Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (10)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

Sport und Buch Strauß, Köln, 2002

G. NISSEN-LIE, P. HEMMERSBACH, E. HAUG E, G. TJØNNFJORD, P. TORJESEN, V. SKIBELI:
Charge Analyses of Human Erythropoietin and Analogues
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (10). Sport und Buch Strauß, Köln, (2002) 147-157

Nissen-Lie, G.*, Hemmersbach, P.*, Tjønnfjord, G.[¤], Torjesen, P.[#], and Skibeli, V.*

Charge analyses of human erythropoietin and analogues

*Section for Doping Analysis, [#]Hormone Laboratory, Aker University Hospital, Oslo, Norway.
[¤]The National Hospital, University of Oslo, Norway

Corresponding author: Venke Skibeli, Section for Doping Analysis, Hormone Laboratory, Aker University Hospital, 0514 Oslo, Norway. Phone: 47-22894914. Fax: 47-22894151. E-mail: venke.skibeli@ioks.uio.no.

Abstract

Charge analyses were performed on human erythropoietin (hEPO) based on two different approaches, namely isoelectric focusing (IEF) (Lasne and De Ceaurriz, 2000) of the intact EPO molecule including the sugar groups, and ion-exchange chromatography of the isolated oligosaccharide structures (Skibeli et al. 2001). The two methods revealed major molecular differences between endogenous hEPO and recombinant hEPO (rhEPO), including the epoetin analog darbepoetin alfa.

EPO from sera obtained from anemic patients was isolated using magnetic beads coated with a hEPO-specific antibody. The charge pattern of urinary hEPO (u-hEPO) extracted by the magnetic beads, revealed a similar charge pattern on IEF as obtained when the urinary samples were concentrated by ultrafiltration. IEF-analyses of human serum EPO (s-hEPO) and u-hEPO from the same individuals, demonstrated distinct differences in charge profiles, revealing u-hEPO with a significantly more acidic pattern than circulating hEPO.

IEF analyses of rhEPO and darbepoetin alfa compared to endogenous hEPO, demonstrated very different charge patterns where both s-hEPO and u-hEPO appeared more acidic than rhEPO. Darbepoetin alfa is focused on the most acidic side of the pH gradient caused by the negatively charged sialic acids attached to the two extra N-linked groups. The negative charges on endogenous hEPO compared to rhEPO cannot be explained by additional sialic acids on the sugar groups, since the most acidic rhEPO variants are reported to be nearly saturated with this negatively charged monosaccharide (Elliot et al, 1994).

EPO from human serum emerged as a broad band on SDS PAGE, with an apparent molecular weight significantly smaller than rhEPO. Darbepoetin alfa, on the other

EPO from human serum emerged as a broad band on SDS PAGE, with an apparent molecular weight significantly smaller than rhEPO. Darbepoetin alfa, on the other hand, was significantly larger than rhEPO, due to the additional sugar groups. The bandwidth of the EPO species corresponded with microheterogeneity due to extensive glycosylation.

Charge analysis by ion-exchange chromatography, demonstrated that the N-linked oligosaccharides from s-hEPO contained only mono-, di- and tri-sialylated structures, being deficient of the tetra-sialylated structures present in N-glycans from rhEPO and darbepoetin alfa. Charge profiling of the N-linked oligosaccharides from darbepoetin alfa revealed that a significant amount of the glycans existed as tetra-sialylated structures. Sugar structures with only one negative charge were not detected in darbepoetin alfa.

IEF, weak anion-exchange chromatography and SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) are all methods that are able to differentiate endogenous hEPO from rhEPO and darbepoetin alfa.

The discrimination between u-hEPO and rhEPO by the IEF method used in routine doping control, is probably possible because renal clearance of s-hEPO in some way leads to more negatively charged forms.

Introduction

Human EPO is a glycoprotein that is synthesised mainly in the kidney and stimulates erythropoiesis through actions on erythroid progenitor cells (Krantz 1991; Jelkmann 1992; Fisher 1997). Human EPO was the first hematopoietic growth factor to be cloned (Jacobs et al. 1985; Lin et al. 1985). Recombinant hEPO has been available as a drug since 1988 and is used in the clinical treatment of anemia. The drug darbepoetin alfa or NESP (novel erythropoiesis stimulating protein) was introduced in 2001 and is a modification of epoetin alfa containing two additional N-linked glycans (Egrie and Browne 2001).

