, IS-CE, High throughput method, internal standard, capillary electrophoresis, insoluble compounds.

ABSTRACT

The Internal Standard Capillary Electrophoresis method (IS-CE) has been implemented in a novel sequential injection – capillary electrophoresis instrument for the high-throughput determination of acidity constants (pK_a) regardless of solubility, number of pK_a s or structure. This instrument comprises a buffer creation system that automatically mixes within a few seconds four reagents for in-situ creation of the separation electrolyte with a pH range of 2-10, ionic strength of 10-100 mM and organic solvent content from 0-40%. Combined with 30 kV, a short capillary (25 cm) and a short effective length (15 cm to the UV detector) fast 20 s electrophoretic separations can be obtained. The low standard deviation of the replicates and the low variation compared to reference values show that this system can perfectly determine acidity constants of drugs by IS-CE. A single pK_a can be determined in two minutes and a set of 20 measurements in half an hour, allowing rapid, simple and flexible determination of pK_a values of pharmaceutical targets.

GRAPHICAL ABSTRACT (TOC)



INTRODUCTION

New technologies and strategies for drug discovery and development have evolved considerably over the last few decades adding new opportunities for gathering and integrating information to increase drug discovery success and efficiency^{1,2}. Consequently, pharmaceutical companies synthesize a great number of potential drugs and chemical precursors in a relative short time. To select those which are the most suitable for further development, there is a need for high throughput screening of potential drug candidates as soon as they are available.^{3,4}

ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) and DMPK (Drug Metabolism and Pharmacokinetic) studies frequently use physicochemical parameters for the understanding of drug properties and processes. One of the main properties that affects the pharmaceutical potential of a compound is the dissociation constant, K_a , (or pK_a in logarithmic scale) since it determines the ionization degree of the compound. In fact, the neutral and ionic forms can have very different physicochemical and biological properties and the pK_a is sometimes decisive for a given application.⁴⁻⁷

The determination of acidity constants can be performed by titration, but it is more convenient to use automated methods such as capillary electrophoresis where multiple targets can be screened rapidly.⁸ This involves measuring the electrophoretic mobility of the target in a number of different pH electrolytes to construct something akin to a titration curve from which the pK_a can be determined. Recently, a new CE approach that uses internal standards (IS-CE) has been developed⁹ and applied for a range of different compounds¹⁰ with only three-minutes required per analyte. The key to this approach is the use of an IS, a compound with a precisely known pK_a similar to that of the analyte (AN). If they are injected together, the differences in the mobilities of the compounds can be directly related to the difference in their acidity. This means that only two electropherograms are needed to determine the acidity constant. Unlike other methods, IS-CE does not need an accurate measure of the electrolyte pH and may correct for interactions of analyte with the buffers and possible experimental and systematic errors – such as temperature or pH buffer variations due to electrolysis during a sequence. This method has been evaluated for pK_a determinations of sparingly soluble compounds reaching solubility limits of $10^{-6} \text{ mol} \cdot \text{L}^{-1}$.¹¹ Furthermore, for those drugs with lower solubility, the use of co-solvents and extrapolation procedures have been performed.¹² Consequently, IS-CE is now a reliable, precise and accurate method applicable to any kind of compound regardless its solubility, number of pK_a s or structure. However, commercial CE

instruments are big, heavy and expensive, and the need to cover all the pH range (from 2 to 12) requires preparation of a lot of different buffers, and electrolyte preparation is tedious and long, particularly for methanol-water buffers. There are also storage issues when multiple electrolytes need to be kept for prolonged periods of time. To overcome these issues, special instrumentation has been designed and adapted to the IS-CE method to simplify and streamline the process for pK_a determination by using a sequential injection – capillary electrophoresis platform.

Sequential injection – capillary electrophoresis uses a flow-based sample and electrolyte introduction system to introduce solutions into the capillary, and is perfectly suited for continuous monitoring applications or the integration of sample handling processes.^{13,14} In the present work, a recently developed sequential injection – capillary electrophoresis system^{15–18} has been adapted for the determination of acidity constants. The detector was changed to a UV absorbance detector and an automated on-line buffer creation system developed in order to prepare fresh buffer at any desired pH and co-solvent mixture. The ability to accurately determine a pK_a value in 2 min is shown in aqueous media and mixed aqueous-methanol conditions.

EXPERIMENTAL

Chemicals

Dimethyl sulfoxide (DMSO), methanol (MeOH), potassium chloride (KCl), sodium formate, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (BisTris), tris(hydroxymethyl)amino-methane (Tris), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) of analytical reagent grade were obtained from Sigma-Aldrich (New South Wales, Australia). Standard solutions of 0.5 M hydrochloric acid and 0.5 M sodium hydroxide were from Riedel-deHaën (Seelze, Germany) and anhydrous sodium acetate from AJAX (Sydney-Melbourne, Australia). Solutions were prepared in water from a Milli-Q water plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩcm. All studied drugs (internal standard set⁹, mefenamic acid and terfenadine) were reagent grade or purer, and were purchased from Sigma-Aldrich, Merck (Darmstadt, Germany) or Carlo Erba (Milan, Italy).

Stock buffer solutions (sodium formate, sodium acetate, BisTrisHCl, TrisHCl, NaCHES and NaCAPS) and pH modifiers (NaOH and HCl) were prepared in aqueous media at $0.25 \text{ mol}\cdot\text{L}^{-1}$. A 0.05 M of KCl aqueous solution was prepared for the outlet vial.

Stock solutions of analytes and ISs were prepared at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$ and 4% DMSO was added as electroosmotic flow (EOF) marker. They were diluted in water or in a methanol/water mixture (when they were not soluble in water itself). Afterwards, a 1/10 dilution of the stock solution in water was prepared for injection ($100 \text{ mg}\cdot\text{L}^{-1}$, 0.4% DMSO).

All stock solutions were filtered through a nylon mesh $0.45 \text{ }\mu\text{m}$ porous size (Agilent Technologies, Santa Clara, CA, USA).

Instrument Design

A schematic diagram of the home-built instrument is shown in Figure 1, based upon a modified design for the rapid separation of inorganic explosive anions recently developed.¹⁵ This IS-CE instrument is composed of five Milligat pumps (MG-5, GlobalFIA, Fox Island, WA, USA). The first four were used to create and deliver the desired BGE and solvent, and the fifth to pump both analyte and internal standard. A buffer selection valve (7-port selector valve; MXP-7970, Rheodyne, Oak Harbor, WA, USA) was connected to pump 1 to select the appropriate stock buffer solution. A pH modifier valve (2-position; MXP-7980, Rheodyne, Oak Harbor, WA, USA) was connected to pump 2 to select acid or base solution to titrate the BGE to the appropriate pH. Pumps 3 and 4 were connected to water and co-solvent containers, respectively, with the water used to ensure constant ionic strength across all pH values, and the co-solvent used to increase the solubility of compounds not soluble in water. Analyte and internal standard trays were connected to pump 5 by a PEEK (poly(ether ether ketone)) Y-shape piece (P-512, Upchurch Scientific, Oak harbor, WA, USA). A 2-position 4-port injection valve (360T041SHH, NResearch, West Caldwell, NJ, USA) was used to direct sample or BGE to the analytical system. A PEEK tee-shape connector (P-727, Upchurch Scientific, Oak harbor, WA, USA) was used to interface the flow system and the CE capillary. The capillary inlet was fixed at a constant position close to the centre of the interface with the help of a piece of capillary ($360 \text{ }\mu\text{m}$ o.d.) introduced through the horizontal part of the tee-piece. The outlet side of the capillary was immersed in a 5 mL vial filled with KCl 50 mM . A stainless steel syringe needle was cut to yield a 2 cm long, 0.51 mm i.d. tube and was employed as outlet and ground electrode and connected to the interface through the

waste tubing. An isolation valve (HP225K021, NResearch, West Caldwell, NJ, USA) was linked on the waste tubing at the T piece outlet to control solution direction either to capillary or to waste.

The four channels used to make the BGE were connected into a microfluidic 3D micromixer, based upon Baker's transformations¹⁹, printed using a Miicraft 3D printer (Hsinchu, Taiwan). The object was designed in CAD software and converted into STL with triangle facets. The digital 3D model was sliced into 2D cross section layers of 50 μm depth and printed using colourless cream resin. The 3D micromixer was designed with four channels (from pump 1 to pump 4) and connected by a "Y" shape as shown in Figure 1, creating laminar flow in four different sections. Subsequently, this 4 laminar-flow was mixed by splitting and recombining the flows at a perpendicular angle in a mixing unit. A previous study²⁰ found that 0.1 $\mu\text{g/mL}$ fluorescein and 0.1 $\mu\text{g/mL}$ rhodamine were mixed completely after passing through 4 mixer units. As there were acid-base reactions and solvent-water mixtures, a total of 6 units in a row were printed to ensure a complete mixing. The entire chip was 32 mm $\times$ 20 mm $\times$ 14 mm (width $\times$ depth $\times$ height), yielding 0.5 mm wide and deep squared micro-channels for the solution and 1.6 mm cylindrical channels to squeeze in the PEEK tubing.

Separation was driven by an EMCO 4300 high-voltage power-supply (Sutter Creek, CA, USA) working with normal polarity with the cathode (-) electrode immersed in the outlet vial. A commercial ActiPix D100 imaging detector was purchased from Paraytec (York, UK). The detector uses a 9 mm $\times$ 7 mm active pixel sensor array made up 1280 $\times$ 1024 individual 7 μm pixels. The xenon light source was filtered using a 214 nm interference filter. The sample rate was 10 Hz. The detector head was positioned 10 cm from the outlet of the CE capillary. The detector was insulated from the high voltage supply using a 3D printed case. Data acquisition was obtained by Paraytec ActiPix D100 Online Mode software.

The system was controlled from a laptop using USB to RS-485 converter (GlobalFIA, Fox Island, WA, USA) serial connections for the Milligat pumps. The injection valve, isolation valve and high voltage power supply were interfaced to the computer using NI DAQ system (USB-6212). BGE and pH modifier valves were also interfaced but using NI DAQ system (USB-6008). Total system control, except data acquisition was achieved using software LabView v11.0. Both interfaces and software were from National Instruments (Austin, TX, USA). The same software was used to monitor both voltage and current provided by the power supply.

The entire system was operated on a small cabinet with a Ranco ETC-211100-000 Digital Temperature Controller (Pain City, OH, USA) connected to a cooler and a heater. Temperature was set to 25 ± 1 °C and homogenized in whole cabinet using a fan. Figure 2 shows the prototype instrument and the main parts.

System operation

Operational sequence steps of the IS-CE system are described in Table 1. Briefly, the tee-interface was filled with the BGE and the capillary rinsed by closing the isolation valve for 20 s. Subsequently, a mixture of IS and AN were injected by firstly filling the interface and secondly closing the isolation valve for 5 s. After injection, the tee-piece was cleaned using the BGE. Finally, the BGE flow rate was changed ($0.02 \mu\text{L} \cdot \text{s}^{-1}$ for weak bases, $0.3 \mu\text{L} \cdot \text{s}^{-1}$ for weak acids), isolation valve closed and HV applied to facilitate a pressure-assisted electrophoretic separation. These conditions were selected in order to minimize analysis time and consumption of reagents.

To prepare the aqueous buffers at the desired pH and constant ionic strength of 50 mM, calculated flows of 0.25 M HCl or 0.25 M NaOH were added to a constant flow of the 0.25 M stock buffer solution (always a 20 % of the total BGE flow rate). Buffer stock solution was chosen according to the buffering pH range of each buffer ($\text{pK}_a \pm 1$). Finally, water was added to dilute the buffer solution until a constant ionic strength of 50 mM. For MeOH-H₂O mixtures, calculations were performed for each specific mixture keeping the same percentage of stock buffer solution. Buffer pK_a values and pH scale shifts due to methanol (when measuring in methanol-water solvents) were taken into consideration. For extremely basic pH (12.7), the appropriate flow rate of 0.25 M NaOH was directly mixed with the desired proportion of MeOH/H₂O maintaining the ionic strength constant. In this manner, all the useful pH range (from 2.8 to 12.7) at any percentage of methanol mixture between 0.0 and 40.0 % (v/v) can be covered.

Electrophoretic conditions

Separations were performed using fused-silica capillaries (25 μm I.D., 360 μm O.D. and 25 cm length, 15 cm to the detector) obtained from Polymicro Technologies (Phoenix, AR, USA). Sample and internal standard were injected hydrodynamically by closing the isolation valve for 5 s at $0.5 \mu\text{L} \cdot \text{s}^{-1}$. Separation was performed at 30 kV and normal polarity under

pressure by closing the isolation valve while BGE was flushing at $0.02 \mu\text{L}\cdot\text{s}^{-1}$ (bases) or $0.3 \mu\text{L}\cdot\text{s}^{-1}$ (acids). Capillaries were conditioned for the first time by flushing 1 M NaOH for 2.0 min at $1 \mu\text{L}\cdot\text{s}^{-1}$ and then 2.0 min with water at same rate. For routine analysis, the capillary was rinsed for 2.0 min with 0.25 M NaOH and 0.5 min with water at the beginning and ending of the session using pump 2 and 3 of the system in Figure 1. When the instrument was not in use, stock solutions were removed and stored in a refrigerator at 4°C and all tubing and pumps were flushed for 2 min with a solution of 15% MeOH/water (v/v).

pK_a determination by IS-CE method

The procedure for acidity constants determination by IS-CE has been previously reported.⁹ Briefly, the method is based on the use of an IS with pK_{a} similar to that of test compound ($\Delta\text{pK}_{\text{a}} < 1$), choosing as first approximation the pK_{a} predicted for the test compound, ACD/Labs software in our case.²¹ Then, mobilities of the IS and the test compound are measured in two different buffers: a buffer in which the analyte and the IS are completely ionized ($\text{pH} \gg \text{pK}_{\text{a}}$ for an acid or $\text{pH} \ll \text{pK}_{\text{a}}$ for a base); and a second buffer in which both are partially ionized (pH in the range $\text{pK}_{\text{a}} \pm 1$). From these mobility measurements the pK_{a} of the test compound can be directly obtained if the pK_{a} of the IS is accurately known.

For determination of aqueous pK_{a} of insoluble compounds, acidity constants (pK_{a}) at different ratios of hydro-organic mixtures are determined. Then, the pK_{a} values are extrapolated to 0% organic solvent by the Yasuda-Shedlovsky equation.²² This mathematical expression performs a lineal extrapolation between pK_{a} values through the eq 1.

$$\text{pK}_{\text{a}} + \log[\text{H}_2\text{O}] = \frac{a}{\varepsilon} + b \quad (1)$$

In this equation $\log[\text{H}_2\text{O}]$ is the logarithm of the molar water concentration of the given solvent mixture, and ε is the electric permittivity of the binary solvent.²³ From the plot of the pK_{a} of a compound in a given methanol/water mixture vs. the inverse of the electric permittivity of the binary solvent a linear relationship should be obtained. Extrapolation to pure water provides the aqueous pK_{a} of the compound using $\varepsilon = 78.3$ and $\log[\text{H}_2\text{O}] = 55.5$.

RESULTS AND DISCUSSION

The IS-CE method recently developed⁹ has become a powerful and high-throughput tool for pK_{a} determination in drug discovery and development. However, there are some

limitations regarding the previous system. A large number of BGEs are to be prepared *a priori* and stored in the instrument autosampler. This reduces sample capacity decreasing the usefulness of this approach. Moreover, pH instability of aqueous and solvent-water buffers is always a problem. Therefore, it would be ideal to have the electrolyte made on-line according to the requirements for each target.

For the system to be functional, it is necessary to be able to generate on-line buffers with pH ranging from 2-12. This could be achieved in two main ways. The first is to mix several different buffer constituents (for instance, NaHCOO, pH 2.60-4.80; BisTrisHCl, pH 5.50-7.50; TrisHCl, pH 7.00-9.00; NaCAPS pH 9.40-11.60) to obtain the desired pH. However, this approach has some disadvantages: the pH and ionic strength are difficult to control as they all are weak acids and basics and ion-pairs between electrolytes have not been studied. In the second and better approach, stock buffer is selected using a valve and pH is adjusted with strong acid or base, which is instrumentally simpler as it requires 2 fewer pumps. Another 2 pumps can be used to add water to dilute the buffer and ensure constant ionic strength and organic solvent to allow pK_a determinations of water-insoluble compounds. This approach also has the added advantage that both acid, base and water are available on-line for conditioning capillaries if necessary.

BGE considerations

Several different buffers have to be used in order to cover the entire pH range from 2-13. These buffers must be adequate as running buffers in CE and the ionic strength has to be kept constant throughout the series.^{24,25} Therefore, it is important to select the most appropriate of the most common CE BGEs. Buffered electrolyte solution can present specific interactions with the analytes. In a previous study²⁶ we evaluated many buffer solutions used in the determination of acidity constants by CZE observing interactions between some buffers and the analytes which led to inaccurate pK_a determinations. As a result of this work, a set of buffers which showed no deviations due to specific interactions were identified. From this, the following 6 BGE components were identified for use in the IS-CE instrument developed here: HCOOH/HCOO⁻ (pK_a 3.75), CH₃COOH/CH₃COO⁻ (pK_a 4.76), BisTrisH⁺/BisTris (pK_a 6.46), TrisH⁺/Tris (pK_a 8.08), CHES/CHES⁻ (pK_a 9.55) and CAPS/CAPS⁻ (pK_a 10.40).

One of the advantages of the IS-CE method is that accurate external measure of the buffer pH is not needed. Small pH variations due to fast capillary conditioning, electrolysis and CO₂

absorption from the atmosphere do not affect to the pK_a determination since the pH is always measured inside the capillary by the internal standard. What cannot be corrected for is changes in ionic strength and this must be the same for the two buffer solutions in which the mobilities are measured. For this reason, buffers must be prepared at the desired pH at constant ionic strength, the latter with a high degree of accuracy. Therefore, the accuracy and precision of the buffer pumps is of vital importance in order to obtain appropriate mixtures. Milligat pumps were selected on their operational and technical specifications: speed between $0.0005\text{--}167\text{ }\mu\text{L}\cdot\text{s}^{-1}$ giving a volume precision of $< 0.08\%$ dispensing $1250\text{ }\mu\text{L}$ and $< 0.3\%$ for $125\text{ }\mu\text{L}$.

Accuracy of on-line aqueous BGE mixtures

The reliability of buffer pH was evaluated by programming 30 different pH values (from 2.8 to 12.7) with 4–6 pH values from each electrolyte constituent into the on-line buffer generation system. The effluent was collected after the 3D micromixer and the pH measured using a conventional pH-meter. The measured pH ($pH_{(m)}$) values were compared to the calculated ones ($pH_{(calc)}$) as shown in Figure 3. Fitting parameters of the overall data is presented in the following equation:

$$pH_{(m)} = (1.000 \pm 0.004) pH_{(calc)} + (-0.01 \pm 0.03)$$

$$N = 30 \quad SD = 0.08 \quad F = 73377 \quad R^2 = 0.9996 \quad (2)$$

The slope and intercept of the correlation are not significantly different from 1 and 0, respectively, for a 95% confidence level. Therefore, these results demonstrate the good agreement and also consolidate the desired pH with respect to the measured one for all the pH range used.

Regarding the maintenance of a constant ionic strength, pK_a values of 8 internal standard (acids and bases) from the reference list⁹ were determined by the classic CE method using the home-built instrument. In the classic method, the effective mobility (μ_{eff}) of the analyte is measured at pH values within the working range of CE. The plot of the mobility vs. pH gives a sigmoidal curve with an inflection point when $pH = pK'_a$ (acidity constant at working ionic strength). Consequently, this acidity constant is determined by fitting the corresponding parameters through eq 3.

$$\mu_{\text{eff}} = \frac{\mu_{\text{HX}^z} + (\mu_{\text{X}^{z-1}})10^{\text{pH}-\text{pK}'_{\text{a}}}}{1+10^{\text{pH}-\text{pK}'_{\text{a}}}} \quad (3)$$

where μ_{HX^z} and $\mu_{\text{X}^{z-1}}$ are the limiting electrophoretic mobilities of the subscript species. pK'_{a} is related to the thermodynamic pK_{a} by the activity coefficients, calculated by means of the Debye-Hückel equation.¹⁰ Figure 4 shows the experimental mobilities for the selected internal standards. For those determinations, the capillary was previously rinsed for 3 min with the buffer to ensure that it was perfectly conditioned at the desired pH. The curve obtained from the fit is plotted as a line in this figure, and the obtained pK'_{a} values calculated by eq 3 are listed in Table 2, where they are compared to those obtained as a reference pK_{a} values for the IS-CE method. The fitting parameters indicate a perfect correlation between pH and mobility and the biggest difference between the two methods for pK_{a} determination was 0.05. From this it can be concluded that the ionic strength remains constant using the on-line buffer generation system.

Determination of acidity constants by IS-CE

Several acidic and basic compounds with reference acidity constant values were selected as test compounds to evaluate the automated IS-CE system. Some of the reference values were taken from the internal standard list for this method,⁹ and some others were previously determined by the IS-CE method.¹² All of these values have been previously established using commercial Beckman (Palo Alto, CA, USA) and Agilent Technologies (Santa Clara, CA, USA) capillary electrophoresis instruments. Compounds listed in Table 3 show the reference acidity constant at zero ionic strength and 25 °C. This table also shows the IS selected in each determination, the number of determinations, the experimental pK_{a} calculated with its standard deviation, and its difference from the reference value.

Very small differences were obtained between the acidity constants determined by IS-CE using the home-built CE system and commercial CE equipment. All values are within 0.07 from the reference value and precision is excellent with standard deviation of less than 0.05. In some cases such as papaverine, *N,N*-dimethyl-*N*-phenylamine, ibuprofen and terfenadine, the pK_{a} were determined using two different internal standards with good agreement.

BGE methanol-water mixtures

In order to determine acidity constants of compounds sparingly soluble in water, the performance of the instrument when using methanol-water mixtures as solvent in the BGE was tested. It is well-known that small proportions of organic solvent in water change the aqueous acidity constant since it affects to the relative permittivity and hydrogen-bond donor capacity. Therefore, hydro-organic buffers can be tested by the respective shifts of acidity constant at different proportion of solvent. The pK_a of ibuprofen was determined using benzoic acid as the internal standard at increasing percentages from 2% MeOH/H₂O (v/v) to 40%. At each level of MeOH mobilities were measured at two pH values as for the completely aqueous system. Figure 5 shows the electropherograms from 2% to 40% of MeOH at pH where ibuprofen and benzoic acid are completely ionized. This figure demonstrates that migration time increases proportionally with methanol ratio. As voltage and ionic strength were constant during each run, these changes can only be attributed to the decreasing conductivity as well as changes in the pH scale and the acidity constants of buffers due to the increasing percentage of MeOH. Consequently, this causes mobility shift and non-linear pK_a variations of ibuprofen with respect to the % MeOH (Figure 6). In a previous work,¹² we established the reference acidity constants of all the IS set at 10, 20, 30 and 40% MeOH-H₂O, including ibuprofen. In order to demonstrate the good accuracy of the experimental pK_a values, Figure 6b also includes the experimental values at 10, 20, 30 and 40% MeOH-H₂O determined by a commercial CE instrument. The overall results and Figures 5 and 6 demonstrate the good mixing of methanol-water buffers as well as the ability to combine in a few seconds different parameters to create a desired pH and organic mixture buffer.

