Groleau, PE., Desharnais, P., Ayotte, C.

Characterization of Glycopeptides Released from pmol Levels of Recombinant Erythropoietin by Nanoflow HPLC-Chip Electrospray Ionization Coupled to Ion Trap Mass Spectrometry

INRS-Institut Armand-Frappier, 245 Boul. Hymus, Pointe-Claire, Québec, Canada

Abstract

Glycopeptides derived from endoproteinase Glu-C digest of recombinant erythropoietin (rhEPO) preparation were analyzed by nanoflow HPLC-Chip electrospray ionization coupled to ion trap mass spectrometry (HPLC-Chip-ESI-MS/MS, Agilent Technologies). The purpose of this work was to evaluate the potential of this new technology to detect the N-linked and O-linked glycopeptides from pmol amount of rhEPO, the level required to eventually allow the analysis of the human endogenous EPO (hEPO). The four theoretical glycosylation sites, Asn24, Asn38, Asn83 and Ser126, were identified as glycopeptides 22-37, 38-55, 73-96 and 118-136 respectively. The instrument allowed the observation of the smallest isoforms in detriment of the largest isoforms. With 3 pmol (100 ng) of starting material on Chip, only the desialylated N-glycopeptides 22-37 and 38-55 can be observed. It is suggested that this new platform enables a partial characterization of the isoforms of rhEPO and the same characterization of hEPO would be possible to find discrimination points between them.

INTRODUCTION

Human erythropoietin (hEPO) is a glycoprotein hormone produced primarily in the kidney, which stimulates erythropoiesis after binding to receptors in the bone marrow [1]. It is composed of 165 amino acids on which 3 asparagines are N-linked glycosylated (Asn24, Asn38 and Asn83) and 1 serine is O-linked glycosylated (Ser126). The oligosaccharide portion of the glycoprotein accounts for 40% of its total molecular weight (30 kDa) [2,3]. Glycans on the recombinant human EPO (rhEPO) have been characterized extensively by LC-MS [4,5,6]. However, the same characterization was never achieved with hEPO because it is

available only in very low amount representing an analytical challenge. It is suspected that the number of antennary branching, N-acetylglucosamine residues, fucosylation, sulfatation, and sialylation may vary on the different glycosylation sites.

Nanospray LC-MS exhibits higher sensitivity and lower sample consumption than conventional or even capillary HPLC and is particularly suitable for proteomic analysis of limited amounts of proteins from biological sources. The HPLC-Chip technology was developed to facilitate the use of the nanospray interface by limiting capillary tubing, column leaking or needle spray adjustment [7]. The integration of the enrichment column onto the Chip allows concentration of the sample prior to being directed onto the analytical column at a flow rate of 300 nL/min, enhancing signal to noise ratio of the analytes. The purpose of this work was to use a nanoflow HPLC-Chip coupled with electrospray ionization ion trap mass spectrometry system (HPLC-Chip-ESI-MS/MS, Agilent Technologies) to characterize glycopeptides released from pmol amount of rhEPO, and evaluate the limit of detection in order to discriminate between the different glycosylation profiles of rhEPO and hEPO.

EXPERIMENTAL

Materials

Biological Reference Preparation of erythropoietin (rhEPO BRP) was purchased from the European Pharmacopoeia Commission (Strasbourg, France). Endoproteinase Glu-C from *Staphylococcus aureus V8* and neuraminidase from *Clostridium perfringens* were purchased from Roche Applied Science (Penzberg, Germany). All reagents were of analytical grade. Pharmaceutial preparations of epoetin alfa and darbepoetin alfa as injection solutions were gifts from Amgen (Thousand Oaks, CA, USA). Partially purified hEPO was obtained in 10 U (about 100 ng) vials from NIBSC (Herts, UK).

