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Matrix-assisted laser-desorption time-of flight ionisation and high-performance liquid chromatography–electrospray ionisation mass spectral analyses of two glycosylated recombinant epoetins

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Abstract

Mass spectrometric analyses of the recombinant proteins in Eprex® and Aranesp® were undertaken with the goal of producing reference mass spectra and evaluating strategies to improve its applicability as a method for equine and canine doping control of these substances. A simple, low chemical noise deglycosylation reaction removed microheterogeneity due to post-translational carbohydrate attachment and both proteins were detectable using MALDI-TOF-MS. Deglycosylated human erythropoietin (hEPO) was also detected using HPLC–ESI-MS. This is the first time that spectra of deglycosylated Eprex and Aranesp have been published. Eight synthetic reference standards, which match peptides produced by endoproteinase Glu-C enzymatic cleavage of Aranesp and/or Eprex, were analysed by ESI-MS and ESI-MS–MS. The E12 Glu-C peptide, common to both proteins, was detected at the low femtomole-level using gradient nano-HPLC–ESI-MS–MS in the positive ion mode.
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1. Introduction

The active ingredient of Eprex® (EPR) is recombinant human erythropoietin (rhEPO), a glycoprotein that has been reported to enhance aerobic capacity in humans by increasing red cell volume [1] and is rumoured to be used by athletes engaging in sports requiring endurance. Anecdotal accounts of EPO abuse in racing animals have been widespread in many jurisdictions around the world. More recently a novel erythropoiesis stimulating protein Aranesp® (NESP) has become available. This glycoprotein, produced by recombinant DNA technology, only differs from human EPO at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn and Pro90Thr). These modifications allow for additional oligosaccharide attachment at residues 30 and 88 [2], increasing its serum half-life in comparison to EPR [2].

The recombinant human EPO in EPR is produced from Chinese hamster ovarian (CHO) cells. This

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particular rhEPO has an average molecular mass of approximately 30 000 and, after the C-terminal arginine is lost due to post-translational modification by carboxypeptidase [3], the mature protein consists of a 165-amino-acid sequence with two disulphide linkages (Cys7–Cys161 and Cys29–Cys33) [4]. From a chemical viewpoint rhEPO produced in Chinese hamster ovarian cells is not a single entity, but a large population of sialoglycoproteins composed of a variety of highly branched N- and O-linked glycan motifs attached at one or more of the four potential glycosylation sites (Asn24, Asn38, Asn83 and Ser126) along the protein backbone [4–8]. The lack of glycosylation pattern completion by the glycosyltransferases of the CHO cells results in a heterogeneous mixture of glycoproteins. NESP is also produced in CHO cells and because it has an additional two sites where N-linked carbohydrate chains can be attached, it is composed of an even greater number of chemical entities [2] than EPR. If this heterogeneity due to the carbohydrate added as a post-translational modification is not reduced or eliminated, it will make it considerably more difficult to detect EPR or NESP in doping control samples using mass spectrometry based analytical techniques.

The main reason behind the development of an accurate method of detecting the administration of these exogenous erythropoietic factors to racing animals is that both EPR and NESP are likely to be performance enhancing, as there is a strong positive correlation [9] between the red-cell mass increase they produce and improved maximal oxygen uptake in horses. They, therefore, would give an unfair advantage over untreated competitors.

Previously reported methods of detecting hEPO in equine and canine samples have all been based on sensitive immunoassays [10–13]. This is probably due to the fact that immunoassay techniques are much less affected by the microheterogeneity of these glycoproteins than instrumental detection methods like mass spectrometry. The microheterogeneity greatly reduces the sensitivity for MS detection, as the detected signal is split between numerous and therefore less intense ions. However, immunoassays lack specificity and are generally only suitable in doping control as a method of screening out those likely to contain the target analyte prior to further, more definitive, analysis.

Lasne and de Ceaurriz [14] have reported a method of detecting the recombinant human epoetins, EPO-α (Eprex) and EPO-β (Neorecormon®), in human urine by using an immunoblotting procedure that can differentiate endogenous hEPO from these recombinant EPOs on the basis of the presence of additional basic bands after electrophoretic separation of a urinary extract. However, we are of the opinion that a MS-based analysis for EPR or NESP protein in racing animal samples will be more suitable. It is reported [15] that there are differences in the amino acid sequence of EPO from rhesus monkey, rat, sheep, pig, dog, cat and, while these researchers did not generate the full coding sequence, they were able to show that there was approximately 80% similarity in part of the sequence between human and equine EPO precursor. We have investigated ways to exploit this difference with a view to providing a definitive test for detecting hEPO and NESP use in racehorses and greyhounds.

We report on our study of methods, which require minimal sample manipulation, for converting these epoetins into a more homogeneous compound or compounds suitable for MS analysis. This has included developing a single-step, low chemical noise, procedure for the removal of the carbohydrate and also the use of endoproteinase Glu-C digestion to produce peptides which can also be used as targets in doping surveillance aimed at detecting epoetin abuse. To assist with the later option, reference peptides, with amino acid compositions matching those published for the Glu-C hydrolysis of rhEPO [5] were synthesised. HPLC (positive and negative)-ESI-MS–MS analysis of the synthetic peptides and peptides obtained by the Glu-C enzymic hydrolysis of EPR and NESP, has shown them to be identical.

E12, an analogue of one of the larger Glu-C derived peptides of EPR and NESP, analysed in the positive ESI mode was found to give the best combination of MS response and information rich spectra. A 50-fmol amount of the synthetic peptide E12 was analysed using a nano-HPLC–ESI system. This analytical methodology is reported to have a detection of limit of 50 amol [16] and should be well suited to screening for EPR and NESP in the concentration range likely to be encountered in racing samples.

2. Experimental

2.1. Reagents and chemicals

Endoproteinase Glu-C (50 μg, sequencing grade), N-glycosidase F (250 U/0.25 ml), O-glycosidase (25 mU/50 μl) and neuraminidase (1 U/100 μl) were purchased from Roche Diagnostics (Sydney, Australia). Amicon® Ultrafree centrifugal filter units with Biomax 10K nmwl membrane (0.5 ml capacity), Amicon Centricon YM-10 centrifugal filter units and C4+C_{18} Zip-tip® were from Millipore (Sydney, Australia). Caffeic acid was from ICN Biomedicals (Sydney, Australia). Trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (4-HCCA) and D-galactono-γ-lactone were purchased from Sigma (Sydney, Australia). The MALDI calibration mixtures were provided in the Sequazyme® peptide mass standards kit purchased from Applied Biosystems (Melbourne, Australia). Eprex rhEPO (10 000 units/ml, batch 01GS2OT) was obtained from Janssen-Cilag (Sydney, Australia) and Aranesp® (darbepoetin alfa 500 μg/ml, batch L000610990151) was a gift from Amgen (Sydney, Australia). All other reagents were of analytical grade. Water was purified by a Millipore water purification system and had a resistivity of >18 MΩ/cm. Mallinckrodt acetonitrile (nanograde®) was supplied by Selby Biolab (Sydney, Australia).