Active hEPO consists of a single 165 amino acid polypeptide chain with three N-glycosylation sites at Asn24, Asn38 and Asn83, respectively, and one O-glycosylation site at Ser126. The average carbohydrate content is about 40 % (Lai et al. 1986; Takeuchi et al. 1988; Tsuda et al. 1988). A glycoprotein generally appears as a mixture of glycoforms. These glycoform populations have been shown to be cell and

species, as well as polypeptide and site specific (Parekh et al. 1987; Parekh et al. 1989 *i*) and *ii*); Zerfaoui and Ronin 1996; Rudd and Dwek 1997). Each glycoprotein therefore has a reproducible and characteristic glycosylation profile or glycosylation pattern.

Glycosylation is important for the biological activity of EPO. Removal or modification of the glycan chains from hEPO results in altered *in vivo* and *in vitro* activity (Dube et al. 1988; Takeuchi et al. 1988; Fukuda et al. 1989; Imai et al. 1990, *i*) and *ii*); Sytkowski et al. 1991; Kamerling 1996). Furthermore, the number of sialic acid residues and branching pattern of the N-linked oligosaccharides modify the pharmacodynamics, speed of catabolism and biological activity of hEPO (Fukuda et al. 1989; Imai et al. 1990, *i*); Higuchi et al. 1992).

Today three pharmaceuticals of rhEPO and the analogue darbepoetin alfa (novel erythropoiesis stimulating protein / NESP) are vailable for clinical use. The rhEPOs are classified as epoetin alfa, beta and omega according to the manufacturing method. Epoetin alfa, beta and darbepoetin alfa are produced in Chinese Hamster Ovary (CHO) cells, while epoetin omega is produced in Baby Hamster Kidney (BHK) cells. Although CHO and BHK cells glycosylate proteins in a qualitatively similar manner to human cells, some human tissue-specific carbohydrate motifs are not synthesised by the hamster cells since they lack the proper sugar-transferring enzymes (Svensson et al. 1990; Bergwerff et al. 1993; Watson et al. 1994; Kamerling 1996; Grabenhorst et al. 1999). Due to the extra sugar groups, darbepoetin alfa becomes more acidic and has a prolonged serum halflife compared to rhEPO (Macdougall et al. 1999).

In addition to the established rhEPO products, a new epoetin is soon available called epoetin delta, which is produced in a human cell line. Since the new drug is obtained from human cells it may reveal a similar charge pattern as endogenous hEPO on IEF that could be a problem with regard to the discrimination of the two EPO species. In view of the differences in glycosylation between proteins produced in different cell systems, structural analysis of natural and rhEPO has become increasingly important. By charge profiling important discrepancies between endogenous hEPO, rhEPO and darbepoetin alfa supported by SDS gel analysis and glycan analyses were demonstrated.

Materials and Methods

Preparation of human erythropoietin-specific magnetic beads

The preparation of the magnetic beads was performed according to Skibeli et al. (1998 and 2001).

Immuno-magnetic extraction

In order to reduce unspecific binding, serum samples were treated with polyethylene glycol (PEG) 6000 (12.5 % (w/v)) to precipitate immunoglobulins (Kuzuya et al. 1977) and centrifuged at 3500 x g for 20 minutes prior to incubation with the antibody-coated magnetic beads. Human EPO-specific magnetic beads were incubated with serum from anemic patients and treated according to Skibeli et al. (2001).

Isoelectric focusing (IEF)

IEF and sample preparation of u-hEPO were performed according to the method developed by Lasne (Lasne 2000; Catlin 2002).

SDS PAGE and immunoblotting

The analyses were performed according to Skibeli et al. (2001).

Enzymatic release of N-linked oligosaccharides

Isolation of N-linked sugar was performed according to Skibeli et al. (2001).

Fluorescent labeling of released oligosaccharides with 2-aminobenzamide (2-AB)

Glycans were labeled with 2-aminobenzamide (2-AB) according to Guile et al. (1994).

Charge analysis of 2-AB-labeled oligosaccharides

Weak anion-exchange chromatography was carried out using a GlycoSep C column (Glyko, USA) according to Guile et al. (1994) . The column was calibrated by the analysis of 2-AB-labeled sialylated N-glycans released from bovine fetuin (Rudd et al. 1997; Rudd et al. 1999 *i)* and *ii)*).

