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RESEARCH ARTICLE

Characterization of Glycoproteins by Capillary Electrophoresis Electrospray Mass Spectrometry (CE-ES-MS). Applications to Diagnosis in Biomedicine

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Characterization of glycoproteins by capillary electrophoresis electrospray mass spectrometry (CE-ES-MS). Applications to diagnosis in biomedicine
Summary

Glycosylation is the most common posttranslational modification in proteins and the carbohydrates participate in many biological processes. The number and type of glycoforms for a certain glycoprotein may change as a consequence of pathological processes. In our work a method for the separation of transferrin sialoforms has been developed, that permits the diagnostic of Congenital Disorders of Glycosylation (CDG) using a polybrene-dextran sulphate coating and CE-ESI-TOF methodologies. In order to improve the sensitivity the use of solid-phase extraction coupled on-line to CE-ESI-MS is studied and the SPE-CE-ESI-MS developed methods are applied for the characterization of rHuEPO glycoforms. The achieved separation and the high mass-resolving power of flight (TOF) mass detection allows to establish the most probable rHuEPO glycoforms.

Key words: *Capillary Electrophoresis, Glycoproteins, Electrospray Mass Spectrometry, Proteins.*
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Glikoproteinlerin Kapilerelektroforez Elektrosprey Kütle spektrometrisi ile Karakterizayonu (KE-ES-KS). Biomedikal Uygulamaları
Özet

Glikozilasyon, çoğu biyolojik süreçte yer alan protein ve karbohidratların posttranslasyonal modifikasyon şeklidir. Glikoproteinin glikozillenme sayısı ve şekli patolojik sürecin sonucu olarak farklılık gösterebilir. Çalışmamızda konjenital glikozillenme bozukluklarının teşhisine olanak veren ve polibren-dekstran sülfat kaplaması ve CE-ESI-TOF kullanılarak transferrin sialoform ayrıştırma yöntemi geliştirildi. Duyarlığı artırmak için katı-faz ekstraksiyonu ile CE-ESI-MS eşzamanlı olarak çalışıldı ve SPE-CE-ESI-MS yönetemi rHuEPO glikoformlarının karakterizasyonuna uyarlandı. Yüksek kütle-ayırım güçlü kütle spektrometrik dedektör kullanılarak en olası rHuEPO glikoformlarının ortaya çıkmasını sağladı.

Anahtar Kelimeler: *Kapiler elektroforez, Glikoproteinler, Electrospray Kütle spektrometrisi, Proteinler.*

INTRODUCTION

Genomics and, even more important, the wide field of proteomic and related clinical applications like biomarkers, dramatically increase the demand of sensitive and selective analytical tools for the analysis of biological samples. The sugar content of proteins has been demostrated to be critical for its biological activity, and it is influenced during its manufacturing process by the cell line and the incubation culture conditions[1]. The polymorphism associated with the amount, the size, and the structur e of the carbohydrate chains is known as microheterogeneity, and the molecular species generated ar e termed glycoforms. In this context, investigations of glycoproteins have become increasing important, in particular with respect to the variations in glycosylation patterns observed in serum from healthy individuals and patiens.

Glycosylation is the most common posttranslational modification in proteins and carbohydrates participate in many biological processes and encode information

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for molecular recognition, protein folding, stability and pharmacokinetics[2]. The number and type of glycoforms for a certain glycoprotein may change as a consequence of pathological processes[3]. For example, patients with Congenital Disor ders of Glycosylation (CDG) or chronic alcoholism present hypoglycosylation of several plasmatic glycoproteins as transferrin (Tf), which is analyzed as a model glycoprotein for CDG diagnosis[4]. Tf is one of the twenty high abundance human plasma proteins. Other different analytical problem is analysis of erythropoietin (EPO) which is found as very low abundance protein. EPO is a glycoprotein hormone, which regulates erythropoiesis and has been extensively used for the treatment of several anemias associated with acute and chronic diseases[5]. Despite the many benefits of EPO in the clinic, it has been most widely publicized on account of its extensive misuse as performance enhancing agent in endurance sports[6].