2.2. Synthetic peptides

Synthetic peptides E5, E7, E8, E9, E10, E11, E12 and E13 were made by Auspep (Parkville, Australia). Prior to being supplied, the peptides were analysed by the manufacturer using Superspher® 250-4 Li-Chrocart 100 RP-18 HPLC column with 0.1% TFA as solvent A and acetonitrile–0.1% TFA in water (90:10, v/v) as solvent B. The flow was 1.0 ml min and a linear gradient of 0% B to 70%B was used to elute the peptides with UV detection at 218 nm.

2.3. Single step deglycosylation of Eprex and Aranesp

Deglycosylation was performed in buffer consisting of 20 m*M* $NaPO_4$ (pH 7.2), 20 m*M* D-galactono-γ-lactone and 0.01% sodium azide. The following components were added to a 0.5 ml polypropylene tube; EPR or NESP (25 pmol/9 μl), deglycosylation buffer (10 μl), N-glycosidase F (3 μl), O-glycosidase (2 μl) and neuraminidase (1 μl). The mixture was then incubated at 37 °C for ~18 h.

2.4. Endoproteinase Glu-C digest of Eprex and Aranesp

One 50-μg vial of sequencing grade endoproteinase Glu-C was reconstituted in 50 μl 100 m*M* ammonium acetate (pH 8.0). The following components were added to a 0.5 ml polypropylene tube; EPR (approximately 0.6 nmol/20 μl) or NESP (10 μg/20 μl) and 5 μl of Glu-C. The mixture was incubated at 27 °C for ~18 h.

2.5. MALDI-TOF-MS analysis of whole Eprex and Aranesp and enzymatic digests of Eprex

MALDI-TOF-MS analyses were performed on a Voyager DE STR mass spectrometer with a nitrogen laser (337 nm; 2 ns pulse) from PE Biosystems (Framingham, MA, USA). All analyses were in the positive ion mode.

The intact glycosylated or deglycosylated epoetins were concentrated and desalted over a C4 Zip-tip using a 0.1% TFA water wash and the analyte eluted directly onto the stainless steel target in 2 μl caffeic acid matrix [10 mg/ml in TFA–water–acetonitrile (0.1:20:80, v/v/v)]. Glu-C digests of EPR or NESP were concentrated and desalted over a C_{18} Zip-tip (Millipore, Sydney, Australia) using 0.1% TFA in water wash and the peptides eluted directly onto the stainless steel target in 2 μl 4-HCCA matrix (5 mg/ml in TFA–water–acetonitrile (0.1:20:80, v/v/v)].

2.6. HPLC–ESI-MS analysis of deglycosylated Eprex

A Thermo Finnigan LCQ^{deca} (San Jose, CA, USA) ion trap mass spectrometer with standard ESI source was calibrated according to the manufacturer's procedure when operating in the normal mass range (100–2000 m/z) and using TFA sodium adducts when operated in the high mass range. A MicroTech™ Ultra II (Sunnyvale, CA, USA) micro-

HPLC and was used to control the injector and provide a gradient for all separations.

Deglycosylated whole hEPO was separated using a MicroTech 50 mm×320 μm fused-silica inside (polyether ether ketone) PEEK column (Kromasil™ C_{18}; 5 μm packing) with mobile phases A=0.03% TFA in water and B=0.03% TFA in acetonitrile at a combined flow-rate of 10 μl/min. After an initial 10 min rapid purge at 80 μl/min and 30 min equilibration period, the injection valve with a 1-μl PEEK loop was switched for 180 s. The starting mobile phase conditions of 95% A were ramped in a linear gradient to 5% A over 5 min and held for 25 min before returning to initial conditions over 2 min. The positive ion ESI conditions used were nitrogen sheath gas at 35 (arb.) and auxiliary setting of 1 (arb.), heated capillary temperature 225 °C and 5 kV source voltage. The ion trap was set up to scan for positive ions 350–4000 m/z with a maximum ionization time of 50 ms.

2.7. HPLC–ESI-MS analysis of synthetic peptides, Glu-C hydrolysed Aranesp and Eprex

2.7.1. HPLC

NESP and EPR, digested with endoproteinase Glu-C, were separated using an Agilent (Sydney, Australia) stainless steel 300 SB-C_8 microbore rapid resolution 1 mm×150 mm column (Zorbax™ C_8; 3.5 μm packing and 300 Å pore). The mobile phase A=0.1% formic acid in water and B=acetonitrile was used at a combined flow-rate of 80 μl/min in splitless mode. An initial 5 min rapid purge at 150 μl/min with the purge valve in the open position proceeded a 10-min equilibration period. The starting mobile phase conditions of 95% A were ramped to 5% A over 15 min and held for 13 min before returning to initial conditions over 2 min. The ESI conditions used were nitrogen sheath gas at 50 (arb.) and auxiliary flow of 2 (arb.), heated capillary temperature 200 °C and 5 kV source voltage.

2.7.2. Conditions used for MS–MS

The conditions used for the MS2 experiments with a maximum ionization time of 200 ms are shown in Table 1.