Preparation of desialylated glycopeptides

rhEPO preparation was washed with 5 volumes of 100 mM ammonium acetate buffer pH 8.0 on 30 KDa Microcon (Millipore Corporation, Bedford, MA) to remove excipient and detergent. For 10 µg of rhEPO, an aliquot of 25 µL of a Glu-C solution at 0.02 µg/µL was added (substrate:enzyme ratio 20:1, w/w) as well as 5 mU (50 ng) of neuraminidase and the mixture was incubated overnight at 37°C. The digestion was stopped by ultrafiltering peptides on a 30 KDa Microcon, in order to remove non hydrolyzed proteins and enzymes. The flow

through containing the peptides was evaporated under a gentle stream of nitrogen at 37°C and reconstituted in 20 µL of 0.1% formic acid in water for LC-MS injection.

HPLC-Chip-ESI-MS/MS

The sample was analyzed using Agilent 1200 HPLC and 6330 Ion Trap system consisting of a nanoflow pump, wellplate sampler, capillary pump, HPLC-Chip/MS interface and 6330 ion trap XCT Ultra mass spectrometer. For the Glu-C digest, 0.5 µL of digests (250 ng) was injected on a Zorbax Chip composed of a 40 nL enrichment column and a 75 µm x 43 mm 5µm C18-SB analytical column. The eluents for both capillary and nanopump consisted of 0.2% formic acid in distilled water (A) and 0.2% formic acid in 90/10 acetonitrile/distilled water (B). The flow rate for the capillary pump was constant at 4 µL/min in 2% B (isocratic), while the flow rate for the nanopump was 0.3 µL/min, following a gradient of 2 to 70% B in 30 min. A flush volume of 4 µL was required to allow the sample to be loaded onto the enrichment column. The mass spectrometer was operated in ESI positive ionization mode, with drying gas flow at 4 L/min and temperature at 325°C. In-source voltage was set at 1800 V, skimmer at 30 V, capillary exit at 100 V. Automatic MS/MS in a data-dependant manner was acquired in enhanced mode at *m/z* 200-2000, in order to monitor carbohydrate ions of low *m/z*. Extraction of the diagnostic ion at *m/z* 366 for HexHexNAc in the product ion spectra was used to monitor glycopeptides. Extraction of precursor ions was also performed to estimate the limit of detection of the system.

SDS-PAGE and immunoblotting

One-dimensional (1D) SDS-PAGE was performed on 10% gels and the proteins were blotted onto polyvinylidene difluoride membranes (0.2 µm: BioRad Laboratories, Hercules, CA) in a semi-dry apparatus. Membranes were then blocked with milk powder in PBS and incubated with the AE7A5 mouse monoclonal anti-hEPO antibody (R&D Systems, Minneapolis, MN) and anti-mouse HRP (Pierce, Rockford, IL). Blots were visualized with a chemiluminescent substrate on X-ray films.

RESULTS AND DISCUSSION

Endoproteinase Glu-C cleaves peptide bonds at Glu (E) and to a lower extent at Asp (D) residues and was chosen to digest rhEPO since it releases glycopeptides with only one

glycosylation site. Neuraminidase was used to remove sialic acids to limit the heterogeneity of the oligosaccharide structures, enhancing the detection of the glycopeptides. Figure 1 presents the base peak chromatogram (BPC) of 250 ng (starting material on Chip) of the desialylated digest of rhEPO. Theoretical peptide mapping is presented in Table 1, and most of the peptides were identified in Figure 1A as singly, doubly or triply charged ions. Peptides [1-8]-S-S-[160-165], 118-136 and 63-72 present the highest intensity and other peptides and glycopeptides are identified in trace amount. The Glu-C digestion releases predominantly glycopeptides 22-37, 38-55, 73-96 and 118-136, but minor glycopeptides arising from further cleavage can also be observed. Glycopeptides were all identified in the digest with the help of the diagnostic ion at *m/z* 366 in the product ion spectra. Minor glycopeptide 38-43 was also used to characterize N38. It results from the specific cleavage of Asp43 but is however in lower abundance being preceded by a proline residue conferring steric hindrance. Since large peptides like glycopeptides ionize in multiply charged ions, the signal intensity is distributed among the different charged states in detriment of the limit of detection. Therefore, MS/MS is used to fragment peptides and monitor diagnostic ions such as *m/z* 204 (HexNAc), *m/z* 366 (HexHexNAc) or *m/z* 292 (NeuAc) for sialylated glycopeptides [8,9]. The high intensity oxonium ion at *m/z* 366 was chosen; the extracted ion chromatogram (EIC) for this ion is shown in Figure 1B.