The addition of MeOH allows pK_a values to be determined for mefenamic acid and terfenadine, which are unable to be determined using 100% aqueous buffers because they are insoluble. For these compounds aqueous pK_a values were extrapolated by Yasuda-Shedlovsky approximation. To validate this approach, ibuprofen was also determined using completely aqueous BGE and also MeOH/H₂O mixtures (from 2% to 40%), obtaining average experimental values of 4.48 and 4.45, respectively. In case of mefenamic acid and terfenadine, comparing the acidity constants determined by this prototype and the commercial CE similar values were obtained. Summarizing, the low standard deviation of the replicates and the low variation respect to the reference values show how the system developed in this work can perfectly determine acidity constants of drug by the use of the IS-CE method.

Throughput of the home-built CE system

Using the home-built automated IS-CE instrument, faster separations were obtained when compared to commercial available instruments from Beckman or Agilent technologies.^{9,12,27} This is primarily due to the much shorter capillaries that can be used (L_D 15 cm) and high voltages (30 kV). With lower internal diameter (25 μ m) and good refrigeration, the detrimental heat due to Joule effect was avoided. Closing the control solenoid and varying the BGE pump speed allows different pressures to be generated to provide faster separations, to avoid current fluctuations and to constantly fill the capillary with fresh buffer. All these conditions allow electropherograms to be obtained within 20 to 60 s, with the MeOH/H₂O mixtures being slightly longer due to the increase in viscosity. Figure 5 shows an example for two acidic compounds in different methanol mixtures.

The on-line buffer generator is the biggest advantage of this IS-CE instrument compared to commercial ones, and is the first to feature on-line electrolyte generation. The mixing system avoids instability, environmental and human errors since it prepares a new buffer each time, consuming low quantities of stock solutions. The software only requires a pH and percentage of methanol to be inserted to fill the capillary with fresh solution within a few seconds.

Using the IS-CE method with this CE system increased the throughput for acidity constant determination. For a single compound just two minutes are needed to determine its acidity constant and for a set of 20 just half an hour. 20 pK_a at 20 different MeOH/H₂O mixtures were determined for ibuprofen in half an hour with no preparation of the buffers beforehand, making this a simple and quick approach to the determination of pK_a values in a range of experimental conditions.

CONCLUSION

A simple automated CE system has been designed and constructed for the implementation of high-throughput IS-CE to determine acidity constants of any bioactive compound. The instrument has been built from commercially available parts with a 3D micromixer designed and printed to combine four different flows into one single homogeneous flow. Good reliability of pH and ionic strength of buffers were obtained from pH 2.8 to 12.7 in aqueous media and several methanol-water mixtures (until 40%). With short capillaries of just 15 cm to the detector, high voltages (30 kV) and applying pressure during the separation, separations of 20 s were obtained, and 2 min required for a pK_a determination. Furthermore,

the ability to determine pK_a using MeOH at 20 different concentrations could be performed in 30 min, allowing the calculation of acidity constants of water-insoluble compounds. The low standard deviation of the replicates and the low variation compared to the reference values show this system can determine acidity constants of drugs by the use of the IS-CE method in a fast and reliable way.

AUTHOR INFORMATION

Corresponding Author

Prof. Michael Breadmore

Australia Centre of Research on Separation Sciences

University of Tasmania

Phone: +61-3-6226-2154

Fax: +61-3-6226-2858

e-mail: mcb@utas.edu.au

Notes:

Authors declare no competing financial interest.

ABBREVIATIONS

CE: capillary electrophoresis; IS: internal standard; IS-CE: internal standard capillary electrophoresis; BGE: background electrolyte.

ACKNOWLEDGMENT

The authors thank the Ministerio de Ciencia e Innovación of the Spanish Government and the Fondo Europeo de Desarrollo Regional (FEDER) of the European Union (Project CTQ2010-19217/BQU) for financial support. Furthermore, J.M. Cabot thanks the Secretaria d'Universitats i Recerca of the Departament d'Economia i Coneixement of the Catalan Government for a supporting scholarship (2013FI_B1 00209). M.C. Breadmore would like to thank the Australian Research Council for a Future Fellowship (FT130100101).

REFERENCES

- (1) Comer, W. T. In *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, **2013**.
- (2) Ganellin, R.; Roberts, S.; Jefferis, R.; Stocks, M. In *Introduction to Biological and Small Molecule Drug Research and Development*, **2013**, 81–126.
- (3) Babić, S.; Horvat, A. J. M.; Mutavdžić Pavlović, D.; Kaštelan-Macan, M. *Trends in Analytical Chemistry* **2007**, 26, 1043–1061.
- (4) Wan, H.; Ulander, J. *Expert Opinion on Drug Metabolism and Toxicology* **2006**, 2, 139–155.
- (5) Kerns, E. *Drug Discovery Today* **2003**, 8, 316–323.
- (6) Reijenga, J.; van Arno, H.; van Antonie, L.; Teunissen, B. *Analytical Chemistry Insights* **2013**, 8, 53–71.
- (7) Taylor, J. B.; Trigg, D. J.; Comer, J. E. A. In *Comprehensive Medicinal Chemistry II*; 2007; pp. 357–397.
- (8) Nowak, P.; Woźniakiewicz, M.; Kościelniak, P. *Journal of chromatography. A* **2015**, 1377, 1–12.
- (9) Cabot, J. M.; Fuguet, E.; Rosés, M. *ACS Combinatorial Science* **2014**, 16, 518–525.
- (10) Cabot, J. M.; Fuguet, E.; Ràfols, C.; Rosés, M. *Journal of Chromatography A* **2013**, 1279, 108–116.
- (11) Cabot, J. M.; Fuguet, E.; Rosés, M. *Electrophoresis* **2014**, 35, 3564–3569.
- (12) Cabot, J. M.; Fuguet, E.; Rosés, M. *Journal of Chromatography A* **2015**. Manuscript submitted.
- (13) Wuersig, A.; Kubán, P.; Khaloo, S. S.; Hauser, P. C. *The Analyst* **2006**, 131, 944–949.
- (14) Kulka, S.; Quintás, G.; Lendl, B. *The Analyst* **2006**, 131, 739–744.
- (15) Blanco, G. a; Nai, Y. H.; Hilder, E. F.; Shellie, R. a; Dicinoski, G. W.; Haddad, P. R.; Breadmore, M. C. *Analytical chemistry* **2011**, 83, 9068–9075.

- (16) Gaudry, A. J.; Guijt, R. M.; Macka, M.; Hutchinson, J. P.; Johns, C.; Hilder, E. F.; Dicinoski, G. W.; Nesterenko, P. N.; Haddad, P. R.; Breadmore, M. C. *Analytica chimica acta* **2013**, *781*, 80–87.
- (17) Gaudry, A. J.; Nai, Y. H.; Guijt, R. M.; Breadmore, M. C. *Analytical chemistry* **2014**, *86*, 3380–3388.
- (18) Alhusban, A. a; Gaudry, A. J.; Breadmore, M. C.; Gueven, N.; Guijt, R. M. *Journal of chromatography. A* **2014**, *1323*, 157–162.
- (19) Carrière, P. *Physics of Fluids* **2007**, *19*, 118110.
- (20) Shallan, A. I.; Smejkal, P.; Corban, M.; Guijt, R. M.; Breadmore, M. C. *Analytical chemistry* **2014**, *86*, 3124–3130.
- (21) ACD/Percepta Platform v2012 Software, 2012.
- (22) Avdeef, A.; Box, K. J.; Comer, J. E.; Gilges, M.; Hadley, M.; Hibbert, C.; Patterson, W.; Tam, K. Y. *Journal of pharmaceutical and biomedical analysis* **1999**, *20*, 631–641.
- (23) Avdeef, A.; Comer, J. E. A.; Thomson, S. J. *Analytical Chemistry* **1993**, *65*, 42–49.
- (24) Poole, S. K.; Patel, S.; Dehring, K.; Workman, H.; Poole, C. F. *Journal of Chromatography A* **2004**, *1037*, 445–454.
- (25) Baker, R. B. *Capillary Electrophoresis*; Wiley-Interscience Publication: New York, **1995**.
- (26) Fuguet, E.; Reta, M.; Gibert, C.; Rosés, M.; Bosch, E.; Ràfols, C. *Electrophoresis* **2008**, *29*, 2841–2851.
- (27) Fuguet, E.; Ràfols, C.; Bosch, E.; Rosés, M. *Journal of Chromatography A* **2009**, *1216*, 3646–3651.

FIGURES

Figure 1. Schematic diagram of the IS-CE system.

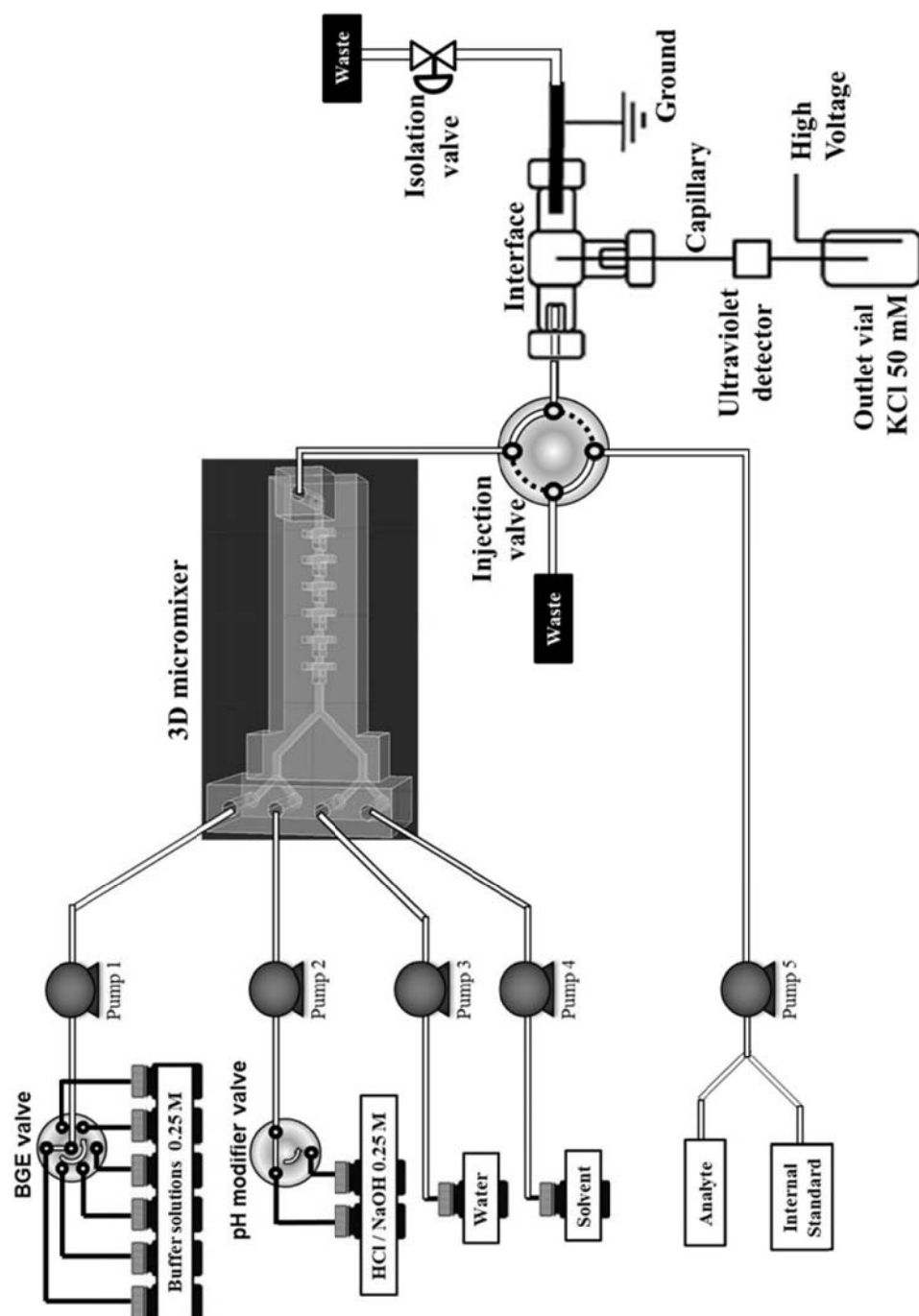


Figure 2. Photograph of the automated Internal Standard Capillary Electrophoresis system.

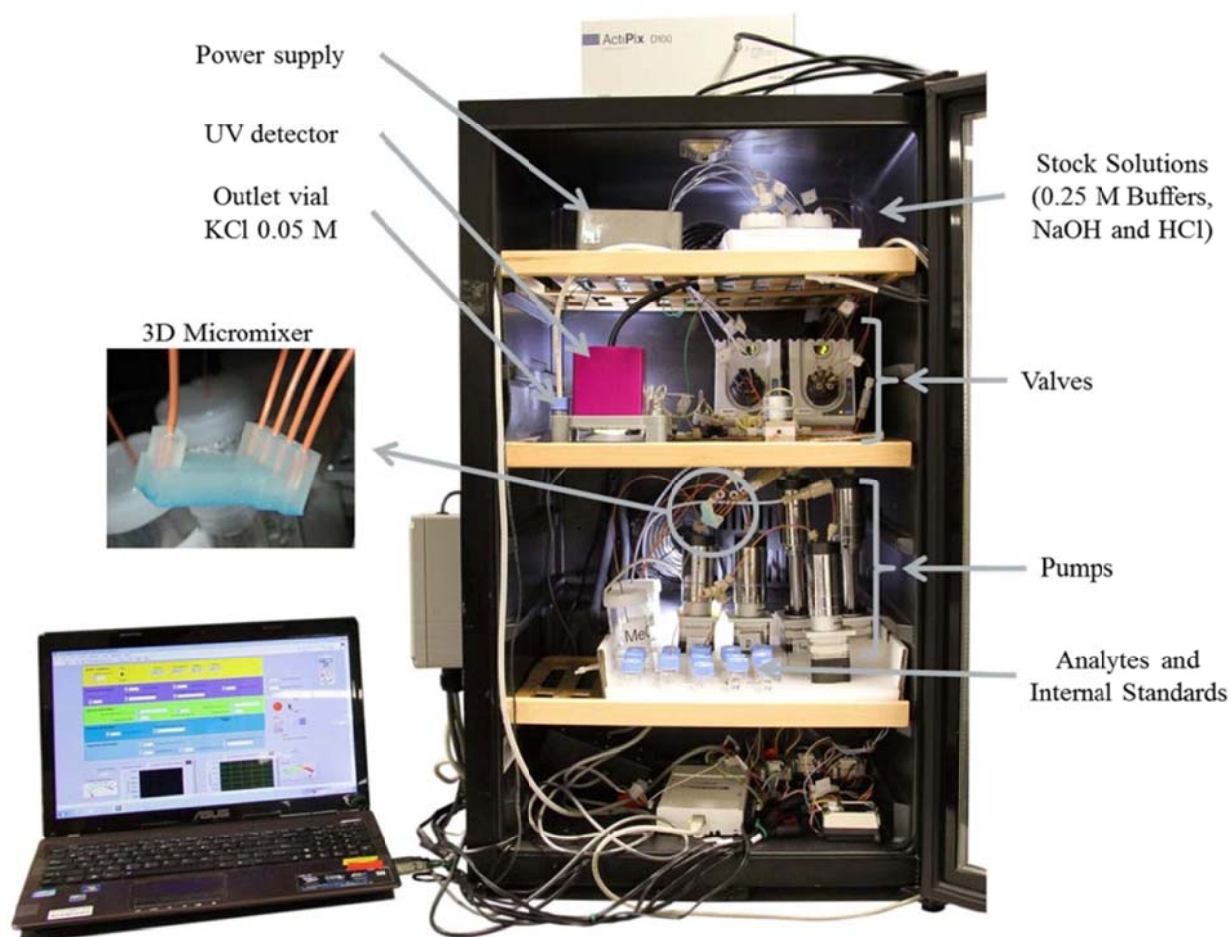


Figure 3. pH of the BGE calculated ($\text{pH}_{(\text{calc})}$) as a function of the measured pH after the 3D micromixer using conventional pH-meter ($\text{pH}_{(\text{m})}$)

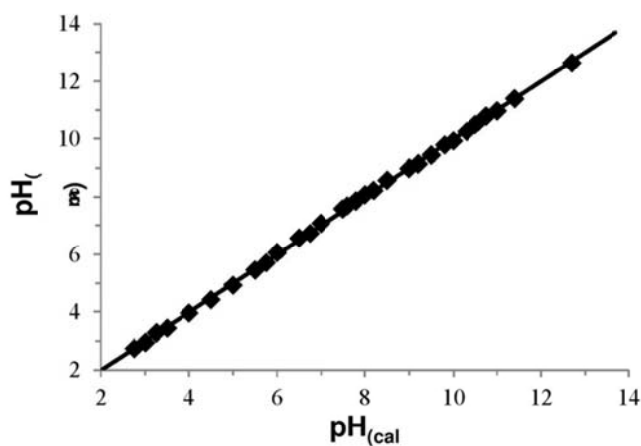


Figure 4. Electrophoretic mobilities ($\times 10^4 \text{ cm}^2 \text{ V}^{-1} \text{ min}^{-1}$) of basic (empty dots) and acidic (full dots) internal standards vs. pH. Dots are experimental mobilities and fitted lines are obtained by eq 3. ($\square$;.....) papaverine; ($\circ$;---) lidocaine; (Δ ;.....) diphenhydramine; ($\diamond$;---) nortryptiline; ($\blacksquare$;.....) benzoic acid; ($\bullet$;---) nicotinic acid; ($\blacktriangle$;.....) methylparaben; ($\blacklozenge$;---) 4-bromophenol. Electrophoretic conditions: 50 mM ionic strength, normal polarity at 30 kV, 25 °C.

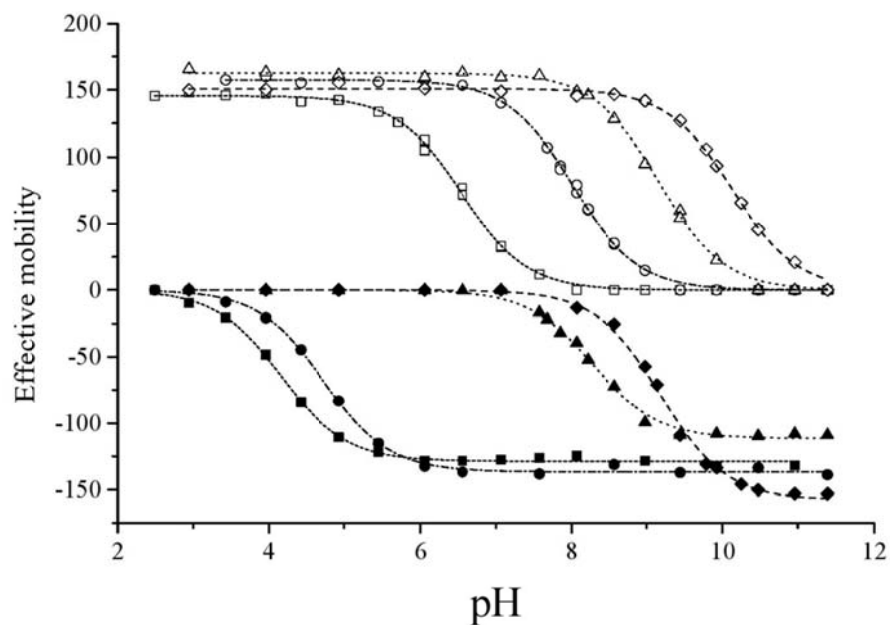


Figure 5. Electropherograms from 2 % to 40 % of MeOH (v/v) of ibuprofen (AN, analyte) and benzoic acid (IS, internal standard) using DMSO as electroosmotic flow marker. Conditions: ionic strength of 0.05 (CHES/CHES⁻), -30 kV, BGE flow rate 0.3 $\mu\text{L}\cdot\text{s}^{-1}$ during separation (isolation valve closed).