2.8. Nano-HPLC–ESI-MS–MS of synthetic peptide E12

Peptide E12 was separated using the MicroTech® Ultra II micro-HPLC (Sunnyvale, CA, USA) connected to a new objective PicoView™ (Cambridge, MA, USA) nanospray source fitted with a 75 μm I.D. fused-silica PicoFrit™ (15 μm tip) column packed in the laboratory to 75 mm with Spherisorb (C_{18}; 5 μm). The PicoFrit tip was positioned approximately 1–2 mm from the heated capillary kept at a temperature of 185 °C and the ESI voltage applied via a stainless steel (SS) zero dead volume T connector was adjusted to provide a stable nanospray from the PicoFrit tip at a split flow of approximately 200 nl

Table 1
Parameters used during ESI-MS–MS experiments

Peptide	Negative ion ESI-MS			Positive ion ESI-MS		
	m/z of the precursor ion	Amplitude of applied RF voltage (V)	Mass range for product ion scan	m/z of the precursor ion	Amplitude of applied RF voltage (V)	Mass range for product ion scan
E1	1502.00	1.50	410–2000	–	–	–
E2	601.50	1.50	165–2000	–	–	–
E3	691.50	1.50	190–2000	–	–	–
E5	1688.50	1.75	460–2000	1689.50	2.00	465–2000
E7	1571.0	1.75	445–2000	1573.0	2.00	450–2000
E8	728.00	2.00	205–2000	729.00	2.00	205–2000
E9	1114.00	1.70	315–2000	1115.00	1.50	315–2000
E10	1334.80	1.90	500–2000	1336.00	1.90	500–2000
E11	2211.00	2.75	610–2000	1108.00	1.85	305–2000
E12	1836.00	1.90	505–2000	920.00	2.40	305–2000
E13	1417.00	2.20	390–2000	1419.00	2.00	390–2000

with a mobile phase composition of A–B (50:50). In general, running voltages were in the region of 2.0 to 2.8 kV. The 1-μl sample was loaded onto the column at a flow-rate of 5 μl/min×2 min, using a Valco (Houston, TX, USA) six-port polymeric stator and Cheminert® rotor as an injector. This was combined with the use of a six-port rapid purge valve supplied with the HPLC to channel the entire flow onto the column by removing the splitting as shown in Fig. 1.

During sample injection the mobile phase was a mixture of A–B (95:5) (A = 0.1% formic acid water and B = acetonitrile) and flow splitting at the T piece (approximately 25:1) was stopped for the duration of the sample-loading step. Before the start of the solvent gradient the actuation of the rapid purge valve using the software allowed the split flow-rate to the PicoFrit column to be decreased to approximately 200 nl/min. The mobile phase conditions of A–B (5:95) were held for 0.75 min. before ramping on a linear gradient to 80:20 at 2.00 min., the mobile phase was changed on a linear gradient to 20:80 at 20 min and held at this composition for 250 min. Thereafter, mobile phase was returned to initial conditions over 2 min. The conditions used for the MS2 experiment is described above.

3. Results and discussion

The spectrum (Fig. 2a) of approximately 1 pmol of glycosylated EPR analysed by MALDI-TOF-MS in a caffeic acid matrix shows a broad peak with a maxima at m/z 29 620 and this is consistent with the average molecular mass of 30 400 stated in the product information [17] supplied. The heterogeneous nature of the material is evident from the peak's width (m/z range from 26 000 to 33 500) and this was in accordance with the varying composition of the carbohydrate attachment reported previously [5]. This result is not consistent with the detection of a 52 000 band [13] during immunoblot assay of rhEPO on polyacrylamide gel. NESP was also analysed by MALDI-TOF-MS and Fig. 2b shows a similar pattern to Fig. 2a with a broad peak with a maximum at m/z 36 054. The increase in mass is consistent with the increased carbohydrate component of NESP as compared to EPR.

While several papers provide for removal of either N- or O-linked glycosyl chains from EPO using enzymatic [4–8] or chemical [7] means, we were unable to find a published one-step deglycosylation procedure for simultaneously removing both the N- and O-linked glycans. Another negative factor was that some published methods for removing the carbohydrate chains include the addition of denaturing agents like sodium dodecylsulphate (SDS) and other detergents (e.g. Nonidet P40®) to facilitate the deglycosylation reaction. We opted for a single step incubation of the unreduced epoetins with N- and O-glycosidases plus neuraminidase enzymes without the addition of these substances as they can suppress ionisation and contribute to the chemical noise. This

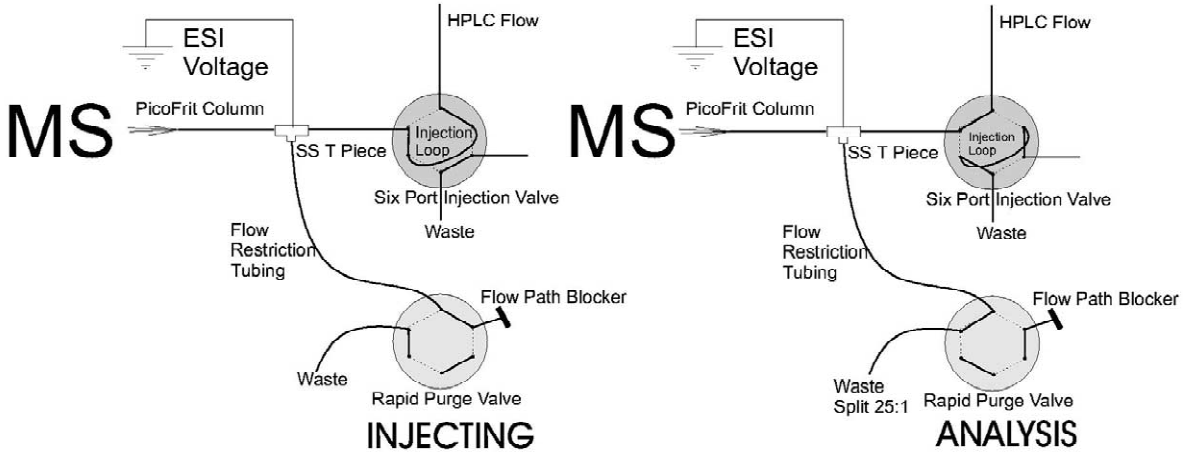

Fig. 1. Configuration of the HPLC injection and rapid purge valves used for nano-HPLC–ESI.