Figure 2 presents the product ion spectra obtained for glycopeptides 22-37, for the glycoforms bi, bi-Lac_1 and tri-Lac_1. The ions resulting from the fragmentation of the oligosaccharide residues of the bi- and tri-antennary glycopeptides are clearly identified as doubly and triply charged ions, as if glycan units were removed one by one in the ion trap (identified as loss of Hex or HexNAc on the Figure). Furthermore, ions at *m/z* 731 confirm the presence of N-acetyllactosamine (Lac) in the glycan structure, allowing differentiation of molecular weight isomers, such as a bi-Lac_1 antennary versus a tri-antennary. These observations in the product ion spectra of glycopeptide 22-37 favours lower antennary structures with Lac repeats, rather than additional antenna structures. The same product ion spectral deconvolution was followed for proposing the structures for glycopeptides 118-136 and 38-55 (38-43) and the results are summarized in Table 2. As expected, since peptide 118-136 is a simple O-linked structure composed of a single N-acetylgalactosamine residue (GalGalNAc), it produces ions at *m/z* 735.4 $^{(+3)}$ and 1102.5 $^{(+2)}$ of very high intensity.

Table 1: Theoretical Glu-C digest mapping with the observed masses from Figure 1.

Residue	Amino acid sequence	Theoretical average mass of the AA sequence [b]	Observed *m/z*	Charge state	Observed mass	Retention time (min)
[1-8]+ [160-165] [a]	APPRLICD+ ACRTGD	1503.7	502.1, 752.4	+3, +2	1503.3	9.5
9-13	SRVLE	602.7	603.3	+1	602.3	2.5
14-18	RYLLE	692.8	693.4	+1	692.4	10.5
19-21	AKE	346.4	-	-	-	-
22-37[a]	**AENITTGCAEHCSLNE**	**1689.8**				10.1-11.0
38-55 [c]	**NITVPD TKVNFYAWKRME**	**2212.6**				17.0-18.0
38-43	**NITVPD**	**657.7**				8.7-9.3
56-62	VGQQAVE	729.8	730.5	+1	729.5	2.8
63-72	VWQGLALLSE	1115.3	1115.6	+1	1114.6	20.5
73-96	**AVLRGQALLVNSSQPWEPLQLHVD**	**2671.1**				19.1-20.3
97-117	KAVSGLRSLTTLLRALGAQKE	2212.6	738.2	+3	2211.6	20.0
118-136	**AISPPDAASAAPLRTITAD**	**1838.1**				14.0-14.7
137-159	TFRKLFRVYSNFLRGKLKLYTGE	2837.4	946.6	+3	2836.8	28.1

[a] peptides [1-8]+[160-165] and 22-37 are linked by a disulfide bridge.
[b] Theoretical average masses calculated from Expasy Biological Server (www.expasy.org)
[c] Glycopeptide 38-55 was partially cleaved to 38-43 and both peptides are observed.

Table 2: Observed *m/z* of the 22-37, 118-136, 38-55 and 38-43 desialylated glycopeptides and proposed oligosaccharide structures.

