Research article

Drug Testing and Analysis

Received: 1 October 2011 Revised: 19 October 2011 Accepted: 20 October 2011 Published online in Wiley Online Library: 2 December 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.388

The overlooked difference between human endogenous and recombinant erythropoietins and its implication for sports drug testing and pharmaceutical drug design

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Sequential deglycosylation by exoglycosidase treatment (Reagent Array Analysis Method, RAAM) and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a profound structural difference between human endogenous and recombinant erythropoietins. While both proteins behaved similarly upon digestion with *Arthrobacter ureafaciens* α-sialidase and *Steptococcus pneumoniae* β-D-galactosidase, the action of N-acetyl-β-D-glucosaminidase from *Steptococcus pneumoniae* was partly blocked by endogenous but not recombinant erythropoietins. Consequently, further treatment with *Jack bean* α-D-mannosidase and *Helix pomatia* β-D-mannosidase led to only very limited additional deglycosylation of endogenous EPO, while rhEPO glycans continued to be degraded. The behaviour was visualized by SDS-PAGE combined with Western blotting. While the apparent molecular masses of most endogenous glycoforms did not further decrease after treatment with the first three enzymes, masses of most rhEPO glycoforms continued to drop after digestion with the two mannosidases. Both, human urinary and serum EPO showed this blocking effect, and all of the tested 28 recombinant epoetins were accessible to further degradation by exo-mannosidases. The majority of EPO pharmaceuticals is produced in Chinese hamster ovary (CHO) cell lines, few in other ones (i.e. baby hamster kidney (BHK) or human fibrosarcoma (HT-1080) cells). Since human endogenous EPO is primarily produced by the kidneys, tissue specific glycosylation might explain the altered deglycosylation behaviour. This difference was overlooked since EPO was first isolated from human urine in 1977. The results might prove useful for anti-doping testing and future EPO drug development. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: erythropoietin; EPO; glycosylation; glycosidase; SDS-PAGE; isoelectric focusing (IEF-PAGE); doping control

Introduction

When Eugene Goldwasser (1922–2010) started research on erythropoietin (EPO) in 1955, the primary goal was to isolate this haematopoietic factor from a then not-yet-defined matrix of the human body as a possible means to cure anaemia caused by radiation sickness.[1] In 1836, it was discovered that anaemia is a kidney disease, but it took until 1957 until Jacobson *et al*. identified the kidneys as the main producers of erythropoietin.[2,3] Goldwasser first focused on plasma for isolating the unknown factor by using blood from anaemic sheep. But the amount and purity of the isolated EPO was rather limited.[4–7] Through his contact with T. Miyake, who collected 2550 litres of urine of aplastic anaemia patients, Goldwasser finally switched to urine as the main source for his EPO-purification strategy. In 1977, Golwasser and Kung managed to purify 8 mg of EPO out of this urine by using a seven-step process. The EPO had an activity of 70400 U/mg.[8] Subsequently, the sequence of the first 26 N-terminal amino acids was determined and published in 1981, but it contained two errors. The correct sequence was derived from cDNA by Jacobs *et al*. in 1985[9] and in the same year Lin *et al*. (Amgen)[10] cloned the human EPO gene, which was the basis for the biotechnological production of EPO in a transfected Chinese hamster ovary (CHO) cell line. Finally, in 1989 Amgen received FDA approval for *Epogen*, the first pharmaceutical containing recombinant human erythropoietin (rhEPO).[11] Rumours about the abuse of rhEPO as erythropoiesis stimulating agent (ESA) for doping purposes already existed in the late 1980s, and rhEPO was subsequently put on the IOC (International Olympic Committee) list of prohibited substances.[12]

Due to the extremely low abundance of EPO in human urine and serum/plasma (typically in the low ng/l range), comprehensive characterization of the glycan structures present on endogenous EPOs remained an elusive task. Only two mass-spectrometry-based articles addressing this subject appeared, in 1987 and 1997, respectively.[13,14] The latter used matrix-assisted laser desorption/ionization (MALDI) mass spectrometry for glycopetide profiling of human urinary EPO (uhEPO) and concluded that no differences between the N-glycan types of uhEPO and rhEPO existed.[14] In one of his last publications, Goldwasser showed in 1997 that recombinant and urinary human erythropoietins might significantly differ in conformation – 20 years after the first

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purification of uhEPO and eight years after FDA-approval of rhEPO. Among the experiments, which Goldwasser led to this conclusion, was the enzymatic removal of EPO's neuraminic acids by sialidase treatment, which resulted in a higher loss in molecular mass for uhEPO than rhEPO on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Table 1). This experiment was the starting point for the exoglycosidase studies presented in this paper.