Until recently, isoelectric focusing electrophoresis was the reference method for glycoforms analysis due to its high selectivity allowing an easy detection of genetic glycoprotein variants. However, this complex and time consuming procedure favoured the introduction of new alternative methods. Capillary electrophoresis (CE), has become one of the most important techniques for glycoform separation[7]. In previous studies Tf sialoforms were resolved by CE-UV [4]. Also the two commercial ready-to-use pharmaceuticals of recombinant human EPO (rHuEPO), epoetin-α and epoetin-β, and the hyperglycosylated EPO analogue NESP was analysed[8, 9] . Separation and characterization of the different glycoforms from each glycoprotein are presented. However endogenous EPO are generally found at subnanomolar levels such as other endogenous hormones and the poor concentration detection sensitivity of CE precludes the direct analysis of these hormones at the levels found in biological fluids.

The high values of the concentration limits of detection in CE are closely related with the small volume capacity of the capillary columns. Several instrumental, electrophoretic and chromatographic modifications have been described in order to overcome this limitation. In solid phase extraction coupled on-line to capillary electrophoresis (SPE-CE), a microcartridge or analyte concentrator is inserted near the inlet of the separation capillary[10]. The analyte concentrator contains a solid phase extraction sorbent which retains the target analyte, enabling large volumes of sample to be intruduced. The captured analyte is eluted in a small volume of an appropiate solution, resulting in sample clean-up and concentration enhancement, with minimum sample handling. Several researchers have perceived the suitability of SPE-CE to perform selective and sensitive analysis of proteins and peptides in complex diluted samples. In addition, the use of solid-phase extraction coupled on-line to capillary electrophoresis electrospray mass spectrometry (SPE-CE-ESI-MS) has demonstrated improved capabilities for characterization of compounds found at low concentration in a complex matrix[10].

Mass spectrometry (MS) has emerged as a powerful tool for the analysis of large biomolecules. However, direct analysis of intact glycoproteins by MALDI-TOF and conventional electrospray ionization mass spectrometry (ESI-MS) has presented some problems in order to resolve microheterogenous structures. Thus, a previous glycoform separation is mandatory to obtain valuable information about the carbohydrate heterogeneity of glycoproteins. CE-ESI-MS has been successfully used for this purpose. [11-13]. However, volatile background electrolytes (BGE) are necessary to provide suitable electrospray ionization and therefore, obtain good sensitivity. The non-volatile BGE used in the current CE-UV methods for glycoform separation preclude the CE-ESI-MS coupling [14-16].

In our work, CE-UV methods for the separation of glycoforms in volatile BGE have been developed. The CE-ESI-MS separation method for intact rHuEPO has been improved as a consequence of the use of a novel acrylamide-based coating that provides a stable suppression of electroosmotic flow (EOF) and allows a succesful glycoform separation[7, 17] Also a method for the separation of Tf sialoforms has been developed, that permits the diagnostic of Congenital Disorders of Glycosylation and chronic alcoholism. A negative modified capillary is obtained by a first amino qua-

ternary coating (Polybrene) attached to the capillary wall, followed by a second anionic coating (Dextran sulphate)[6, 7]. In order to improve detection limits of CE-ESI-MS methodologies, the use of solid-phase extraction coupled on-line to capillary electrophoresis using electrospray mass spectrometry detection (SPE-CE-ESI-MS) is studied for the analysis of peptide hormones in dilute solutions[10]. This resulted in sample clean-up and concentration enhancement, with minimum sample handling. The CE-ESI-MS developed methods ar e applied for the characterization of rHuEPO glycoforms.