Peptide	**Observed *m/z***	**Charge (+)**	**Calc. mass**	**Proposed oligosaccharide structures[a]**	**Theor. mass[b]**
22-37	865.5, 1154.3	4, 3	3459.3	Bi	3458.6
	957.1, 1275.8	4, 3	3824.4	Bi-Lac_1	3823.9
	1048.7, 1397.1	4, 3	4189.5	Bi-Lac_2/Tri-Lac_1	4189.3
	1139.4	4	4553.6	Tri-Lac_2/Tetra-Lac_1	4554.6
118-136	1102.5, 735.4	2, 3	2203.0	GalGalNAc	2203.4
38-55	996.4, 1328.5	4, 3	3982.5	Bi	3982.2
	870.2, 1087.8, 1449.9	5, 4, 3	4347.2	Tri	4346.9
	942.2, 1179.4	5, 4	4713.6	Tri-Lac_1/Tetra	4712.3
	1016.7, 1270.2	5, 4	5076.8	Tetra-Lac_1	5077.6
38-43	809.3	3	2424.9	Bi	2427.3
	930.1, 1397.0	3, 2	2792.0	Tri	2792.6
	1053.3	3	3156.9	Tri-Lac_1/Tetra	3158.0
	881.2, 1175.0	4, 3	3522.0	Tetra-Lac_1	3523.3
	972.6, 1296.8	4, 3	3887.4	Tetra-Lac_2	3888.6

[a]All N-glycans contain fucosylated core

[b]Theoretical mass calculated from the addition of the average mass of the peptide and the average mass of the glycan.

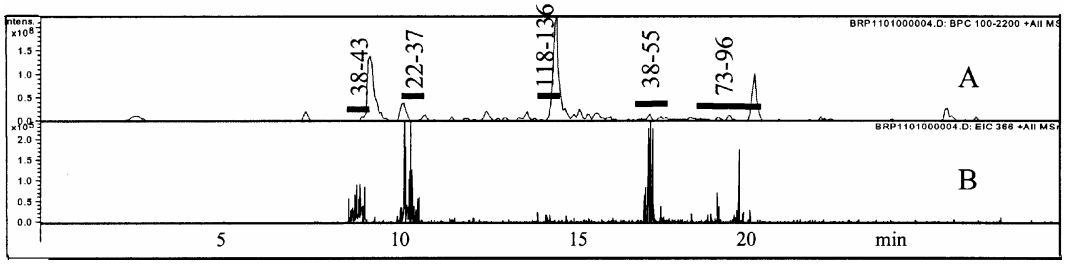

Figure 1: BPC (A) and EIC (B) at *m/z* 366 of 250 ng of the desialylated rhEPO digest (~7.8 pmol). Proposed structures are summarized in Table 2.

Previous studies revealed that using µg or mg amount of rhEPO, tetra-antennary structures are predominant over smaller bi-antennary structures [5,6,10,11]. The HPLC-Chip coupled with the ion trap mass spectrometer allows the detection of lower molecular weight glycopeptides where charge state is low. The proposed instrumental setup does definitely not allow a complete characterization of the rhEPO, but it could be suitable for partial isoform identification at the level required to analyze the authentic standard of hEPO, available only in 10 U vials (3 pmol). To do so, the limit of detection for the markers producing the monitored oxonium ions was estimated with different volumes injected on Chip. Extraction of the precursor ions for the lower antennary N-linked glycopeptides 22-37 and 38-55 with an acceptable signal to noise ratio could be achieved for 100 ng (about 3 pmol) on Chip. Furthermore, in the targeted MS/MS mode, we observed that only 10 ng (about 300 fmol) of epoetin alfa was needed to identify the most intense isoforms (Figure 3). Different products can be chosen for extraction to mimic MRM acquisition from data acquired on the ion trap. Glycopeptide 118-136 presents the highest signal, with the peptide core as product. N-linked glycopeptides 22-37 was extracted with the oxonium ion at *m/z* 366 as product, and 38-55 was extracted with the fragmentation of the oligosaccharide ramification as products. It proposes that at this level, these isoforms could be used to differentiate between rhEPO and hEPO.