Experimental

Materials

Reference standards for human recombinant (BRP-EPO batch 3) and human urinary erythropoietin (uhEPO; second international reference preparation) were bought from the European Directorate for the Quality of Medicines (Strasbourg, France) and the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK), respectively. Pharmaceutical formulations of recombinant erythropoietins were from Janssen-Cilag (*Erypo*; Vienna, Austria), Roche (*NeoRecormon*, MIRCERA; Mannheim, Germany), Amgen (*Aranesp (NESP)*; Thousand Oaks, CA, USA), Shire (*Dynepo*; Hampshire, UK), Bioclones (*Repotin*; Sandton, South Africa), Blausiegel (*Alfaepoetina*; Cotia, Brazil), Biosintetica [distributor]/Bio Sidus [manufacturer] (*Hemax*; Sao Paulo, Brazil/ Buenos Aires, Argentina), Biopharma (*Epocrin*; Kyiv, Ukraine), Microgen (*Erythrostim*, Moscow, Russia), Beijing Four Rings Biopharmaceutical Co. (Beijing 4 Rings; Beijing, China), Shenyang Sunshine Pharmaceutical (*Epiao*; Shenyang, China), NCPC GeneTech (*Jimaixin*; Shijiazhuang, China), VHB Life Sciences/Shenzhen Sciprogen Biopharma (*Epotop*; Mumbai, India/ShenZhen, China), Shantha Biotechnics (Shanpoietin; Medchal, India), Wockhardt (Wepox-2000 and Wepox-4000; Nani Daman, India), Gennova Biopharmaceuticals (*Vintor*; Pune, India), Emcure Pharmaceuticals/ Gennova Biopharmaceuticals (*Epofer*; Pune, India/Pune, India), LG Life Sciences/LG Life Sciences (*Espogen*; New Dehli, India/ Seoul, Korea), Gland Pharma/Intras Biopharmaceuticals (*Eposure*; Hyderabad, India/ Ahmedabad, India), Cadila Healthcare/Bio Sidus (*Zyrop*; Ahmedabad, India/Buenos Aires, Argentina), Intras Biopharmaceuticals (*Epofit*; Ahmedabad, India), Shandong Kexing Bioproducts (*Eposino*; Shandong, China), Panacea Biotech/Intras Biopharmaceuticals (*Epotrust*; Dehli, India/Ahmedabad, India), Micro Labs/Shanta Biotechnics (*Eposis*; Bangalore, India/Medchal, India), Hexal Biotech ForschungsGmbH (*Epoetin alfa Hexal*; Holzkirchen, Germany), Medice Arzneimittel Pütter GmbH/ Hexal Biotech ForschungsGmbH (*Abseamed*; Iserlohn, Germany/ Holzkirchen, Germany), Sandoz/Hexal Biotech ForschungsGmbH (*Binocrit*; Kundl, Austria/Holzkirchen, Germany), and Hospira/ Stada Arzneimittel (*Retacrit*; Hoofddorp, Netherlands/Bad Vilbel, Germany).