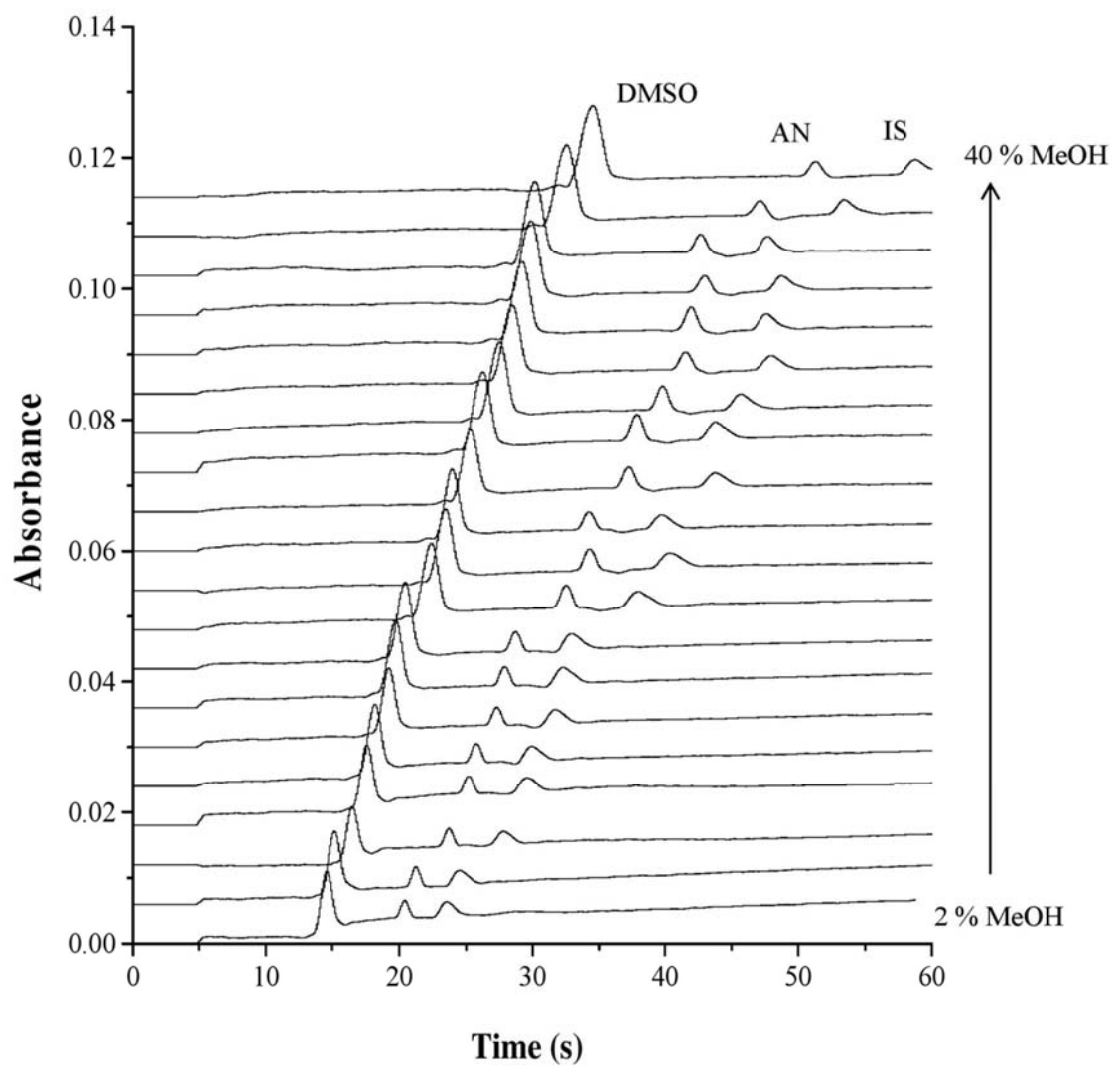
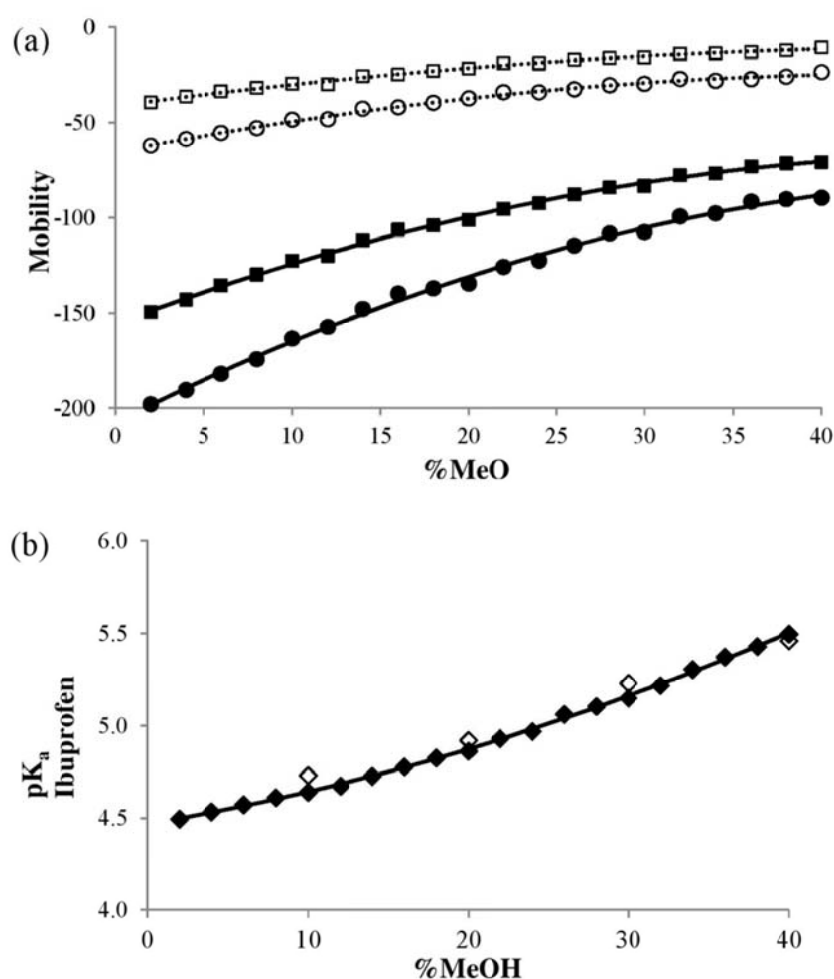


Figure 6. Acidity constants (pK_a) at several MeOH/H₂O mixtures (from 2% to 40%). (a) Mobility ($\times 10^4 \text{ cm}^2 \text{ V}^{-1} \text{ min}^{-1}$) versus % MeOH (v/v) of ibuprofen ($\square$; $\blacksquare$) and benzoic acid ($\circ$; $\bullet$) at pH values where they are partially and totally ionized. Fitted solid lines show the mobility at pH when they both are completely ionized, and fitted dashed lines when they are partially ionized. (b) pK_a of ibuprofen at 0 ionic strength and 25 °C as a function of % MeOH (v/v). Solid line is the fit to a quadratic polynomial equation ($R^2 = 0.9992$) and empty symbols ($\diamond$) are the reference pK_a of ibuprofen at 10, 20, 30 and 40% of MeOH-H₂O determined by commercial CE equipment.¹²



TABLES

Table 1. Sequence operation for the IS-CE system.

Setup operation	Injection valve position	Isolation Valve	BGE Total Flow-rate ($\mu\text{L}\cdot\text{s}^{-1}$)	Sample Flow-rate ($\mu\text{L}\cdot\text{s}^{-1}$)	Time (s)	Volume dispensed (μL)
1 [*] BGE system cleaning	1	Open	30	-	5	150.0
2 [*] BGE capillary cleaning	1	Close	1	-	20	30.0
3 Sample interface cleaning	2	Open	-	30	10	300.0
4 Equilibrate	2	Open	-	0.5	2	1.0
5 Sample injection	2	Close	-	0.5	5	2.5
6 BGE interface cleaning	1	Open	30	-	5	150.0
7 Separation: Bases	1	Close	0.02	-	60	1.2
Acids	1	Close	0.30	-	60	18

* Steps 1 and 2 were operated when buffer pH was changed.

Table 2. Comparison of experimental pK_a values at 25 °C and zero ionic strength, obtained through the IS-CE pK_a reference list⁹ and the classic capillary electrophoresis method (statistics of the fittings to eq 3 are also shown).

Compound	IS-CE method		Classic method			
	pK_a		$pK_a \pm s$	R^2	SD	F
Base						
Papaverine	6.41		6.46 ± 0.01	0.9989	4.39	23151
Lidocaine	7.93		7.93 ± 0.01	0.9990	4.02	31428
Diphenhydramine	9.08		9.07 ± 0.02	0.9982	8.32	15809
Nortryptiline	10.08		10.05 ± 0.02	0.9976	8.18	13442
Acid						
Benzoic acid	4.22		4.22 ± 0.02	0.9980	4.85	17430
Nicotinic acid	4.85		4.82 ± 0.02	0.9984	5.66	13366
Methylparaben	8.35		8.37 ± 0.03	0.9959	9.15	4380
4-Bromophenol	9.28		9.27 ± 0.02	0.9973	11.54	6346

Table 3. Thermodynamic acidity constants determined by the Internal Standard Capillary Electrophoresis system in aqueous media. Comparison between experimentally determined values ($pK_{a(\text{exp})}$) and the reference ones ($pK_{a(\text{ref})}$). $pK_{a(\text{ref})}$ values were established using commercial instruments. N: number of determinations.

Analyte	$pK_{a(\text{ref})}$	IS	N	$pK_{a(\text{exp})} \pm s$	$(pK_{a(\text{ref})} - pK_{a(\text{exp})})$
Aqueous Buffers					
Papaverine	6.41 ^a	4- <i>tert</i> -butylpyridine	8	6.43 $\pm$ 0.04	-0.02
		2,4-Lutidine	3	6.43 $\pm$ 0.02	-0.02
Clonidine	8.10 ^a	Lidocaine	12	8.07 $\pm$ 0.04	0.03
<i>N,N</i> -dimethyl- <i>N</i> -phenylamine	8.95 ^a	Diphenhydramine	12	8.97 $\pm$ 0.05	-0.02
		Propranolol	7	9.02 $\pm$ 0.05	-0.07
Diphenhydramine	9.08 ^a	<i>N,N</i> -dimethyl- <i>N</i> -phenylamine	13	9.06 $\pm$ 0.05	0.02
Ibuprofen	4.49 ^a	Benzoic acid	5	4.47 $\pm$ 0.04	0.02
		Nicotinic acid	5	4.48 $\pm$ 0.06	0.01
Phenol	9.89 ^a	4-Bromophenol	3	9.92 $\pm$ 0.03	-0.03
		Paracetamol	3	9.89 $\pm$ 0.02	0.00
MeOH/H₂O Buffers					
Ibuprofen	4.49 ^a	Benzoic acid	20	4.45 $\pm$ 0.06	0.04
Mefenamic acid	4.17 ^b	Benzoic acid	4	4.11 $\pm$ 0.03	0.06
Terfenadine	9.19 ^b	<i>N,N</i> -dimethyl- <i>N</i> -phenylamine	4	9.26 $\pm$ 0.04	-0.07
		Diphenhydramine	4	9.22 $\pm$ 0.03	-0.03

^a Values taken from reference.⁹

^b Values taken from reference.¹²

Capítol III

Resultats i Discussió

El mètode anomenat IS-CE va ser desenvolupat recentment per a la determinació de constants d'acidesa de compostos monopròtics simples [75]. No obstant això, cal desenvolupar completament aquest mètode per tal de proporcionar una metodologia d'alt rendiment, robusta, simple, fiable i apta per a la determinació de la constant d'acidesa de qualsevol tipus de substància bioactiva.

Per a desenvolupar aquest mètode cal una llista de compostos de diferent estructura i naturalesa química amb valors de pK_a de referència per tal de facilitar el procés de selecció dels patrons interns. Així mateix, cal adaptar el mètode IS-CE per a la determinació de constants d'acidesa de compostos amb més d'una constants d'acidesa i posteriorment optimitzar-lo per tal de convertir-lo en un mètode *high throughput*,

D'altre banda, cal avaluar la capacitat del mètode IS-CE per a la determinació de compostos de baixa solubilitat i fer servir medis hidro-orgànics per a aquells amb solubilitats més baixes.

Per últim, cal dissenyar un nou prototip perfectament automatitzat i específic per aquest mètode, per així poder-lo implementar per a determinacions rutinàries en laboratoris de mesures fisicoquímiques i de *drug discovery and development*.

3.1. ESTABLIMENT D'UNA LLISTA DE PATRONS INTERNS DE REFERÈNCIA

La selecció d'un estàndard apropiat amb un valor de pK_a conegut i ben determinat és de vital importància per a l'aplicació d'aquest mètode, degut a que l'exactitud en la determinació de la constant d'acidesa de l'anàlit dependrà en bona part de la certesa dels valors de pK_a dels estàndard i de la similitud entre els valors de pK_a d'ambdós. Així doncs, per tal de facilitar el procés de selecció de patrons interns adequats, s'estableix el pK_a d'un llistat de patrons interns àcids i bàsics de diferent estructura que cobreix tot l'interval útil en electroforesi capil·lar.

Per tal de proporcionar una llista de patrons interns àcids, se seleccionaren diversos compostos d'aquesta naturalesa com a possibles candidats i s'establiren els valors pK_a de referència. En poques paraules, aquest procediment consisteix en un procés iteratiu en què es determina el pK_a d'un compost de la llista utilitzant els compostos veïns (amb una diferència inferior a ± 1) com a IS. Per iniciar el procés s'utilitzen valors de pK_a bibliogràfics o estimats, i un cop obtinguts uns nous valors de pK_a per a cada compost, aquests s'utilitzen novament en una nova iteració, juntament amb les dades de mobilitat. Aquest procediment es realitza per a cada compost del conjunt i es recalculen les constants fins a obtenir valors de pK_a concordants entre els valors de pK_a consecutius ($\Delta pK_a \leq 0.01$). Un exemple amb una explicació més detallada es pot observar a l'article I, juntament amb la Taula 3. A la Taula 2 del mateix article es mostren els compostos escollits i les seves constant d'acidesa a 25°C i a força iònica zero. La bona concordança dels valors trobats amb els valors de la literatura demostra com aquesta llista proveeix una sèrie de patrons interns àcids amb valors de pK_a de referència útil per a determinar pK_a d'anàlits a través del mètode IS-CE.

Per a una validació més acurada dels valors de pK_a determinats per als patrons interns, es va determinar el pK_a d'alguns dels compostos a partir del mètode clàssic. Tal com explica la secció 1.3.2 de la introducció, el mètode clàssic relaciona la mobilitat de l'anàlit amb el pH en un interval aproximat de $pK_a \pm 4$. Subseqüentment, les dades experimentals s'ajusten a l'equació no lineal que relaciona la mobilitat efectiva amb el pH, equació (1.13), i es troben els paràmetres de pK'_a i μ_{A^-} . El pK_a termodinàmic es determina corregint per la força iònica seguint les equacions (1.5) i (1.7).

Particularment, ens les determinacions del paracetamol i del fenol es van veure anomalies al final de la corba. La Figura 3.1 mostra com els punts obtinguts pel tampó de NaOH presenten un desplaçament de la mobilitat cap a valors més negatius que els punts determinats amb els altres tampons.

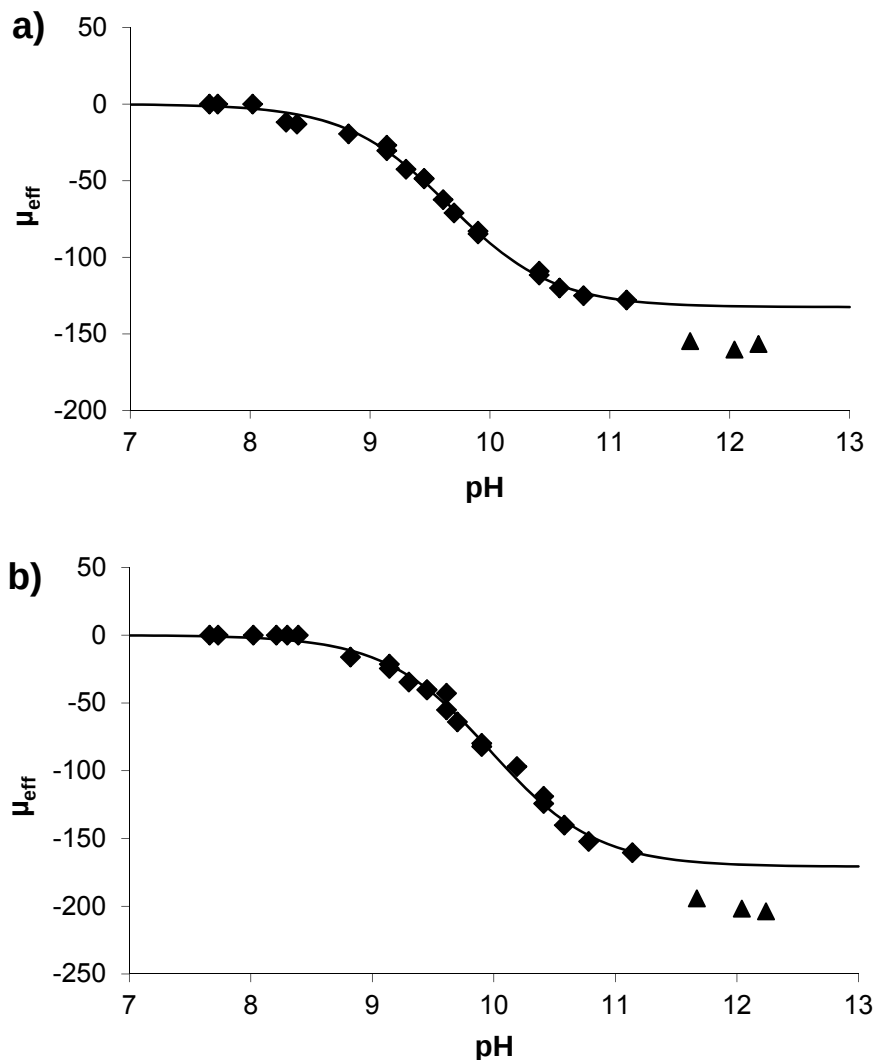


Figura 3.1. Gràfic de la mobilitat $\times 10^4 \text{ (cm}^2 \text{ s}^{-1} \text{ V}^{-1}\text{)}$ en funció del pH pel (a) paracetamol i pel (b) fenol. Els tampons utilitzats són: (♦) TrisH⁺/Tris, CHES/CHES⁻, CAPS/CAPS⁻; (▲) tampó NaOH; (—) Corba obtinguda a l'ajustar els punts a l'equació (1.13), sense tenir en compte el tampó NaOH.

Aquests resultats permeten concloure que el tampó de NaOH pot ser considerat incorrecte per a la determinació de pK_a pel mètode clàssic. Ajustant les mateixes dades sense tenir en compte els punts realitzats amb el tampó de NaOH, es va observar com els valors determinats pel mètode clàssic no presenten diferències significatives respecte dels del mètode IS-CE. Aquest fet és degut a que l'augment de

la mobilitat que produeix el NaOH en l'anàlit, es veu compensat amb el produït per al patró intern, obtenint llavors el mateix valor de pK_a . Tot això prova que, a diferència del mètode clàssic, la utilització del mètode IS-CE pot fer front als canvis de mobilitat causats per efectes inesperats i interaccions que afecten en un grau similar a l'anàlit i patró intern.

De la suma d'aquests patrons interns àcids i de la llista de patrons bàsics confeccionada i presentada paral·lelament per E. Fuguet i col·laboradors [77], es pot crear una llista uniformada d'IS àcids i bàsics. D'aquesta forma, s'estableix un conjunt de patrons interns de diferent naturalesa àcid-base i estructura amb valors de pK_a de referència que cobreixen tot l'interval de pH útil en electroforesi capil·lar. La llista dels patrons amb els valors de pK_a corresponents es troba a la Taula 3.1.

Partint d'aquesta llista, els analistes tenen a disposició un ampli conjunt de fins a 48 compostos amb valors de pK_a consolidats per escollir de forma ràpida i simple el millor patró intern per a una determinació donada.

Taula 3.1. Conjunt de patrons interns per a la determinació de constants d'acidesa pel mètode IS-CE. Constants d'acidesa termodinàmiques a 25°C. (A): patró intern àcid. (B): patró intern bàsic.

IS	pK _a	IS	pK _a
Àcid 2-clorobenzoic	2.84 (A)	4-Nitrofenol	7.09 (A)
2,6-Dibromo-4-nitrofenol	3.31 (A)	Vanil·lina	7.36 (A)
Àcid 4-nitrobenzoic	3.37 (A)	2,4,6-Trimetilpiridina	7.51 (B)
2,6-Dinitrofenol	3.69 (A)	Fenobarbital	7.53 (A)
Àcid 3-bromobenzoic	3.79 (A)	4-Hidroxibenzaldehid	7.61 (A)
2,4-Dinitrofenol	4.12 (A)	Lidocaïna	7.93 (B)
Àcid benzoic	4.22 (A)	Clonidina	8.10 (B)
Ibuprofè	4.49 (A)	3,5-Diclorofenol	8.18 (A)
Anilina	4.63 (B)	Bupivacaïna	8.19 (B)
Àcid nicotínic	4.85 (A)	Metilparabè	8.35 (A)
Quinolina	4.93 (B)	2-Clorofenol	8.50 (A)
4-terc-Butilnilina	4.93 (B)	1-Fenilpiperazina	8.75 (B)
Warfarina	5.17 (A)	N,N-Dimetil-N-benzilamina	8.95 (B)
N,N-Dimetil-N-fenilamina	5.17 (B)	3-Clorofenol	9.04 (A)
Piridina	5.28 (B)	Difenhidramina	9.08 (B)
2,5-Dinitrofenol	5.30 (A)	Procaïnamida	9.26 (B)
Sulfacetamida	5.42 (A)	4-Bromofenol	9.28 (A)
Acridina	5.55 (B)	Imipramina	9.37 (B)
4-terc-Butilpiridina	6.03 (B)	Propranolol	9.47 (B)
2,4,6-tribromofenol	6.04 (A)	1-Aminoetilbenzenè	9.52 (B)
Papaverina	6.41 (B)	Paracetamol	9.58 (A)
2,4-Lutidina	6.81 (B)	Efedrina	9.72 (B)
Trazodona	6.84 (B)	Fenol	9.89 (A)
Pilocarpina	7.08 (B)	Nortriptilina	10.08 (B)

3.2. MÈTODE IS-CE PER A COMPOSTOS POLIPRÒTICS

En l'equilibri àcid-base general per a una substància polipròtica la mobilitat electroforètica pot expressar-se segons l'equació (1.15), anomenada (3.1) en aquest Capítol:

$$\mu_{eff} = \frac{\mu_{H_n A^z} + \sum_{i=1}^n 10^{ipH - \sum_{j=1}^i pK'_{aj}} \mu_{H_{n-i} A^{z-i}}}{1 + \sum_{i=1}^n 10^{ipH - \sum_{j=1}^i pK'_{aj}}} \quad (3.1)$$

Les mobilitats de les diferents espècies es poden calcular a través dels temps de migració mitjançant l'equació (1.16) i la constant termodinàmica a partir dels coeficients d'activitat seguint l'equació (1.10).

Seguint el mateix procediment que es feia servir per a substàncies monopròtiques, a cada constant d'acidesa que tingui l'anàlit polipròtic se li assigna un patró intern monopròtic de la Taula 3.1 amb un valor de pK_a similar. Posteriorment s'injecten cadascuna de les mescles d'AN i IS a dos valors de pH diferents: un proper al valor de pK_a d'ambdós; i un altre on s'obtingui la mobilitat límit de l'AN i IS, en altres paraules, a un $pH = pK_a \pm 2$, segons si es tracta d'un àcid o d'una base.