Results and Discussion

EPO was successfully isolated from human serum samples using magnetic beads coated with a rabbit polyclonal anti-hEPO antibody (Skibeli et al. 1998). The magnetic beads were capable of binding endogenous hEPO from serum (Skibeli et al 2001) as well as urine samples (data not shown) obtained from anemic patients. The immuno-magnetic extraction of EPO from serum was demonstrated by gel analyses (figure 1 and 2) and EPO was analysed with respect to electrophoretic properties, glycosylation and oligosaccharide structures (Skibeli et al. 2001). The study by Skibeli et al. (2001) was the first report describing sugar profiling of the oligosaccharide structures of s-hEPO including their comparison with rhEPO glycans. Previously, only the urinary species of endogenous hEPO had been isolated and characterised (Miyake et al. 1977; Lai et al. 1986, Takeuchi et al. 1988; Tsuda et al. 1988).

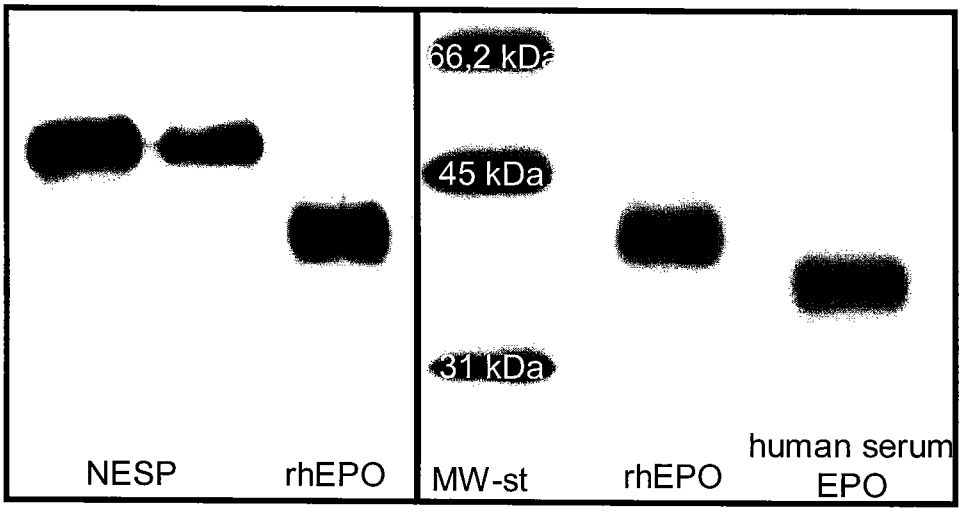

Figure 1. Molecular size differences between s-hEPO, rhEPO and darbepoetin alfa (NESP = novel erythropoiesis stimulating protein), demonstrated by SDS PAGE followed by immunoblotting with a monoclonal anti-hEPO antibody. (MW-st. = molecular weight standards).