Table 1. Mass differences between uhEPO and rhEPO as observed by Kung and Goldwasser in 1997 on SDS-PAGE. Sialidase treatment led to a greater loss in apparent molecular mass for uhEPO than for rhEPO

	rhEPO		uhEPO	
	M_r [kDa]	ΔM_r [kDa]	M_r [kDa]	ΔM_r [kDa]
Intact EPO	38.2	-	37.0	-
Asialo-EPO	34.8	***3.4***	32.9	***4.1***
O-glycanase treated EPO	31.9	2.9	31.9	1.0
N-glycanase treated EPO	21.6	10.3	21.6	10.3

Sets of five exoglycosidases, namely α-sialidase (*Arthrobacter ureafaciens*; EC 3.2.1.18), β-D-galactosidase (*Steptococcus pneumoniae* expressed in *E. coli*; EC 3.2.1.23), N-acetyl-β-D-glucosaminidase (*Steptococcus pneumoniae* expressed in *E. coli*; EC 3.2.1.30), α-D-mannosidase (*Jack bean*; EC 3.2.1.24), and β-D-mannosidase (*Helix pomatia*; EC 3.2.1.25), were bought from two suppliers (Sigma-Aldrich; St Louis, MO, USA, and Glyko/ PROzyme; San Leandro, CA, USA). N-glycosidase F (peptide-N-glycosidase F, PNGase F; *Flavobacterium meningosepticum* expressed in *E. coli*; EC 3.5.1.52) was from Roche. All glycosidase digests were performed in an incubator from Heraeus (Hanau, Germany).

Pre-cast BisTris gels (NuPAGE; 10% T, 1.5 mm, 10 wells), lithium dodecyl sulfate (LDS) sample buffer, 4-morpholinepropanesulfonic acid (MOPS) electrophoresis running buffer as well as an Xcell SureLock Mini-Cell and PowerEase 500 power supply were from Invitrogen (Carlsbad, CA, USA).

For IEF-PAGE, ammonium peroxidisulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), acrylamide/bisacrylamide solution (40% T, 3% C; PlusOne ReadySol IEF), tris(hydroxymethyl) aminomethane (Tris; PlusOne), urea (PlusOne), glycine (PlusOne), sodium dodecyl sulfate (SDS; PlusOne), casting moulds including clamps (FlexiClamps) and gel support film (GelBond PAGfilm), as well as blotting papers (NovaBlot, and Blotting Paper 21 x 26 cm) were bought from GE Healthcare. The flat-bed electrophoresis system (Multiphor II) and EPS 3500 XL power supply were from GE Healthcare, too. Servalyte carrier ampholytes (3–10 Iso-Dalt) were received from Serva (Heidelberg, Germany).

All Western transfers were performed on a semi-dry blotter from BioRad (Trans-Blot SD; Hercules, CA, USA). Extra thick blotting paper (BioRad) was used for the blotting of SDS-PAGE gels. The non-fat milk for preparing membrane blocking and washing solutions was also obtained from BioRad. Clone AE7A5 anti-EPO antibody was received from R&D Systems (Minneapolis, MN, USA) and the streptavidin horseradish peroxidase complex from Biospa (Milano, Italy). Tween-80 (Surfact-Amps; 10%), the biotinylated secondary antibody (ImmunoPure goat anti-mouse IgG (H + L)) and the substrates for enhanced chemiluminescence detection (West Pico, West Femto) were from Pierce (Rockford, IL, USA). Images of Western blots were acquired on a LAS-4000 CCD-camera (Fujifilm; Tokyo, Japan). GASepo (version 1.3b2; ARC; Seibersdorf, Austria) was used as image analysis software.

Methanol (LiChrosolv, gradient grade), glacial acetic acid (p.a.), and sodium hydroxide (NaOH; 1 N, Titrisol) were obained from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) tablets, DL-dithiothreitol (DTT), sodium chloride (NaCl), sucrose (electrophoresis grade), sodium metabisulfite ($Na_2S_2O_5$), sodium citrate, citric acid, bovine serum albumin (BSA, ELISA grade), L-aspartic acid, Tris buffered saline (TBS), human serum, methyl red, and decane were purchased from Sigma-Aldrich (St Louis, MO, USA).

Devices for micro- (Steriflip; 0.2 μm) and ultrafiltration (Amicon Ultra-0.5; nominal molecular weight limit (NMWL) 10 kDa) and membranes for blotting (Durapore, Immobilon-P) were from Millipore (Billerica, MA). Centrifugal microfilters (0.2 μm; Nanosep MF) were from PALL (Ann Arbor, MI, USA). MilliQ (MQ) water was used for preparing all buffers and solutions (Millipore). Protein low-bind sample tubes (0.5 and 1.5 ml), GELoader tips, the microcentrifuge, and Thermomixer were from Eppendorf (Hamburg, Germany).

