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Analysis of recombinant human erythropoietin and novel erythropoiesis stimulating protein digests by immunoaffinity capillary electrophoresis–mass spectrometry

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ABSTRACT

In this work, we demonstrate that detection of a specific peptide marker by immunoaffinity capillary electrophoresis–mass spectrometry (IA-CE–MS) could be used to confirm the presence of recombinant human erythropoietin (rhEPO) in solution. Besides the carbohydrate content, the amino acid sequence of novel erythropoiesis stimulating protein (NESP) differs from human erythropoietin (hEPO) at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr). After digesting both glycoproteins in solution by trypsin and PNGase F, two specific proteotypic peptides, EPO (77–97) and NESP (77–97) which differ in three amino acids, were selected as rhEPO and NESP markers, respectively. Both digests and their mixtures were analyzed by IA-CE–MS. The IA stationary phase was prepared from a custom made polyclonal anti-EPO (81–95) antibody immobilized on a solid support of CNBr-Sepharose 4B and was packed in a microcartridge near the inlet of the separation capillary. As the antibody was directed to a synthetic peptide EPO (81–95), only the proteotypic peptide EPO (77–97) was retained. The retained peptide was eluted, separated by electrophoresis and detected by MS. The method was specific to confirm the presence of rhEPO in solution. Although the limits of detection for the peptide marker were similar to those obtained with CE–MS (a few mg/L), these results show the potential of this novel approach to detect in the future rhEPO and its analogues selectively and unambiguously at the levels expected in biological fluids.

1. Introduction

Erythropoietin (EPO) is a hormone produced mainly by the kidney involved in the maturation and proliferation of red blood cell precursors in bone marrow. EPO is a part of a physiological feedback mechanism that maintains red blood cell number and tissue oxygen supply at adequate levels [1]. Human EPO (hEPO) is a highly glycosylated protein of 30 kDa [1,2] that shows three N-glycan (in Asn24, Asn38 and Asn83) and one O-glycan chains (in Ser126) attached to the polypeptide backbone. Recombinant human EPO (rhEPO) has been extensively used to treat various forms of anaemia [2]. However, it has become especially well known due to its misuse in endurance sports disciplines, being forbidden, together with its analogues, by sport authorities since 1989 [3,4]. The novel erythropoiesis stimulating protein (NESP or darbepoetin alpha) is a hyperglycosylated analogue of rhEPO that differs in five amino acid residues (Ala30Asn, His32Thr, Pro87Val, Trp88Asn and Pro90Thr) [5]. These modifications allow NESP to bear five N-linked carbohydrate chains instead of three, as rhEPO does. Hence, the molecular mass of NESP is around 37 kDa, and it shows a higher number of sialic acid residues [4,5]. The higher sialic acid content of NESP provides a longer circulating half-life and extended biological activity, thus requiring less frequent administration than rhEPO [5]. Due to its efficacy in relieving anaemia in patients, NESP was the fifth best-selling pharmaceutical product in the world in 2007 with worldwide sales of about US$ 5×10^9 [6]. Recently, a synthetic analogue of hEPO named CERA (continuous erythropoietin receptor activator) has emerged in the market, providing longer circulating half-life and thus being much more effective than hEPO and NESP [7].

The official method approved by the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA) for the detection of rhEPO and its analogues is the *Urine EPO Test* [8]. This method is based on the separation of urine EPO glycoforms by iso-

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electric focusing (IEF), followed by a double-blotting procedure and chemiluminescence detection [8]. At present, it is the only method of detection that differentiates between endogenous and recombinant forms of EPO at the low concentration levels found in biological fluids (≤200 ng/L of hEPO in human urine or plasma). However, this method is long, tedious and does not permit confirmation by mass spectrometry (MS). Therefore, new methods for a simple and rapid screening and confirmation of these agents in human urine and plasma would be useful not only to control their abuse in sports, but also to gain an insight into their therapeutic and biological functions.

The sensitivity of the methodologies based on MS for the direct detection of intact glycoproteins in solution is limited because of their high molecular weight and the detrimental effect of sugars on ionization [9–11]. The detection of low molecular weight specific markers (i.e. peptides, glycopeptides or carbohydrates) could be explored as an alternative to detect EPO in dilute solutions. This would also allow discrimination between the different erythropoietin molecules [12,13]. rhEPO or NESP administration may be confirmed with glycopeptide or carbohydrate markers, as the two recombinant hormones show differences from endogenous EPO at the carbohydrate level [13,14]. However, in the case of NESP, screening for the enzymatically released peptides that show some of the amino acid differences between EPO and NESP (i.e. T21–45 and T77–97) could be a better choice, as peptides are better ionizable and detected by MS than glycopeptides or carbohydrates.

Capillary electrophoresis–mass spectrometry (CE–MS) has become a powerful hyphenated technique for the separation of a great number of biomolecules enabling characterization of analytes in complex samples from molecular mass information. However, CE shows limited concentration sensitivity as a consequence of the small sample volume capacity of the capillary columns. Several instrumental, electrophoretic and chromatographic approaches have been described in order to overcome this limitation [15]. Among the chromatographic approaches, the use of solid-phase extraction coupled on-line to capillary electrophoresis (SPE–CE) is worth highlighting, given its advantages and versatility [16–19]. In SPE–CE, an extraction microcartridge or analyte concentrator is inserted near the inlet of the separation capillary. This microcartridge contains the solid sorbent, which retains the target analyte, enabling large volumes of sample to be introduced. The retained analyte is eluted in a small volume of an appropriate solution, resulting in sample clean-up and concentration enhancement. The technique is usually called immunoaffinity capillary electrophoresis (IA-CE) when the solid sorbent in the microcartridge contains an immobilized antibody (Ab). IA-CE is only at early stage of development. Recently, an attempt was made to selectively preconcentrate intact rhEPO from dilute solutions by IA-CE with ultraviolet detection [20]. Nevertheless, IA-CE has most often been demonstrated with laser-induced-fluorescence detection, for the selective preconcentration of low molecular weight immunoreactive compounds from biological fluids and tissues [17,21]. In this sense, immunoaffinity capillary electrophoresis–mass spectrometry (IA-CE–MS) may be an excellent alternative to detect and identify rhEPO and its analogues at the levels expected in biological fluids from low molecular weight specific markers.

In this work, we used IA-CE–MS to detect a peptide marker which confirmed the presence of rhEPO in solution. The immunoaffinity sorbent was prepared by immobilizing a custom made polyclonal anti-EPO (81–95) Ab [22] to a CNBr-Sepharose 4B solid support (Amersham Biosciences, Piscataway, USA). CE–MS and IA-CE–MS methods were developed for the analysis of synthetic peptides derived from rhEPO and NESP sequences and their whole enzymatic digests. The results showed the advantages and also the limitations of the proposed method for the detection of EPO via a specific peptide marker.