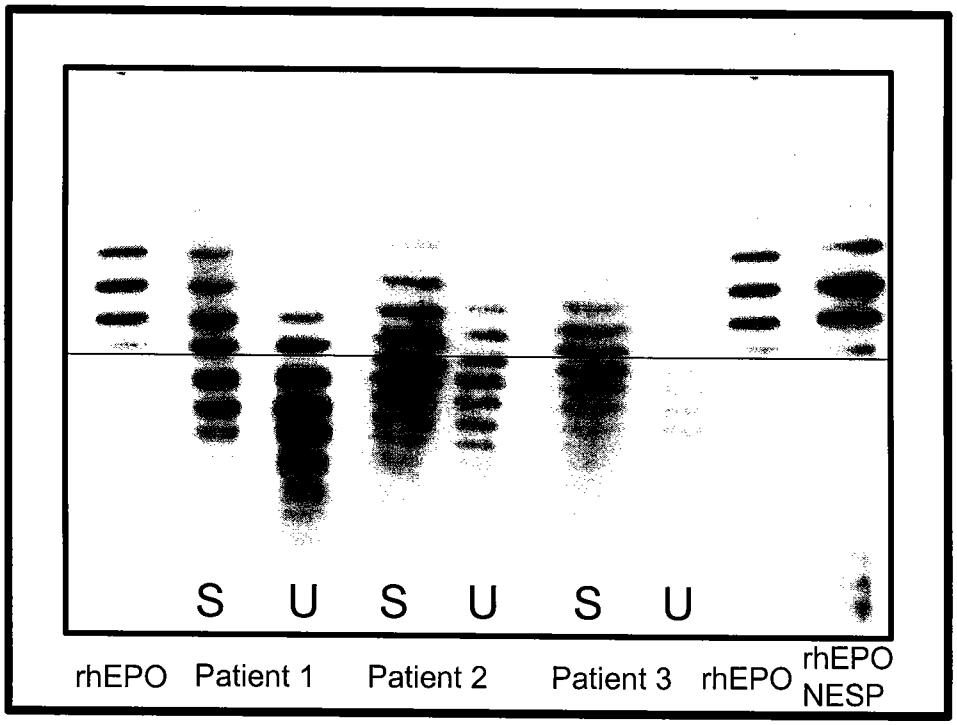

Figure 2. Isoelectric focusing (IEF) and subsequent immunoblotting of endogenous hEPO from serum (S) and urine (U). (NESP = novel erythropoiesis stimulating protein = darbepoetin alfa).

The binding of s-hEPO to the magnetic beads was demonstrated both by SDS PAGE and IEF followed by immunoblotting. On SDS PAGE s-hEPO appeared as a broad immuno-stained band with a molecular size slightly, but significantly, smaller than the size of rhEPO (figure 1). The bandwidth of the monomer indicated microheterogeneity of s-hEPO. The apparent molecular weights of rhEPO (epoetin alfa) and s-hEPO as revealed by SDS PAGE, were found to be 40 kDa and 34 kDa, respectively, while darbepoetin alfa in this system corresponded to 47 kDa (figure 1), which is compatible with two additional N-linked complex glycans (Egrie and Browne 2001).

The IEF analyses (Lasne 2001) revealed extensive charge heterogeneities within the endogenous hEPO populations both from serum and urine (figure 2). The charge variations within the epoetins, on the other hand, were considerably more uniform. A wider spectrum of charge variants for s-hEPO compared to rhEPO has previously been shown by two-dimensional gel electrophoresis (Skibeli et al 2001).

IEF of endogenous and rhEPO revealed significant differences in charge patterns (figure 2), where both s-hEPO and u-hEPO are more acidic than rhEPO. According to the literature, the oligosaccharides of the most acidic forms of rhEPO are nearly saturated with sialic acids (N-linked and O-linked)(Elliot et al. 1994), leaving the

additional negative charges on endogenous hEPO to be of another origin than sialic acids. The acidic IEF pattern of endogenous hEPO is difficult to explain by the present knowledge of the molecular structure of both natural and rhEPO, but could be due to negatively charged groups such as sulphate, phosphate or deamidations. Further structural investigations have to be performed of both s-and u- hEPO to elucidate the nature of the charge discrepancy between natural and rhEPO.
When serum and urinary EPO from the same patients (three) were run in parallel lanes on IEF, a charge switch towards the acidic end of the pH gradient was observed for u-hEPO compared to s-hEPO (figure 2 and 3).

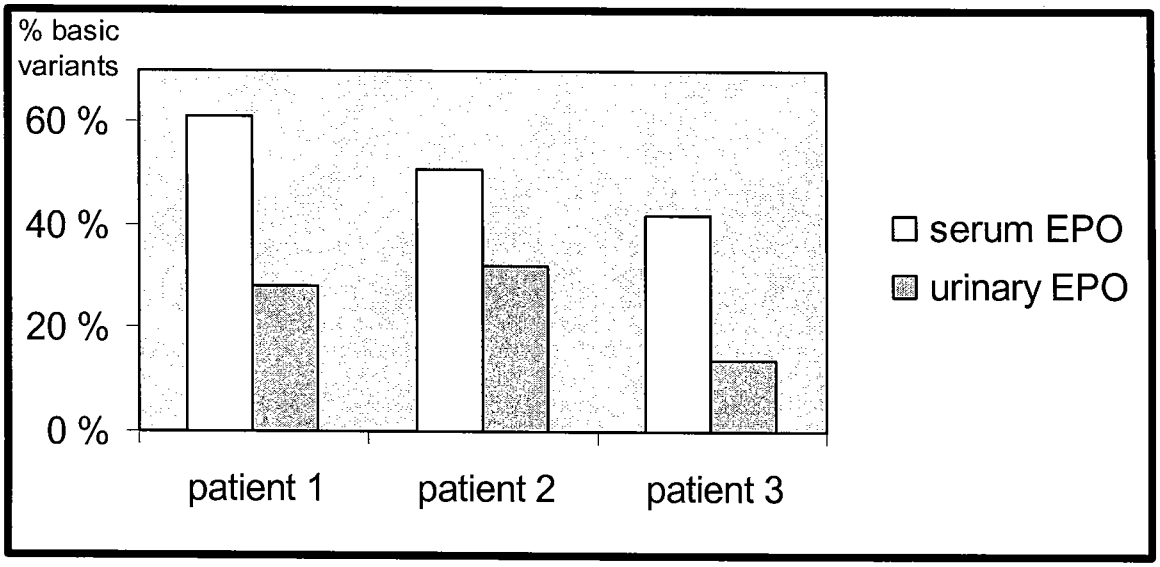

Figure 3. The relative amount of "basic variants" of s-hEPO compared to u-hEPO within the same individuals with anemia. The % "basic EPO variants"-calculations is defining the amount of the sample that is corresponding to a standard preparation of rhEPO relative to total EPO.