For immunoaffinity purification of EPO a purification kit from MAIIA Diagnostics (Upsala, Sweden) was used in combination with the QIAvac 24 Plus system from QIAGEN (Hilden, Germany).

Methods

Exoglycosidase digestion of erythropoietins (RAAM)

Before performing exoglycanase digests, solutions of recombinant and endogenous EPOs were rebuffered to 100 mM sodium citrate buffer (pH 5.5) using Amicon ultra-0.5 devices (NMWL 10 kDa). Briefly, between 0.2 and 100 IU (in < 60 µl) of various erythropoietins were diluted with 470 µl citrate buffer and supplemented with 100 µg BSA. After brief vortexing, ultrafiltration was performed at 14000 *rcf* for 30 min in a microcentrifuge. Retentates were recovered by inverting ultrafilters and subsequent centrifugation at 2000 *rcf* for 5 min. Before digestion EPO retentate concentrations were adjusted with citrate buffer to *ca* 0.01–1 IU/µl. Typically, 10 µl of the adjusted retentates were digested by addition of *ca* 2.5 mU of α-sialidase (*Arthrobacter ureafaciens*), *ca* 1.5 mU of β-D-galactosidase (*Steptococcus pneumoniae*), *ca* 25 mU of N-acetyl-β-D-glucosaminidase (*Steptococcus pneumoniae*), *ca* 20 mU of α-D-mannosidase (*Jack bean*), and *ca* 35 mU of β-D-mannosidase (*Helix pomatia*). Deglycosylation reactions with more than one exoglycosidase were performed simultaneously by adding up to five exoglycanases to the reaction tubes (Eppendorf 0.5 ml low protein binding tubes) and overnight incubation at 37 °C (Reagent Array Analysis Method, RAAM).[15] In order to address quality of glycosidases and reproducibility, enzymes of two different suppliers were tested (Sigma-Aldrich, Glyko/PROzyme). However, digestion with β-D-mannosidase was preferably performed after overnight deglycosylation with the four other enzymes and subsequent adjustment to pH 4 with 1 M citric acid in order to establish optimal conditions for the action of *Helix pomatia* glycanase (*vide infra*). The reaction mixture was then typically incubated for an additional 3 to 16 h at 37 °C.

PNGase F digestion of uhEpo

Digestion of uhEPO by PNGase F was performed by first incubating 0.1 IU of uhEPO in 20 mM sodium phosphate buffer pH 7.5/20 mM DTT for 5 min at 95 °C. After cooling down to room temperature, 1 unit of PNGase F was added and the solution was incubated at 37 °C overnight.

Immunoaffinity purification of shEpo

For deglycosylation of human serum EPO (shEPO) with exoglycosidases, shEPO was first immunoaffinity purified using a kit from MAIIA Diagnostics.[16] Serum samples were diluted 1:10 with TBS containing 0.1% Tween-20 (detergent aid; provided with the kit) and then microfiltrated using 0.2 µm centrifugal filters (Nanosep MF; 14000 *rcf*, 2 min) or Steriflip units. Anti-EPO columns were equilibrated with 1 ml of washing buffer (supplied with the kit) at a flow rate of *ca* 1 ml/min (QIAvac system), and then the diluted serum samples were passed through the columns at the same flow rate. Typically, between 2 and 20 ml of diluted serum samples (0.2–2 ml undiluted serum) were used per column. Immunoaffinity columns were then washed again with 1 ml of washing buffer and spun for 1 min at 1000 *rfc* in a microcentrifuge. Bound shEPO was eluted by adding 50 µl of desorption buffer (prepared by mixing of 50 µl desorption buffer component A with 0.5 µl detergent aid) and collected in 1.5 ml tubes by centrifugation at 1000 *rcf*/1 min. Immediately after desorption, eluates were neutralized by addition of 5 µl adjustment buffer (provided with the kit). For exoglycosidase digestion, desorbed shEPO was rebuffered by diafiltrating the eluate with 500 µl citrate buffer (100 mM, pH 5.5) and in Amicon ultra-0.5 devices (10 kDa NMWL) as described earlier.