2. Materials and methods

2.1. Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Isopropanol (iPrOH), acetic acid (HAc, glacial), formic acid (HFo, 98–100%), ammonia (NH_3, 25%), hydrogen chloride (HCl, 37%), sodium chloride (NaCl), glycine and sodium hydroxide (NaOH) were supplied by Merck (Darmstadt, Germany). Sodium azide (NaN_3), 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), DL-dithiothreitol (DTT, ≥99%), iodoacetamide (IAA), ammonium hydrogencarbonate (NH_4HCO_3) and glycine were supplied by Sigma–Aldrich (Madrid, Spain). Ammonium acetate (NH_4Ac) was obtained from Prolabo (Fontenay-sous-bois, France) and tris(hydroxymethyl)aminomethane (Tris) from Baker (Phillipsburg, NJ, USA). Dynorphin A (1–7) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg, $[M+H]^+$ = 868.5 Da) was supplied by Bachem (Bubendorf, Switzerland). Trypsin (Sequencing grade modified) from Promega (Madison, WI, USA) and peptide *N*-glycosidase F (PNGase F) was obtained from Roche (Mannheim, Germany). Water with a conductivity lower than 0.05 mS/cm was obtained using a Milli-Q water purification system from Millipore (Molsheim, France).

2.2. Samples

2.2.1. Synthetic peptides

Solid-phase methodologies were used for the synthesis of the C-terminal carboxamide peptides corresponding to the EPO (81–95) and NESP (86–104) sequences, which were assembled manually in polypropylene syringes fitted with a polyethylene disk, following the Fmoc/tBu chemistry. As in previous work [22], both sequences had an extra Cys residue at the C-terminus to allow conjugation to keyhole limpet hemocyanin carrier protein or an immunoaffinity chromatography (IAC) resin.

The peptide EPO (81–95) was prepared on a *p*-methylbenzhydrylamine resin (0.98 mmol/g, 0.05 mmol scale), to which 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]-phenoxyacetic acid (Rink-amide handle, 3 equiv.) was coupled by two treatments of 1 h with 1,3-diisopropylcarbodiimide (DIPCDI, 3 equiv.) and *N*-hydroxybenzotriazole (HOBt, 3 equiv.) in *N*,*N*-dimethylformamide (DMF). A final loading of 0.44 mmol/g was achieved by reduction of the functionalization using 1 equiv. of reagents for the coupling of the first amino acid (DIPCDI/HOBt) and further capping of the remaining free amino groups with Ac_2O/pyridine 50 equiv. (30 min). Amino acids (3 equiv.) were anchored to the polymeric support with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU, 3 equiv.) in the presence of *N*,*N*-diisopropylethylamine (DIEA, 6 equiv.) in DMF after Fmoc removal with 20% piperidine in DMF (1 min + 10 min). Final deprotection and cleavage from the resin were performed with trifluoroacetic acid (TFA)/triisopropylsilane/water (95:3:2) for 1.5 h at room temperature. The crude peptide was purified by reverse-phase preparative HPLC on C_{18} silica. The final product was homogeneous by analytical HPLC and gave a satisfactory mass by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (22% yield) with a Voyager-RP-DE system from Perseptive Biosystems (Framingham, MA, USA).

The peptide NESP (86–104) was grown on an aminomethyl poly(ethylene glycol)-based ChemMatrix support (0.53 mmol/g, 0.06 mmol scale), which was functionalized with the Rink-amide handle (3 equiv.) using TBTU (3 equiv.) and DIEA (6 equiv.) in DMF (1 h). Each amino acid was incorporated by double coupling under similar conditions, followed by a capping step with Ac_2O/pyridine 50 equiv. (30 min). Amino groups were deprotected under similar conditions to those described for EPO (81–95), except

for residues 93–86 where 3 extra 10 min treatments with 20% piperidine in DMF were utilized. Full deprotection and cleavage of the peptide from the resin was accomplished by treating the resin with TFA/thioanisol/ethanedithiol/anisol (90:5:3:2) for 1.5 h at room temperature. Purification was performed by reversed-phase preparative HPLC on C_{18} silica, affording a homogeneous product by analytical HPLC that gave the mass of the target peptide by MALDI-TOF-MS (5% yield).

Lyophilized peptides were stored at −20 °C when not in use. An aqueous stock solution of 1000 mg/L of each peptide was prepared and stored at −20 °C. Working peptide solutions were obtained by mixture and dilution with 50 mM HAc and 50 mM HFo (pH 2.2) for CE–MS analysis, and with 20 mM NH_4Ac (pH 7.0) for IA-CE–MS experiments.

2.2.2. rhEPO and NESP samples

rhEPO produced in a CHO cell line was provided by the European Pharmacopoeia as a Biological Reference Product (BRP-lot2). Each sample vial contained 250 μg of EPO (a mixture of epoetin alpha and beta), 0.1 mg of Tween 20, 30 mg of trehalose, 3 mg of arginine, 4.5 mg NaCl, and 3.5 mg of Na_2HPO_4. The content of each vial was dissolved in purified water to obtain a 1000 mg/L solution of rhEPO. NESP (darbepoetin alpha, Aranesp) was purchased from Amgen (Thousand Oaks, CA, USA). Each pre-filled syringe contained 300 μg of NESP, Na_2HPO_4, NaH_2PO_4, NaCl, Tween 80 and 0.6 mL of purified water. Excipients of low molecular weight were removed from rhEPO and NESP samples by passage through a Microcon YM-10 centrifugal filter device from Millipore (MW cut-off 10 kDa, Bedford, MA, USA) [11]. The filter was initially washed with purified water for 10 min in a centrifuge at 13,000 × g, and the sample was centrifuged after that for 10 min under the same centrifugal force. The residue was washed three times for 10 min in the same way with an appropriate volume of purified water. The final residue was recovered from the upper reservoir by upside-down centrifugation in a new vial (3 min at 1000 × g) and sufficient purified water was added to adjust the glycoprotein concentration to 1000 mg/L.

rhEPO and NESP were reduced, alkylated and subsequently subjected to enzymatic digestion with trypsin. Next, N-glycans were released by enzymatic deglycosylation with PNGase F. Briefly, 2.5 μL of 0.5 M DTT in 50 mM NH_4HCO_3 (pH 7.9) were added to an aliquot of 100 μL of desalted rhEPO or NESP at 1000 mg/L (100 μg). The mixture was incubated in a water bath at 56 °C for 30 min, and then alkylated with 50 mM IAA for 30 min at room temperature in the dark. Excess of low molecular weight reagents were removed with Microcon YM-10, following the same procedure explained

Table 1
List of synthetic peptides and enzymatically released rhEPO and NESP peptides detected by CE–MS.