A diferència de les substàncies monopròtiques, les mobilitats límit i efectives dels patrons interns s'utilitzen per a calcular el pH dins del capil·lar. Així doncs, si s'aïlla el pH de l'equació (1.19) i es corregeix la força iònica utilitzant l'equació (1.5) s'obté, per a un IS de naturalesa àcida, que el pH es calcula a partir de l'equació (3.2):

$$pH = pK_{a(IS)} - \log \frac{\mu_{IS^-} - \mu_{eff(IS)}}{\mu_{eff(IS)}} + \log \gamma_{IS^-} \quad (3.2)$$

I per a un IS bàsic, s'utilitza l'equació (1.24) i es corregeix per la força iònica mitjançant l'equació (1.6) per a obtenir l'equació (3.3).

$$pH = pK_{a(IS)} + \log \frac{\mu_{IS^+} - \mu_{eff(IS)}}{\mu_{eff(IS)}} - \log \gamma_{IS^+} \quad (3.3)$$

Aquest simple càlcul del pH es pot fer a partir de la mobilitat límit (μ_{IS^-} o μ_{IS^+}) i la mobilitat efectiva ($\mu_{eff(IS)}$) mesurades als dos valors de pH corresponents, i del $pK_{a(IS)}$ present a la llista de patrons interns de la Taula 3.1.

Com que la mobilitat efectiva de l'anàlit, $\mu_{eff(AN)}$, es mesura conjuntament amb la mobilitat efectiva del patró intern, $\mu_{eff(IS)}$, el pH calculat per al patró intern correspon al pH on també es mesura la $\mu_{eff(AN)}$. D'aquesta manera s'obtenen una sèrie de valors de $\mu_{eff(AN)}$ en funció del pH, i fent un ajust no lineal d'aquests parells de valors a l'equació (3.1) es poden trobar els valors de pK'_{ai} .

Per a l'estudi del mètode IS-CE en substàncies polipròtiques es seleccionaren un total de 20 fàrmacs, des de compostos simples com aminofenols fins a substàncies com cefalosporines o derivats de les tetraciclins amb 3 constants d'acidesa. Els compostos es van escollir en funció de les seves característiques àcid-base i de la importància farmacològica, així com també per la seva dificultat en la determinació de les constants d'acidesa. La llista de compostos es troba a la Taula 3.2 i les estructures d'aquestes a la Figura 1 de l'article II.

Un cas interessant per comprovar la robustesa del mètode és el dels compostos amb valors de pK_a molt alts o molt baixos. En comparació amb els casos anteriors apareix una dificultat, ja que no és possible mesurar directament les mobilitats límit. En aquests casos, s'ha d'utilitzar un IS de naturalesa diferent, és a dir, per a un pK_a molt àcid un IS de naturalesa bàsica i per a un pK_a molt bàsic un IS de naturalesa àcida.

El mètode IS-CE també és aplicable a compostos amb valors de pK_a propers entre ells. El procediment a seguir és el mateix que en els exemples anteriors. No obstant això, es necessiten menys electroferogrames ja que tant els patrons com el compost d'assaig s'injecten tots junts en la mateixa anàlisi per a una determinació simultània de dos punts d'ajust (un amb cada patró).

Els resultats obtinguts de l'aplicació del mètode d'IS-CE als 20 compostos polipròtics estudiats es presenten a la Taula 3.2 juntament amb els valors trobats a la literatura. Les referències bibliogràfiques es troben resumides a la Taula 8 de l'article II. En general, les desviacions estàndard associades als valors de pK_a obtinguts són inferiors a 0.1 unitats de pK_a , sent per a la majoria dels valors 0.05 o menys.

Taula 3.2. Constants d'acidesa termodinàmiques a 25°C determinades a partir del mètode IS-CE ($pK_{a(\text{exp})}$), i trobades a la literatura ($pK_{a(\text{lit})}$).

Compost	$pK_{a(\text{exp})}$	S	$pK_{a(\text{lit})}$
Furosemida	3.83	0.05	3.78; 3.87; 3.64; 3.76; 3.60
	10.25	0.07	10.62; 10.75; 10.6; 10.32; 10.35
Àcid ftàlic	3.02	0.05	3.06; 2.73; 2.94; 2.95
	5.38	0.05	5.38; 4.78; 5.43; 5.41
Àcid 4-hidroxibenzoic	4.65	0.02	4.45; 4.57
	9.45	0.04	9.32; 9.46
Acetazolamida	7.55	0.03	7.52; 7.78
	9.27	0.04	9.41; 9.57
Pirilamina	4.01	0.03	4.14; 4.02
	8.99	0.02	9.12; 8.92
Histamina	5.67	0.04	5.80; 5.89
	9.91	0.04	9.96; 9.85
Clorfeniramina	3.64	0.04	3.86
	9.27	0.03	9.13; 9.18
Quinina	4.11	0.07	3.95; 3.92; 3.95; 4.29; 4.14
	8.43	0.04	8.46; 8.43; 8.60; 8.53; 8.39
Levodopa	2.16	0.18	2.31; 2.30
	9.47	0.04	9.74; 9.70
Histidina	5.99	0.01	5.96; 5.94; 5.67
	9.32	0.02	9.27; 9.31; 8.99
Fenilalanina	2.20	0.09	2.16; 3.23; 2.23
	9.35	0.02	9.01; 9.39; 9.32
2-Aminofenol	4.71	0.01	4.63; 4.78; 4.72
	9.80	0.01	9.42; 9.97; 9.71
Piridoxina	4.89	0.01	4.67; 4.87; 4.77; 4.87
	9.01	0.02	9.02; 9.11; 9.04; 8.99
Labetalol	7.27	0.03	7.28; 7.36; 7.35
	9.69	0.04	9.49; 9.40; 9.16
Sotalol	8.36	0.05	8.38; 8.28
	9.92	0.05	9.47; 9.75
Ampicil·lina	2.76	0.07	2.50; 2.41; 2.42
	7.48	0.02	7.04; 6.94; 7.30
Cefalexina	2.68	0.13	2.40; 2.61; 2.53; 2.93
	7.17	0.03	7.27; 7.08; 7.13; 7.18
Cefadroxil	2.72	0.04	2.55; 2.52; 2.48
	7.67	0.02	7.21; 7.65; 7.37
	10.02	0.04	9.71; 9.64
Clortetraciclina	3.28	0.02	3.33; 3.30; 3.3
	7.19	0.04	7.55; 7.44; 7.4
	8.86	0.04	9.33; 9.27; 9.3
Oxitetraciclina	3.28	0.02	3.22; 3.10; 3.04; 3.27; 3.3
	7.48	0.05	7.46; 7.35; 8.00; 7.32; 7.3
	8.88	0.06	8.94; 9.20; 9.11; 9.1

Quan es comparen els resultats obtinguts pel mètode IS-CE amb els de la literatura, es pot observar que per a la majoria de compostos existeix una diferència < 0.1 unitats. Per a compostos tals com clorfeniramina, labetalol, sotalol, ampicil·lina, cefadroxil, clortetraciclina o levodopa hi ha diferències al voltant de 0.2-0.3 unitats de pK_a per algunes de les seves constants d'acidesa. No obstant això, la informació bibliogràfica d'aquests compostos és escassa i dispersa, de manera que l'estimació real de l'exactitud d'aquests valors bibliogràfics no és possible. La Figura 7 de l'article II il·lustra la bona correlació entre els valors experimental i bibliogràfics, demostrant novament que el mètode IS-CE pot aplicar-se per a compostos amb més d'una constant, independentment de la seva complexitat.

3.3. IS-CE COM A MÈTODE *HIGH THROUGHPUT*

Per aconseguir convertir el mètode IS-CE en *high throughput*, s'ha analitzat i millorat el rendiment dels paràmetres instrumentals i de les condicions experimentals, segons 6 apartats principals: (i) la tria d'una instrumentació adequada; (ii) l'optimització dels paràmetres instrumentals de l'equip seleccionat; (iii) la millora del rendiment en la separació electroforètica i en la determinació del pK_a ; (iv) l'optimització de condicionaments del capil·lar; (v) la utilització de DMSO com a marcador d'EOF; (vi) i finalment l'avaluació general de la precisió i exactitud del mètode un cop optimitzat.

En primer lloc l'instrument comercial seleccionat és el P/ACE MDQ de Beckman, ja que es presenta com el més adequat pel desenvolupament del mètode. Les safates de mostres i tampons estan ubicades en llocs diferents i es poden treure fàcilment de la seva posició i a més es poden emmagatzemar directament a 4°C a l'interior de l'instrument. D'altra banda, l'instrument permet l'aplicació de pressió durant la separació, així com també la injecció seqüencial de mostres.

En segon lloc, per a l'optimització dels paràmetres instrumentals cal tenir en compte l'equació (1.16), anomenada en aquest Capítol, equació (3.4):

$$\mu = \frac{L_T L_D}{V} \left(\frac{1}{t_m} - \frac{1}{t_0} \right) \quad (3.4)$$

Degut a que la mobilitat roman constant, l'única manera d'aconseguir temps de migració més curts (t_m i t_0) és utilitzant longituds de capil·lar curtes (L_D i L_T) i aplicant voltatges grans (V). Així doncs, el capil·lar es va tallar a la longitud mínima que la configuració de l'instrument permet (25.0 cm fins al detector i 35.2 cm de longitud total) i es va seleccionar un voltatge de 20 kV per a mantenir la linealitat entre la tensió aplicada i el corrent generat per a tots els tampons (on encara es mantenia la llei d'Ohm). En aquestes condicions de capil·lar i voltatge, els temps de migració es redueixen en comparació amb aquells obtinguts anteriorment. S'arriba fins a temps d'anàlisi d'entre 1 i 3 min, essent les determinacions més llargues aquelles a valors de pH baixos i amb compostos aniònics; en altres paraules, anàlisis amb EOF baix i compostos amb mobilitat negativa.

El tercer pas va ser implementar l'anomenada injecció seqüencial. Aquesta injecta un rere l'altre l'anàlit i tots els patrons interns seleccionats abans de començar a aplicar la

diferència de potencial. Així mateix, per accelerar la migració dels compostos, es va aplicar pressió durant la separació: com més gran sigui la pressió, més curt serà el temps de migració. No obstant això, l'alta pressió pot provocar pics superposats. L'exemple mostrat a la Figura 1 de l'article III il·lustra que quan la pressió augmenta, disminueix el temps de migració així com també la resolució entre pics. Així doncs, es va aplicar 0.5 psi com a millor compromís entre un temps d'electroferograma curt i alta resolució.

Les metodologies de pre- i post-condicionament del capil·lar, el quart punt, van ser optimitzades a fi de reduir el temps de rentat i, com a conseqüència, el temps total d'anàlisi. La Taula 2 de l'article III mostra les seqüències de condicionament després dels processos d'optimització. La reducció va ser dràstica, gairebé de 12 vegades, sense afectar a la repetibilitat dels resultats.

En cinquè lloc es va utilitzar el DMSO com a marcador de l'EOF. El DMSO s'utilitza àmpliament per a la síntesi de nous fàrmacs i per a crear biblioteques de compostos en la indústria farmacèutica [11,83,84], per tant molts dels fàrmacs potencials per caracteritzar normalment ja el tenen incorporat abans de l'anàlisi [46,85]. Tanmateix, el DMSO té una absortivitat molar elevada i pot causar problemes en la detecció dels pics si es troba a concentracions altes. Així doncs, 0.4% de DMSO al vial d'injecció va ser la proporció òptima per a la determinació eficaç de compostos en CE (Figura 2c de l'article III).

En sisè i últim lloc, es va avaluar la precisió i exactitud del mètode optimitzat. La precisió dels valors de pK_a obtinguts es va avaluar de dues maneres: la primera determinant les mobilitats efectives, μ_{eff} , a diversos valors de pH pròxims al pK_a de l'anàlit (en lloc de fer-ho a un únic valor); i la segona manera, més ràpida, a partir de diversos patrons interns injectats seqüencialment en el mateix electroferograma. L'article III juntament amb la Figura 5 mostra un exemple utilitzant la mepivacaïna com a anàlit. Pel que fa a l'exactitud, el mètode IS-CE fins al moment s'ha utilitzat per a determinar fins a 129 valors de pK_a de 93 fàrmacs d'estructures diverses. Per estimar l'exactitud dels valors de pK_a experimentals, $pK_{a(IS-CE)}$, aquests s'han correlacionat amb els valors trobats a la literatura, $pK_{a(lit)}$, a 25°C i força iònica zero o mesurats en el nostre laboratori per altres mètodes. Les dades numèriques d'aquesta correlació es troben a la secció de *Supporting Information* de l'article III. La Figura 3.2 mostra la representació entre $pK_{a(IS-CE)}$ i $pK_{a(lit)}$, i els paràmetres de la correlació entre ambdós estan resumits a l'equació (3.5):

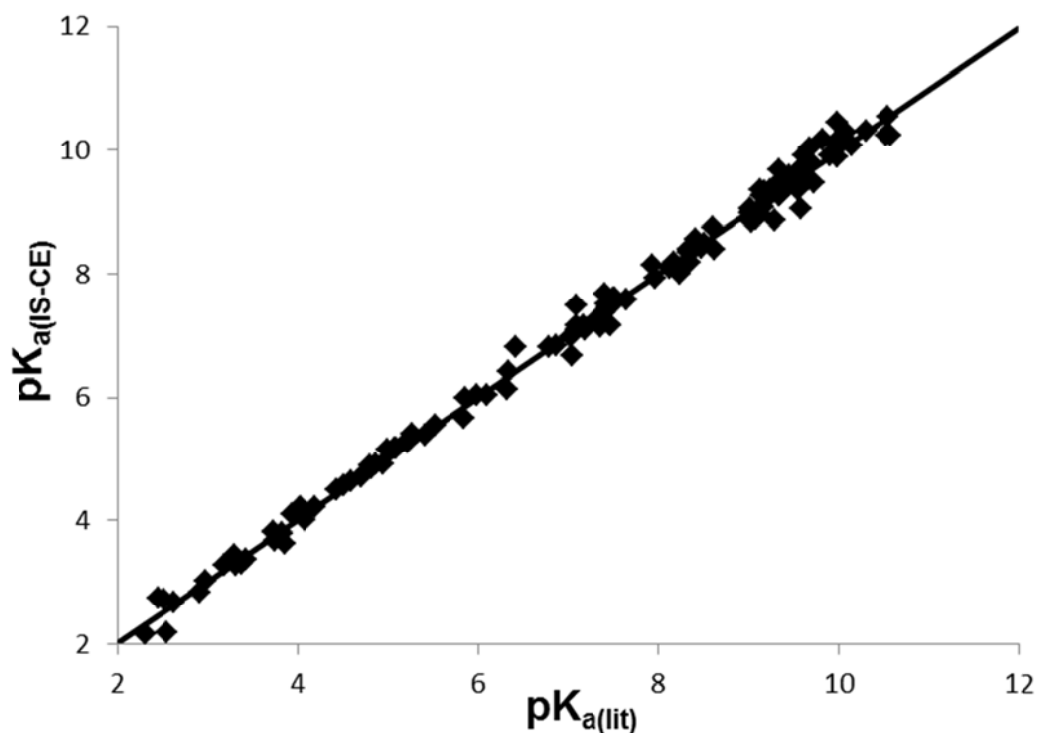


Figura 3.2. Correlació entre els valors de pK_a determinats pel mètode IS-CE ($pK_{a(IS-CE)}$) i dels trobats a la literatura ($pK_{a(lit)}$). La línia continua representa d'ajust lineal associat.

$$pK_{a(IS-CE)} = (0.995 \pm 0.006)pK_{a(lit)} + (0.05 \pm 0.05)$$

$$N = 129 \quad SD = 0.17 \quad F = 24783 \quad (3.5)$$

El pendent i la intersecció de la correlació no són significativament diferents de 0 i 1 respectivament, per a un nivell de confiança del 95%. Aquests resultats demostren la bona exactitud del mètode IS-CE en comparació amb els valors de referència de la literatura.

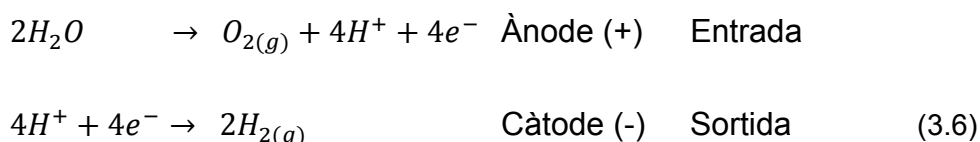
Arribat a aquest punt, amb els paràmetres instrumentals i les diferents etapes del mètode optimitzades, es van comprovar altres aspectes importants per a l'aplicació rutinària del mètode IS-CE en laboratoris de mesures fisicoquímiques o de *Drug Discovery and Development*, com per exemple l'estudi de l'estabilitat dels patrons interns i dels tampons de separació.

3.3.1. Estudi d'estabilitat dels tampons i dels patrons interns

Per a obtenir bons resultats experimentals s'han de complir els requisits sobre els BGE i els patrons interns ja comentats a la secció 1.4.3 del Capítol I. El pH del tampó pot modificar-se principalment per tres factors: el primer és la volatilització, com passa per exemple amb els tampons d'amoniac; el segon és l'electròlisi quan s'aplica una diferencia de potencial; i per últim la dissolució de CO₂ en tampons de NaOH o altament bàsics. Tot i que en el mètode IS-CE el pH de la solució tampó a l'interior del capil·lar es pot calcular fàcilment, és important saber quantes vegades es pot utilitzar la mateixa parella de tampons, mantenint les seves propietats dins d'un interval acceptable. En conseqüència, es va monitoritzar el pH dels tampons recomanats per a la utilització de l'IS-CE (presentats a la Taula 1 de l'article I) en funció del temps d'electròlisi. El pH es va calcular utilitzant el pK_a i les mobilitats límit i efectiva del patró intern a partir de l'equació (3.2) per a un IS àcid i l'equació (3.3) per un IS bàsic.

El pH de tots els BGE va ser monitoritzat al llarg del temps, fent anàlisis cada cinc minuts durant un temps total de 16 hores d'electroseparació. La determinació del pH es va dur a terme utilitzant tres IS per a cada BGE. La mitjana del pH obtingut per a cada electròlit es representa gràficament durant les primeres 10 hores d'electroseparació a la Figura 3.3.

Com es pot observar, en tots els casos el pH disminueix amb el temps d'anàlisi. Això pot explicar-se fàcilment pels processos d'electròlisi produïts en els vials de separació a causa de les reaccions que impliquen la generació (tampó d'entrada) o el consum (tampó de sortida) d'ions d'hidrogen a través dels elèctrodes [86]. L'equació (3.6) descriu els processos redox que tenen lloc als vials de separació de l'equip electroforètic.



Com s'ha explicat abans, el mètode IS-CE minimitza alteracions en les condicions d'anàlisi gràcies a que utilitza un IS que pateix la mateixa alteració que l'anàlit. Això vol dir que aquestes variacions en el pH no afecten a la determinació del pK_a del compost, sempre i quan l'AN i l'IS estiguin degudament ionitzats.

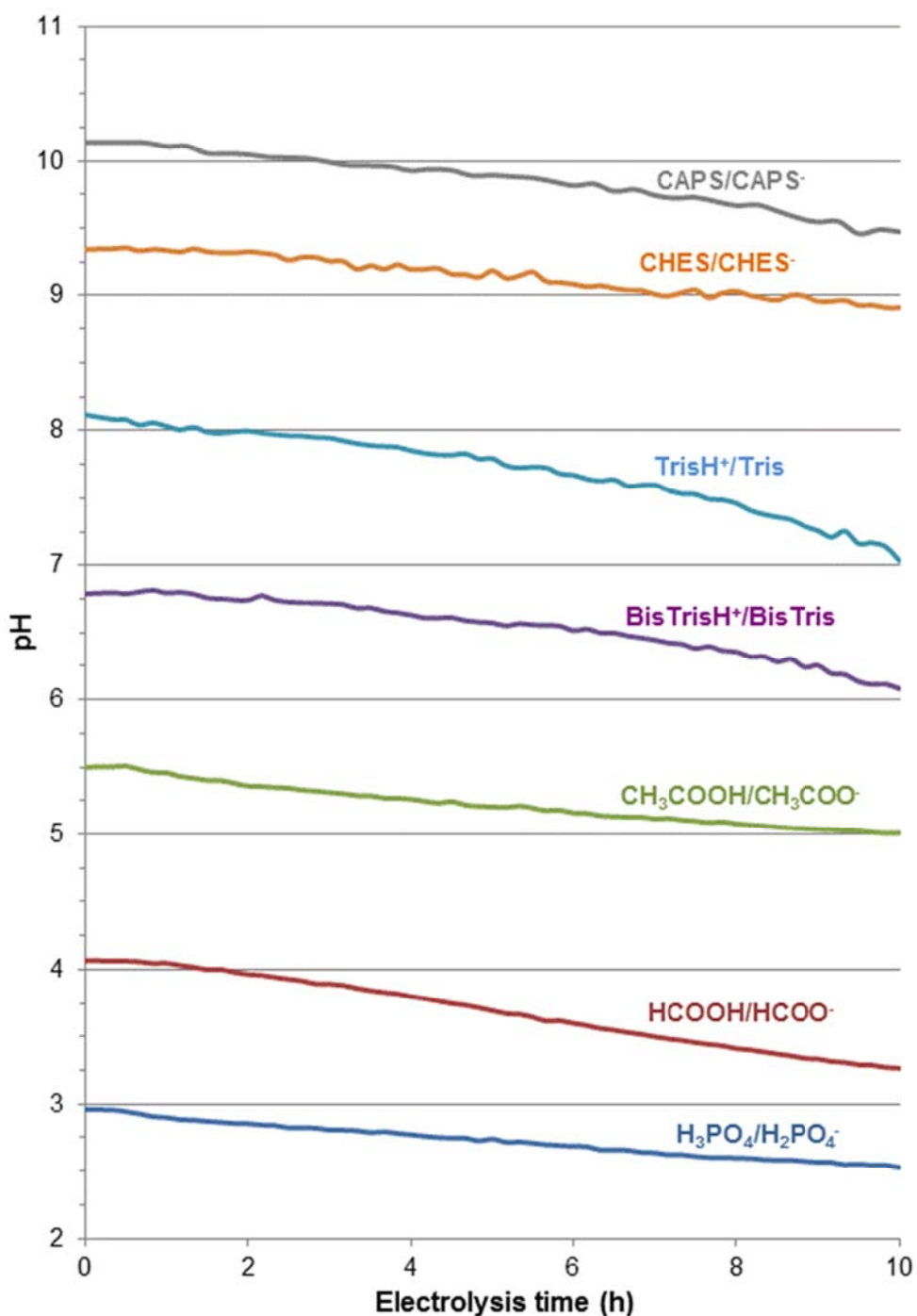


Figura 3.3. pH dels BGE en funció del temps d'electròlisi (vials amb 1.6 mL de BGE a 50 mM). pH calculat a través de les equacions (3.2) i (3.3) utilitzant alguns dels patrons interns llistat a la Taula 3.1.