SDS-PAGE of exoglycosidase-digested EPOs

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of glycosidase-digested erythropoietins precast BisTris-gels (10 % T, 1.5 mm, 10 wells) with MOPS running buffer were used. Samples were diluted with 0.05% BSA/PBS solution and *ca* 0.05–0.1 ng of digested EPO was applied on the gel. SDS-PAGE was performed as described elsewhere.[17] Briefly, digests were heated for 5 min at 95 °C (Thermomixer, 800 rpm) in LDS sample buffer (100 mM DTT). The catholyte was supplemented with 5 mM sodium metabisulfite as antioxidat and gels were run at constant voltage (200 V) for *ca* 55 min (maximum 120 mA and 25 W). Intact standards (BRP, NIBSC) were used on most gels as band positional references and at approximately the same concentration as the digested EPOs. Electrophoresis was stopped as soon as the dye-front reached the bottom of the gel.

IEF-PAGE of exoglycosidase-digested EPOs

Polyacrylamide gels for separating EPO isoforms by isoelectric focusing (IEF) were prepared as described earlier.[18] An acrylamide-bisacrylamide solution (5% T/3% C) containing 7 M urea and 4% (*w/v*) carrier ampholytes (Servalyt 3–10 Iso-Dalt) was used for casting gels (25 x 11.5 cm, 1 mm) with preformed wells on a plastic film (GelBond PAG).[19] Electrolytes were sodium hydroxide (1 N; catholyte) and L-aspartic acid (0.04 M; anolyte).[20] Decane was used instead of kerosene for establishing close contact between GelBond and the cooling plate of the Multiphor II unit. Gels were prefocused at constant voltage (250 V, 30 min; 10 °C), then samples were pipetted into the wells and focusing was continued for 2000 Vh at constant current (25 mA; maximum 25 W and 2000 V; 10 °C). Tween-80 (1%) was added to all samples before electrophoresis. Typically, 0.2 ng of intact and digested EPOs were applied on each lane. Dilutions were prepared using 0.05% BSA/PBS solution. Methyl red was added to the catholyte as marker dye. The inter-electrode distance was approximately 10 cm.

Western double-blots of SDS- and IEF-PAGE gels

After electrophoresis proteins were transferred to PVDF-membranes (Immobilon-P) by semi-dry Western blotting. SDS-PAGE gels were first equilibrated in Bjerrum buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol; 3 x 5 min)[21] and then blotted at constant current (1.0 mA/cm^2) for 60 min using extra thick blotting paper (one layer on each side).[17] Towbin buffer (25 mM Tris, 192 mM glycine, no methanol) was used as transfer buffer for IEF-PAGE gels. Three layers of thick blotting paper were used on each side of the sandwich. IEF-gel and Immobilon-P membrane were separated by a Durapore membrane for easier removal of the soft gel from the Immobilon-P membrane after the blot.[18] Transfers were done at 1 mA/cm^2 and for 30 min. All subsequent steps were identical for SDS- and IEF-PAGE separations and were already described in detail elsewhere.[17,18] In short, membranes were incubated in 5 mM DTT/PBS (37 °C, 60 min), blocked in 5% non-fat milk (NFM)/PBS (60 min), and incubated in primary antibody (clone AE7A5; 1:1000 (*v/v*) in 1% NFM/PBS) overnight in a cold room. After washing in 0.5% NFM/PBS

(3 x 7 min) the primary antibody was transferred under acidic conditions (0.7% acetic acid) to a second Immobilon-P membrane (double-blot; 0.8 mA/cm^2, 10 min).[18] The membrane was blocked (5% NFM/PBS; 60 min) and sequentially incubated with a secondary antibody (biotinylated goat anti-mouse IgG; 1:2000 (*v/v*), 1% NFM/PBS, 60 min) and a streptavidin horseradish peroxidase (HRP) complex (1:2000 (*v/v*), 1% NFM/PBS; 60 min) for detection of the transferred primary antibody. Before incubation in HRP the membrane was washed 3 x 7 min in 0.5% NFM/PBS, and after the incubation 3 x 7 min in PBS. Depending on the required sensitivity either West Pico or West Femto were used as chemiluminescence substrates. Images were acquired with a LAS-4000 CCD-camera and analysed with GASepo software.