The difference in the content of "basic" variants between s- and u-EPO calculated relatively to rhEPO (figure 3), were from 25 to 30 %. This may indicate structural modifications of endogenous hEPO in the kidneys that also include a selective clearance of the most acidic forms. When terminal sialic acids, which are negatively charged, are removed from circulating hEPO, it is rapidly cleared by the galactose receptor in the liver, and this may promote the more negatively charged forms of s-hEPO to be secreted into the urine. Even a slight modification of the terminal sialic acids reduces the half-life of hEPO to a few minutes (Fukuda et al. 1989). Several reports on endogenous hEPO have shown that circulating hEPO contains less acidic glycoforms than u-hEPO (Tam et al. 1991; Wide et al. 1995). Wide and colleagues also showed that rhEPO secreted in the urine is more acidic than rhEPO analysed

from serum of the same individual. Wide concluded that the charge difference could be due to a difference in renal handling of the various glycoforms of hEPO (Wide et al. 1995).

Charge analyses of isolated N-linked sugar structures from different species of hEPO are shown in figure 4. Both s-hEPO and rhEPO contained several acidic oligosaccharide structures. The charge distribution analyses of the 2-AB glycan pools revealed unique patterns for all the hEPO species, including darbepoetin alfa. The most prominent difference between s-hEPO, rhEPO and darbepoetin alfa, was the lack of tetra-acidic oligosaccharide structures in s-hEPO (figure 4) (Skibeli et al. 2001). Glycans from epoetin alfa, beta and omega, all contained such structures. Several reports have demonstrated that sialic acids are the sole contributors to the negative charges in rhEPO (Sasaki et al. 1987; Takeuchi et al.1988; Tsuda et al. 1988; Imai et al. 1990, *ii*)). The study by Skibeli et al (2001) indicated that the acidic charges detected on the glycans from s-hEPO are sialic acids. Nearly all glycans from darbepoetin alfa are composed of tetra-sialylated oligosaccharides (figure 4)(Egrie and Browne 2001).

The increasing clinical use of rhEPO underlines the importance of structural comparison with s-hEPO for the understanding of its pharmacodynamics. Furthermore, the known misuse of rhEPO in sports renders the ability to detect significant molecular differences between endogenous hEPO and rhEPO important (Erslev 1987; Birkeland and Hemmersbach 1999; Rivier and Saugy 1999; Lasne and de Ceaurriz 2000; Lippi and Guidi 2000; Parisotto et al. 2000).