Sequence*	Acronym	Position		m/z** calculated	m/z observed
Synthetic peptides					
LVNSSQ**PWEP**LQLHVC-NH_2	EPO (81–95)	81–95	$[M+2H]^{2+}$	924.9	924.9
Q**VNET**LQLHVDKAVSGLRSC-NH_2	NESP (86–104)	86–104	$[M+2H]^{2+}$	1098.5	1098.5
			$[M+3H]^{3+}$	732.7	732.7
rhEPO and NESP digests					
1. APPR	–	1–4	$[M+H]^{+}$	440.2	440.2
2. LICDSR-(Cys-CAM)[a]	–	5–10	$[M+H]^{+}$	763.3	763.3
			$[M+2H]^{2+}$	382.1	382.1
3. VLER	–	11–14	$[M+H]^{+}$	516.3	516.2
4. YLLEAK	–	15–20	$[M+H]^{+}$	736.4	736.4
			$[M+2H]^{2+}$	368.7	368.6
5. VNFYAWK	–	46–52	$[M+H]^{+}$	927.4	927.4
			$[M+2H]^{2+}$	464.2	464.1
6. MEVGQQAVEVWQGLALLSEAVLR	–	54–76	$[M+2H]^{2+}$	1263.6	1263.3
			$[M+3H]^{3+}$	842.7	842.8
7. AVSGLR	–	98–103	$[M+H]^{+}$	602.3	602.3
8. SLTTLLR	–	104–110	$[M+H]^{+}$	803.4	803.4
			$[M+2H]^{2+}$	402.2	402.1
9. ALGAQK	–	111–116	$[M+H]^{+}$	587.3	587.3
10. TITADTFR	–	132–139	$[M+H]^{+}$	924.4	924.4
			$[M+2H]^{2+}$	462.7	462.6
11. LFR	–	141–143	$[M+H]^{+}$	435.2	435.2
12. VYSNFLR	–	144–150	$[M+H]^{+}$	898.4	898.4
			$[M+2H]^{2+}$	449.7	449.6
13. LYTGEACR-(Cys-CAM)[a]	–	155–162	$[M+H]^{+}$	969.4	969.4
			$[M+2H]^{2+}$	485.2	485.1
14. TGDR	–	163–166	$[M+H]^{+}$	448.2	447.2
15. GQALLV<u>D</u>SSQ**PWEP**LQLHVDK (rhEPO)[b,***]	15-EPO	77–97	$[M+2H]^{2+}$	1180.6	1180.5
			$[M+3H]^{3+}$	787.3	787.3
15. GQALLV<u>D</u>SSQ**V<u>D</u>ET**LQLHVDK (NESP)[c,***]	15-NESP	77–97	$[M+2H]^{2+}$	1148.0	1148.0
			$[M+3H]^{3+}$	765.7	765.7

[a] Cysteine was treated with iodoacetamide to form carbamidomethyl-cysteine (Cys_CAM).
[b] Peptide corresponding to rhEPO digest that differs from NESP in 3 amino acids (bold).
[c] Peptide corresponding to NESP digest that differs from rhEPO in 3 amino acids (bold).
* Amino acid sequence of EPO from SwissProt and of NESP according to [5].
** m/z values are monoisotopic values.
*** Peptides 15-EPO and 15-NESP have been modified from the native sequence of rhEPO and NESP, respectively, as PNGase F treatment converts a glycosylated asparagine (N) to aspartic acid (<u>D</u>).

above, and reconstituted in 100 μL of 50 mM NH_4HCO_3 (pH 7.9). Trypsin in an enzyme to sample ratio of 1:40 (w/w) was added, and the mixture was carefully vortexed and incubated at 37 °C for 18 h. Trypsin digestion was stopped by heating to 100 °C for 5 min. Subsequently, 4 μL of PNGase F (4 U) were added, and solution was incubated at 37 °C. To ensure total deglycosylation of tryptic digests, after 24 h another 4 μL of PNGase F were added and incubation was continued for another 24 h. Finally, rhEPO and NESP digests were again heated to 100 °C for 5 min and stored at −20 °C until used.

2.3. CE–MS

All CE–MS experiments were performed in an HP ^{3D}CE capillary electrophoresis instrument (Agilent Technologies) coupled on-line to a LC/MSD Trap SL (Agilent Technologies, Wilmington, DE, USA) with a G1603A sheath-flow capillary electrophoresis–electrospray mass spectrometry (CE–ESI-MS) interface (Agilent Technologies). CE instrument was controlled using ChemStation Rev. A.08.03 software (Agilent Technologies). MS instrument control, data acquisition and data processing were performed using the LC/MSD Trap software 5.2 (Bruker Daltonik, Bremen, Germany). Sheath liquid was delivered by a KD Scientific 100 Series infusion pump (Holliston, MA, USA) at a flow rate of 3.3 μL/min. The tuning of the mass spectrometer parameters was performed automatically by direct infusion, at 50 mbar through the separation capillary, of a 25 mg/L solution of peptide EPO (81–95) (see Table 1 for the structure) in the separation electrolyte maximizing the signal for the double charged molecular ion. Full scan mass spectra were acquired in the m/z range from 250 to 1500 m/z, averaging every 7 spectra (speed = 13,000 m/z/s). All experiments were performed in positive mode and the ESI voltage and end plate offset were set at 4000 and −500 V, respectively. Voltages on capillary exit and skimmer were 240 and 45 V, respectively. Octopole voltages were set at 10 and 3 V and octopole radiofrequency at 200 Vpp. Lens were −10.8 and −79.3 V and trap drive 75 (arbitrary units). Nebulizer gas (N_2) pressure was 7 psi, drying gas (N_2) flow rate was 2 L/min and drying temperature was set at 300 °C [23,24].

A Polymicro bare fused-silica capillary of 75 cm total length (L_T) × 75 μm internal diameter (I.D.) × 360 μm outer diameter (O.D.) supplied by Composite Metals Service (Worcester, UK) was used for CE–MS analysis. New capillaries were activated by flushing them sequentially with the following solutions for 30 min each: 1 M NaOH, water and background electrolyte (BGE). Activation and conditioning procedures were performed off-line in order to avoid NaOH entering the mass spectrometer. Separations were carried out at 25 °C under normal polarity. A voltage of 18 kV was applied for optimum electrophoretic separation. Between runs the capillary was rinsed for 5 min with BGE. A sheath liquid of iPrOH:H_2O (60:40, v/v) with 0.5% of HFo was used in order to obtain optimum detection sensitivity. Columns were stored overnight, filled with BGE. If longer storage was necessary, the capillary was rinsed with water for about 10 min and dried by air flushing for 10 min. Before CE–MS analysis, all solutions were passed through a 0.45-mm nylon filter (MSI, Westboro, MA, USA). The sheath liquid was degassed for 10 min by sonication before use. All samples were kept at 4 °C and stored at −20 °C when not in use for a long period.

A neutral BGE of 20 mM NH_4Ac (adjusted to pH 7 with ammonia) was used for the analysis of peptides EPO (81–95) and NESP (86–104). Synthetic peptide solutions were injected hydrodynamically at 33 mbar for 3 s. For the analysis of rhEPO and NESP digests, injection was performed hydrodynamically at 50 mbar for 15 s and two BGE were used: the neutral BGE of 20 mM NH_4Ac (pH 7.0) and, an acidic BGE of 50 mM HAc and 50 mM HFo (pH 2.2).

pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain).