Monitoritzant la determinació del pK_a per a un compost també cada 5 minuts durant 16 hores d'electroseparació pel mètode IS-CE, es va comprovar que el valor del pK_a es manté constant després de 500 injeccions aproximadament (considerant anàlisis d'uns

2 minuts, Figura 4 de l'article III). Aquest fet demostra que l'ús del mètode IS-CE no requereix una substitució freqüent dels vials de separació, mentre que en el mètode clàssic la variació pH es minimitza canviant els vials aproximadament cada 1-2 hores d'electroseparació.

No obstant això, la Figura 3.3 mostra que el pH del TRIS o CAPS disminueix gairebé una unitat després de deu hores. Aquest fet fa disminuir la capacitat amortidora i, el que és més important, variar l'estat d'ionització de l'AN i IS. Per tal d'evitar aquest fet, es recomana la substitució dels tampons després d'aproximadament 10 hores d'ús o després d'unes 300 injeccions, considerant un temps d'anàlisi mitjà de 2 minuts.

Tal com s'ha comentat anteriorment, la Taula 3.1 mostra la llista de valors de pK_a dels 48 patrons interns. Aquesta llista proporciona un conjunt de compostos de referència amb constants d'acidesa perfectament conegudes i ajuda en el procés de selecció d'un IS apropiat per a una donada determinació de pK_a . Així doncs, quan s'ha de determinar el pK_a d'un anàlit, un o diversos patrons interns de la safata de mostres s'injecten seqüencialment amb el compost d'assaig. No obstant això, pot ser que les solucions patró es degradin amb el temps i per això cal fer un estudi sobre la seva estabilitat.

Per a aquesta avaluació, cada compost es va injectar periòdicament a un pH on es troba totalment ionitzat, controlant l'àrea i la mobilitat del compost al llarg del temps. L'àcid nicotínic, la *N,N*-dimetil-*N*-fenilamina, la piridina i la 4-*terc*-butilpiridina mostraven una disminució del 10% de l'àrea total en uns pocs dies per la qual cosa s'han de canviar diàriament. En el cas de la vanil·lina, l'anilina, la quinolina, la 4-*terc*-butilanilina, la 2,4-lutidina, la trazodona, la pilocarpina, el 2,4,6-trimetilpiridina, la bupivacaïna, la imipramina i el propranolol, la substitució s'ha de fer setmanalment. La warfarina, el 2,4,6-tribromofenol, l'acridina, la papaverina, i la procaïnamina, requereixen la substitució mensual. Per a la resta dels patrons interns, l'àrea del pic continuava sent del 90% o més després d'un període de quatre mesos.

3.4. APLICACIÓ DEL MÈTODE IS-CE PER A LA DETERMINACIÓ HIGH THROUGHPUT DE PERFILS DE LIPOFILICITAT

En el Capítol I s'ha explicat com són d'importants les determinacions *high throughput* per als paràmetres fisicoquímics de processos de *drug discovery and development*. S'ha comentat també que, així com la constant d'acidesa, la lipofilicitat ($\log P$) també juga un paper molt important en l'estudi de l'activitat biològica. Així doncs, la seva determinació ràpida també és rellevant en l'estudi de fàrmacs. De la mateixa manera, la determinació ràpida del pK_a i de la lipofilicitat ($\log P$) permet la determinació ràpida del perfil de lipofilicitat ($\log D$) en funció del pH.

Tradicionalment, la lipofilicitat d'una substància neutre s'expressa com a logaritme del coeficient de partició octanol-aigua o en la seva forma logarítmica, $\log P_{o/w}$. Ara bé, per a molècules amb propietats àcid-base el coeficient de partició varia amb el pH, anomenant-se llavors el coeficient de lipofilicitat a qualsevol pH com a $\log D_{o/w}$ (coeficient de distribució).

Per a substàncies ionitzables, el $\log D_{o/w}$ es pot estimar a partir de les constants d'acidesa i del coeficient de partició de la substància neutra i totalment ionitzada [87]. Tanmateix, no es pot realitzar la determinació directa del coeficient de partició de l'espècie totalment iònica, però segons un treball publicat per S. Donovan i M. Pescatore [88] es pot assumir que aquest valor és típicament 3.15 unitats logarítmiques més baix que el de l'espècie neutra. Així doncs, el $\log D_{o/w}$ es pot expressar seguint l'equació (3.7), per a un àcid monopròtic i seguint l'equació (3.8), per a una base monopròtica.

$$\log D_{o/w} = \log \left(\frac{P_{o/w} + (P_{o/w} - 3.15) \cdot 10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \right) \quad (3.7)$$

$$\log D_{o/w} = \log \left(\frac{P_{o/w} + (P_{o/w} - 3.15) \cdot 10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \right) \quad (3.8)$$

Conseqüentment, coneixent la constant d'acidesa (pK_a) i el coeficient de partició ($\log P_{o/w}$), es pot calcular el $\log D_{o/w}$ a qualsevol pH.

Tal com comenta l'article IV, s'ha desenvolupat un mètode que permet la determinació del $\log P_{o/w}$ en pocs minuts utilitzant la tècnica d'UHPLC (acrònim anglès de *ultra high*

performance liquid chromatography). Degut a que el desenvolupament i metodologia d'aquest mètode no forma part d'aquesta tesi, no s'explicarà ni es discutirà en profunditat. No obstant això, se'n fa una breu descripció. Aquest mètode utilitza el càlcul de retencions cromatogràfiques en gradient per UHPLC per calcular els anomenats índexs d'hidrofobicitat cromatogràfica (CHI_{MeCN}) de les espècies neutres de substàncies ionitzables. Un cop trobat els valors de CHI , aquests serveixen per calcular el valor de $\log P_{o/w}$ a través de l'equació descrita per Klara Valkó et al. [89],

$$\log P_{o/w} = 0.054CHI_{MeCN} + 1.319A - 1.877$$

$$N = 86 \quad R = 0.70 \quad SD = 0.29 \quad F = 655 \quad (3.9)$$

involucrant un descriptor d'acidesa per pont d'hidrogen (paràmetre A). Resumint, en tant sols una anàlisi per UHPLC de menys de 4 minuts es pot determinar el valor del coeficient de partició de la forma neutra d'una substància qualsevol.

En aquest treball, tant el mètode IS-CE per a la determinació de valors de pK_a com el de UHPLC per a la determinació de valors de $\log P_{o/w}$ es van combinar per descriure una alternativa *high throughput* per a la determinació dels perfils de lipofilitat de compostos bioactius. Per a aquest estudi, es va determinar el pK_a i el $\log P_{o/w}$ d'una sèrie de 28 substàncies àcides i bàsiques. La Taula 3.3 llista totes les substàncies amb els corresponents valors de pK_a i $\log P_{o/w}$ determinats així com també mostra els valors de CHI i A en la determinació. Seguint les equacions (3.7-3.8), la taula també presenta la determinació d'un paràmetre biològic interessant com el coeficient de partició al pH sanguini de 7.4, $\log D_{7.4}$. Aquests valors són contrastats amb els valors de referència trobats a la literatura, $\log D_{7.4(ref)}$. Comparant ambdós valors de la taula es pot observar que hi ha una molt bona similitud entre els valors de $\log D_{7.4}$, amb excepció de la lidocaïna, que presenta una desviació major, però de només d'una unitat de $\log D$ aproximadament.

Taula 3.3. Determinació del $\log P_{o/w}$ pel mètode basat en UHPLC, de pK_a pel mètode IS-CE i del coeficient de partició a pH 7.4, $\log D_{7.4}$, a partir de les equacions (3.7-3.8).

Compost	CHI	A	$\log P_{o/w}$	pK_a	$\log D_{7.4}$	$\log D_{7.4}(\text{lit})$
<i>Substàncies bàsiques</i>						
Papaverina	66.93	0.00	1.74	6.41	1.69	-
Reserpina	98.70	0.31	3.86	6.64	3.79	3.89 ^{a)}
2,4-Lutidina	49.35	0.00	0.79	6.81	0.69	-
Ketoconazol	83.90	0.00	2.65	6.81	2.55	3.42 ^{a)}
Trazodona	76.34	0.00	2.25	6.84	2.14	2.54 ^{b)}
Pilocarpina	20.86	0.00	-0.75	7.08	-0.92	-
2,4,6-Trimetilpiridina	57.17	0.00	1.21	7.51	0.85	-
Lidocaïna	86.40	0.26	3.13	7.89	2.52	1.61 ^{a)}
Clonidina	51.30	0.42	1.44	8.10	0.67	0.62 ^{b)}
Bupivacaïna	101.75	0.26	3.96	8.19	3.11	-
Quinina	66.42	0.23	2.01	8.45	0.93	-
Haloperidol	88.90	0.31	3.34	8.56	2.15	2.77 ^{a)}
Procaïna	62.90	0.23	1.82	9.08	0.14	0.24 ^{a)}
Propranolol	80.90	0.29	2.88	9.48	0.83	1.23 ^{a)}
Metoprolol	61.50	0.29	1.83	9.53	-0.26	-0.25 ^{a)}
Atenolol	32.10	0.78	0.89	9.67	-1.33	-1.81 ^{a)}
<i>Substàncies àcides</i>						
Paracetamol	21.20	0.91	0.47	9.58	0.46	0.35 ^{a)}
2-Clorofenol	63.64	0.33	2.00	8.50	1.96	-
Teofil·lina	21.40	0.35	-0.27	8.39	-0.31	-0.07 ^{a)}
Metilparabè	52.33	0.66	1.82	8.35	1.78	-
3,5-Dinitrofenol	85.97	0.83	3.86	8.18	3.80	-
4-Hidroxibenzaldehid	38.36	0.66	1.07	7.61	0.86	-
Fenobarbital	51.84	0.52	1.61	7.53	1.37	1.51 ^{b)}
Vanil·lina	41.37	0.44	0.94	7.36	0.61	-
4-Nitrofenol	55.31	0.67	1.99	7.09	1.51	-
Warfarina	82.90	0.31	3.01	5.17	0.83	0.89 ^{a)}
Àcid benzoic	48.00	0.57	1.47	4.22	-1.40	-1.27 ^{a)}
Indometacina	90.70	0.57	3.77	4.16	0.88	-

a) Mètode Shake-flask. Ref. [90]. b) Ref. [2].

3.5. DETERMINACIÓ DE CONSTANTS D'ACIDES DE COMPOSTOS POC SOLUBLES

A diferència d'altres mètodes, l'IS-CE presenta un gran avantatge per als fàrmacs poc solubles. Normalment la mobilitat límit és fàcil de mesurar, ja que la solubilitat augmenta a mesura que augmenta el grau de ionització, tal i com s'ha comentat a la secció 1.1.1.2 de la introducció. En el cas de les mobilitats efectives, si es detecta la precipitació del fàrmac, la determinació de la mobilitat es pot tornar a provar a un nou pH on el grau de ionització sigui més alt.

En aquesta secció del treball es presenta i s'analitza la determinació de valors de pK_a de fàrmacs en solució aquosa pel mètode IS-CE. El conjunt de compostos compilats inclou 38 substàncies amb un total de 51 valors de pK_a , essent molts dels compostos fàrmacs comercials. La seva selecció es va realitzar en funció de les respectives solubilitats intrínseques. La Taula 3.4 mostra els compostos juntament amb la forma logarítmica de la seva solubilitat intrínseca ($\log S_{(lit)}$, en $\text{mol} \cdot \text{L}^{-1}$) i la constant d'acidesa determinada pel mètode IS-CE ($pK_{a(IS-CE)}$). Els compostos s'enumeren en ordre de solubilitat decreixent, començant pels molt solubles com ara la teofil·lina amb $\log S$ de -1.10, i acabant pels molt insolubles com el tamoxifè amb $\log S$ de -8.49. Aquests valors de solubilitat foren obtinguts a partir de diverses fonts [37,63,91,92], o a través del programa de predicció ACD/Labs v2012 [80] quan no hi havia dades experimentals disponibles a la literatura. En el cas de que hi hagués més d'un valor bibliogràfic, la taula presenta la seva mitjana.

Per a un fàrmac amb solubilitat suficientment alta, el pK_a es determina pel mètode IS-CE habitual sense gaire problemes. A mesura que la solubilitat baixa, la determinació del pK_a del fàrmac esdevé més difícil cada vegada. El diclofenac, amb $\log S$ de -5.45 unitats, va ser el compost més soluble que precipitava quan es preparava la solució a injectar, és a dir, quan es preparava la dilució 1/10 en aigua a partir de la solució mare de $1 \text{ mg} \cdot \text{mL}^{-1}$ dissolta en metanol. Es pot dir per tant, que no s'observen problemes de precipitació en tot el procés de determinació de pK_a a través del mètode IS-CE per fàrmacs amb valors de $\log S$ superiors a -5, aproximadament.

Taula 3.4. Compostos d'estudi amb els respectius logS de la literatura i els valors de pK_a determinats pel mètode IS-CE. Valors de pK_a a 25°C i força iònica zero.

Compost	logS _(lit)	pK _a (IS-CE)	Compost	logS _(lit)	pK _a (IS-CE)
Teofil·lina	-1.10 ^{b)}	8.39	Galangina	-3.93 ^{b)}	7.25
Atenolol	-1.29 ^{c)}	9.67			9.54
Procaïna	-1.72 ^{c)}	9.08	Diflunisal	-4.20 ^{b)}	2.97
Alprenolol	-2.63 ^{c)}	9.37	Flurbiprofè	-4.30 ^{c, d)}	4.19
Diltiazem	-2.94 ^{c)}	7.99	Ketoconazol	-4.40 ^{b)}	3.45
Fisetina	-3.00 ^{b)}	7.14			6.81
		8.39	Maprotilina	-4.69 ^{c)}	10.23
		10.28	Ciproheptadina	-4.94 ^{b)}	8.90
Morin	-3.02 ^{b)}	5.15	Reserpina	-5.05 ^{b)}	6.64
		8.15	Fenbufè	-5.19 ^{e)}	4.73
		10.46	Clorpromazina	-5.29 ^{c, d)}	9.26
Campferol	-3.37 ^{b)}	7.16	Diclofenac	-5.45 ^{c, d)}	4.22
		9.05	Haloperidol	-5.47 ^{c)}	8.5
		10.54	Miconazole	-5.62 ^{c)}	6.14
Quercetina	-3.40 ^{b)}	7.15	Mifepristona	-5.75 ^{c, e)}	5.23
		8.84	Indometacina ^{a)}	-5.77 ^{d, e)}	4.16
		10.31	Niclosamida	-6.15 ^{f)}	7.25
Apigenina	-3.47 ^{b)}	7.60	Àcid mefenàmic	-6.34 ^{c)}	4.17
		9.35	Àcid meclofenàmic	-6.86 ^{c)}	4.09
Naringenina	-3.48 ^{b)}	6.68	Seraconazol	-6.99 ^{b)}	6.07
		10.16	Gliburida	-7.05 ^{e)}	5.44
Hesperetina	-3.50 ^{b)}	6.97	Loperamida	-7.13 ^{c)}	8.79
		10.13	Terfenadina	-7.74 ^{c)}	9.19
Oxifenciclimina	-3.58 ^{b)}	11.01	Amiodarona	-8.17 ^{c)}	8.59
Dibucaïna	-3.68 ^{b)}	8.57	Tamoxifè	-8.49 ^{e)}	8.40
Fluoxetina	-3.92 ^{c)}	10.08			

a) Degradació observada quan es prepara a 1mM d'àcid o base. b) ACD/Labs 2012. [80]. c) Ref. [63]. d) Ref. [37]. e) Ref. [91]. f) Ref. [92].

3.5.1. Fàrmacs amb solubilitat lleugerament baixa

Els compostos amb logS més baixos que el de la clorpromazina, inferiors a -5.3 aproximadament, precipitaven en diluir la solució mare en aigua. No obstant això, es poden adoptar diverses estratègies per superar aquest problema. La primera opció és afegir la quantitat necessària de HCl o NaOH per tal de ionitzar el compost al vial de mostra augmentant així la seva solubilitat. Atès que el mètode de mesura és molt ràpid, moltes vegades la precipitació es pot evitar si el compost és ionitzat i solubilitzat

prèviament, i encara que hi hagi canvis de pH a l'interior del capil·lar el pK_a es mesura mentre la solució es manté sobresaturada. De totes maneres, si es detecta precipitació al capil·lar, una segona opció és realitzar la mesura de la mobilitat efectiva a un nou pH més a prop del pH on predomina la forma iònica, és a dir, a un major grau de ionització del fàrmac. Una tercera estratègia és diluir els fàrmacs a aproximadament a 5-10 ppm, ja que la detecció en aquestes concentracions encara es pot realitzar correctament.

Les estratègies explicades, soles o combinades, permeten la determinació de constants d'acidesa de fàrmacs directament en solució aquosa fins a $\log S$ de -6 aproximadament. De fet, la niclosamida va ser l'últim fàrmac en ordre decreixent de solubilitat determinat d'aquesta manera. Tal i com mostra la Taula 1 de l'article V, el grau de ionització per a aquest grup de compostos es troba al voltant del 70% o més. Aquest percentatge de ionització és suficient per mantenir el fàrmac en solució, però prou baix com per permetre el càlcul de pK_a .

3.5.2. Fàrmacs amb solubilitat molt baixa

Pel que fa als compostos amb solubilitat molt baixa, $\log S < -6.34$, aquests precipiten durant les anàlisis realitzades en medi aquós, fins i tot quan s'adopten les estratègies presentades a l'apartat anterior. Així doncs, la utilització de cosolvents i procediments d'extrapolació són estrictament necessaris.

Treballar amb barreges hidro-orgàniques implica establir nous valors de pK_a de referència per al conjunt dels IS en tota la gamma de composicions metanol-aigua de treball (0-40% v/v). El procediment seguit per a establir aquests valors de pK_a es basa en la mateixa metodologia utilitzada anteriorment per a establir la llista d'IS àcids, explicada a la primera secció d'aquest Capítol. El procediment es va aplicar separatament per IS àcids i IS bàsics i en ambdós casos els valors de pK_a es van determinar a quatre composicions diferents de metanol-aigua, 10, 20, 30 i 40% (v/v). Al començament de la iteració, es van utilitzar com a constants de partida els valors de pK_a estimats per a cada mescla MeOH-H₂O a partir d'equacions empíriques [93,94]. En cada cas, es va aplicar una equació específica depenent del grup funcional implicat a la constant d'acidesa i del percentatge de metanol.

La Taula 1 de l'article VI llista els valors de pK_a obtinguts per a les diferents barreges MeOH-H₂O així com també els valors aquosos i les desviacions estàndards associades. Per tal de conèixer el valor de pK_a de referència a qualsevol composició de metanol-aigua entre 0-40%, els quasi 200 valors experimentals d'aquesta taula es van ajustar a equacions quadràtiques per a cada IS. Aquests ajustos es resumeixen a la Taula 2 de l'article VI i permeten el càlcul de la constant d'acidesa a qualsevol volum per a qualsevol IS de la llista entre un interval de 0-40% de metanol.

Els valors de pK_a publicats a la literatura ($pK_{a(lit)}$) van ser comparats amb els valors obtinguts a través de les equacions de la Taula 2 de l'article VI. Els valors de la literatura s'obtingueren a partir de diverses fonts, la majoria de les quals es troben a l'excel·lent compilació de Palm et al. [95]. Particularment, 101 valors bibliogràfics en composicions de metanol que van entre 11-45% van ser correlacionats amb els establerts pels patrons interns. La Taula 3 i la Figura 1 del mateix article presenten les correlacions entre els valors de pK_a . L'escassetat i dispersió de la informació bibliogràfica d'aquests valors fa que sigui difícil realitzar una estimació real de l'exactitud. No obstant això, els valors absoluts de ΔpK_a no superen les 0.25 unitats i en la majoria dels casos és inferior a 0.1. D'altra banda, no es van arribar a trobar valors fiables d'alguns dels patrons interns. Tanmateix, aquests valors també són indirectament avaluats ja que el mètode de mesura implica contrastar conjuntament els valors de pK_a mesurats i per tant estan tots relacionats entre si. D'aquesta manera, un bon ajust amb els valors de la literatura implica una bona relació general, fins i tot per a aquells casos en què la comparació directa amb valors bibliogràfics no ha estat possible.

Els 8 últims fàrmacs de la Taula 3.4 van ser els seleccionats per a l'avaluació del mètode en medis hidro-orgànics, des de l'àcid mefenàmic amb logS de -6.34 fins al tamoxifè amb un valor de -8.49. Els valors pK_a d'aquests fàrmacs es van determinar a quatre percentatges de metanol com a cosolvent (10, 20, 30 i 40% v/v), i l'extrapolació al 0% seguint l'equació lineal de Yasuda-Shedlovsky tal com s'ha explicat anteriorment a la introducció. La Taula 4 de l'article VI mostra les constants d'acidesa determinades en les mescles, els paràmetres d'ajust a l'equació de Yasuda-Shedlovsky i el pK_a extrapolat per a cada fàrmac amb la corresponent desviació associada a la determinació.

3.5.3. Comparació amb altres mètodes

La Taula 3.5. mostra una comparació de metodologies per a la determinació pK_a d'aquests fàrmacs poc solubles, particularment aquells que presentaven problemes i que han estat mencionats anteriorment. Les substàncies s'han separat en dos blocs, les que es poden analitzar en medi aquós i les que necessiten de mescles hidrò-orgàniques. Per a la majoria de compostos es van trobar forces valors a la literatura ($pK_{a(lit)}$) determinats per a una gran varietat de mètodes i condicions experimentals. Tots els valors de pK_a de la taula estan a força iònica zero i a 25°C. No es van trobar fonts fiables dels valors de pK_a de la mifepristona, la niclosamida i el sertaconazol de manera que no es poden incloure en la comparació. La taula també inclou informació sobre el cosolvent i l'interval utilitzat (% v/v) en cada determinació. Algunes de les referències no especifiquen l'interval de cosolvent, o fins i tot el cosolvent utilitzat (n.e. a la taula).

En general s'observa que els resultats obtinguts pel mètode IS-CE s'aproximen molt als dels altres mètodes. Quan s'analitzen les dades de la literatura per a les determinacions en medi aquós, es veu que a aquests nivells de solubilitat les valoracions potenciomètriques requereixen l'ús de cosolvents i mètodes d'extrapolació per obtenir valors de pK_a aquosos. Pel que fa a les valoracions espectrofotomètriques, llevat de diclofenac totes les mesures també es realitzen en medis hidro-orgànics. El mètode clàssic CE està en el límit entre l'ús o no de cosolvents. Per exemple, la constant d'acidesa de l'indometacina s'ha determinat en aigua per *multiplexed* CE, mentre que el de miconazole requereix l'ús de mescles de metanol-aigua i l'extrapolació a través de l'equació Yasuda-Shedlovsky.