EXPERIMENTAL PART

Chemicals

All chemicals used in the preparation of buffers and solutions are analytical reagent grade. Standard human transferrin (partially saturated, min 98%), insulin, sodium dextran sulphate (M=500000), and hexadimethrin bromide (Polybrene, PB, M=15000) are purchased from Sigma Ultra Tol™ Dynamic PreCoat LN was provided by Target Discovery (Palo Alto, CA, USA). Trypsin Gold, Mass Spectrometry Grade, was obtained by Promega (Madison, WI, USA). Standard rHuEPO was obtained as BRP from Pharmacopoeia (EDQM, European Pharmacopoeia, Council of Europe, Strasbourg, France). Epoetin-α (Eprex) 6000 IU from Janssen-Cilag (Neuss, Germany) and epoetin-β (NeoRecormon) 4000 IU from Roche (Mannheim, Germany) were obtained as ready-to-use drugs. Deionised and organic-eliminated water was obtained using a Milli-Q water purification system (Millipore, Schwalbach, Germany). All solutions and background electrolytes were degassed by ultrasonication before use.

Instrumental

CE-ESI-MS

CE analysis was performed on a Hewlett Packard CE (Agilent Technologies, Waldbronn, Germany). For CE-ESI-MS coupling, a coaxial sheath-liquid sprayer was used (Agilent Tecnologies). For intact EPO glycoprotein analysis, separation was performed in capillaries coated with polybren (PB) or ultra Tol™ Dynamic Pre-Coat LN (LN). For Tf analysis the separation capillary is coated with a polybren-dextran sulphate (PB-DS) double layer coating.

MS

Mass spectrometric Tf analysis is carried out in a Mariner TOF mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) coupled to the CE system, whereas the hormone spectrometric investigations an EPO characterization are performed with the CE system coupled to a MSD Ion Trap mass spectrometer (Agilent Technologies) and an orthogonal accelerated TOF mass spectrometer (oaTOF-MS) (MicroTOF, Bruker Daltonik), respectively.

RESULTS AND DISCUSSION

Transferrin glycoform analysis by CE-UV and CE-ESI-MS. Application to CDG chronic alcoholism diagnosis.

A CE-UV separation method has been developed using a MS compatible buffer with 25 mM NH_4Ac at pH 8.5. Best separation conditions have been obtained in a coated capillary based on a Successive Multiple Ionic Layer (SMIL) performed by a first layer of PB and a second layer of dextran-sulphate (DS). This coating provides a constant and cathodic EOF, and minimizes the interactions between Tf and capillary walls.

In order to deplete albumin and the most abundant immunoglobulins from serum, a commercial kit based on dyes and immunoaffinity capture has been used prior to electrophoretic analysis. Figure 1 shows the obtained electropherograms in two different sera, one from a healthy individual and the other from a CDG patient. A clear difference on the electrophoretic profiles is observed. The CE-UV developed method is now applied in clinical diagnosis.

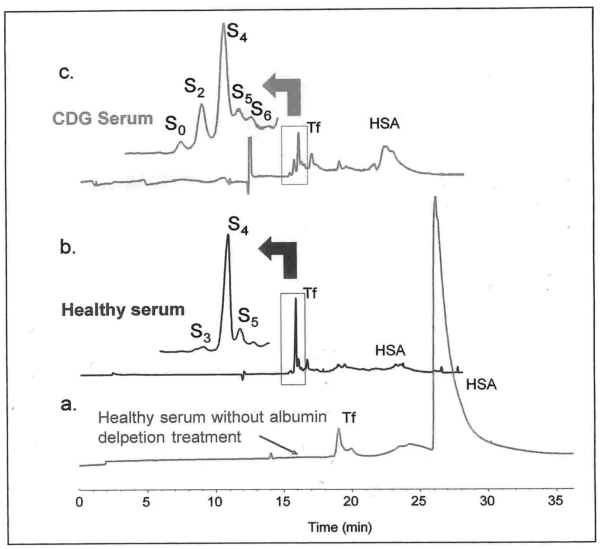

Figure 1. CE-UV electropherograms obtained from different serum samples. a) Non-treated healthy, b) healthy serum and c) CDG serum both passed through the albumin depletion kit.