2.4. IA-CE–MS

2.4.1. Preparation of the IA sorbent

A custom made anti-peptide EPO (81–95) polyclonal Ab [22] was immobilized on a CNBr-activated Sepharose 4B solid support (Amersham Biosciences). Two millilitres of anti-peptide EPO (81–95) antiserum were purified by IAC against the synthetic peptide using an EAH Sepharose 4B column activated with the peptide as described in [22]. Polyclonal Abs were eluted in one fraction of 8 mL. Ab concentration of the eluted fraction was determined by measuring the absorbance of the solution at 280 nm and applying the following correction factor: $C_{Ab} = A_{280}/1.44$, where C_{Ab} is the concentration of Ab in mg/mL, and A_{280} the measured absorbance at 280 nm [25]. Eight millilitres of purified anti-EPO (81–95) Ab solution, corresponding to around 1 mg of Ab, was preconcentrated to a final volume of 95 μL with Centricon YM-3 centrifugal filter devices from Millipore (MW cut-off 3 kDa, Bedford, MA, USA). Subsequently, 100 μL of swollen CNBr-activated Sepharose 4B resin was activated, following the manufacturer's recommended protocol. Briefly, after washing the resin with 1 mM HCl and later with the coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl at pH 8.3), anti-EPO (81–95) Ab diluted in coupling buffer was mixed with the resin. The mixture was allowed to react for 2 h at room temperature with end-over-end rotation. After the removal of the supernatant and washing (five times with 150 μL of coupling buffer), 1 mL of 0.1 M Tris–HCl end-capping solution (0.1 M of Tris–HCl pH 8.0) was added to the particles, and the mixture was shaken for a further 2 h. The supernatant was removed and Sepharose particles were successively washed five times with 0.5 mL of 0.1 M NaAc (pH 4) with 0.5 M of NaCl followed by 0.5 mL of 0.1 M Tris–HCl (pH 8) with 0.5 M of NaCl. Finally, the IA sorbent was washed with 10 mM Tris–HCl (pH 7.6) with 0.01% NaN_3 and stored in the same buffer at 4 °C ready to use.

2.4.2. Construction of the IA-CE column

The construction of the IA-CE column was adapted from a procedure described elsewhere [16,20]. The main change was that no frits were necessary in the IA microcartridges, due to the size and swelling ability of the derivatized Sepharose beads. A 75 cm L_T × 75 μm I.D. × 360 μm O.D. fused-silica capillary was prepared and activated as in Section 2.3. Capillary was cut into two pieces, of 7.4 and 67 cm, in order to insert the IA microcartridge. The construction was monitored under a stereomicroscope. A piece of bare fused-silica capillary (0.7 cm L_T × 250 μm I.D. × 360 μm O.D., Polymicro Technologies) was used as the body of the microcartridge. The body was coupled to the 7.4 cm piece of capillary using a 0.5 cm polyethylene sleeve (280 μm I.D. × 610 μm O.D., "Intramedic" PE 10, Clay Adams division of Becton Dickinson, NJ, USA). Thus, it was easily vacuum-filled with the IA sorbent. When it was completely full, the IA microcartridge connected to the 7.4 cm piece was filled with 10 mM Tris–HCl with 0.01% NaN_3 and stored at 4 °C until use. Before IA-CE–MS experiments, the column was extended to full length with the 67 cm piece of capillary using another polyethylene sleeve. The tight junction provided made no adhesive sealing necessary and the microcartridge completely replaceable. The joining ends connected by the polyethylene sleeves must be cleanly cut in order to minimize the dead volume and leaking at the joints. The 75 cm full-length column, containing the IA microcartridge at 7.4 cm from the inlet, was prepared to fit into the cartridge-cassette device. The IA-CE column was washed with neutral BGE (20 mM NH_4Ac pH 7.0) for 5 min to remove Tris and NaN_3. Finally, the system was checked for

abnormal flow restrictions and current stability before IA-CE–MS analysis.

2.4.3. IA-CE–MS methodology

All IA-CE–MS columns were first conditioned with 3 min of neutral BGE at 930 mbar. Under optimum conditions, samples of synthetic peptides or rhEPO/NESP digests were then hydrodynamically introduced at 50 mbar for 10 min (3.1 μL using the Hagen–Poiseuille equation [26]). A rinse with neutral BGE containing 0.5 M of NaCl (4 min at 930 mbar) removed non-specifically retained peptides and finally, a flush of neutral BGE (5 min at 930 mbar) equilibrated and filled the column before the elution and the electrophoretic separation. Peptides retained by antibody–antigen interactions were eluted after injecting acidic BGE (50 mM HAc and 50 mM HFo, pH 2.2, the so-called elution plug) for 100 s at 50 mbar. Immediately after the injection of the elution plug, the separation was carried out by applying a voltage of 18 kV (normal polarity). Between runs, the capillary was rinsed for 4 min with elution plug and 5 min with BGE in order to avoid carry-over between consecutive analyses. Pre-conditioning and post-conditioning were performed off-line, leaving the MS system in stand-by mode and switching off the nebulizer gas in order to avoid NaCl entrance into the MS.

3. Results and discussion

3.1. CE–MS

The development of IA-CE–MS methods is a challenging task that requires the use of a volatile separation BGE for ESI-MS detection, but also with near physiological pH for appropriate antibody–antigen interactions. Some authors have described the use of *N*-(carbamoylmethyl)-2-aminoethanesulfonic acid (ACES), NH_4HCO_3, NH_4Fo or *N*-methyl morpholine at neutral pH as BGEs for CE–MS [16,27–29]. However, neutral BGEs have rarely been employed for on-line SPE–CE–MS [16,27]. To the best of our knowledge, IA-CE–MS at neutral pH has only been described by once for determination of immunoreactive gonadotropin-releasing hormone in serum and urine [29]. In that case the separation buffer consisted of 60 mM NH_4HCO_3 pH 8 with 1% (v/v) of acetonitrile. In our case, after some preliminary experiments, 20 mM NH_4Ac at pH 7.0 was selected for CE–MS and IA-CE–MS of the synthetic peptides EPO (81–95) and NESP (86–104) and rhEPO and NESP digests under neutral conditions. Fig. 1 shows in (a) the base peak electropherogram (BPE) obtained for a mixture of 100 mg/L of each synthetic peptide by CE–MS, and in (b) and (c) NESP (86–104) and EPO (81–95) mass spectra, respectively. Double and triple protonated molecular ions were the main peaks observed for NESP (86–104) peptide, whereas EPO (81–95) only showed the double protonated molecular ion, probably because this peptide had a lower number of ionizable groups in its sequence. The obtained peak for EPO (81–95) was very narrow. This fact could be explained by a dynamic pH junction mechanism, as synthetic peptides were injected in a low pH sample solution and separated in a higher pH BGE. The focusing electrophoretic effect was promoted by the differences in the ionization state of each peptide at low and neutral pH values [30]. Furthermore, EPO (81–95) was a more acidic peptide (p*I* of EPO (81–95) is 5.2 vs. 6.7 of NESP (86–104), Table 2) and it was focused to a higher extent at the neutralization boundary resulting in narrower electrophoretic peaks [30]. Successive analyses confirmed that migration times of peptides EPO (81–95) and NESP (86–104) increased from run-to-run, probably due to the lack of buffer capacity of the 20 mM ammonium acetate BGE at pH 7.0, as other authors noted for ammonium formiate [28]. This problem was solved by changing BGE solution of the inlet vial for fresh BGE every 4–5 analyses. Repeatability, intermediate precision and limits of detection (LODs) of the proposed method were determined. Table 2 shows LODs, and repeatability and intermediate precision in terms of RSDs of migration times and peak area values, measured from the extracted ion electropherogram (EIE) of each synthetic peptide (EPO (81–95): 924.9 *m*/*z*; NESP (86–104): 732.7 + 1098.5 *m*/*z*). The RSD values for repeatability were slightly better than for intermediate precision, as expected. The RSD values for peak areas were excellent for peptide NESP (86–104) but not for EPO (81–95). A likely explanation for this result is that peptide EPO (81–95) would be more difficult to ionize in positive mode than NESP (86–104) because it might be negatively charged at neutral pH (see p*I* values on Table 2) [31]. Consequently, despite the acidic sheath liquid being