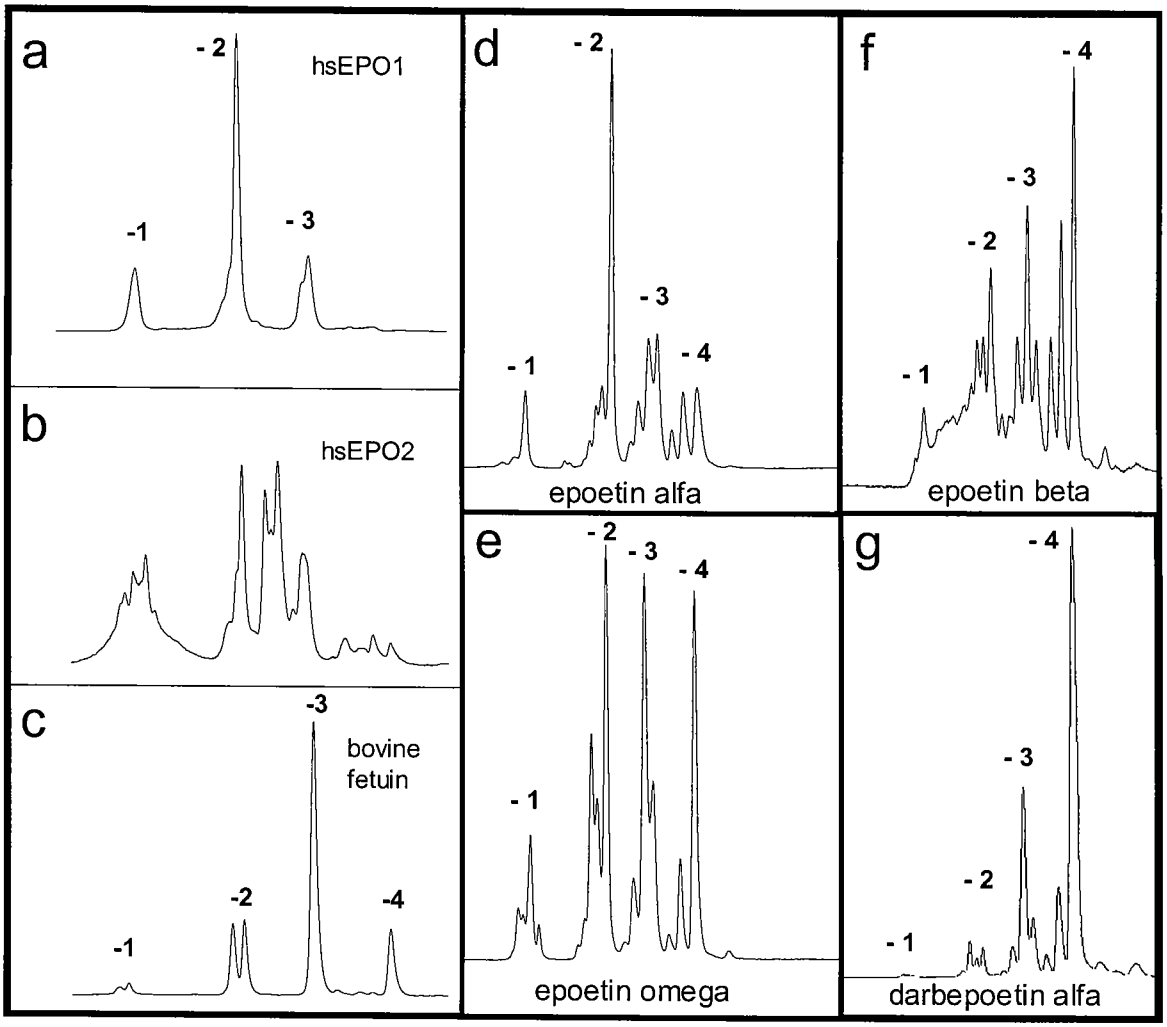

Figure 4. Charge patterns of N-linked oligosaccharides from s-hEPO obtained according to Guile et al. (1994). The classes of charged N-glycans, named mono-, di-, tri-, and tetra-acidic structures, were assigned by comparison with standard bovine fetuin sugars released and labeled with 2-aminobenzamide (2-AB) in the same way (Rudd et al. 1997; Rudd et al. 1999 *i*) and *ii*)). Panel a) and b) represent charged 2-AB-labeled glycans from s-hEPO from two patients with aplastic anemia. Panel c) represents 2-AB glycans from bovine fetuin (20 pmol). Panel d), e), f), and g) represent 2-AB glycans from epoetin alfa (20 pmol), epoetin omega (20 pmol), epoetin beta (20 pmol), and darbepoetin alfa (20 pmol), respectively.

The discrepancy in the glycosylation charge profiles for the various hEPO species is in agreement with other studies showing that glycoproteins synthesised by different cell systems display unique repertoires of oligosaccharides (Parekh et al. 1989 *i*) and *ii*); Fischer et al. 1996).

In conclusion, the current investigation revealed that s-hEPO emerged with a significantly lower molecular weight and a more acidic charge pattern than rhEPO. Darbepoetin alfa appeared considerably larger than both rhEPO and s-hEPO on SDS

PAGE and revealed the most acidic charge profile. In addition, the charge profiles of u-hEPO appeared more negative on IEF than the profiles of s-hEPO. These findings suggest structural differences between endogenous hEPO and rhEPO, and also between s-hEPO and u-hEPO. The observed charge dissimilarities between s-hEPO and u-hEPO are possibly caused by charge modifications of s-hEPO in the kidneys, a process that may also include a different renal reabsorption of the charge variants of EPO (Wide et al. 1995). The size and charge distinctions between s-hEPO and rhEPO demonstrated by SDS PAGE, IEF, and ion-exchange chromatography, are suggesting major structural discrepancies. The elucidation of the nature of these structures would be of vital importance with respect to the validation and development of EPO-methods in doping control.