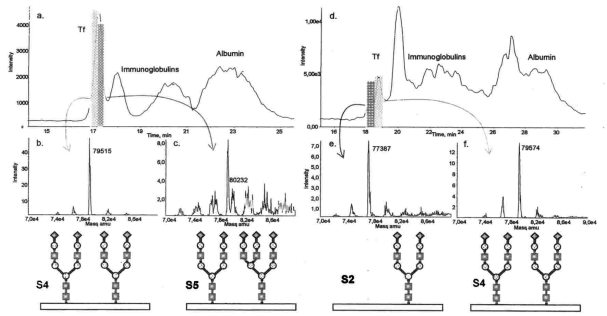

Figure 2. a) Total Ion Electropherogram obtained from a serum from a healthy individual in the CE-ESI-MS optimized conditions. b) and c) deconvoluted mass spectra obtained from the beginning and the end of the Tf peak respectively. d) Total Ion Electropherogram obtained for a serum from a CDG patient. e) and f) deconvoluted mass spectra obtained from the two partial resolved glycoforms of Tf. The most probable glycan composition is displayed below the deconvoluted mass spectra.

Using CE-ESI-MS methodologies the best sensitivity is obtained with a sheath liquid containing 90% of methanol and 0.5 % formic acid. Other experimental parameters as sheath liquid flow rate, nebulizer gas and ionization potentials have been optimized in order to achieve good sensitivity and separation. Separation of different proteins present in serum has been achieved and mass spectra can be deconvoluted. A glycoform of 77387 Da is obtained in a serum from a CDG patient that is not observed for a control serum (Figure 2).

Analysis of hormones by on line SPE-CE-ESI-MS

Endogenous hormones are generally found at subnanomolar levels in biological samples. In our studies, solid-phase extraction coupled on-line to capillary electrophoresis electrospray mass spectrometry (SPE-CE-ESI-MS) is explored for the preconcentration and separation of dilute solutions of peptide hormones. First, a CE-ESI-MS methodology is developed and validated. Limits of detection (LOD) of around 1 μg mL are obtained for all the studied hormones. For SPE-CE-ESI-MS experiments, a home-made SPE microcartridge containing a C18 sorbent is constructed near the inlet of the separation capillary. (Figure 3). After optimizing the on-line preconcentration methodology, LOD between 0.1 and 10 ng mL^{-1} are achieved. The preconcentration methodologies have been applied to rHuEPO analysis using an on-line inmunoaffinity solid phase extraction (IA-CE-ESI-MS).The preliminary results obtained using a custom-made inmunoaffinity sorbent prepared from an anti-human EPO polyclonal antibody and glutaraldehyde-glass beads show the potential of this novel approach.

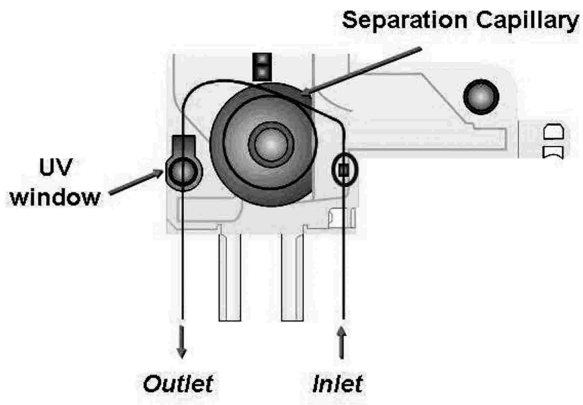

Figure 3. Scheme of a cartridge containing a capillary column with a C_{18} analyte concentrator (C_{18} AC) for SPE-CE-UV analysis.

Characterization of rHuEPO glycoforms by CE-ESI-MS

Several recombinant human erythropoietins (rHuEPO) from different origin have been analysed. Coated

capillaries are mandatory in order to decrease or suppress the adsorption of glycoproteins to the silica capillary wall. In our works, two different capillary coatings have been used: [1] polybrene (PB), an amino quaternary polymer with positive charges that reverses the EOF, and[2] UltraTol Dynamic Pre-Coat Low Normal (LN), an acrylamide polymer that suppresses the EOF at low pH. The best sensitivity has been obtained with sheath liquids containing 1% acetic acid and high resolution TOF-MS has been found to be the most suitable mass analyzer for detection of intact glycoproteins differing in a few Da. In CE-ESI-MS analyses of intact glycoproteins, numerous and complex data are obtained and therefore, extracting useful and valuable information from spectra is not as obvious as in more straightforward compounds. Figure 4 shows the separation and mass spectra obtained from rHuEPO in a LN coated capillary, summarizing the procedure performed in the data analysis.