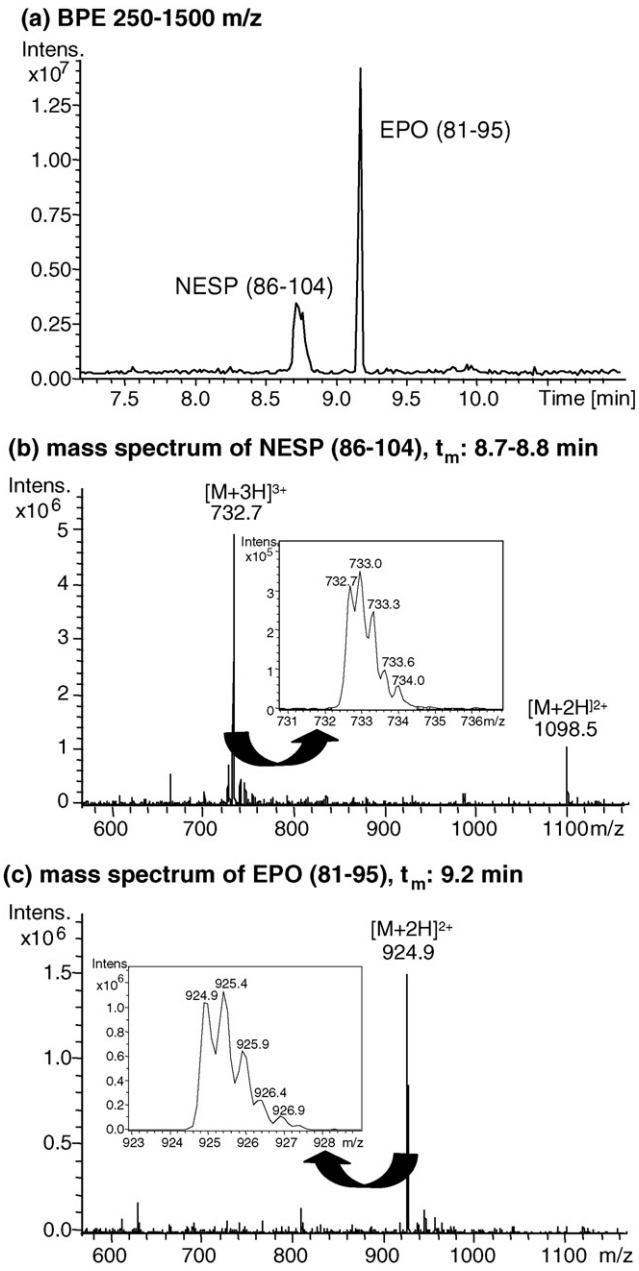

Fig. 1. CE–MS analysis of a mixture of the synthetic peptides (sample: 100 mg/L solution of peptides EPO (81–95) and NESP (86–104); injection: 3 s at 33 mbar; voltage: +18 kV; *T*: 25 °C; neutral BGE: 20 mM NH_4Ac pH 7.0). (a) Base peak electropherogram (BPE). (b) and (c) mass spectra obtained for NESP (86–104) and EPO (81–95) peptides, respectively. Insets: isotopic patterns of triple and double protonated ions for each synthetic peptide.

Table 2
Repeatability, intermediate precision reproducibility and LOD for the analysis of the synthetic peptides by CE–MS at neutral pH.

Synthetic peptide	pI[a]	Repeatability[b] (n = 6), %RSD		Intermediate precision[b] (n = 18), %RSD		LOD (mg/L)
		Peak Area	Migration time	Peak Area	Migration time	
EPO (81–95)	5.2	13	1.2	26	1.7	6 (S/N = 9)
NESP (86–104)	6.7	6	0.8	9	1.5	6 (S/N = 5)

[a] Isoelectric points (p*I*) were estimated with p*I*/Mw compute [31].
[b] Repeatability (n = 6/1 day) and intermediate precision (n = 18/3 days) studies were performed with a synthetic peptide mixture at 100 mg/L using the same separation capillary.

delivered at a high flow compared to the liquid flow coming-up from the column, ion production would be compromised to some extent leading to extra variability of peak area values.

Similarly, tryptic-PNGase F digests of rhEPO and NESP were analyzed by CE–MS at neutral pH. The disulfide bonds of rhEPO and NESP were reduced and alkylated before digestion in order to increase trypsin cleavage yields [14]. Table 1 lists the peptides detected for rhEPO and NESP with their corresponding amino acid position in the native protein sequence, and their calculated and observed m/z values. Sequence coverage was around 70% for both glycoproteins. In both cases, only one proteotypic peptide that was expected was not detected: the de-*N*-glycosylated peptide in position (21–45). As can be observed in Table 1, the digestion pattern of rhEPO and NESP was almost identical. All peptides were common to both digests, except for peptide 15 (15-EPO for rhEPO and 15-NESP for NESP) which showed three of the five amino acid differences between rhEPO and NESP (Table 1, in bold). PNGase F treatment converted glycosylated asparagine residues (N) to aspartic acid (D), (Table 1, underlined). This is why peptides 15-EPO and 15-NESP were slightly modified from the native sequence of both glycoproteins (see Table 1). As they were specific for rhEPO and NESP, they were selected as peptide markers for their differentiation. Fig. 2 shows the CE–MS electropherograms for a solution of 50 mg/L of rhEPO (Fig. 2a) and NESP (Fig. 2b) digests. Concentration of the digests is expressed in mg/L of 15-EPO peptide and 15-NESP, respectively. Fig. 2a (i) and b (i) illustrates the separation of the digests obtained using the neutral BGE of 20 mM of NH_4Ac (pH 7.0). Numbers depicted in the electropherograms correspond to peptides listed in Table 1. As expected, except for peptides 15-EPO and 15-NESP, the migration orders of the detected peptides were similar. All digested peptides in Table 1 were detected with the exception of peptide 14. For comparison with standard conditions employed in CE–MS for peptides in positive ion mode, an acidic BGE of 50 mM HAc and 50 mM HFo (pH 2.2) was also used for digest separation. Fig. 2a (ii) and b (ii) shows the separation achieved with this acidic BGE. The increase in analysis times produced by the lower electroosmotic flow (EOF) and the better peak shape compared to those obtained with the neutral BGE, resulted in increased separation resolution. In addition, the acidic pH of the BGE had a slightly favourable effect on sensitivity (e.g. compare y-scales of EIEs in Fig. 2a (i) and (ii)), leading to the detection of peptide 14, which was not detected with the neutral BGE. Fig. 2a (iii) and b (iii) shows the mass spectra of 15-EPO and 15-NESP peptides with the neutral BGE. In both cases, the main signals corresponded to triple and double protonated ions. As can be observed in the insets, poorer isotopic resolution was obtained for the triple protonated ions because of the lower resolving power of the ion trap for these higher protonated ions.