Results and discussion

Buffer selection for RAAM deglycosylation with exoglycosidases

According to literature the optimal pH-working ranges of the five exoglycosidases are significantly different, namely pH 4.5–5.5 for *Arthrobacter ureafaciens* α-sialidase, pH 6.0–6.5 for *Steptococcus pneumoniae* β-D-galactosidase, pH 5.0-6.0 for *Steptococcus pneumoniae* N-acetyl-β-D-glucosaminidase, pH 4.0–5.0 for *Jack bean* α-D-mannosidase, and pH 4.0–4.5 for *Helix pomatia* β-D-mannosidase (Table 2).[22–25] Typically, phosphate buffers are recommended for deglycosylation with the first three enzymes and acetate, citrate, or citrate-phosphate buffers for the two mannosidases.[22,26–29] In addition, *Jack bean* α-D-mannosidase requires Zn^{2+} ions for optimal activity and stability below pH 5.5.[30] However, Zn^{2+} ions precipitated with phosphate-containing buffers. Hence, these buffers were avoided for RAAM reactions. In order to compromise all these requirements, 100 mM citrate buffer pH 5.5 was used for all RAAM experiments.[30,31] Due to the fact that the pH optimum of *Helix pomatia* β-D-mannosidase is below pH 4.5, but α-D-mannosidase from *Jack bean* is instable without Zn^{2+} ions below pH 5.5, digests were preferably performed in citrate buffer at pH 5.5 overnight with subsequent pH-adjustment to pH 4.0 for additional digestion with *Helix pomatia* β-D-mannosidase (*vide supra*).

Structural basis for the interpretation of RAAM exoglycosidase digestions of EPOs

Numerous studies were performed in order to elucidate the glycan structures of various erythropoietins. The results were mainly obtained by mass spectrometry coupled with electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), nuclear magnetic resonance (NMR) spectroscopy, or were based on the binding behaviour of various plant lectins.[32–35] However, due to the scarceness of purified human endogenous EPO, the majority of these studies were performed with recombinant erythropoietins. Only two mass spectrometric investigations were undertaken using human urinary erythropoietin and none with human serum EPO. Based on the results of all these experiments it was concluded that EPO contains three N-glycans (on amino acid positions N24, N38, and N83) and one O-glycan (on S126). The N-glycans are of the complex type and are present in various combinations of bi-, tri-, and tetraantennary structures. Recombinant EPOs contain small amounts of N-glycolylneuraminic acid (Neu5Gc),[34] mannose-6-phosphate,[36] and sulfated monosaccharides.[37,38] Sialic acids (Neu5Gc, Neu5Ac) of recombinant human erythropoietins are exclusively in α2-3 linkage to D-galactose,[39] while uhEPO was shown to also possess Neu5Ac in α2-6 linkage.[40] Since tissues of humans do not express CMP-N-acetylneuraminic acid hydroxylase due to evolutionary loss,[41,42] it is generally assumed that human endogenous EPOs do not contain Neu5Gc. A fact, which was also supported by structural investigations performed on Dynepo, a recombinant EPO expressed in a human cell line (HT-1080 fibrosarcoma cells).[43,44] Depending on cell line and culture conditions, sialic acids of rhEPOs exhibit additional O-acetylations, preferably on 9-O position.[39,45] L-fucose is primarily α1-6 linked to the terminal N-acetylglucosamine (GlcNAc) residue of the pentasaccharide core on N-glycans.[39] In some cases, the presence of fucose in sialyl Lewis^x structures could be shown.[44] Most rhEPOs contain a significant but variable amount N-acetyllactosamine repeating units on the branches of their N-glycans.[39,46,47]