Si s'analitzen les dades experimentals de les determinacions amb el mètode IS-CE utilitzant cosolvents i es comparen amb les bibliogràfiques, es pot observar que per a aquests nivells de solubilitat tots els mètodes requereixen l'ús de cosolvent i procediments d'extrapolació excepte el CE-MS. La tècnica CE-MS permet la determinació directa en tampons aquosos, probablement a causa de que l'alta sensibilitat del mètode li permet treballar a molt baixa concentració. No obstant això, aquest mètode és lent i car a no ser que s'agrupin molts fàrmacs per a una determinació simultània.

Taula 3.5. Comparació entre els valors de pK_a termodinàmics a 25°C determinats pel mètode IS-CE i pels mètodes de la literatura, per als compostos més insolubles de la Taula 3.4.

Compost	pK_a (IS-CE)	pK_a (lit)	Mètode	Cosolvent	% v/v
<i>Fàrmacs analitzats en medi aquós</i>					
Diclofenac	4.22	4.16	Pot. [96]	Isopropanol	100
		4.09	Pot. [38]	MDM*	17 – 48
		4.03	GLpK _a espect. [63]	No	0
		4.21	CE-MS [74]	No	0
Haloperidol	8.56	8.65	Pot. [21]	MeOH	46 – 66
		8.55	Pot. [97]	MDM*	37 – 56
		8.42	GLpK _a pot. [63]	n.e.	n.e.
		8.96	CE-MS [74]	No	0
Miconazole	6.14	6.32	GLpK _a espect. [63]	n.e.	n.e.
		6.38	Multiplexed CE [49]	MeOH	30 – 60
Mifepristona	5.23	-	-	-	-
Indometacina ^{e)}	4.16	4.53	Clàssic CE [24]	MeOH	24 – 66
		4.15	CE-MS [74]	No	0
		4.18	Multiplexed CE [49]	No	0
Niclosamida	7.25	-	-	-	-
<i>Fàrmacs analitzats en medi metanol/aigua (10-40% MeOH/H₂O)</i>					
Àcid mefenàmic	4.17	4.22	GLpK _a espect. [63]	MeOH	n.e.
Àcid meclofenàmic	4.09	4.10	GLpK _a n.e. [63]	n.e.	n.e.
Sertaconazol	6.07	-	-	-	-
Gliburida	5.44	5.22	CE-MS [74]	No	0
Loperamida	8.79	8.90	GLpK _a espect. [63]	MeOH	n.e.
Terfenadina	9.19	9.25	GLpK _a pot. [63]	MeOH	50 – 66
		9.74	Pot. [98]	n.e.	n.e.
		9.31	Multiplexed CE [49]	MeOH	40 – 60
		9.21	CE-MS [74]	No	0
Amiodarona	8.59	8.85	Pot. [25]	MeOH	46 – 60
		8.80	Pot. [38]	MDM*	40 – 60
		8.73	Pot. [38]	MeOH	n.e.
		8.94	Pot. [2]	n.e.	n.e.
		8.73	GLpK _a espect. [63]	MeOH	40 – 50
		8.85	Clàssic CE [25]	MeOH	50
Tamoxifè	8.40	8.62	Multiplexed CE [49]	MeOH	50 – 60
		8.36	Pot. [2]	n.e.	n.e.
		8.51	Multiplexed CE [49]	MeOH	40 – 60
		8.71	CE-MS [74]	No	0

Espect.: espectrofotometria; Pot.: potenciometria. n.e.: no s'especifica a la referència.

a) Ref. [63]. b) Ref. [37]. c) Ref. [91]. d) Ref. [92]. e) Degradació observada quan es prepara a 1mM d'àcid o base.

Un dels avantatges del mètode IS-CE quan es treballa en medis hidro-orgànics és que el pH de la solució tampó es calcula dins del capil·lar a partir de l'IS, obtenint directament el ${}^s\text{pH}$ (pH en l'escala de la barreja hidro-orgànica) si el pK_a de l'IS és troba a l'escala ${}^s\text{pH}$. En altres mètodes com la potenciometria, l'espectrofotometria i el CE amb el mètode clàssic el pH es mesura amb un elèctrode de vidre en general calibrat amb tampons aquosos, mentre que les mesures de la mostra es realitzen en presència de cosolvent. Per tant, aquests mètodes necessiten una correcció per canviar els valors de l'escala aquosa a l'escala de la mescla [49]. Aquestes correccions, es poden evitar en el mètode IS-CE.

Pel que fa al cosolvent utilitzat, es demostra que el metanol és el preferit degut a que les seves propietats són molt similars a les de l'aigua i és capaç de solubilitzar molts compostos insolubles en aigua. Observant l'interval de composicions (% v/v), gairebé tots els mètodes de referència (valoracions potenciomètriques, espectrofotomètriques i el mètode clàssic CE) requereixen l'ús de volums de metanol més alts que el mètode IS-CE. Aquest fet presenta un avantatge ja que per a determinacions aquoses de pK_a mitjançant l'extrapolació Yasuda-Shedlovsky, s'obté un resultat tant més precís com més baix sigui el percentatge de solvent orgànic utilitzat. L'ús de MDM (volums iguals de MeOH, 1,4-dioxà i MeCN) en lloc del metanol permet generalment disminuir el contingut de solvent orgànic, tal i com s'observa per al diclofenac, l'haloperidol i l'amiodarona. Tot i així, els resultats obtinguts demostren que el mètode IS-CE permet la determinació dels valors de pK_a al 10% en metanol mentre que altres mètodes requereixen al voltant del 40-50%, fent que la fiabilitat del mètode IS-CE resulti superior a la dels altres.

3.6. DISSENY D'UN INSTRUMENT ESPECÍFIC PER AL MÈTODE IS-CE

En les seccions anteriors s'ha anat desenvolupant el mètode IS-CE, aconseguint a hores d'ara que aquest mètode esdevingui ràpid, fiable, precís i acurat per a determinar constants d'acidesa de compostos d'interès biològic, sense importar la seva solubilitat, nombre de constants o complexitat. No obstant això, els instruments comercials d'electroforesi capil·lar són grans, cars i tenen algunes limitacions. Per exemple la necessitat de cobrir tot l'interval de pH (2-12) requereix la preparació, d'entrada, d'una gran quantitat de tampons diferents, fent la preparació d'electròlits feixuga i llarga especialment per als tampons de mescles metanol-aigua. A més, el fet de disposar tots els tampons a les safates de l'injector, redueix la capacitat per a mostres i constitueix una limitació de la utilitat d'aquest mètode per a l'anàlisi de rutina. Per a superar aquests problemes, en aquesta tesi s'ha dissenyat una instrumentació específica adaptable pel mètode IS-CE, tot simplificant i agilitzant el procés per a la determinació del pK_a .

3.6.1. Disseny de l'equip

A la Figura 3.4 es mostra un diagrama d'un instrument desenvolupat recentment per a la separació ràpida d'anions o cations inorgànics, conegut en anglès com a *sequential injection – capillary electrophoresis* [99–104]. Aquest sistema està compost de dues bombes, una per a la mostra i l'altra per al tampó, connectades alhora a una vàlvula de dues posicions anomenada vàlvula d'injecció. Aquesta vàlvula tria automàticament a través d'un software quina solució deixa passar a la interfase. Aquesta interfase és una peça en forma de "T", que connecta a la seva entrada amb la vàlvula d'injecció i perpendicularment amb el capil·lar (Figura 3.5). A la sortida connecta amb una altra vàlvula, anomenada vàlvula d'aïllament, que obre o tanca el canal en funció de si volem o no fer passar líquid pel capil·lar. La part final del capil·lar està submergida a un vial que conté el mateix tampó, on s'hi col·loca un elèctrode per tal d'aplicar la diferència de potencial. L'elèctrode de terra es troba a la interfase.

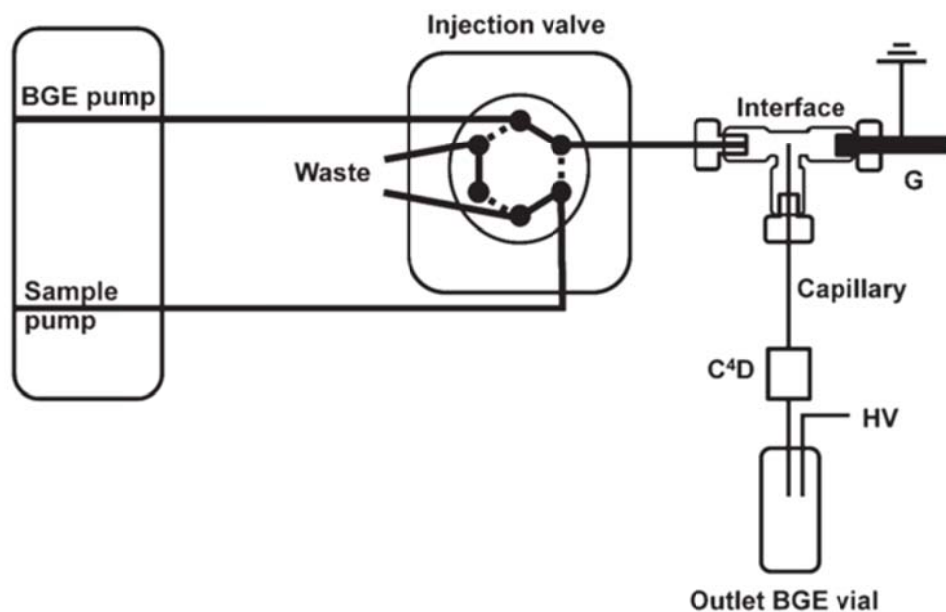


Figura 3.4. Diagrama esquemàtic d'un sistema *sequential injection – capillary electrophoresis* per a la determinació ràpida d'explosius inorgànics [101]. G: terra; HV: alt voltatge; C⁴D: detector *capacitively coupled contactless conductivity*.

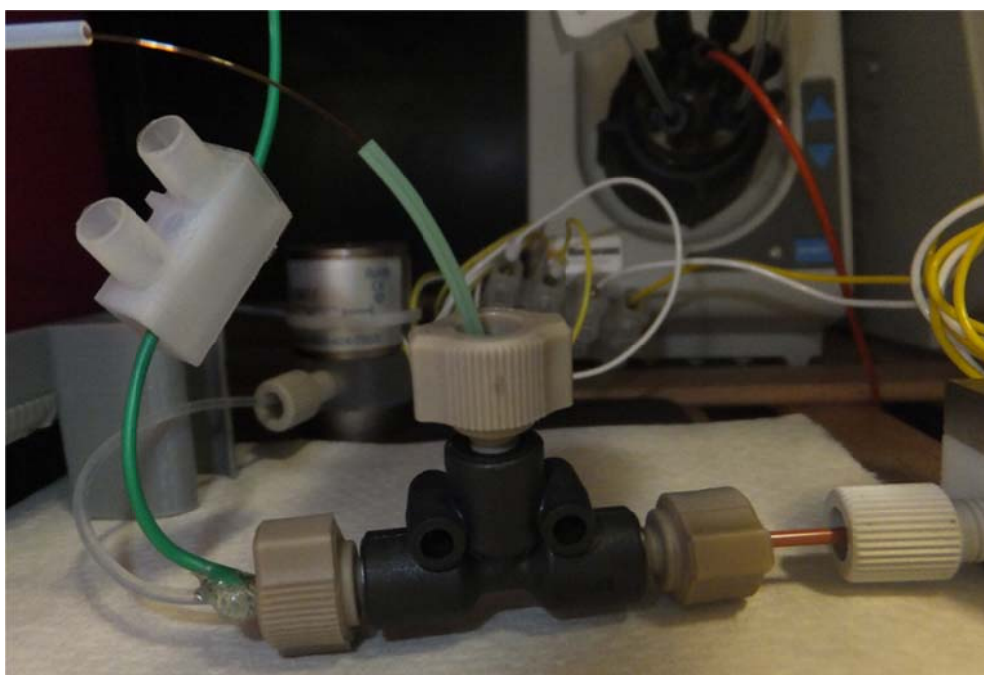


Figura 3.5. Fotografia de la interfase. La solució entra per la dreta i surt per l'esquerra, el capil·lar es troba connectat perpendicularment al flux de solució. El final del tub de sortida està connectat amb la vàlvula d'aïllament. El cable verd és la connexió de terra.

El sistema funciona seguint 6 passos principals: (i) s'activa la bomba del BGE per rentar la interfase; (ii) es tanca la vàlvula d'aïllament per fer entrar el BGE dintre del capil·lar; (iii) un cop condicionat el capil·lar amb BGE, es gira la vàlvula d'injecció i s'acciona la bomba de la mostra per rentar la interfase amb mostra; (iv) es tanca la vàlvula d'aïllament o s'aplica voltatge uns segons per a la injecció de la mostra al capil·lar; (v) es torna a canviar la vàlvula d'injecció, s'acciona la bomba del BGE i es renta la interfase; (vi) s'aplica alt voltatge per a la separació.

Com s'ha comentat anteriorment, d'acord amb les necessitats i requisits del mètode IS-CE, i basat en el *sequential injection – capillary electrophoresis*, es va dissenyar un sistema per a generar l'electròlit en continu, anomenat en anglès *on-line buffer generation system*. Aquest funciona tal i com mostra la Figura 3.6.

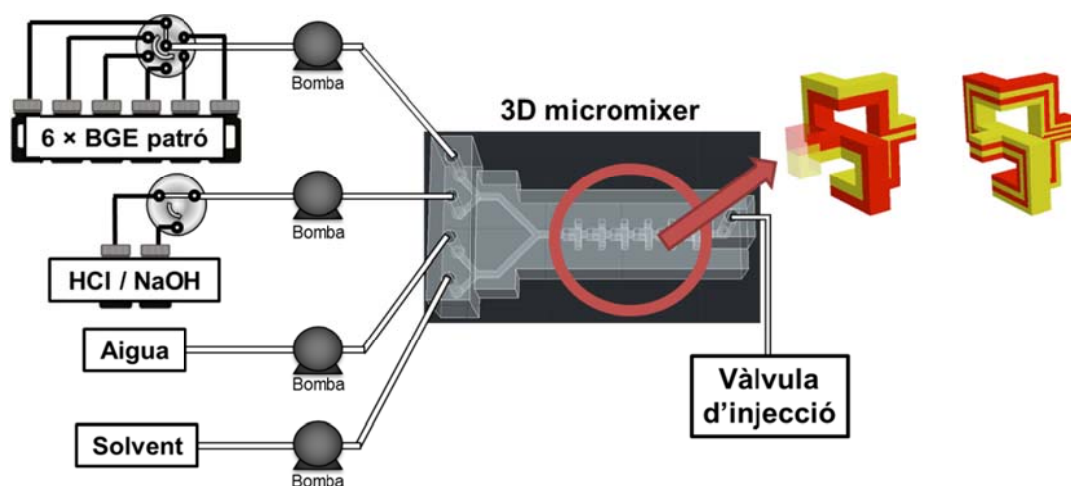


Figura 3.6. Diagrama del *on-line buffer generation system*.

El procediment per a preparar els tampons en el mètode IS-CE convencional es realitza de la següent manera: es mescla la proporció adequada d'una solució d'electròlit patró amb la quantitat adequada de HCl o NaOH i finalment es dilueixen en aigua o metanol-aigua segons convingui fins a un volum controlat. En el cas del *on-line buffer generation system* la solució tampó s'aconsegueix a partir de la mescla de quatre canals. Se selecciona el BGE i el modificador (HCl o NaOH) i es bombeja per a cada una de les bombes el flux desitjat per a generar qualsevol tampó (a qualsevol pH a força iònica controlada i constant) i/o qualsevol mescla hidro-orgànica. El mesclador de 3 dimensions o *3D micromixer*, basat en les transformacions tridimensionals de Baker [105], aconsegueix un flux únic i uniforme separant el flux en una dimensió i tornant-lo a unir en una segona dimensió (senyalat amb una fletxa vermella a la Figura

3.6). D'aquesta manera es va dissenyar digitalment un model tridimensional i va ser imprès amb una impressora 3D utilitzant resina incolora. Aquest 3D micromixer va ser dissenyat amb quatre canals d'entrada (des de la bomba 1 a la bomba 4) connectats en forma de "Y" com es mostra a la Figura 3.6, creant un flux laminar de quatre seccions diferents. Posteriorment, aquests quatre fluxos laminars es barregen dividint i recombinant els fluxos en un angle perpendicular. Un estudi previ [106] va demostrar que es necessiten un mínim de 4 unitats en el *mixer* per aconseguir una bona mescla. Degut a que aquest sistema té reaccions àcid-base i mescles d'aigua i solvent, es van aplicar un total de 6 unitats per assegurar una barreja completa. La mida del xip és de 32 mm x 20 mm x 14 mm (amplada x alçada x profunditat).

Tenint en compte les consideracions ja comentades sobre els BGE a la secció 1.4.3 del Capítol I, les solucions mare dels electròlits seleccionats són 6 dels estudiats fins ara: $\text{HCOOH}/\text{HCOO}^-$ (pK_a 3.75), $\text{CH}_3\text{COOH}/\text{CH}_3\text{COO}^-$ (pK_a 4.76), $\text{BisTrisH}^+/\text{BisTris}$ (pK_a 6.46), $\text{TrisH}^+/\text{Tris}$ (pK_a 8.08), $\text{CHES}/\text{CHES}^-$ (pK_a 9.55) i $\text{CAPS}/\text{CAPS}^-$ (pK_a 10.40). Els patrons de BGE es preparen a concentració 0.25 M. Per a seleccionar el BGE desitjat s'utilitza una vàlvula de 6 posicions amb 7 ports, i una vàlvula de 2 posicions amb 3 ports per a seleccionar el reactiu per ajustar el pH i la força iònica (HCl o NaOH 0.25 M). La Figura 3.7 mostra una imatge de la vàlvula que controla els electròlits. Els canals de l'aigua i el metanol es connecten directament als recipients receptius.



Figura 3.7. Fotografia de la vàlvula de 6 posicions amb 7 ports que selecciona la solució mare d'electròlit. Els tubs transparents són l'entrada i el tub taronja la sortida. En aquest cas, la fotografia mostra la selecció 6, que representa la succió d'electròlit NaCAPS 0.25 M.

Combinant el sistema *sequential injection – capillary electrophoresis* i el *on-line buffer generation system* es va dissenyar l'instrument d'electroforesi capil·lar per la determinació de constants d'acidesa pel mètode IS-CE, Figura 3.8. Una fotografia d'aquest sistema, tot indicant les parts més destacades, s'il·lustra a la Figura 3.9.

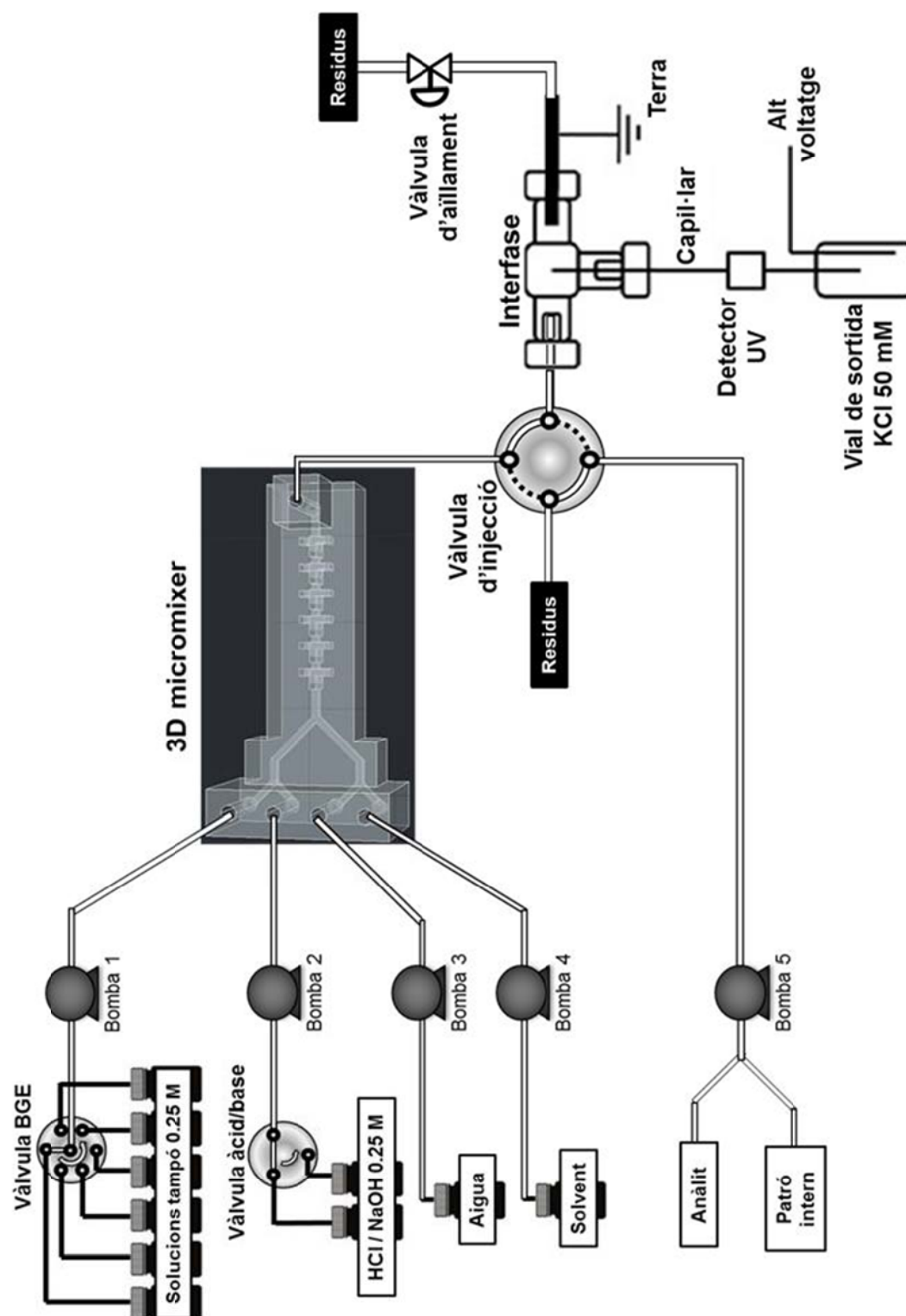


Figura 3.8. Diagrama esquemàtic del prototip d'instrument pel mètode IS-CE.