3.2. IA-CE–MS

After testing and validation of the CE–MS methodology using a neutral volatile BGE, IA-CE–MS was explored for the analysis of the synthetic peptides EPO (81–95) and NESP (86–104) and rhEPO and NESP digests. The IA sorbent was prepared in-house, immobilizing a custom made polyclonal anti-EPO (81–95) Ab [22] on a CNBr-activated Sepharose 4B solid support. Then, it was packed in a microcartridge following the procedure described in Section 2.4.2 in order to construct the IA-CE column. The major goal of an IA-CE–MS method is to optimize the extraction process, maximizing the recovery of the target analyte and minimizing the amount of co-eluted impurities, under conditions which provide efficient and reproducible CE separations compatible with on-line ESI-MS detection. A generic IA-CE protocol consists of five basic steps (equilibration, sample loading, washing, elution and electrophoretic separation) [16,17,20,29]. As the polyclonal Ab was raised against peptide EPO (81–95), it was confirmed by immunochemical analytical methods (e.g. enzyme-linked immunosorbent assay (ELISA)) that it presented improved affinity for the EPO (81–95) synthetic peptide. Hence, a 50 mg/L solution of the EPO (81–95) synthetic peptide was employed to study the influence of several parameters on the IA-CE–MS method.

First, the IA microcartridge was washed with the neutral BGE in order to remove trapped air, eliminate residual salts from the storing buffer, such as Tris and NaN_3, and equilibrate and prepare the IA sorbent for the interaction with the target analyte. As explained above, a BGE near to physiological pH was used in order to avoid Ab denaturation and permit antigen–antibody interaction. After this equilibration step, suitable sample loading was required in order to retain the maximum amount of target on the sorbent. In our case, the sample solution was hydrodynamically injected for 10 min at three different pressures: 930, 50 and 25 mbar (i.e. 57.7, 3.1, 1.5 μL of sample, respectively [26]). The amount of EPO (81–95) eluted at 50 mbar was significantly higher than the amount eluted at 930 mbar. Even though less sample volume was loaded into the IA column, contact time between the antigen and the Ab was probably prolonged, and consequently, the immobilized Ab was able to recognize and retain higher amounts of peptide EPO (81–95) at 50 mbar than at 930 mbar. These results differ from those described for non-specific sorbents, such as reverse-phase C18, where sample loading at 930 mbar was preferred [16,24]. In contrast to the results at 50 mbar, at 25 mbar, the amount of EPO (81–95) eluted was less than that at 50 mbar or 930 mbar, suggesting that the amount of peptide loaded into the system per unit time was not enough to saturate the IA sorbent. Sample loading time was also evaluated at 50 mbar. Finally, 10 min was selected as an appropriate sample loading time since the peak area of EPO (81–95) peptide showed no significant increase after introducing sample solution for 20 or 30 min (i.e. 6.2 or 9.3 μL [26]).

Once the sample was successfully loaded, a washing step with neutral BGE containing 0.5 M NaCl was required. This type of washing is widely used in conventional IAC to remove undesired molecules retained by non-specific interactions and preserve antigen–antibody bonds [22]. In order to eliminate residual NaCl, the system was equilibrated before the electrophoretic separation with a rinse of neutral BGE for 5 min. Rinsing times lower than 5 min resulted in the detection of NaCl cluster ions at the beginning of the run. Once the IA-CE column was filled with neutral BGE, several approaches could be used for analyte elution from the IA sorbent. The antibody–antigen interaction can be destabilized by

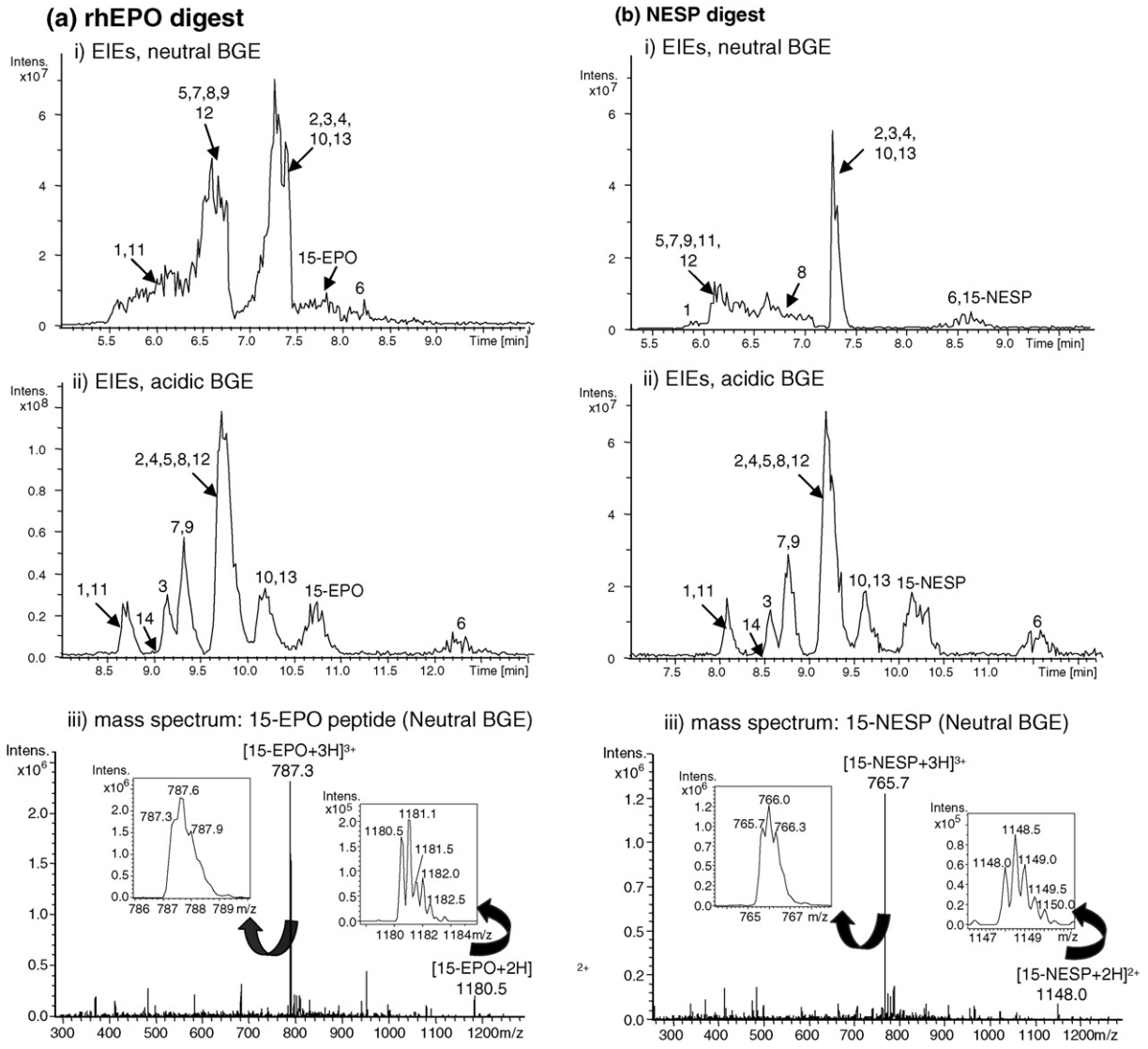

Fig. 2. CE–MS analysis of (a) rhEPO and (b) NESP digests (sample: 50 mg/L solution of 15-EPO or 15-NESP peptides; injection: 15 s at 50 mbar; voltage: +18 kV; *T*: 25 °C). Extracted ion electropherograms (EIEs) using: (i) neutral BGE: 20 mM NH_4Ac pH 7.0, (ii) acidic BGE: 50 mM HAc and 50 mM HFo pH 2.2 and (iii) mass spectra of 15-EPO or 15-NESP peptides obtained with the neutral BGE. Insets: isotopic patterns of triple and double protonated ions for 15-EPO or 15-NESP peptides.