Table 2. Optimum pH working ranges of the five exoglycosidases used in the RAAM studies of this paper

Enzyme	optimal pH range
α-sialidase (*Arthrobacter ureafaciens*)	4.5 - 5.5
β-D-galactosidase (*Steptococcus pneumoniae*)	6.0 - 6.5
N-acetyl-β-D-glucosaminidase (*Steptococcus pneumoniae*)	5.0 - 6.0
α-D-mannosidase (*Jack bean*)	4.0 - 5.0
β-D-mannosidase (*Helix pomatia*)	4.0 - 4.5

SDS-PAGE analyses of exoglycosidase digested endogenous and recombinant human EPOs

While SDS-PAGE and fluorophore-assisted carbohydrate electrophoresis (FACE) analyses of exoglycosidase treated recombinant epoetins were occasionally published,[48–50] so far no comprehensive SDS-PAGE analyses of sequentially exoglycosidase digested endogenous human EPOs in direct comparison to rhEPOs was performed. In 1997, and in one of his last publications, Goldwasser demonstrated that after sialidase-treatment the loss of apparent molecular mass on SDS-PAGE was greater for uhEPO than rhEPO (Table 1).[48] However, no further SDS-PAGE analyses were published on sequentially deglycosylated uhEPO after galactosidase, N-acetyl glucosamindase, and mannosidase treatment. A possible reason might have been a lack of availability of highly purified sequencing-grade enzymes and subsequent inconsistent results due to enzymatic side activities and batch variability. Unfortunately, enzyme quality is still an issue today and has to be kept in mind when results are interpreted.

RAAM analysis of uhEPO and rhEPO reference standards

Figure 1 shows the results obtained after RAAM deglycosylation of reference standards for uhEPO (NIBSC) and rhEPO (BRP-EPO). Under reducing conditions the apparent molecular mass of intact uhEPO and BRP-EPO on SDS-PAGE is *ca* 34 kDa and *ca* 36–38 kDa, respectively (lanes 1 and 2).[17] Hence, the mass of endogenous human EPO (uhEPO) is slightly lower than the mass of rhEPO (*ca* 2–4 kDa).[17,51] After treatment with *Arthrobacter ureafaciens* α-sialidase the difference in the two masses was slightly increased as was already shown by Kung and Goldwasser in 1997 (Table 1), i.e. the removal of sialic acids led to an apparently

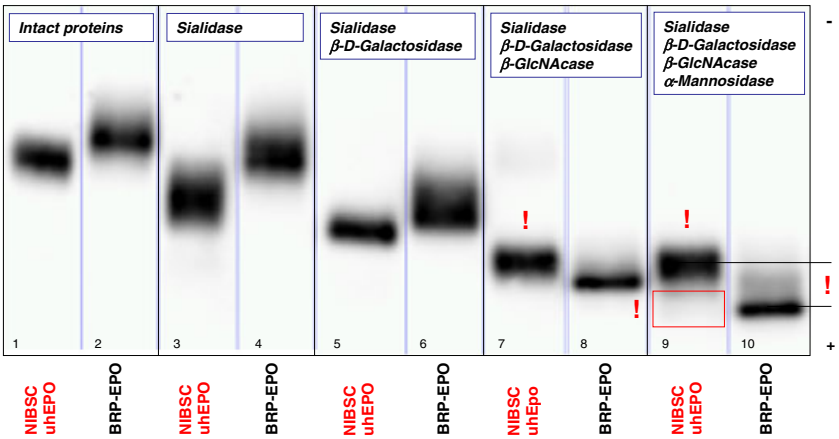

Figure 1. Comparison of sequentially treated uhEPO and rhEPO with up to four exoglycosidases by RAAM. Due to partial blockade of *Steptococcus pneumoniae* β-GlcNAcase by uhEPO (lane 7) no further degradation with α-mannosidase (lane 9) occurs. This effect is not observed for rhEPO (lanes 8 and 10), which continues to be enzymatically degraded. SDS-PAGE analysis with Western double-blot.