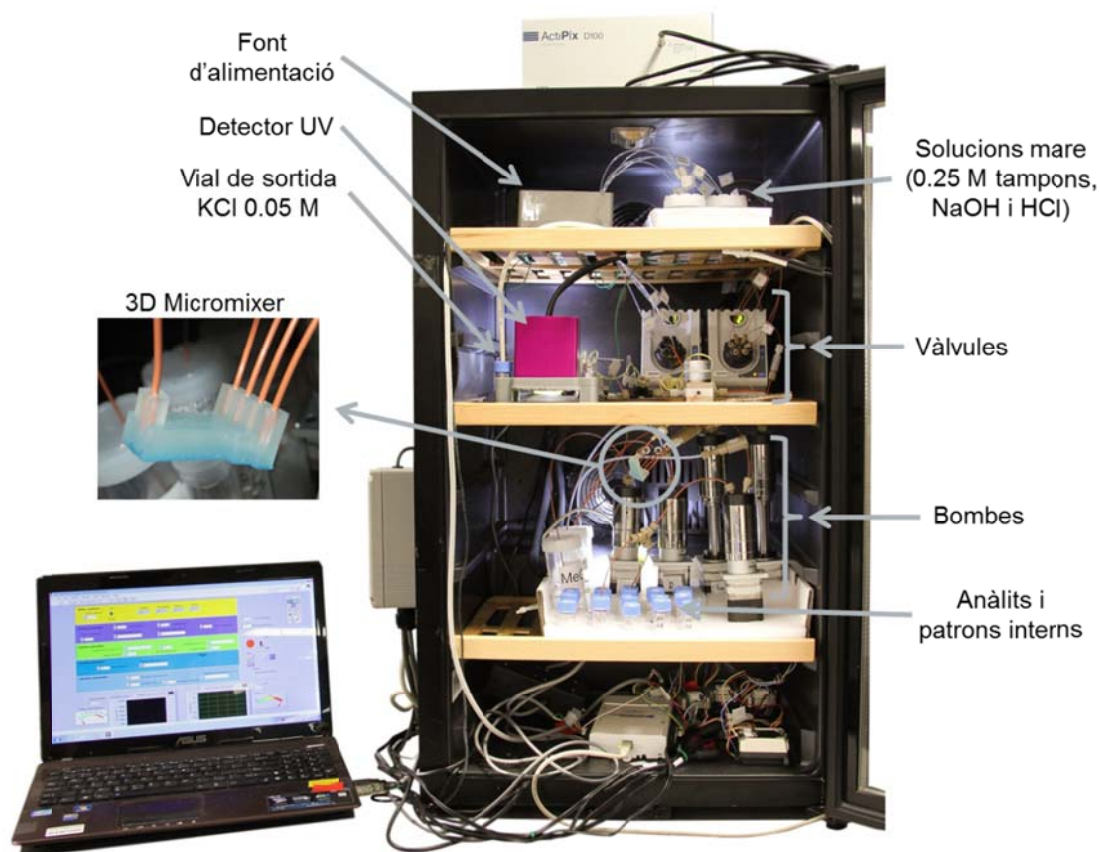


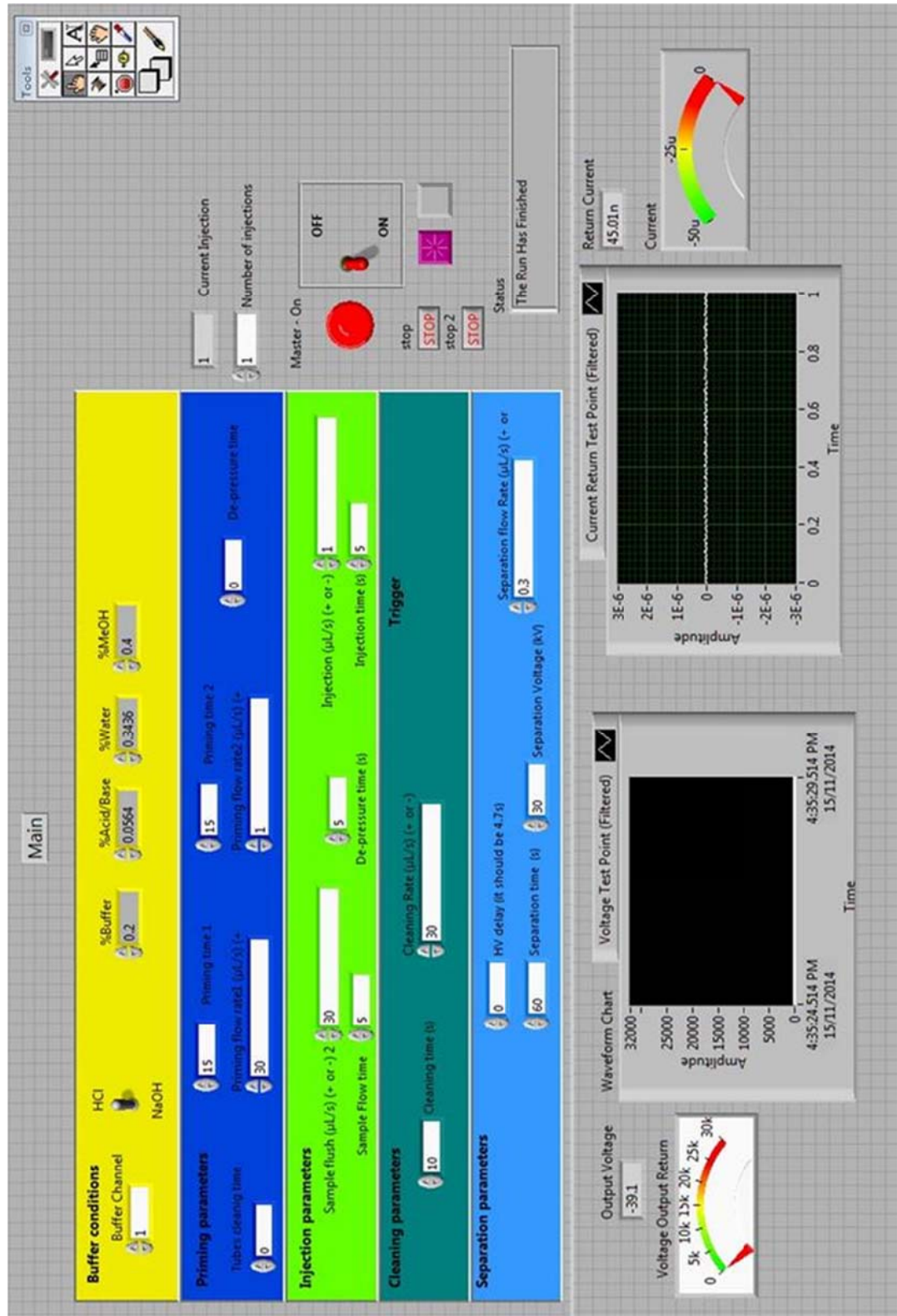
Figura 3.9. Fotografia del sistema per a la determinació automatitzada de constants d'acidesa pel mètode IS-CE.

El sistema d'introducció de la mostra es fa a partir de la succió simultània d'ambdós compostos a través d'una peça en forma de "Y" que els connecta. D'altra banda, degut a la naturalesa dels compostos d'estudi i dels patrons interns, aquest sistema incorpora per primera vegada un detector UV. Aquest detector, en ser metàl·lic i estar a prop de l'elèctrode es va aïllar mitjançant una caixa impresa en 3D tal i com es veu a la Figura 3.10. Altres especificacions tècniques i mecàniques així com també el funcionament del sistema i les condicions electroforètiques es troben àmpliament desglossades a l'article VII. Cal destacar que tot el sistema està connectat amb un portàtil i controlat a través del software LabView v11.0. La Figura 3.11 mostra la pàgina d'execució del software.



Figura 3.10. Fotografia del detector UV ActiPix D100. Les peces fúcsia i gris presents a la part superior i inferior formen la caixa d'aïllament per al detector. La finestra del capil·lar es troba al cartutx negre. El vial blau és on es submergeix la sortida del capil·lar i a on es col·loca l'elèctrode per a l'aplicació d'alt voltatge. A la dreta del detector es pot observar la interfase (peça negra).

Figura 3.11. Pantalla de comandament d'execució del software LabView. En aquest cas es pot veure com el programa genera un tancament de fòrmic al 40% en volum de metanol, barrejant un 20% del tancament NaHCOO 0.25 M, 5.64% de HCl, 34.36% d'aigua i 40% de MeOH. A la part inferior de la figura es veuen els controladors de corrent i voltatge. En el cas que sigui necessari, es poden triar el nombre de rèpliques o canviar els paràmetres de pre- i post-condicionament així com també el voltatge aplicat i el temps de separació.



3.6.2. Determinació automatitzada del pK_a pel mètode IS-CE

Com s'ha comentat al llarg d'aquest treball, un dels avantatges del mètode IS-CE és que no necessita una mesura acurada del pH del tampó. No obstant això, com a qualsevol mètode, cal controlar la força iònica en totes les determinacions. Per aquesta raó, els tampons s'han de preparar amb exactitud a un pH determinat i, sobretot, a força iònica constant.

El pH de les mescles de tampó generades es van comparar amb el pH calculat a 30 valors diferents, entre 2.8 i 12.7 i amb 4-6 valors diferents de pH per a cada electròlit del sistema. La mescla que sortia del 3D *micromixer* es recollia i es mesurava el pH amb un pH-metre convencional. La bona concordança que presenta la Figura 3 de l'article VII i els ajustos de la recta confirmen que la mescla dels 4 fluxos és bona per a tot l'interval de treball. Altrament, per assegurar que la força iònica es manté constant, es van determinar pel mètode clàssic però utilitzant el nou disseny instrumental, els valors de pK_a de 8 àcids i bases de la llista de patrons interns. Els valors d'aquestes constants es van comparar amb els valors de referència de la llista, obtenint variacions inferiors a 0.05 unitats entre ambdós mètodes. En mescles metanol-aigua es va estudiar com la mobilitat i el pK_a de l'ibuprofè variava en funció del % de MeOH. Així mateix, també es va comparar el pK_a experimental d'algunes de les mescles MeOH/H₂O amb el determinat a partir d'equips comercials. A la Figura 6 de l'article VII s'il·lustra la bona concordança entre els valors. Tots aquests resultats demostren la bona barreja de tampons tant aquosos com hidro-orgànics, confirmant el bon funcionament del *on-line buffer generation system*, així com també que el pH i la força iònica dels tampons generats es mantenen constants.

Diversos compostos àcids i bàsics foren seleccionats com a compostos d'assaig per a avaluar el sistema automatitzat IS-CE. Se'n van determinar les constants d'acidesa diverses vegades i per a alguns d'ells es va realitzar amb diversos patrons interns. Els valors resultants es van comparar amb els obtinguts a partir d'equips comercials com ara l'electroforesi capil·lar Beckman (Palo Alto, CA, EUA) i Agilent Technologies (Santa Clara, CA, EUA). Tal com mostra la Taula 3 de l'article VII, els resultats presenten petites diferències entre les constants d'acidesa determinades per l'instrument automatitzat IS-CE i l'instrument comercial de CE, però que no superen una diferència de 0.07 unitats respecte els valors de referència, i la precisió és excel·lent, amb una desviació estàndard de menys de 0.05.

Utilitzant l'instrument automatitzat s'obtenen separacions més ràpides en comparació amb els instruments comercials de Beckman o d'Agilent tecnologies. Això es deu principalment a la utilització de capil·lars molt més curts (L_D 15 cm) i voltatges més alts (30 kV). Degut a que s'utilitza un menor diàmetre intern (25 μ m) i una bona refrigeració, s'evita la calor causada per l'efecte Joule. D'altra banda, el fet d'aplicar pressió a la separació dóna electroferogrames més ràpids, elimina possibles fluctuacions de la corrent i omple el capil·lar de tampó fresc constantment. Totes aquestes condicions permeten obtenir electroferogrames d'entre 20 i 60 s, essent els de les mescles de metanol-aigua els més llargs, a causa de l'augment de la viscositat i la disminució del corrent.

El *on-line buffer generation system* és el major avantatge d'aquest instrument en comparació amb els comercials, i és el primer a incorporar la generació automàtica d'electròlit en línia. El sistema evita la inestabilitat i els errors ambientals i humans, ja que es prepara un tampó nou cada vegada consumint petites quantitats de solucions mare. En altres paraules, el software només requereix un pH i el percentatge de metanol (o qualsevol altre solvent orgànic) per omplir el capil·lar de solució fresca en pocs segons.

Utilitzant el mètode d'IS-CE amb aquest sistema automatitzat d'electroforesi capil·lar, s'augmenta el rendiment per a la determinació de constants d'acidesa. Per a un sol compost es necessiten només dos minuts per a determinar la seva constant d'acidesa i només mitja hora per a un conjunt de 20 compostos. El pK_a de l'ibuprofè s'ha determinat en 20 mescles diferents de MeOH/H₂O en tant sols mitja hora sense la necessitat d'una preparació de tampons prèvia. Tots aquests avantatges fan d'aquest instrument, juntament amb els avantatges propis del mètode IS-CE, una eina simple i ràpida per a la determinació de valors de pK_a en una àmplia gamma de condicions experimentals.

CONCLUSIONS

Del treball realitzat en aquesta Tesi es poden extreure les següents conclusions:

1. La llista general desenvolupada de 48 compostos de naturalesa àcida i bàsica, de diferent estructura i que cobreix tot l'interval de treball en electroforesi capil·lar és una eina útil i fiable per tal de seleccionar de forma simple i ràpida el millor patró intern per a una determinació donada. D'acord amb els bons resultats en comparació amb els valors de la literatura, aquesta llista és adequada per a la determinació de constants d'acidesa de fàrmacs pel mètode IS-CE.
2. El mètode IS-CE és vàlid per a la determinació de compostos polipròtics, fins i tot per a aquelles determinacions on els valors de pK_a són molts extrems o molt pròxims entre ells, i dona bons resultats en termes de precisió i d'exactitud en relació amb els valors de la literatura.
3. Amb un capil·lar curt de 25 cm fins al detector, temps de condicionaments reduïts, un 0.4% de DMSO com a marcador, injecció seqüencial de 0.5 psi per 3 segons, i l'aplicació de 20 kV i 0.5 psi durant la separació, es poden determinar constants d'acidesa en un temps d'entre 3 i 5 minuts si s'utilitzen els tampons i patrons interns recomanats. La determinació ràpida de pK_a i la correlació satisfactòria amb els valors bibliogràfics dels 129 valors de pK_a de 93 compostos conclouen que l'IS-CE és vàlid per a la determinació *high throughput* de constants d'acidesa.
4. La capacitat del mètode IS-CE per a determinar constants d'acidesa de fàrmacs parcialment solubles en medi aquós és del voltant d'una solubilitat intrínseca de 10^{-6} M. No obstant això, per a compostos amb solubilitats des d'aproximadament 10^{-5} M, cal adoptar diverses estratègies simples per vèncer la precipitació durant la determinació del pK_a . Quan la solubilitat és més baixa de 10^{-6} M, la utilització de cosolvent i de procediments d'extrapolació són estrictament necessaris.
5. Les valoracions potenciomètriques i espectrofotomètriques requereixen l'ús de cosolvent per a les determinacions de constants d'acidesa de compostos amb solubilitats més altes que pel mètode IS-CE. Per a les determinacions utilitzant la tècnica CE, en alguns casos el mètode clàssic necessita l'ús cosolvents mentre que el mètode IS-CE permet treballar encara en solució aquosa.

6. Els valors de pK_a del conjunt de 48 patrons interns àcids i bàsics desenvolupats per a mescles de metanol-aigua són vàlids per a la determinació sistemàtica de constants d'acidesa de fàrmacs entre el 0 i 40% de metanol.
7. Treballant en composicions metanol-aigua, el mètode *high throughput* IS-CE pot determinar constants d'acidesa aquoses de fàrmacs comercials molt poc solubles, amb solubilitats per sota de 10^{-6} M. En comparació amb altres mètodes, la potenciomètria, l'espectrofotometria i els mètodes basats en CE requereixen l'ús de percentatges de metanol més alts per a aquestes determinacions (40% mínim). El mètode IS-CE pot determinar les constants d'acidesa d'aquests compostos utilitzant només el 10% de metanol, obtenint d'aquesta manera extrapolacions més bones quan es determina la constant d'acidesa aquosa.
8. Amb 5 bombes, 4 vàlvules i un 3D micromixer per combinar 4 fluxos diferents en un flux únic i homogeni, l'instrument dissenyat i construït per a l'IS-CE determina constants d'acidesa de forma ràpida i automatitzada, sense importar la complexitat, nombre de constants d'acidesa o solubilitat. El *on-line buffer generation system* produeix tampons de pH entre 2.8 a 12.7 i a força iònica constant en medis aquosos i medis metanol-aigua (fins al 40% de metanol) en pocs segons. Amb capil·lars curts de 15 cm fins al detector, alts voltatges (30 kV) i l'aplicació de pressió durant la separació, s'obtenen separacions de 20 segons i determinacions de pK_a en 2 minuts. La baixa desviació estàndard dels replicats i la baixa variació en comparació amb els valors de referència mostren que aquest sistema pot determinar constants d'acidesa de fàrmacs utilitzant el ràpid i fiable mètode IS-CE.
9. Com a conclusió general, l'IS-CE per a la determinació de pK_a per a electroforesi capil·lar és un mètode *high throughput*, robust, simple i fiable. Així mateix, el disseny del nou prototip CE automatitzat juntament amb el *on-line buffer generation system* es presenta com a una instrumentació idònia per a l'anàlisi rutinari de fàrmacs potencials en laboratoris de mesures fisicoquímiques i de *drug discovery and development*.

The results obtained throughout the Thesis have led to the following conclusions:

1. The developed reference set of 48 compounds with different structures that cover all the useful pH range in capillary electrophoresis is a useful and fast tool that allows selecting in a simple and fast way the best internal standard for a given determination. The good agreement with the literature values proves that this set is suitable to the pK_a determination of drug by the IS-CE method.
2. The IS-CE method is able to determine pK_a values of polyprotic compounds, even though for those compounds with extreme or very close pK_a values, and gives good accuracy and precision when there are related with literature.
3. With a short capillary of 25 cm to the detector, reduced capillary conditioning times, 0.4% DMSO marker, hydrodynamic sequential injection at 0.5 psi for 3s, and the application of 20 kV and 0.5 psi during separation, a pK_a can be obtained in about 3-5 min using the proposed buffers and ISs. The fast pK_a determination and the good agreement with the 129 pK_a values from 93 drugs, conclude that the IS-CE method is meaningful for high throughput determination of acidity constants.
4. The ability of the high throughput IS-CE method to determine the pK_a values of poorly soluble drugs is around an intrinsic solubility value of 10^{-6} M. However, from compounds with solubility values around 10^{-5} M, different simple strategies should be adopted to avoid precipitation of the compound during the pK_a determination process. When the drug has solubility lower than 10^{-6} M, the use of cosolvents and extrapolation procedures is strictly necessary.
5. Potentiometry and spectrophotometric titrations require the use of cosolvents for determination of acidity constants of compounds with higher solubilities than the IS-CE method. Regarding other CE-based methods, the classic CE method needs in some cases the use of cosolvents, whereas in the IS-CE method it is still not needed.
6. The developed pK_a values of the 48 acidic and basic internal standards set for methanol-water mixtures is suitable to determine acidity constants of drugs ranging from 0 to 40% (v/v) of methanol.

7. Working with methanol-water mixtures, the high throughput IS-CE method is able to determine acidity constants of sparingly soluble commercial drugs, with aqueous solubility under 10^{-6} M. Compared to other methods, potentiometry and spectrophotometry titrations as well as other CE-based methods require the use of higher methanol percentages in the determinations (40% minimum). The IS-CE method is able to determine the acidity constants of these water sparingly soluble compounds at only 10% of methanol, obtaining therefore better extrapolations when aqueous pK_a is determined.
8. With 5 pumps, 4 valves and a 3D micromixer to combine four different flows into one single homogeneous flow, the instrument designed and built for the IS-CE method determines acidity constants quickly and automatically, regardless its complexity, number of pK_a values or solubility. The on-line buffer generation system produces buffers from pH 2.8 to 12.7 at constant ionic strength in aqueous media and methanol-water mixtures (until 40% of methanol) in few seconds. With short capillaries of just 15 cm to the detector, high voltages (30 kV) and applying pressure, separations of 20 s are obtained, and 2 min are required for a pK_a determination. The low standard deviation of the replicates and the low variation compared to the reference values show this system can determine acidity constants of drugs by the use of the fast and reliable IS-CE method.
9. As a general conclusion, the IS-CE for the pK_a determination by capillary electrophoresis is a high throughput, robust, simple and reliable method. Furthermore, the design of the novel CE prototype together with the on-line buffer generation system is presented as an adequate instrumentation for the routine analysis in physicochemical measurements and drug discovery and development laboratories.