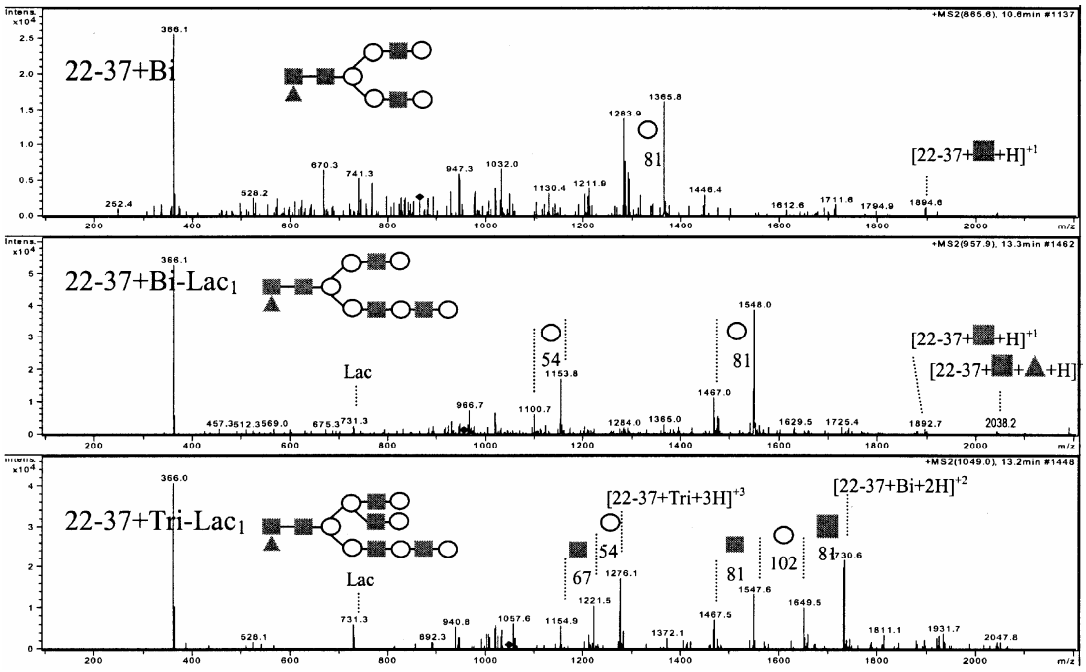

Figure 2: Product ion spectra of precursors of glycopeptide 22-37, bi, bi-Lac_1 and bi-Lac_2/tri-Lac_1 (respectively *m/z* 865.6 $^{(+4)}$, 957.9 $^{(+4)}$ and 1049.0 $^{(+4)}$). Hex are represented by open circle, HexNAc by closed squares and fucose by closed triangle.

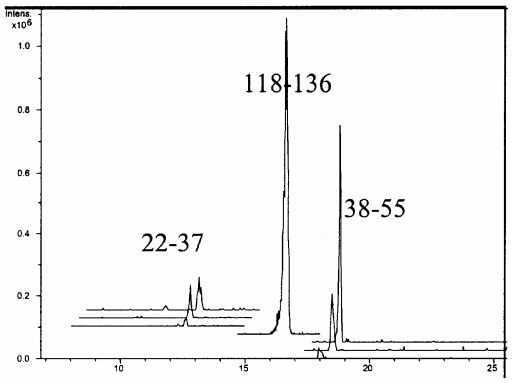

Figure 3: MRM detection for 1U of epoetin alfa (10 ng~300 fmol) for chosen diagnostic *m/z* for glycopeptides 22-37 (1275→366, 1048→366, 1154→366), 118-136 (1102→1838) and 38-55 (1179→1450, 1272→1572, 1087→1328). Unit mass resolution was used for extraction.