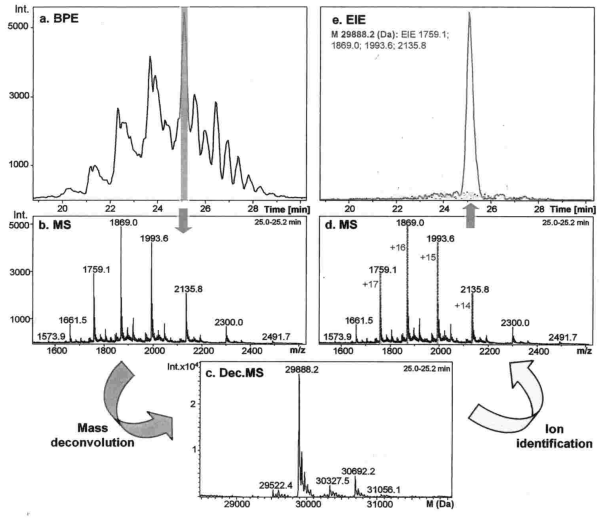

Figure 4. Schematic view for data processing of intact CE-ESI-MS glycoprotein analysis in a LN coated capillary, BGE: 2M acetic acid, separation voltage + 30kV. Sample: Pharmacopoeia rHuEPO 2.5 g/ L injected for 15s, at 50 mbar. a) Base Peak Electropherogram (BPE) obtained, b) mass spectrum obtained in 25.0-25.2 min, c) deconvoluted mass spectrum, d) ion identification for 29888.2 Da glycoform, e) Extracted Ion Electropherogram (EIE) obtained for 29888.2 Da glycoform (in purple) and for some of the different sialoforms present in rHuEPO (in grey).

In order to obtain a more complete information about the carbohydrate moiety of glycoproteins, a study of the composition and structure of the glycans is necessary. In our work a CE-ESI-MS separation method has been developed for the analysis of the N-glycans obtained by PNGase F release from the glycoproteins (Figure 5). Also the sialic acid content of the O-glycosylation site has been characterized based on the glycopeptides obtained by trypsin digest and CE-ESI-MS analysis. Therefore, the probability of every intact sialoform has been calculated taking into account the number of glycosylation sites, and the percentages of the N-glycans and the O-glycans depending on their sialic acid content. The data have been compared with the normalized areas obtained from the EIE of the intact sialoforms of Pharmacopoeia rHuEPO, and therefore the sialic acid assignment has been performed. Once sialic acid number has been assigned, a global carbohydrate composition is deduced in high confidence. Thus, a main molecular mass of 29888.2 Da, consists of the protein backbone (165 amino acids, 18235.8 Da), 22 hexoses, 19 N-acetylhexosamines, 3 fucoses and 13 sialic acids (Figure 6). Table 1 shows the main observed molecular masses and the respective carbohydrate composition.

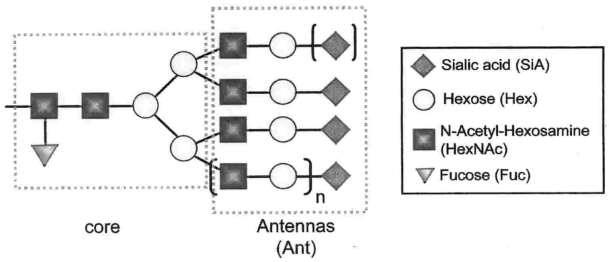

Figure 5. Typical tetra-antennary N-glycan of rHuEPO.