REFERÈNCIES

- [1] R.F. Cookson, The Determination of Acidity Constants, Chemical Reviews. 1972 (1972).
- [2] A. Avdeef, Absorption and drug development: Solubility, permeability and charge state, Second Ed., Hoboken, NJ: John Wiley & Sons, New Jersey, 2012.
- [3] E.H. Kerns, High throughput physicochemical profiling for drug discovery, Journal of Pharmaceutical Sciences 90 (2001) 1838–1858.
- [4] E. Kerns, Pharmaceutical profiling in drug discovery, Drug Discovery Today 8 (2003) 316–323.
- [5] C.E. Kibbey, S.K. Poole, B. Robinson, J.D. Jackson, D. Durham, An integrated process for measuring the physicochemical properties of drug candidates in a preclinical discovery environment., Journal of Pharmaceutical Sciences 90 (2001) 1164–75.
- [6] H. Wan, J. Ulander, High-throughput pK_a screening and prediction amenable for ADME profiling, Expert Opinion on Drug Metabolism and Toxicology 2 (2006) 139–155.
- [7] J.I. Wells, Pharmaceutical Preformulation: The Physicochemical Properties of Drug Substances, Ellis Horwood, London, 1998.
- [8] J.B. Taylor, D.J. Triggle, J.E.A. Comer, Ionization Constants and Ionization Profiles, Comprehensive Medicinal Chemistry II, 2007, 357–397.
- [9] J.B. Dressman, G.L. Amidon, C. Reppas, V.P. Shah, Dissolution Testing as a Prognostic Tool for Oral Drug Absorption: Immediate Release Dosage Forms, Pharmaceutical Research 15 (1998) 11–22.
- [10] P.A. Shore, B.B. Brodie, C.A.M. Hogben, The gastric secretion of drugs; a pH partition hypothesis, Journal of Pharmacology and Experimental Therapeutics 119 (1957) 361–369.
- [11] A. Avdeef, Physicochemical profiling (solubility, permeability and charge state), Current Topics in Medicinal Chemistry 1 (2001) 277–351.
- [12] L. Castro, I. Collins, M.G.N. Russell, A.P. Watt, B. Sohal, D. Rathbone, M.S. Beerm J.A. Stanton, Enhancement of Oral Absorption in Selective 5-HT 1D Receptor Agonists: Fluorinated 3-[3-(Piperidin-1-yl)propyl]indoles, Journal of Medicinal Chemistry 2623 (1998) 2667–2670.
- [13] G. Camenisch, G. Folkers, H. van de Waterbeemd, Review of theoretical passive drug absorption models: Historical background, recent developments and limitations, Pharmaceutica Acta Helvetiae 71 (1996) 309–327.
- [14] N. Nagahara, S. Tavelin, P. Artursson, Contribution of the paracellular route to the pH-dependent epithelial permeability to cationic drugs, Journal of Pharmaceutical Sciences 93 (2004) 2972–84.
- [15] T. Kimura, T. Nakayama, Y. Kurosaki, Y. Suzuki, S. Arimoto, H. Hayatsu, Absorption of 3-amino-1-methyl-5H-pyrido[4,3-b]indole, a mutagen-carcinogen

- present in tryptophan pyrolyzate, from the gastrointestinal tract in the rat, *Japanese Journal of Cancer Research* 76 (1985) 272–277.
- [16] A.G. Prentice, A. Glasmacher, Making sense of itraconazole pharmacokinetics, *The Journal of Antimicrobial Chemotherapy* 56 (2005) 17–22.
 - [17] V. Gobry, G. Bouchard, P.A. Carrupt, B. Testa, H.H. Girault, Physicochemical Characterization of Sildenafil: Ionization, Lipophilicity Behavior, and Ionic-Partition Diagram Studied by Two-Phase Titration and Electrochemistry, *Helvetica Chimica Acta* 83 (2000) 1465–1474.
 - [18] L.J. Henderson, *Americal Journal of Physiology* 21 (1908) 173–179.
 - [19] K.A. Hasselbalch, *Biochemische Zeitschrift* 78 (1916) 112–144.
 - [20] J. Alsenz, M. Kansy, High throughput solubility measurement in drug discovery and development, *Advanced Drug Delivery Reviews* 59 (2007) 546–567.
 - [21] K. Takács-Novák, K.J. Box, A. Avdeef, Potentiometric pK_a determination of water-insoluble compounds: validation study in methanol/water mixtures, *International Journal of Pharmaceutics* 151 (1997) 235–248.
 - [22] A. Avdeef, K.J. Box, J.E. Comer, M. Gilges, M. Hadley, C. Hibbert, W. Patterson, K.Y. Tam, PH-metric log P 11. pK_a determination of water-insoluble drugs in organic solvent-water mixtures, *Journal of Pharmaceutical and Biomedical Analysis* 20 (1999) 631–641.
 - [23] A. Avdeef, J.E.A. Comer, S.J. Thomson, pH-Metric log P 3. Glass electrode calibration in methanol-water, applied to pK_a determination of water-insoluble substances, *Analytical Chemistry* 65 (1993) 42–49.
 - [24] S. Bellini, M. Uhrova, Z. Deyl, Determination of the thermodynamic equilibrium constants of water-insoluble (sparingly soluble) compounds by capillary electrophoresis, *Journal of Chromatography A* 772 (1997) 91–101.
 - [25] V. de Nogales, R. Ruiz, M. Rosés, C. Ràfols, E. Bosch, Background electrolytes in 50% methanol/water for the determination of acidity constants of basic drugs by capillary zone electrophoresis, *Journal of Chromatography A* 1123 (2006) 113–120.
 - [26] F.Z. Erdemgil, S. Sanli, N. Sanli, G. Ozkan, J. Barbosa, J. Guiteras, J.L. Beltrán, Determination of pK_a values of some hydroxylated benzoic acids in methanol-water binary mixtures by LC methodology and potentiometry, *Talanta* 72 (2007) 489–496.
 - [27] M. Yasuda, Dissociation constants of some carboxylic acids in mixed aqueous solvents, *Bulletin of the Chemical Society of Japan* 32 (1959) 429–432.
 - [28] T. Shedlovsky, The behavior of carboxylic acids in mixed solvents, *Electrolytes, Proc. International Symposium, Trieste, Yugoslavia* (1962) 146–151.
 - [29] W.T. Comer, *Perspectives in Drug Discovery, Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, 2013.

- [30] R. Ganellin, S. Roberts, R. Jefferis, M. Stocks, The small molecule drug discovery process – from target selection to candidate selection, *Introduction to Biological and Small Molecule Drug Research and Development*, 2013, 81–126.
- [31] S.K. Poole, S. Patel, K. Dehring, H. Workman, C.F. Poole, Determination of acid dissociation constants by capillary electrophoresis, *Journal of Chromatography A* 1037 (2004) 445–454.
- [32] A. Albert, E.P. Serjeant, *The Determination of Ionization Constants: A Laboratory Manual*, 3rd Ed, Chapman & Hall, London, 1984.
- [33] M.A. Herrador, a G. González, Potentiometric titrations in acetonitrile-water mixtures: evaluation of aqueous ionisation constant of ketoprofen, *Talanta* 56 (2002) 769–775.
- [34] Z. Qiang, C. Adams, Potentiometric determination of acid dissociation constants (pK_a) for human and veterinary antibiotics, *Water Research* 38 (2004) 2874–2890.
- [35] G. Garrido, V. de Nogales, C. Ràfols, E. Bosch, Acidity of several polyprotic acids, amiodarone and quetiapine hemifumarate in pure methanol, *Talanta* 73 (2007) 115–120.
- [36] K. Box, C. Bevan, J. Comer, A. Hill, R. Allen, D. Reynolds, High-Throughput Measurement of pK_a Values in a Mixed-Buffer Linear pH Gradient System, *Analytical Chemistry* 75 (2003) 883–892.
- [37] B. Bard, S. Martel, P.A. Carrupt, High throughput UV method for the estimation of thermodynamic solubility and the determination of the solubility in biorelevant media, *European Journal of Pharmaceutical Sciences* 33 (2008) 230–240.
- [38] K.J. Box, G. Völgyi, R. Ruiz, J.E. Comer, K. Takács-Novák, E. Bosch, C. Ràfols, M. Rosés, Physicochemical properties of a new multicomponent cosolvent system for the pK_a determination of poorly soluble pharmaceutical compounds, *Helvetica Chimica Acta* 90 (2007) 1538–1553.
- [39] K.Y. Tam, K. Takács-Novák, Multi-wavelength spectrophotometric determination of acid dissociation constants: a validation study, *Analytica Chimica Acta* 434 (2001) 157–167.
- [40] J.A. Cleveland Jr., M.H. Benko, S.J. Gluck, Y.M. Walbroehl, Automated pK_a determination at low solute concentrations by capillary electrophoresis, *Journal of Chromatography A* 652 (1993) 301–308.
- [41] S.J. Gluck, J.A. Cleveland Jr., Capillary zone electrophoresis for the determination of dissociation constants, *Journal of Chromatography A* 680 (1994) 43–48.
- [42] S.J. Gluck, J.A. Cleveland Jr., Investigation of experimental approaches to the determination of pK_a values by capillary electrophoresis, *Journal of Chromatography A* 680 (1994) 49–56.

- [43] S.J. Gluck, K.P. Steele, M.H. Benkö, Determination of acidity constants of monoprotic and diprotic acids by capillary electrophoresis, *Journal of Chromatography A* 745 (1996) 117–125.
- [44] Z. Jia, T. Ramstad, M. Zhong, Medium-throughput pK_a screening pharmaceuticals by pressure-assisted capillary electrophoresis, *Electrophoresis* 22 (2001) 1112–1118.
- [45] Y. Ishihama, M. Nakamura, T. Miwa, T. Kajima, N. Asakawa, A rapid method for pK_a determination of drugs using pressure-assisted capillary electrophoresis with photodiode array detection in drug discovery, *Journal of Pharmaceutical Sciences* 91 (2002) 933–942.
- [46] H. Wan, A. Holmén, M. Någård, W. Lindberg, Rapid screening of pK_a values of pharmaceuticals by pressure-assisted capillary electrophoresis combined with short-end injection, *Journal of Chromatography A* 979 (2002) 369–377.
- [47] C. Zhou, Y. Jin, J.R. Kenseth, M. Stella, K.R. Wehmeyer, W.R. Heineman, Rapid pK_a estimation using vacuum-assisted multiplexed capillary electrophoresis (VAMCE) with ultraviolet detection, *Journal of Pharmaceutical Sciences* 94 (2005) 576–589.
- [48] H. Pang, J. Kenseth, S. Coldiron, High-throughput multiplexed capillary electrophoresis in drug discovery, *Drug Discovery Today* 9 (2004) 1072–1080.
- [49] M. Shalaeva, J. Kenseth, F. Lombardo, A. Bastin, Measurement of dissociation constants (pK_a values) of organic compounds by multiplexed capillary electrophoresis using aqueous and cosolvent buffers, *Journal of Pharmaceutical Sciences* 97 (2008) 2581–2606.
- [50] S.M.C. Buckenmaier, D. V McCalley, M.R. Euerby, Determination of ionization constants of organic bases in aqueous methanol solutions using capillary electrophoresis, *Journal of Chromatography A* 1026 (2004) 251–259.
- [51] J.M. Herrero-Martinez, M. Sanmartin, M. Roses, E. Bosch, C. Rafols, Determination of dissociation constants of flavonoids by capillary electrophoresis, *Electrophoresis* 26 (2005) 1886–1895.
- [52] E. Fuguet, M. Reta, C. Gibert, M. Rosés, E. Bosch, C. Ràfols, Critical evaluation of buffering solutions for pK_a determination by capillary electrophoresis, *Electrophoresis* 29 (2008) 2841–2851.
- [53] A. Šlampová, L. Křivánková, P. Gebauer, P. Boček, Standard systems for measurement of pK_s and ionic mobilities: 1. Univalent weak acids, *Journal of Chromatography A* 1213 (2008) 25–30.
- [54] A. Šlampová, L. Křivánková, P. Gebauer, P. Boček, Standard systems for measurement of pK values and ionic mobilities: 2. Univalent weak bases, *Journal of Chromatography A* 1216 (2009) 3637–3641.
- [55] P. Nowak, M. Woźniakiewicz, P. Kościelniak, Application of capillary electrophoresis in determination of acid dissociation constant values, *Journal of Chromatography A* 1377 (2015) 1–12.

- [56] P. Wiczling, P. Kawczak, A. Nasal, R. Kaliszan, Simultaneous determination of pK_a and lipophilicity by gradient RP HPLC, *Analytical Chemistry* 78 (2006) 239–249.
- [57] J. Barbosa, D. Barrón, E. Jiménez-Lozano, V. Sanz-Nebot, Comparison between capillary electrophoresis, liquid chromatography, potentiometric and spectrophotometric techniques for evaluation of pK_a values of zwitterionic drugs in acetonitrile–water mixtures, *Analytica Chimica Acta* 437 (2001) 309–321.
- [58] C. Foulon, N. Duhal, B. Lacroix-Callens, C. Vaccher, J.P. Bonte, J.F. Goossens, Determination of pK_a values of benzoxa-, benzothia- and benzoselena-zolinone derivatives by capillary electrophoresis. Comparison with potentiometric titration and spectrometric data, *European Journal of Pharmaceutical Sciences* 31 (2007) 165–171.
- [59] J. Reijenga, H. van Arno, L. van Antonie, B. Teunissen, Development of methods for the determination of pK_a values, *Analytical Chemistry Insights* 8 (2013) 53–71.
- [60] M. Andrasi, P. Buglyo, L. Zekany, A. Gaspar, A comparative study of capillary zone electrophoresis and pH-potentiometry for determination of dissociation constants, *Journal of Pharmaceutical and Biomedical Analysis* 44 (2007) 1040–1047.
- [61] J.L. Beltrán, N. Sanli, G. Fonrodona, D. Barrón, G. Özkan, J. Barbosa, Spectrophotometric, potentiometric and chromatographic pK_a values of polyphenolic acids in water and acetonitrile–water media, *Analytica Chimica Acta* 484 (2003) 253–264.
- [62] K.J. Box, R.E. Donkor, P. a Jupp, I.P. Leader, D.F. Trew, C.H. Turner, The chemistry of multi-protic drugs Part 1: a potentiometric, multi-wavelength UV and NMR pH titrimetric study of the micro-speciation of SKI-606, *Journal of Pharmaceutical and Biomedical Analysis* 47 (2008) 303–311.
- [63] K.J. Box, J.E.A. Comer, Using measured pK_a , LogP and solubility to investigate supersaturation and predict BCS class, *Current Drug Metabolism* 9 (2008) 869–878.
- [64] D-PAS Instruction Manual, Revision 3, Sirius Analytical Instruments Ltd, Riverside, UK.
- [65] R.B. Baker, *Capillary Electrophoresis*, Wiley-Interscience Publication, New York, 1995.
- [66] D. Heiger, *High Performance Capillary Electrophoresis. An Introduction*, Agilent Technologies, Waldbronn, 2000.
- [67] J.M. Miller, A.C. Blackburn, Y. Shi, A.J. Melzak, H.Y. Ando, Semi-empirical relationships between effective mobility, charge, and molecular weight of pharmaceuticals by pressure-assisted capillary electrophoresis: applications in drug discovery, *Electrophoresis* 23 (2002) 2833–2841.

- [68] J.L. Wang, X.J. Xu, D.Y. Chen, Determination of pK_a values of a triptolide derivative and its impurities by pressure-assisted capillary electrophoresis, *Journal of Pharmaceutical and Biomedical Analysis* 88 (2014) 22–26.
- [69] L. Geiser, Y. Henchoz, A. Galland, P.A. Carrupt, J.L. Veuthey, Determination of pK_a values by capillary zone electrophoresis with a dynamic coating procedure, *Journal of Separation Science* 28 (2005) 2374–2380.
- [70] C. a Currie, W.R. Heineman, H.B. Halsall, C.J. Seliskar, P. a Limbach, F. Arias, K.R. Wehmeyer, Estimation of pK_a values using microchip capillary electrophoresis and indirect fluorescence detection, *Journal of Chromatography B* 824 (2005) 201–205.
- [71] H. Ren, L. Wang, X. Wang, X. Liu, S. Jiang, Measurement of acid dissociation constants and ionic mobilities of 3-nitro-tyrosine and 3-chloro-tyrosine by capillary zone electrophoresis, *Journal of Pharmaceutical and Biomedical Analysis* 77 (2013) 83–87.
- [72] X. Gong, M. Figus, J. Plewa, D.A. Levorse, L. Zhou, C.J. Welch, Evaluation of Multiplexed CE with UV Detection for Rapid pK_a Estimation of Active Pharmaceutical Ingredients, *Chromatographia* 68 (2008) 219–225.
- [73] C.H.R. Martínez, C. Dardonville, Rapid Determination of Ionization Constants pK_a by UV Spectroscopy Using 96-Well Microtiter Plates, *ACS Medicinal Chemistry Letters* 4 (2013) 142–145.
- [74] H. Wan, A.G. Holmén, Y. Wang, W. Lindberg, M. Englund, M.B. Någård, R.A. Thompson, High-throughput screening of pK_a values of pharmaceuticals by pressure-assisted capillary electrophoresis and mass spectrometry, *Rapid Communications in Mass Spectrometry: RCM* 17 (2003) 2639–2648.
- [75] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, A Fast Method for pK_a Determination by Capillary Electrophoresis, *Chemistry & Biodiversity* 6 (2009) 1822–1827.
- [76] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, Fast high-throughput method for the determination of acidity constants by capillary electrophoresis: I. Monoprotic weak acids and bases, *Journal of Chromatography A* 1216 (2009) 3646–3651.
- [77] E. Fuguet, C. Ràfols, M. Rosés, A fast high throughput method for the determination of acidity constants by capillary electrophoresis. 3. Basic internal standards, *Journal of Chromatography A* 1218 (2011) 3928–3934.
- [78] J.L. Beckers, P. Bocek, The preparation of background electrolytes in capillary zone electrophoresis: Golden rules and pitfalls, *Electrophoresis* 24 (2003) 518–535.
- [79] S.P. Porras, M.L. Riekkola, E. Kenndler, The principles of migration and dispersion in capillary zone electrophoresis in nonaqueous solvents, *Electrophoresis* 24 (2003) 1485–1498.
- [80] ACD/Percepta Platform v2012 Software, ACD/Labs 2012.
- [81] SPARC Online Calculator: <http://archemcalc.com/sparc/pKa/>.

- [82] R.S. Gravador, E. Fuguet, C. Ràfols, M. Rosés, Temperature variation effects on the determination of acidity constants through the internal standard-capillary electrophoresis method, *Electrophoresis* 34 (2013) 1203–1211.
- [83] C.A. Lipinski, Solubility in water and DMSO: Issues and potential solutions, *Biotechnology: Pharmaceutical Aspects* 1 (2004) 93–125.
- [84] C. Lipinski, Drug solubility in water and dimethylsulfoxide, *Methods and Principles in Medicinal Chemistry* 37 (2008) 257–282.
- [85] E. Lipka, M. Folly-Klan, J. Charton, M.P. Vaccher, J.P. Bonte, C. Vaccher, Determination of pK_a values of benzimidazole derivatives from mobility obtained by capillary electrophoresis, *Electromigration Techniques in Pharmaceutical and Biomedical Analysis - In Honour of Professor Jacques Crommen* 53 (2010) 1267–1271.
- [86] T. Zhu, Y. Sun, C. Zhang, D. Ling, Z. Sun, Variation of the pH of the background electrolyte as a result of electrolysis in capillary electrophoresis, *Journal of High Resolution Chromatography* 17 (1994) 563–564.
- [87] C. Horváth, W. Melander, I. Molnár, Liquid Chromatography of Ionogenic Substances with Nonpolar Stationary Phases, *Analytical Chemistry* 49 (1977) 142–154.
- [88] S.F. Donovan, M.C. Pescatore, Method for measuring the logarithm of the octanol – water partition coefficient by using short octadecyl – poly(vinyl alcohol) high-performance liquid chromatography columns, *Journal of Chromatography A* 952 (2002) 47–61.
- [89] K. Valko, Ch.M. Du, C. Bevan, D.P. Reynolds, M.H. Abraham, Rapid method for estimating octanol-water partition coefficient (log POCT) from isocratic RP-HPLC and a hydrogen bond acidity term (E_{a2H}), *Current Medicinal Chemistry* 8 (2001) 1137–1146.
- [90] A. Andrés, M. Rosés, C. Ràfols, E. Bosch, S. Espinosa, V. Segarra, J.M. Huerta, Manuscript in preparation.
- [91] C.M. Wassvik, A.G. Holmén, C. a S. Bergström, I. Zamora, P. Artursson, Contribution of solid-state properties to the aqueous solubility of drugs, *European Journal of Pharmaceutical Sciences* 29 (2006) 294–305.
- [92] W. Yang, M.M. de Villiers, Effect of 4-sulphonato-calix[n]arenes and cyclodextrins on the solubilization of niclosamide, a poorly water soluble anthelmintic, *The AAPS Journal* 7 (2005) E241–248.
- [93] M. Rosés, F. Rived, E. Bosch, Dissociation constants of phenols in methanol–water mixtures, *Journal of Chromatography A* 867 (2000) 45–56.
- [94] F. Rived, I. Canals, E. Bosch, M. Rosés, Acidity in methanol–water, *Analytica Chimica Acta* 439 (2001) 315–333.
- [95] V. Palm, *Tables of Rate and Equilibrium Constants of Heterolytic Organic Reactions*, VINITI, 1975.

- [96] C. Ràfols, M. Rosés, E. Bosch, A comparison between different approaches to estimate the aqueous pK_a values of several non-steroidal anti-inflammatory drugs, *Analytica Chimica Acta* 338 (1997) 127–134.
- [97] G. Völgyi, R. Ruiz, K. Box, J. Comer, E. Bosch, K. Takács-Novák, Potentiometric and spectrophotometric pK_a determination of water-insoluble compounds: validation study in a new cosolvent system, *Analytica Chimica Acta* 583 (2007) 418–428.
- [98] A. Avdeef, High-throughput measurements of solubility profiles, B. Testa, H. Van de Waterbeemd, G. Folkers, R. Guy, *Pharmacokinetic Optimization in Drug Research*, Verlag Helvetica Chimica Acta, Weinheim, 2001, 305–325.
- [99] S. Kulka, G. Quintás, B. Lendl, Automated sample preparation and analysis using a sequential-injection-capillary electrophoresis (SI-CE) interface, *The Analyst* 131 (2006) 739–744.
- [100] A. Wuersig, P. Kubán, S.S. Khaloo, P.C. Hauser, Rapid electrophoretic separations in short capillaries using contactless conductivity detection and a sequential injection analysis manifold for hydrodynamic sample loading, *The Analyst* 131 (2006) 944–949.
- [101] G. a Blanco, Y.H. Nai, E.F. Hilder, R. a Shellie, G.W. Dicinoski, P.R. Haddad, M.C. Breadmore, Identification of inorganic improvised explosive devices using sequential injection capillary electrophoresis and contactless conductivity detection, *Analytical Chemistry* 83 (2011) 9068–9075.
- [102] A.J. Gaudry, R.M. Guijt, M. Macka, J.P. Hutchinson, C. Johns, E.F. Hilder, G.W. Dicinoski, P.N. Nesterenko, P.R. Haddad, M.C. Breadmore, On-line simultaneous and rapid separation of anions and cations from a single sample using dual-capillary sequential injection-capillary electrophoresis, *Analytica Chimica Acta* 781 (2013) 80–87.
- [103] A.J. Gaudry, Y.H. Nai, R.M. Guijt, M.C. Breadmore, Polymeric microchip for the simultaneous determination of anions and cations by hydrodynamic injection using a dual-channel sequential injection microchip electrophoresis system, *Analytical Chemistry* 86 (2014) 3380–3388.
- [104] A. a Alhusban, A.J. Gaudry, M.C. Breadmore, N. Gueven, R.M. Guijt, On-line sequential injection-capillary electrophoresis for near-real-time monitoring of extracellular lactate in cell culture flasks, *Journal of Chromatography A* 1323 (2014) 157–162.
- [105] P. Carrière, On a three-dimensional implementation of the baker's transformation, *Physics of Fluids* 19 (2007) 118110.
- [106] A.I. Shallan, P. Smejkal, M. Corban, R.M. Guijt, M.C. Breadmore, Cost-Effective Three-Dimensional Printing of Visibly Transparent Microchips within Minutes, *Analytical Chemistry* 86 (2014) 3124–3130.