Comparison of commercially available ESPs (Erythropoiesis stimulating proteins)

In order to evaluate this technique on different rhEPO variants, two commercially available rhEPO preparations were digested and desialylated. Since pharmaceutical rhEPO preparations also contain other proteins as stabilizer, the BPC is more complex than for standard solutions. The glycopeptide map can however be compared using the diagnostic ions. Figure 4 presents

the EIC at *m/z* 366 of glycopeptides from rhEPO BRP preparation, epoetin alfa (Amgen, CA) and NESP (darbepoetin alfa, Amgen, CA), for 250 ng of starting material injected on Chip. Since the BRP preparation is a mixture of alpha and beta rhEPO, the glycopeptide profile of the epoetin alfa is similar to that of BRP rhEPO, as expected. The region for the peptide 22-37 also presents predominant ions for bi-, bi-Lac_1 and bi-Lac_2 structures. An intense ion eluting after peptide 22-37 was observed only with the epoetin alfa presenting ions at *m/z* 817.2 $^{(+4)}$ and 1089.7 $^{(+3)}$, that is attributed to the bi-antennary glycopeptide 24-37 (MW 3266.1), resulting from the specific but not usually observed cleavage of Glu24. This cleavage is not likely to occur because of steric hindrance conferred by the large glycan structures. The observance of this cleavage site on epoetin alfa solely may indicate a conformational difference in the pharmaceutical preparations of the rhEPO.

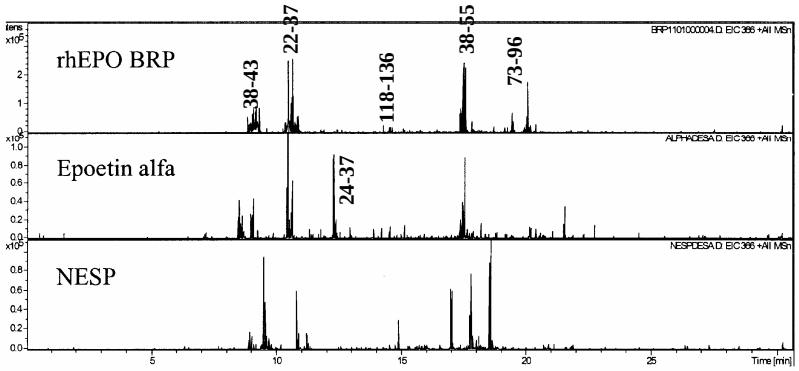

Figure 4: EIC at *m/z* 366 of glycopeptides from rhEPO BRP, epoetin alfa and NESP.

The NESP glycopeptides are different from the rhEPO. Five amino acids are changed in the NESP sequence allowing the presence of two additional N-linked glycans on position 30 and 88 [2]. Glycopeptides 22-37 and 73-96 then present two oligosaccharide structures and as a result the EIC profiles are changed in those regions. Since adding oligosaccharide moieties adds heterogeneity, the signal is consequently reduced for 22-37, and is absent for 73-96. The O-linked glycopeptide 118-136 at *m/z* 1102.5 $^{(+2)}$ was found unchanged in the spectrum (not shown). Glycopeptides 38-43 and 38-55 are both found in the NESP hydrolysate but the profiles of the EIC at *m/z* 366 are only indicating the presence of the tri-Lac_1 isoform as if less heterogeneity was found on the NESP compared to rhEPO.

It is suggested that the presence of Lac residue may become relevant for potential biomarkers. This last hypothesis is supported by the apparent molecular weight of the standard proteins on SDS-PAGE (Figure 5). It was previously shown that hEPO has a lower apparent molecular

weight than rhEPO (34 KDa versus 40 KDa) [12], which was explained by lower sialic acid content. However, Figure 5 shows that sialic acids are not the sole cause of the difference in mass since even desialylated rhEPO presents a higher molecular weight than desialylated hEPO. It has already been suggested by NMR study that Lac ramification were more common in rhEPO from BHK cells (omega) than in hEPO [3]. It is then possible that the greater number of Lac residues may account for this mass difference, and the occurrence of a diagnostic ion at *m/z* 731 would be relevant.

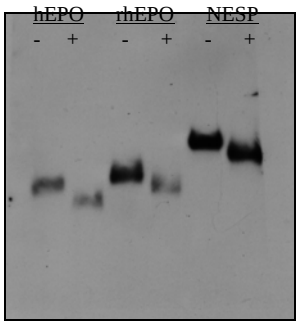

Figure 5: SDS-PAGE and immunoblotting of hEPO, rhEPO and NESP before (-) and after (+) neuraminidase treatment.