Table 1 Main intact rHuEPO glycoforms and the respective carbohydrate composition obtained in a LN coated capillary

	15 SiA	14 SiA	13 SiA	12 SiA	11 SiA	10 SiA	9 SiA	8 SiA
16Hex13HexNAc3Fuc							*26513.4	*26221.9
17Hex14HexNAc3Fuc							*26878.6	*26588.6
18Hex15HexNAc3Fuc						27553.0	27262.3	26972.1
19Hex16HexNAc3Fuc					28208.9	27918.1	27627.8	27336.8
20Hex17HexNAc3Fuc			29158.1	28865.6	28574.1	28283.6	27991.8	27700.6
21Hex18HexNAc3Fuc		29813.3	29522.5	29231.4	28939.8	28648.9	28356.3	
22Hex19HexNAc3Fuc	30473.8	30178.6	29888.2	29596.5	29305.3	29014.5	28722.6	
23Hex20HexNAc3Fuc	30836.3	30543.8	30252.6	29961.8	29670.7	29378.9	29087.9	
24Hex21HexNAc3Fuc	31197.9	30910.2	30618.7	30327.0	30035.6	29744.4		
25Hex22HexNAc3Fuc	31567.6	31274.6	30983.9	30692.2	30400.8			
26Hex23HexNAc3Fuc	31934.3	31640.2	31349.7	31057.5	30768.1			
27Hex24HexNAc3Fuc	32298.8	32006.4	31714.3	31424.4	31132.3			
28Hex25HexNAc3Fuc		32370.5	32080.1	31787.1				
29Hex26HexNAc3Fuc		32735.9	32444.0					
30Hex27HexNAc3Fuc			32810,5					

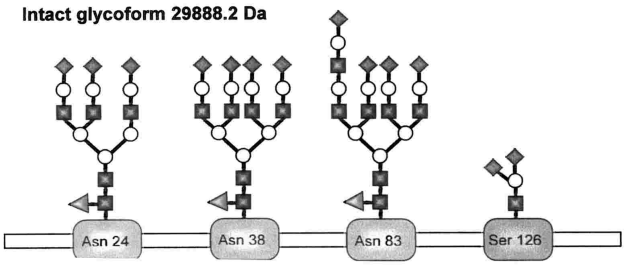

Figure 6. One possible glycan composition for an intact rHuEPO glycoform of 29888.2 Da containing 22 hexoses (O), 19 N-acetylhexosamines (□), 3 fucoses (_) and 13 sialic acids (♦).

CONCLUSIONS

A CE-ESI-MS method for the separation of Tf glycoforms has been developed, that permits the diagnostic of CDG and chronic alcoholism. The method uses a SMIL coated capillary performed by a first layer of polybrene and a second layer of dextran and a sheath liquid interface containing 90 % of methanol and 0,5 % formic acid. Also, solid-phase extraction coupled on-line to capillary electrophoresis electrospray mass spectrometry (SPE-CE-ESI-MS) is explored in order to improve sensitivity of the method. LOD between 10 and 0,1 ng mL are achieved using an on-line preconcentrator. All these methodologies are applied for characterization of intact rHuEPO glycoforms. Numerous and complex data are obtained and therefore a study of the composition and structure of the N-glycans, obtained by PNGase F digestion, is necessary. Also the sialic acid content of O-glycosylation site has been characterized based on the glycopeptides obtained by trypsin digest and CE-ESI-MS analysis. Therefore, the probability of every intact sialoform has been calculated taking into account the number of glycosylation sites, and the percentages of the N-glycans and the O-glycans depending on their sialic acid content. The data have been compared with the normalized areas obtained from the EIE of the intact sialoforms, and therefore the sialic acid assignment has been performed. Once sialic acid number has been assigned, a global carbohydrate composition is deduced in high confidence. Thus, for example, a main molecular mass of 29888.2 Da, consists of the protein backbone (165 amino acids, 18235.8 Da), 22 Hexoses, 19 N-acetylhexosamines, 3 fucoses and 13 sialic acids.