References

Bergwerff, A. A., J. van Oostrum, et al. (1993). "Primary structure of N-linked carbohydrate chains of a human chimeric plasminogen activator K2tu-PA expressed in Chinese hamster ovary cells." Eur J Biochem 212(3): 639-56.

Birkeland, K. I. and P. Hemmersbach (1999). "The future of doping control in athletes. Issues related to blood sampling." Sports Med 28(1): 25-33.

Catlin, D. H., A. Breidbach, et al. (2002). "Comparison of the isolelectric focusing patterns of darbepoetin alfa, recombinant human erythropoietin, and endogenous erythropoietin from human urine." Clinical Chemistry 48(11): 2057-9.

Dube, S., J. W. Fisher, et al. (1988). "Glycosylation at specific sites of erythropoietin is essential for biosynthesis, secretion, and biological function." J Biol Chem 263(33): 17516-21.

Egrie, J. C. and J. K. Browne (2001). "Development and characterization of novel erythropoiesis stimulating protein (NESP)." Nephrol Dial Transplant 16: 3-13.

Elliot, E., T. Bartley, et al. (1994). "Structural requirements for addition of O-linked carbohydrate to recombinant erythropoietin." Biochemistry 33: 11237-45.

Erslev, A. (1987). "Erythropoietin coming of age." N Engl J Med 316(2): 101-3

Fischer, B., A. Mitterer, et al. (1996). "Comparison of N-Glycan Pattern of Recombinant Human Coagulation Factors II and IX Expressed in Chinese Hamster Ovary (CHO) and African Green Monkey (Vero) Cells." J Thromb Thrombolysis 3(1): 57-62.

Fisher, J. W. (1997). "Erythropoietin: physiologic and pharmacologic aspects." Proc Soc Exp Biol Med 216(3): 358-69

Fukuda, M. N., H. Sasaki, et al. (1989). "Survival of recombinant erythropoietin in the circulation: the role of carbohydrates." Blood 73(1): 84-9.

Grabenhorst, E., P. Schlenke, et al. (1999). "Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells." Glycoconj J 16(2): 81-97.

Guile, G. R., S. Y. Wong, et al. (1994). "Analytical and preparative separation of anionic oligosaccharides by weak anion-exchange high-performance liquid chromatography on an inert polymer column." Anal Biochem 222(1): 231-5.

Higuchi, M., M. Oh-eda, et al. (1992). "Role of sugar chains in the expression of the biological activity of human erythropoietin." J Biol Chem 267(11): 7703-9.

Imai, N., M. Higuchi, et al. (1990). "Physicochemical and biological characterization of asialoerythropoietin. Suppressive effects of sialic acid in the expression of biological activity of human erythropoietin in vitro." Eur J Biochem 194(2): 457-62. *i*)

Imai, N., A. Kawamura, et al. (1990). "Physicochemical and biological comparison of recombinant human erythropoietin with human urinary erythropoietin." J Biochem (Tokyo) 107(3): 352-9. *ii*)

Jacobs, K., C. Shoemaker, et al. (1985). "Isolation and characterization of genomic and cDNA clones of human erythropoietin." Nature 313(6005): 806-10.

Jelkmann, W. (1992). "Erythropoietin: structure, control of production, and function." Physiol Rev 72(2): 449-89.

Kamerling, J. P. (1996). "Carbohydrate features of recombinant human glycoproteins." Biotecnologia Aplicada 13: 167-80.

Krantz, S. B. (1991). "Erythropoietin." Blood 77(3): 419-34.

Kuzuya, H., P. M. Blix, et al. (1977). "Determination of free and total insulin and C-peptide in insulin-treated diabetics." Diabetes 26(1): 22-9.

Lai, P. H., R. Everett, et al. (1986). "Structural characterization of human erythropoietin." J Biol Chem 261(7): 3116-21.

Lasne, F. and J. De Ceaurriz (2000). "Recombinant erythropoietin in urine." Nature 405(6787): 635.

Lasne, F. (2001). Double-blotting: "Double-blotting: a solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures." J Immunol Methods 253.

Lin, F. K., S. Suggs, et al. (1985). "Cloning and expression of the human erythropoietin gene." Proc Natl Acad Sci U S A 82(22): 7580-4.

Lippi, G. and G. Guidi (2000). "Laboratory screening for erythropoietin abuse in sport: an emerging challenge." Clin Chem Lab Med 38(1): 13-9.