changing the pH of the running electrolyte though brief exposure to high or low pH, or by adding agents such as chaotropic ions or organic solvents that reduce hydrophobic interactions between the molecules [17]. In our case, the target analyte was eluted by introducing a small volume (i.e. a plug) of an acidic buffer. Acidic plugs of different composition were tested. 100 mM glycine–HCl (pH 2.5) is popular in IAC, and it has also been described for IA-CE–MS [29]. However, we rapidly rule out this non-volatile solution as it had a negative influence on ESI-MS detection. Maximum peak areas were achieved after eluting the EPO (81–95) peptide with a volatile mixture of 50 mM HAc and 50 mM HFo (pH 2.2). Solutions with higher acidity and ionic strength (e.g. 100 mM HAc and 100 mM HFo, pH 2.1) did not improve this result. In contrast, the elution plug volume was critical, and large volumes were required to elute the peptide retained in the column. Several injection volumes were tested: 10, 20, 50, 100 and 150 s at 50 mbar (i.e. 0.05, 0.1, 0.25, 0.5 and 0.77 μL [26]), but the peptide was eluted only after 50 s or longer. An injection of 100 s at 50 mbar was selected for further investigations as it provided the highest peak area of eluted peptide. Finally, after the electrophoretic separation, the IA-CE column was rinsed with elution plug solution. This acidic washing step could degrade the Ab immobilized on the IA sorbent to some extent. However, it was necessary in order to avoid carry-over between consecutive analyses. Under these conditions, the durability of the IA-CE column was roughly 8–10 analyses.

Individual solutions and 50:50 binary mixtures of EPO (81–95) and NESP (86–104) synthetic peptides at 5, 25 and 50 mg/L were analyzed by the established IA-CE–MS method. Concentrations of peptides below the LODs in CE–MS (see Table 2) were not detected by IA-CE–MS. EPO (81–95) was detected analyzing individual solutions and binary mixtures at 25 mg/L. In contrast, NESP (86–104) was not detected either in individual solutions or in mixtures. This confirmed the specificity of the IA-CE–MS method for the EPO (81–95) synthetic peptide [22]. Fig. 3a (i) shows the BPE obtained for a mixture of EPO (81–95) and NESP (86–104) at 25 mg/L. The EIEs of both synthetic peptides (Fig. 3a (ii)), and the mass spectrum obtained at 9.6 min, showing the typical isotopic peak pattern of $[EPO\ (81–95)+2H]^{2+}$, confirmed that the peak overlapping with the elution plug corresponded only to peptide EPO (81–95). This last fact was confirmed after a blank analysis. The electrophoretic separations and currents obtained by IA-CE–MS were different from those obtained by CE–MS because of the large volume of acidic

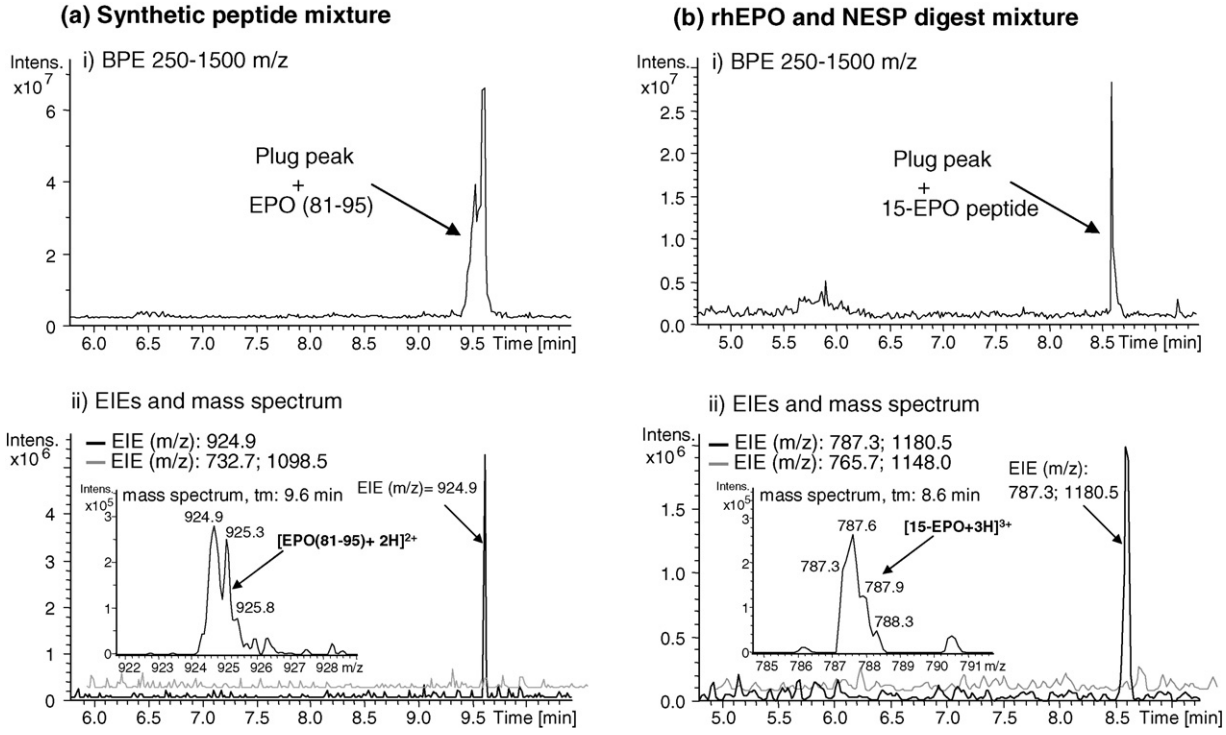

Fig. 3. IA-CE–MS analysis of (a) a mixture of the synthetic peptides (sample: 25 mg/L solution of peptides EPO (81–95) and NESP (86–104); sample introduction: 50 mbar for 10 min; injection of acidic elution plug: 100 s at 50 mbar; voltage: +18 kV; *T*: 25 °C; BGE: 20 mM NH_4Ac pH 7.0). (i) BPE and (ii) EIE of EPO (81–95): 924.9 *m/z* (in black), and EIE of NESP (86–104): 732.7 + 1098.5 *m/z* (in grey). Inset: isotopic pattern of double protonated ion for peptide EPO (81–95). (b) Mixture of rhEPO and NESP digests (sample: 25 mg/L solution of 15-EPO and 15-NESP peptides; rest of experimental conditions as in (a)). (i) BPE and (ii) EIE of 15-EPO: 787.3 + 1180.5 *m/z* (in black), and 15-NESP: 765.7 + 1148.0 *m/z* (in grey). Inset: isotopic pattern of triple protonated ion for 15-EPO peptide.