REFERENCES

1. Skibeli V, Nissen-Lie G, Torjesen P. Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin, *Blood*, 98, 3626-3634, 2001.
2. Mechref Y, Novotny V. Structural investigations of glycoconjugates at high sensitivity, *Chem. Rev.*, 102, 321-369, 2002.
3. Sanz-Nebot V, Balaguer E, Benavente F, Neussus C, Barbosa J. Characterization of transferrin glycoforms in human serum by CE-UV and CE-ESI-MS, *Electhrophoresis*, 28, 1949–1957, 2007.
4. Sanz-Nebot V, González P, Toro I, Ribes A, Barbosa J. Characterization of human transferrin glycoforms by capillary electrophoresis and electrospray ionization mass spectrometry, *J. Chromatogr. B*, 798, 1-7, 2003.
5. Fisher JW. Erythropoietin: physiology and pharmacology update, *Exp. Biol. Med*., 228, 1-14, 2003.
6. Sanz-Nebot V, Benavente F, Vallverdú A, Guzmán NA, Barbosa J. Separation of recombinant human erythropoietin glycoforms by capillary electrophoresis using volatile electrolytes. Assessment of mass spectrometry for the characterization of erythropoietin glycoforms, *Anal. Chem*., 75, 5220-5229, 2003.
7. Balaguer E, Demelbauer U, Pelzing M, Sanz-Nebot V, Barbosa J, NeusüB C. Glycoform characterization of erythropoietin combining glycan and intact protein analysis by capillary electrophoresis - electrospray - time-of-flight mass spectrometry, *Electrophoresis*, 27, 2638-2350, 2006.
8. Sanz-Nebot V, Benavente F, Giménez E, Barbosa J. Capillary electrophoresis and matrix-assisted laser desorption / ionization-time of flight-mass spectrometry for analysis of the novel erythropoiesis-stimulating protein (NESP), *Electrophoresis*, 26, 1451-1456, 2005.
9. Benavente F, Giménez E, Olivieri, AC, Barbosa J, Sanz-Nebot V. Estimation of the composition of recombinant human erythropoietin mixtures using capillary electrophoresis and multivariate calibration methods, *Electrophoresis*, 27, 4008-4015, 2006.
10. Benavente F, Vescina MC, Hernández E, Sanz-Nebot V, Barbosa J, Guzman N. Lowering the

concentration limits of detection by on-line solid-phase extraction-capillary electrophoresis-electrospray mass spectrometry, *J. Chromatogr. A*, 1140, 205-212, 2007.

11. Kelly JF, Locke SJ, Ramaley L, Thibault P. Development of electrophoretic conditions for the characterization of protein glycoforms by capillary electrophoresis-electrospray mass spectrometry, *J. Chromatogr. A*, 720, 409-427, 1996.
12. Demelbauer UM, Plematl A, Kremser L, Allmaier G, Josic D, Rizzi A. Characterization of glyco isoforms in plasma-derived human antithrombin by on-line capillary zone electrophoresis-electrospray ionization-quadrupole ion trap-mass spectrometry of the intact glycoproteins, *Electrophoresis*, 25, 2026-2032, 2004.
13. Neusüss C, Demelbauer U, Pelzing M. Glycoform characterization of intact erythropoietin by capillary electrophoresis-electrospray-time of flight-mass spectrometry, *Electrophoresis*, 26, 1442-1450, 2005.
14. Klampfl, CW . Recent advances in the application of capillary electrophoresis with mass spectrometric detection, *Electrophoresis*, 27, 3-34, 2006.
15. Stutz H. Advances in the analysis of proteins and peptides by capillary electrophoresis with matrix-assisted laser desorption / ionization and electrospray-mass spectrometry detection, *Electrophoresis*, 26, 1254-1290, 2005.
16. Hernández-Borges J, NeusüB C, Cifuentes A, Pelzing M. On-line capillary electrophoresis-mass spectrometry for the analysis of biomolecules, *Electrophoresis*, 25, 2257-2281, 2004.
17. Benavente F, Gimenéz E, Olivieri AC, Barbosa J, Sanz-Nebot V. Estimation of the composition of recombinant human erythropoietin mixtures using capillary electrophoresis and multivariate calibration methods, *Electrophoresis*, 27, 4008-4015, 2006.