Macdougall, I. C., S. J. Gray, et al. (1999). "Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients." J Am Soc Nephrol 10: 2392-95.

Miyake, T., C. K. Kung, et al. (1977). "Purification of human erythropoietin." J Biol Chem 252(15): 5558-64.

Parekh, R. B., A. G. Tse, et al. (1987). "Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1." Embo J 6(5): 1233-44.

Parekh, R. B., R. A. Dwek, et al. (1989). "N-glycosylation and in vitro enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells and a murine cell line." Biochemistry 28(19): 7670-9. *i*)

Parekh, R. B., R. A. Dwek, et al. (1989). "Cell-type-specific and site-specific N-glycosylation of type I and type II human tissue plasminogen activator." Biochemistry 28(19): 7644-62. *ii*)

Parisotto, R., C. J. Gore, et al. (2000). "A novel method utilising markers of altered erythropoiesis for the detection of recombinant human erythropoietin abuse in athletes." Haematologica 85(6): 564-72.

Rivier, L. and M. Saugy (1999). "Peptide hormones abuse in sport: State of the art in the detection of growth hormone and erythropoietin." Journal of Toxicology-Toxin Reviews 18(2): 145-176.

Rudd, P. M. and R. A. Dwek (1997). "Glycosylation: heterogeneity and the 3D structure of proteins." Crit Rev Biochem Mol Biol 32(1): 1-100.

Rudd, P. M., B. P. Morgan, et al. (1997). "The glycosylation of the complement regulatory protein, human erythrocyte CD59." J Biol Chem 272(11): 7229-44.

Rudd, P. M., T. S. Mattu, et al. (1999). "Glycosylation of natural human neutrophil gelatinase B and neutrophil gelatinase B-associated lipocalin." Biochemistry 38(42): 13937-50. *i*)

Rudd, P. M., M. R. Wormald, et al. (1999). "Oligosaccharide analysis and molecular modeling of soluble forms of glycoproteins belonging to the Ly-6, scavenger receptor, and immunoglobulin superfamilies expressed in Chinese hamster ovary cells." Glycobiology 9(5): 443-58. *ii*)

Sasaki, H., B. Bothner et al. (1987). "Carbohydrate structure of erythropoietin expressed in Chinese Hamster Ovary cells by a human erythropoietin cDNA." J Biol Chem 262(25) 12059-76.

Skibeli. V., G. Nissen-Lie et al. (1998). "Immunomagnetic extraction of erythropoietin from human serum by magnetic beads." In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck-Engelke, U. eds. Recent Advances in Doping Analysis: Proceedings of the Manfred Donike Workshop. Vol. 6. Cologne, germany: Sport & Buch Strauß; 1998: 313-29.

Skibeli, V., G. Nissen-Lie, et al. (2001). "Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin." Blood 98(13): 3626-3634.

Svensson, E. C., B. Soreghan, et al. (1990). "Organization of the beta-galactoside alpha 2,6-sialyltransferase gene. Evidence for the transcriptional regulation of terminal glycosylation." J Biol Chem 265(34): 20863-8.

Sytkowski, A. J., L. Feldman, et al. (1991). "Biological activity and structural stability of N-deglycosylated recombinant human erythropoietin." Biochem Biophys Res Commun 176(2): 698-704.

Takeuchi, M., S. Takasaki, et al. (1988). "Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells." J Biol Chem 263(8): 3657-63.

Tam, R. C., S. L. Coleman, et al. (1991). "Comparisons of human, rat and mouse erythropoietins by isoelectric focusing: differences between serum and urinary erythropoietins." Br J Haematol 79(3): 504-11.

Tsuda, E., M. Goto, et al. (1988). "Comparative structural study of N-linked oligosaccharides of urinary and recombinant erythropoietins." Biochemistry 27(15): 5646-54.

Watson, E., A. Bhide, et al. (1994). "Structure determination of the intact major sialylated oligosaccharide chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells." Glycobiology 4(2): 227-37.

Wide, L., C. Bengtsson, et al. (1995). "Detection in blood and urine of recombinant erythropoietin administered to healthy men." Med Sci Sports Exerc 27(11): 1569-76.

Zerfaoui, M. and C. Ronin (1996). "Glycosylation is the structural basis for changes in polymorphism and immunoreactivity of pituitary glycoprotein hormones." Eur J Clin Chem Clin Biochem 34(9): 749-53.