solution injected to elute the peptides. This fact was verified by CE–MS analysis of a mixture of EPO (81–95) and NESP (86–104) after injecting increasing amounts of a sample diluted in the acidic elution solution. Fig. 4 shows the BPE obtained at neutral pH injecting for 10, 50 and 100 s at 50 mbar (i.e. 0.05, 0.25 and 0.5 μL [26]). With an injection time of 10 s, peaks from EPO (81–95) and NESP (86–104) were separated from each other with a stable current value of 25 μA. Longer injection times resulted in poorer separation resolution to the point that peptides comigrated with the elution plug peak when injection times were increased to over 75 s. Under these conditions, in CE–MS and IA-CE–MS, the current fell to a few μA during the first minute of the run, then reached a constant value of 15 μA, and recovered when the injected elution plug left the separation capillary, coinciding with detection of the eluted peptide. To completely confirm that retention of EPO (81–95) was due to antibody–antigen interactions and not to non-specific adsorption on the IA sorbent, solutions of dynorphin A (1–7) at 25–50 mg/L were also analyzed. Dynorphin A (1–7) is an opioid peptide that appears in a similar *m/z* range to EPO (81–95) and NESP (86–104) and its peptide sequence is completely different from any tryptic peptide of rhEPO or NESP. As expected, we did not observe any peak for dynorphin A (1–7). However, when individual solutions of NESP (86–104) at 50 mg/L were analyzed, a peak corresponding to this peptide appeared in the IA-CE–MS electropherogram, but with lower intensity than that observed for EPO (81–95) at 25 mg/L. This weak response of the polyclonal anti-EPO (81–95) Ab against NESP (86–104) in concentrated solutions was also observed by ELISA in previous work [22]. This suggested that the polyclonal Ab contained some specific Abs that could recognize several epitopes present in both peptide sequences. Nevertheless, Ab cross-reactivity for NESP (86–104) was not observed when we analyzed 25 mg/L solutions or binary mixtures of synthetic peptides at 50 mg/L.

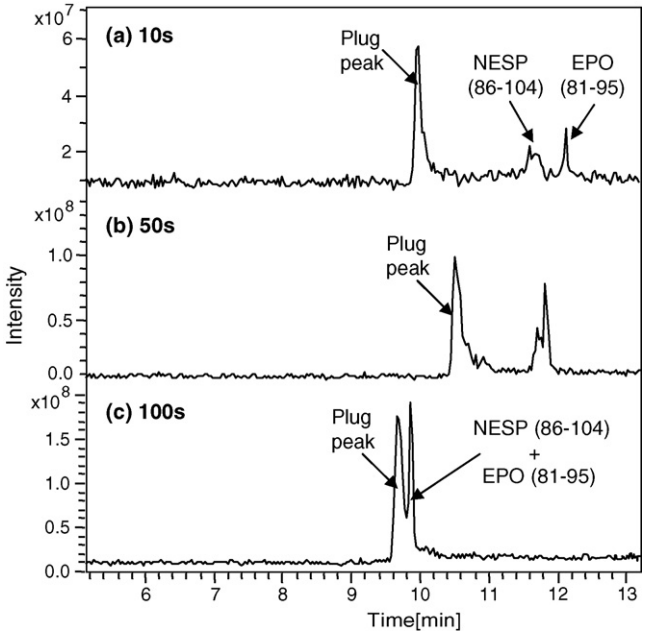

Fig. 4. CE–MS BPE 250-1250 *m/z* for a mixture of synthetic peptides increasing the amount of injected sample diluted in acidic elution plug. (a) 10 s, (b) 50 s and (c) 100 s at 50 mbar (sample: 6 mg/L solution of EPO (81–95) and NESP (86–104) peptides; voltage: +18 kV; *T*: 25 °C; BGE: 20 mM NH_4Ac pH 7.0).

Once we had demonstrated that the polyclonal anti-EPO (81–95) Ab immobilized in the IA microcartridge was highly specific for EPO (81–95) peptide, rhEPO and NESP digests and their 50:50 binary mixtures were analyzed following the same IA-CE–MS methodology. Fig. 3b (i) shows the BPE obtained for a mixture of rhEPO

and NESP digests at 25 mg/L. As can be observed in the EIEs and in the mass spectrum in Fig. 3b (ii) only the proteotypic peptide 15-EPO, which contained the sequence of the EPO (81–95) synthetic peptide, was retained and detected from the mixture of 16 enzymatically released peptides from rhEPO and NESP. Furthermore, peptides 15-EPO and 15-NESP only differ in three aminoacids (87–88, 90) (Table 1) and the Ab was able to distinguish between them. Our results suggest that the method specifically confirms the presence of the 15-EPO peptide in solution even in the presence of peptides from one of its analogues. However, as indicated before, the LODs in IA-CE–MS were slightly higher than those obtained by CE–MS using the neutral BGE (Table 2).

4. Concluding remarks

A CE–MS method using a volatile BGE of ammonium acetate at neutral pH was developed for the separation of EPO (81–95) and NESP (86–104) synthetic peptides. Despite the lack of buffer capacity at pH 7.0, ammonium acetate BGE provided stable electrospray conditions in positive ion mode, good ESI-MS response and intermediate precision and appropriate separation performance if the BGE solution was refreshed frequently. The same method was used for the analysis of rhEPO and NESP tryptic-PNGase F digests. The proteotypic peptides EPO (77–97) and NESP (77–97), which differ in three amino acids, were selected as potential peptide markers for rhEPO and NESP confirmation. A selective IA-CE–MS method was developed for the analysis of the synthetic peptides and rhEPO and NESP digests. As the polyclonal Ab of the IA sorbent was raised against peptide EPO (81–95), only this synthetic peptide and peptide EPO (77–97) from the rhEPO digest were detected. In addition, in spite of differing from EPO (77–97) in only three amino acids, NESP (77–97) was not detected. However, although the IA column was specific, peptides were not detected at low concentrations (LOD $\approx$ 25 mg/L). The most likely explanations for the limited extraction efficiency attained might be the reduced affinity of the polyclonal Ab to the antigenic peptide, the poor yield of the Ab coupling to the CNBr-activated solid support, the deficient orientation of the Ab binding sites or the limited amount of IA sorbent packed inside the microcartridge. We are currently evaluating other Abs, solid supports and coupling chemistries in order to increase the IA active surface and adequately orientate the Ab on the IA sorbent. Nevertheless, in the future, even under optimum IA-CE–MS conditions, biological fluid samples may need some previous off-line immunoaffinity purification before digestion so that the analysis by IA-CE–MS using anti-EPO and anti-NESP specific antibodies can be a sensitive and reliable way of confirming rhEPO and its analogues at the levels expected in biological fluids ($\leq$200 ng/L of hEPO in urine or plasma).

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