CONCLUSION

The smaller isoforms on the 4 glycosylation sites could be identified through extraction of oligosaccharide oxonium ions, but N-linked glycopeptides 22-37 and 38-55/38-43 are proposed for monitoring in different EPO preparations in order to identify structural differences. 100 ng of starting material (approximately 3 pmol of rhEPO) were sufficient to identify the most intense glycopeptides following desialylation as the HPLC-Chip nanospray interface coupled with Agilent's Ion Trap allowed partial characterization of rhEPO. Future work will include a purification process of about 3 pmol of the endogenous hEPO standard; we will then verify if discriminating points (such as Lac repeats) can be found in the most intense isoforms observed through this technique.

Acknowledgements

This work was financed in part by the World Anti-Doping Agency. The contribution of Agilent Technologies is gratefully acknowledged for the HPLC-Chip system.

REFERENCES

[1] Jelkmann, W. (1992) Erythropoietin: structure, control of production and function. *Physiol. Rev*. **72**, 449-89.

[2] Egrie, J.C., Browne, J.K. (2001). Development and characterization of novel erythropoiesis stimulating protein (NESP). *Nephrol., Dial., Transplant* **16 suppl.3,** 3-13.

[3] Tsuda, E., Goto, M., Murakami, A., Akai, K., Ueda, M., Kawanishi, G. (1988) Comparative structural study of N-linked oligosaccharides of urinary and recombinant erythropoietins. *Biochem.* **27**, 5646-5654.

[4] Kawasaki, N., Otha, M., Itoh, S., Hyuga, M., Hyuga, S., Hayakawa, T. (2002) Usefulness of sugar mapping by liquid chromatography/mass spectrometry in comparability assessments of glycoprotein products. *Biologicals* **30**, 113-123.

[5] Otha, M., Kawasaki, N., Itoh, S., Hayakawa, T. (2002) Usefulness of glycopeptide mapping by liquid chromatography/mass spectrometry in comparability assessment of glycoprotein products. *Biologicals* **30**, 235-244.

[6] Stubiger, G., Marchetti, M., Nagano, M., Grimm, R., Gmeiner, G., Reichel C, Allmaier, G. (2005) Characterization of N- and O-glycopeptides of recombinant human erythropoietins as potential biomarkers for doping analysis by means of microscale sample purification combined with MALDI-TOF and quadrupole IT-RTOF mass spectrometry. *J. Sep. Sci*. **28**, 1764-1778.

[7] Yin, H., Killeen, K., Brennen, R., Sobek, D., Werlich, M., van de Goor T. (2005) Microfluidic chip for peptide analysis with an integrated HPLC column, sample enrichment column, and nanoelectrospray tip. *Anal Chem*.**77**, 527-533.

[8] Harazano A, Kawasaki N, Kawanishi T, Hayakawa G. (2005) Site-specific glycosylation analysis of human apolipoprotein B100 using LC-ESI MS/MS. *Glycobiol*. **15**, 447-462.

[9] Harazano, A., Kawasaki, N., Itoh, S., Hashii, N., Ishii-Watabe, A., Kawanishi, T., Hayakawa, T. (2006) Site-specific N-glycosylation analysis of human plasma ceruloplasmin using liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal. Biochem*. **348**, 259-268.

[10] Kawasaki, N., Otha, M., Itoh, S., Hyuga, S., Hyuga, M., Hayakawa, T. (2000) Application of liquid chromatography/mass spectrometry and liquid chromatography with tandem mass spectrometry to the analysis of the site-specific carbohydrate heterogeneity in erythropoietin. *Anal. Biochem*. **285**, 82-91.

[11] Otha, M., Kawasaki, N., Hyuga, S., Hyuga, M., Hayakawa, T. (2001) Selective glycopeptide mapping of erythropoietin by on-line high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. A*, **910**, 1-11.

[12] Skibeli, V., Nissen-Lie, G., Torjesen, P. (2001) Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin. *Blood* **98**, 3626-3634.