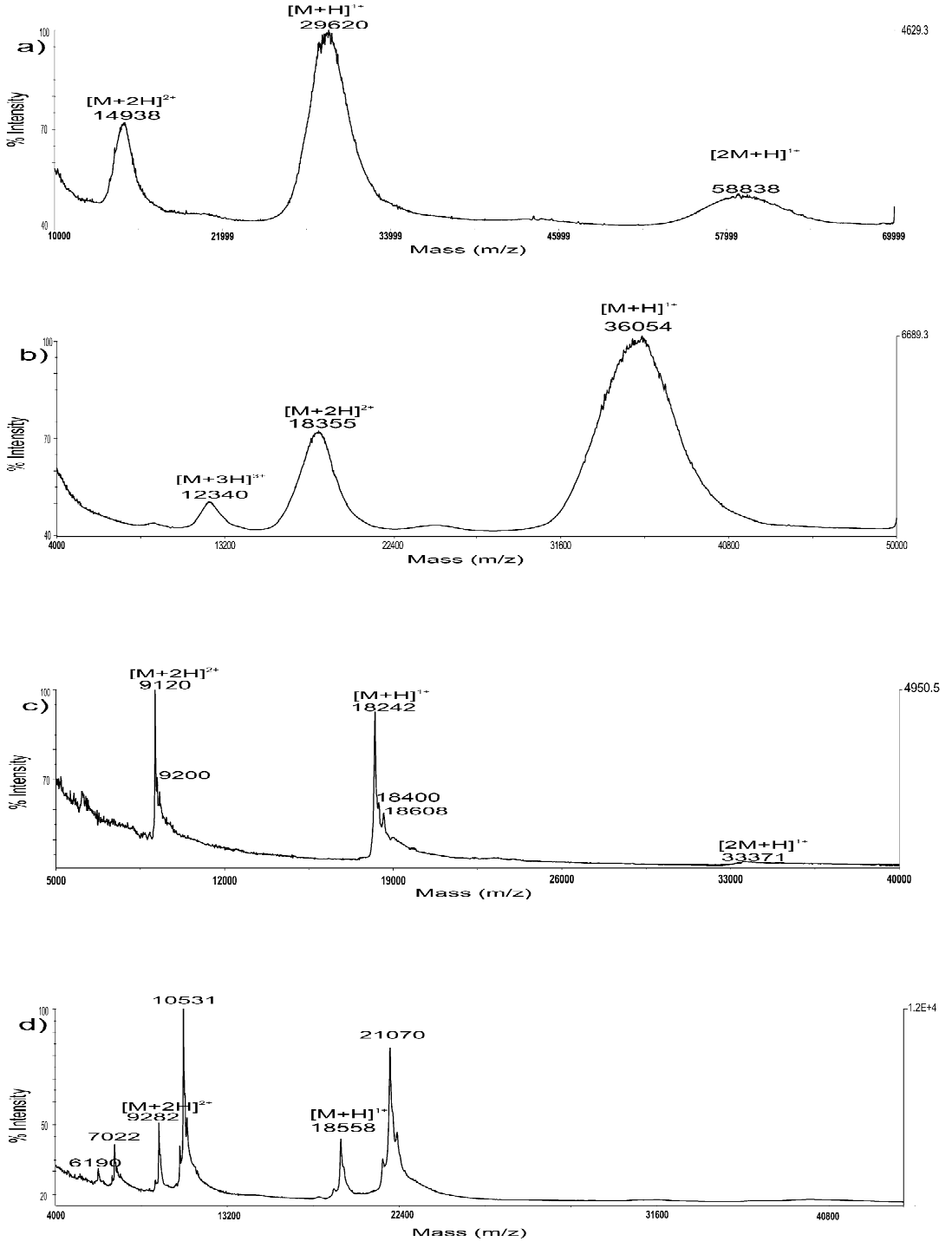

Fig. 2. MALDI-TOF-MS positive ion spectrum of (a) EPR, (b) NESP, (c) deglycosylated EPR and (d) deglycosylated NESP.

approach proved successful for EPR where little or no glycosylated material was detected by MALDI-TOF after the reaction and partially successful for NESP where approximately one third was converted to the fully deglycosylated product.

The MALDI-TOF-MS spectrum (Fig. 2c) of deglycosylated EPR shows peaks at m/z 9120 and m/z 18 242, and this is consistent with both the published amino acid sequence (average molecular mass, M_r, 18 240) and the molecular mass information [17] for the polypeptide provided by the producer of the rhEPO. Fig. 2d shows an $[M+H]^{1+}$ ion at m/z 18 558 and this is consistent with the structure of the deglycosylated NESP. Despite the fact that the reaction has not gone to completion with all of the NESP, it has still enhanced the spectral quality by reducing the heterogeneity and one pseudomolecular ion ($[M+2\ H]^{2+}$, m/z 10 531) characteristic of the partially deglycosylated protein (M_r 21 070 Da) predominates. This is consistent with a previous study [18] where partial deglycosylation of glycoproteins enhanced MALDI-TOF-MS peptide matching. These authors noted that partial deglycosylation also overcame resistance to protease digestion that can occur with glycoproteins.

Testing will have to target the femtomole range at the very least and optimally should be capable of detecting attomol of analyte if a prolonged period of detection after administration is desired. Previous research administering a dose of 125 I.U. per kg body mass of hEPO to racehorses gave blood samples that showed a maximal concentration of 335 mI.U./ml (83 fmol/ml; 2.8 ng/ml) EPO equivalents on immunoassay and these values returned to baseline after 60 h [12]. The authors noted that there was no significant increase in red-cell volume at low doses and propose that doses up to 300 I.U./kg might be required to elicit a visible haematological change. Greyhounds that were given 125 I.U./kg rhEPO reached peak serum levels of 400 mI.U./ml EPO equivalents and this value returned to baseline after approximately 7 days [10]. In many thoroughbred racing jurisdictions the typical volume of equine blood collected would provide approximately 20 ml of serum or plasma for analysis. Therefore at peak concentration there will be up to a total of 2 pmol of hEPO present in an equine sample, but it is more likely that the actual levels encountered will be considerably less than this, as rhEPO is cleared rapidly from the system. However, even with the reduction of complexity by deglycosylation, obtaining a successful MALDI-TOF-MS analysis on our equipment required amounts of analyte in the picomolar range and, therefore, was not sufficiently sensitive to provide an effective method of screening for these epoetins at the level anticipated in doping control samples. Techniques for improving MALDI-TOF-MS sensitivity with specialised equipment, for example ''spot-on-a chip'' [19] and piezoelectric microdispensing [20] for on-target enrichment, were not readily available for our use and so other analytical methods were investigated instead.

Deglycosylated EPR was analysed by HPLC–ESI-MS in the positive ion mode using an LCQ^{Deca} quadrupole ion trap tuned in the high mass range (m/z 100–4000) using a solution of sodium trifluoroacetate produced according to a published method [21]. The spectrum obtained (Fig. 3a) is consistent with the results from the MALDI-TOF-MS analysis and, based on an average of the mass calculated from each of the three strongest ions, the average molecular mass is approximately 18 254. As observed by MALDI-TOF-MS, the removal of the glycan from EPR produced a relatively homogeneous material which could be analysed by MS with greater sensitivity than would be possible with the heterogeneous compound. The extracted ion chromatogram from the analysis of approximately 250 fmol on column is shown in Fig. 3b. Analysis using a HPLC column packed with C_8 packing material produced a sharper peak but also resulted in very low intensity of the ions of interest in the spectrum. We speculate that the lower organic content at which the polypeptide elutes from the C_8 packing, compared to a C_{18} material, does not favour ionization and/or detection of this protein.

Peptides (Table 2) corresponding to eight of the thirteen reported [5] to be produced from hEPO were synthesised and used to develop HPLC–MS methodology. When using an acidic mobile phase gradient (for example, with 0.1% formic acid in water and acetonitrile) all except the E11 peptide were detected under negative ion ESI conditions. One possible reason that these peptides could also be analysed in the ESI ''wrong way round'' mode [22], where negative ions are produced under acidic conditions,

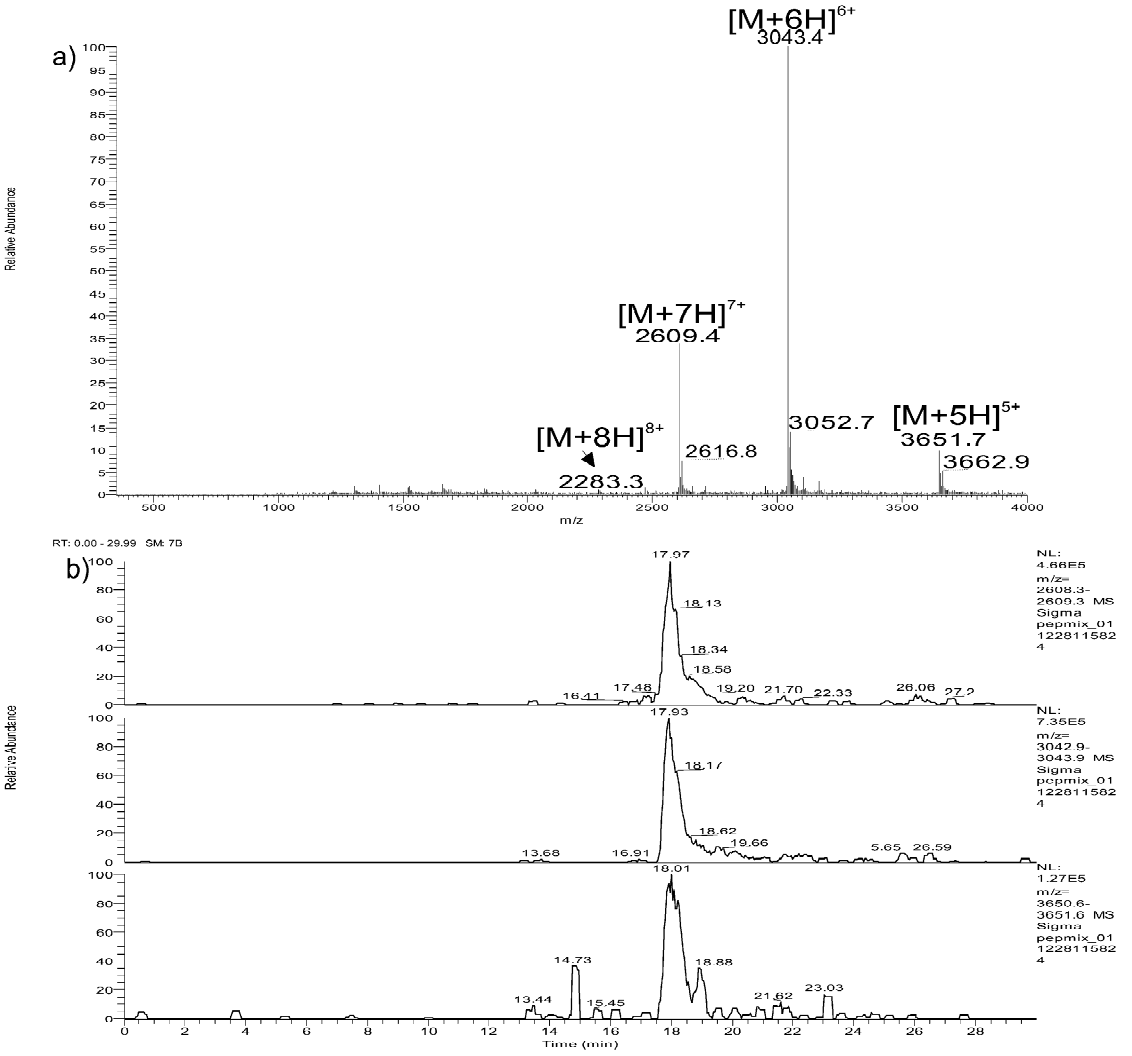

Fig. 3. Results from the analysis of deglycosylated EPR analysed by HPLC–+ESI-MS; (a) spectrum of approximately 3 pmol on column and (b) reconstructed ion chromatograms of $[M+5H]^{5+}$, $[M+6H]^{6+}$, $[M+7H]^{7+}$ ions from 250 fmol of polypeptide.

may be the presence of an acidic (either D or E) C terminal residue on all these peptides. Abundant structural information was provided in both the positive and negative ESI-MS–MS spectra. ESI MS–MS analyses of either the singly or doubly charged positive ion provided a spectrum (Fig. 4) with characteristic b and/or y ions for each peptide analysed. The loss of water and CO_2 from the ions was also common. In the case of negative ion ESI MS–MS spectra, many of the peptides with a glutamic acid C terminus underwent a distinctive neutral loss of 129 via the elimination of 5-aza-caprolactam from the intermediate [23]. The elimination of lactone (M_r 58) from one peptide was also noted.

A Glu-C digest of EPR (Table 3), gave retention time matches that were within ±1% and the absolute relative abundance of the three most intense ions to the base peak for each peptide's spectrum was within ±20% of that obtained from the analysis of the synthetic peptide. No significant (>10%) ions observed in the MS–MS spectra of the synthetic

Table 2
Mass spectral data from synthesised peptides

Peptide	Amino acid sequence	Average mass of $[M+H]^+$	MALDI-TOF-MS Observed m/z	RRT[a]	ESI-TRAP-MS					
					Positive ion ESI			Negative ion ESI		
					Ion	Observed m/z	Major product ion	Ion	Observed m/z	Major product ion
E5	AENITTGCAEHCSLNE[b]	1690.8	1689.6	1.00	$[M+H]^{1+}$	1689.5	1672.5	$[M-H]^{1-}$	1688.7	1670.6
E7	TKVNFYAWKRME	1573.9	1572.4	1.24	$[M+H]^{1+}$	1572.8	1555.5	$[M-H]^{1-}$	1572.0	1441.9
E8	VGQQAVE	730.8	729.7	0.81	$[M+H]^{1+}$	730.2	583.1	$[M-H]^{1-}$	728.8	600.3
E9	VWQGLALLS.E.	1115.3	1114.9	1.38	$[M+H]^{1+}$	1115.4	881.4	$[M-H]^{1-}$	1113.7	984.9
E10	AVLRGQALLVNSSQPWEPLQLHVD	2672.1	2671.0	1.33	$[M+2H]^{2+}$	1336.0	1270.2	$[M-2H]^{2-}$	1334.7	1276.9
E11	KAVSGLRSLTTLLRALGAQKE	2213.6	2212.6	1.48	$M+2H]^{2+}$	1107.2	1033.2	–	NO	NA
E12	AISPPDAASAAPLRTITAD	1839.1	1838.1	1.14	$[M+2H]^{2+}$	920.0	784.4	$[M-2H]^{2-}$	1836.9	1817.7
E13	TFRKLFRVYSNFLRGKLKLYTGE	2838.4	2837.8	1.43	$M+2H]^{2+}$	1419.0	1354.9	$[M-2H]^{2-}$	1417.4	NO

NO, not observed; NA, not analysed.
[a] Relative retention time of peptides on C_{18}—data supplied by manufacturer.
[b] Includes a disulphide bond between peptide amino acids 8 and 12.

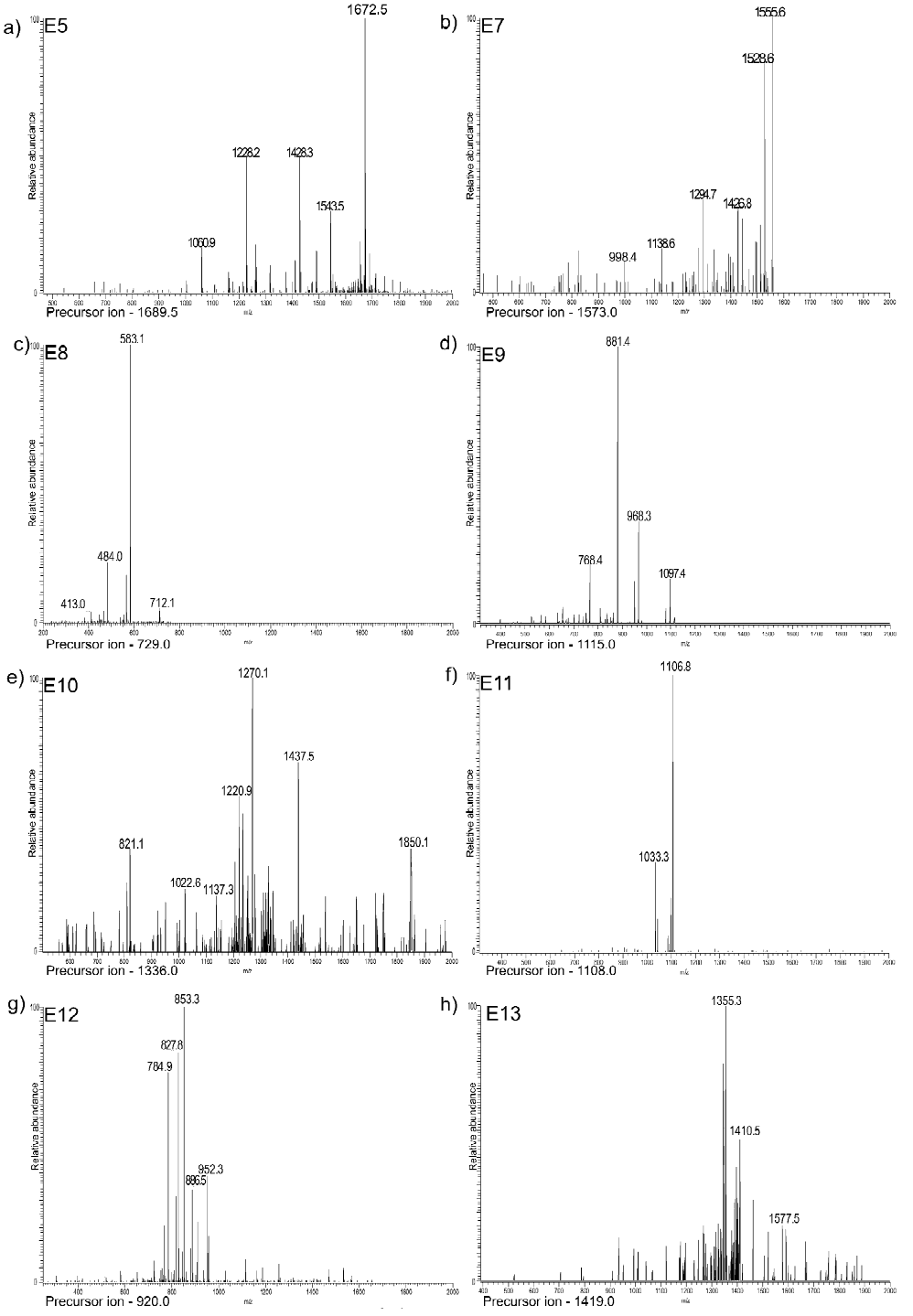

Fig. 4. Positive ion MS-MS spectra of (a) E5, (b) E7, (c) E8, (d) E9, (e) E10, (f) E11, (g) E12 and (h) E13 synthetic peptides.

Table 3
Peptides obtained by Glu-C enzyme hydrolysis of Eprex and Aranesp

Peptide	E1[a]	E2[a]	E3[a]	E4[a]	E5[b]	E6[a]	E7[b]	E8[b]	E9[b]	E10[b]	E11[b]	E12[b]	E13[b]	E1[a]
1.1 Eprex														
Amino acid residue	1–8	9–13	14–18	19–21	22–37	38–43	44–55	56–62	63–72	73–96	91–117	118–136	137–159	160–165
Average molecular mass	1503.7	602.7	692.8	346.4	1689.8	657.7	1572.8	729.8	1115.3	2671.1	2212.6	1838.1	2837.4	
Post-translational modifications	Disul: 7–161				Carb: 24 Disul: 29–33	Carb: 38				Carb: 83		Carb: 126		
1.2 Aranesp														
Peptide	E1[a]	E2[a]	E3[a]	E4[a]	E5′	E6[a]	E7[b]	E8[b]	E9[b]	E10′	E11[b]	E12[b]	E13[b]	E1[a]
Amino acid residue	1–8	9–13	14–18	19–21	22–37[c]	38–43	44–55	56–62	63–72	73–96[c]	91–117	118–136	137–159	160–165
Average molecular mass	1503.7	602.7	692.8	346.4	1707.8[c]	657.7	1572.8	729.8	1115.3	2603.4[c]	2212.6	1838.1	2837.4	
Post-translational modifications	Disul: 7–161				Carb: 24; 30 Disul: 29–33	Carb: 38				Carb: 83; 88		Carb: 126		

Disul., disulphide bond; Carb., carbohydrate attachment.
[a] Glu-C peptides common to both Eprex and Aranesp.
[b] Glu-C peptides unique to Eprex.
[c] Glu-C peptides unique to Aranesp.

peptides were missing from the Glu-C digest's peptide spectra and vice versa. Using the same basis, a Glu-C digest of NESP (Table 3) produced matches to the E7, E8, E9, E11, E12 and E13 synthetic peptides. It was notable that a significant amount of E12, which contains the site for O-glycosylation, was detected in both these digests without deglycosylation of the epoetins being required. However, the N-glycosylated EPR Glu-C peptides E5 and E10 were only poorly detected and to investigate this situation more fully, Glu-C hydrolysed EPR was analysed by MALDI-TOF-MS. The peptides that were detected by MALDI-TOF-MS have been labelled (Fig. 5) in a manner consistent with the publication by Kawasaki et al. [5]. Deglycosylation of the mixture gave a similar spectrum (Fig. 5b) to the spectrum (Fig. 5a) obtained from the untreated mixture, plus an $[M+H]^+$ ion corresponding to E10 at m/z 2673.0 and this confirms that this peptide is only detected in significant quantities after removal of carbohydrate chains attached to the asparagine amino acid residue (Asn83) of this peptide. E5 was not detected in the MALDI-TOF-MS analysis of the deglycosylated digest and, as it lacks an arginine residue, this may be due to the ionization bias against this peptide under MALDI conditions. Arginine containing peptides are reported exhibit a four to eighteen fold increase in signal intensity compared to those with only lysine [24].

Three Glu-C peptides, for which no analogues were synthesised, were also detected during the negative ESI-MS–MS analysis of the Glu-C hydrolysed EPR. These spectra are shown in Fig. 6.

A method of using small diameter HPLC columns to improve the sensitivity of the ESI-MS–MS analysis of these peptides was investigated. A PicoFrit fused-silica column (75 μm I.D., 15 μm tip), packed in-house to a length of 75 mm with C_{18} (5 μm) material and running at a flow of approximately 200 nl/min, was employed for the analysis of the synthetic E12 peptide by nano-HPLC-ESI using a 0.1% formic acid in water and acetonitrile gradient. The results from the analysis of 50 fmol of E12 in the positive ESI mode and are shown in Fig. 7. How-

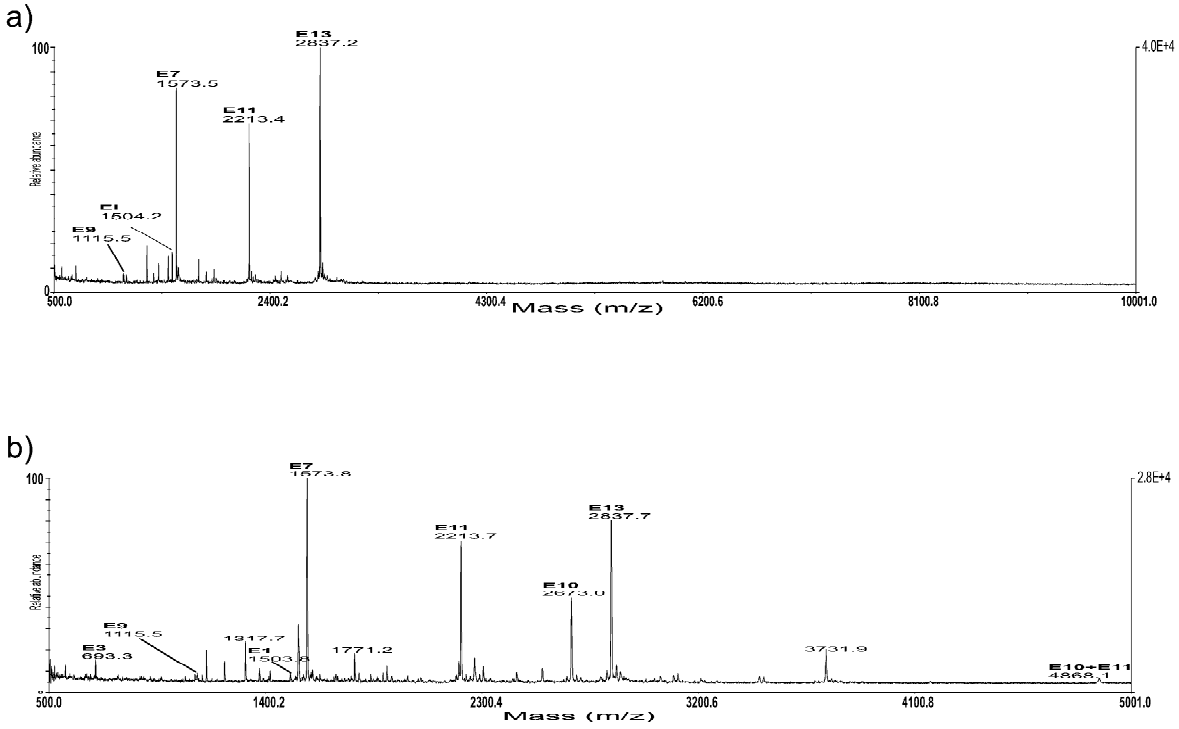

Fig. 5. MALDI-TOF-MS spectrum of (a) Glu-C digested EPR and (b) Glu-C digested and deglycosylated EPR.

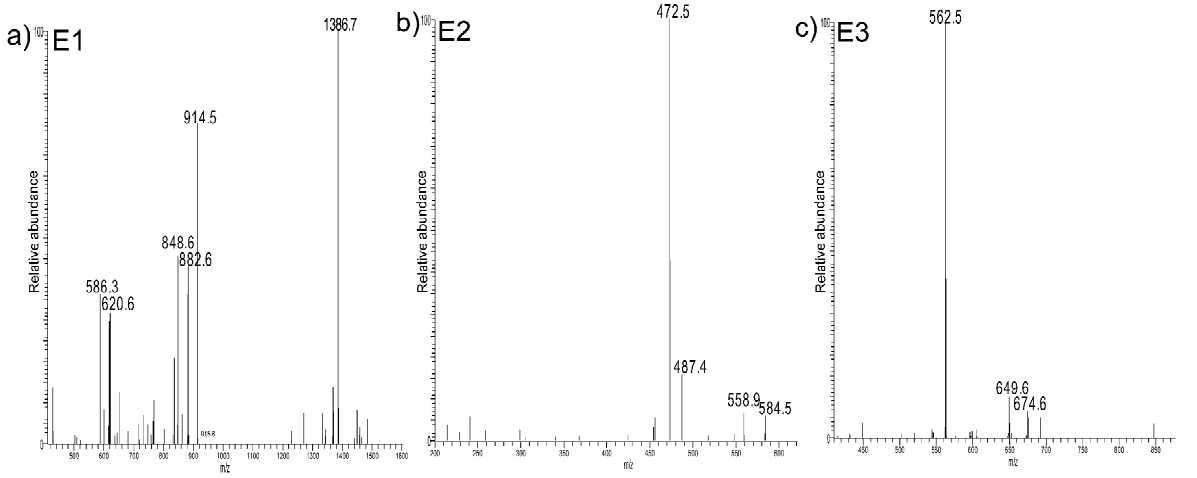

Fig. 6. Negative ion ESI-MS-MS spectra from EPR Glu-C derived peptides; (a) E1-precursor ion m/z 1502.00, (b) E2-precusor ion m/z 601.50 and (c) E3-precusor ion m/z 691.50.

ever, while there is a significant improvement in sensitivity compared to the 1-mm columns we used previously, the time taken to elute from the column (108 min) is too long to be acceptable for routine use. The lag-time is related to the length of time it takes for the change in gradient composition from the HPLC mixing chamber to reach the CEC column and we are currently investigating techniques to reduce the dead volume in our system.

A search of peptide sequences derived from Glu-C digested hEPO against sequences held in the Protein Information Resourcedatabase [25] (http://www.nbrf.georgetown.edu/pirwww/search/patmatch.html) produced a single match to human erythropoietin precursor for the sequences of E1, E5, E10, E11, E12, E13, and only a limited number of hits were produced for the other peptides. A similar result was obtained when the mass fingerprint data from the

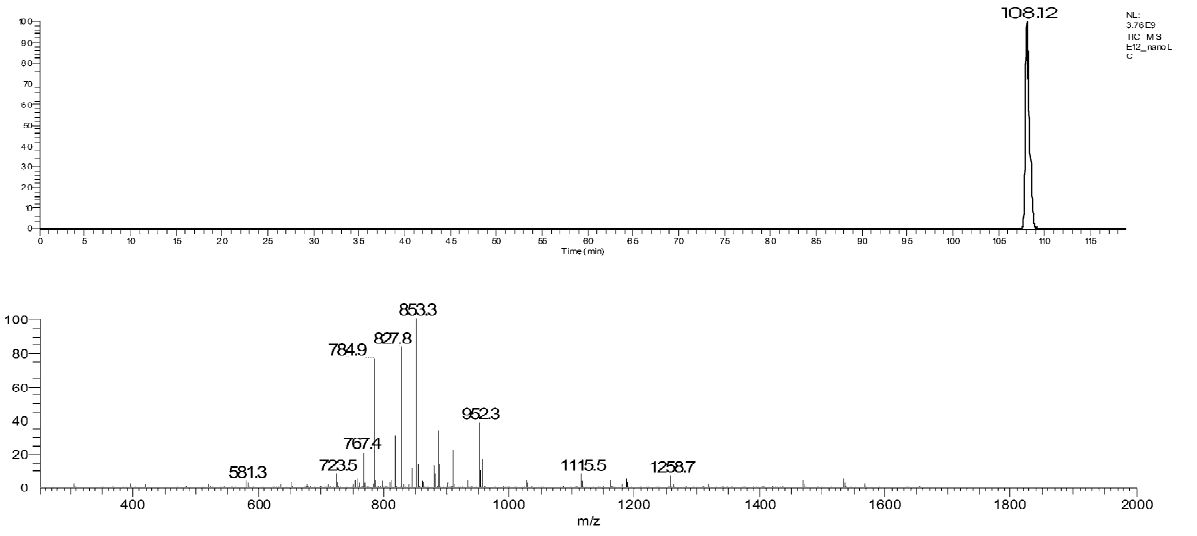

Fig. 7. Nano-HPLC-ESI-MS analysis of 50 fmol of E12 in the positive ion mode; (a) total ion chromatogram and (b) spectrum at 108.1 min.

MALDI-MS analysis of the tryptic and Glu-C digests was searched against the OWL.7.2.2001database using MS-FIT [26] (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm). This indicates that these particular peptides are very characteristic of the protein (Table 3).

4. Conclusion

Deglycosylation of the EPR and NESP and enzymic digestion greatly reduces the heterogeneity of the analyte and thereby enhances detection by MS.

We propose that detection of whole deglycosylated hEPO or NESP in a sample, along with the detection of one of the characteristic Glu-C derived peptides (E1, E11, E12 and E13), will provide sufficient proof of the presence of exogenous epoetin use. In this regard E12 would be the preferred target as the characteristic spectrum is obtained with good sensitivity. An alternative to this strategy would be the detection of two or more of the Glu-C peptides and establishing the presence or absence of either E5 or E10, so that the analyst is able to differentiate between the detection of NESP and EPR.

The reference peptides that we have had synthetised have been shown to be suitable reference standards for use with either strategy of detecting the epoetins Eprex and Aranesp.

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