higher decrease in molecular mass of uhEPO than rhEPO.[48] *Arthrobacter ureafaciens* α-sialidase used for the RAAM experiments is an α(2–3,6,8,9) neuraminidase, which is generally applied for cleaving off all non-reducing terminal and also internal (branched) neuraminic acids from glycan structures, including Neu5Ac and Neu5Glc.[23] While a preference for α(2–6)-linked sialic acids is demonstrable, α(2–3), α(2–8), and α(2–9)-linkages are nevertheless cleaved by this enzyme but with decreasing relative cleavage rates.[23] Typically, this is overcome by increasing enzyme concentration and incubation time at 37 °C from a few hours to overnight incubations. Neuraminic acids on internal residues also slow down the cleavage reaction. The observed higher loss of sialic acids for uhEPO as shown in Figure 1 (lanes 3 and 4) suggests that either additional (e.g. sialyl Lewis structures) or more easily cleavable sialic acids are accessible to the enzyme on uhEPO. However, another explanation for the enhanced decrease in apparent molecular mass of α-sialidase treated uhEPO on SDS-PAGE could be the appearance of additional (internal) negative charges on the glycan structures of uhEPO after removal of the terminal sialic acids. Since SDS hardly interacts with oligosaccharides on glycoproteins as observed by the higher molecular mass of EPO on SDS-PAGE compared to the mass spectrometrically determined mass, the presence of internal negative charges might be seen on SDS-PAGE only after removal of the charged terminal (sialic acid-based) structures. In consequence, this might lead to the observed relative increase in migration speed of sialidase-treated uhEPO in comparison with desialylated rhEPO due to different net-charge densities. Differences in the presence of internal charges are also supported by the results obtained by comparative analysis of exoglycosidase treated rhEPO/uhEPO on IEF-PAGE (*vide infra*).

Combined treatment of EPO with *Arthrobacter ureafaciens* α-sialidase and *Steptococcus pneumoniae* β-galactosidase (Figure 1, lanes 5 and 6) led to a further decrease in apparent molecular mass on SDS-PAGE. The loss in mass was greater for rhEPO than for uhEPO and dominated the electrophoretic behaviour, which in consequence abolished the observed relative increase in mass difference after treatment with sialidase only. In result, a similar mass difference as for the intact proteins was seen after cleavage with the two enzymes. One possible explanation for this behaviour is the higher degree of tri- and tetraantennary glycan structures on rhEPO, which results in a higher amount of cleavable terminal galactose units after removal of the sialic acids.[14] *Steptococcus pneumoniae* β-galactosidase is a β(1–4) galactosidase, which only cleaves β(1–4)-linked terminal and non-reducing galactose residues. This type of linkage dominates the structures found on complex N-glycans. However, no cleavage will occur if a Lewis^x epitope is present, i.e. galactose which is β(1–4)-linked to N-acetylglucosamine carrying an additional fucose.[52] Since a considerable amount of galactose is removed from uhEPO by *Steptococcus pneumoniae* β-galactosidase, the Lewis^x motif cannot be the dominating structure on uhEPO.

In contrast to that, substrate specificity of *Steptococcus pneumoniae* β-N-acetylglucosaminidase is more complex.[53] The enzyme removes non-reducing terminal β-linked N-acetylglucosamine residues, but with different cleavage rates depending on for example, linkage-type (β(1–2), β(1–4), β(1–6)), glycan-type (bi-, tri-, or tetraantennary), and linkage-type of neighbouring sugars or possible steric hindrance. Specificity of this enzyme is also concentration-dependant. At concentrations below 50 mU/ml, only GlcNAc in β(1–2)-linkage is cleaved – but only if the linked mannose is not substituted at C-6. In case a bisecting β(1–4)-linked GlcNAc is present, only the β(1–2)-linkage between GlcNAc and the α(1–3)-linked core-mannose will be hydrolyzed. Above 50 mU/ml, the specificity for β(1–2)-linkage may get lost, which results in additional cleavage of β(1–4) and β(1–6)-linked GlcNAc residues.[23,24] The rate of the enzymatic reaction depends on steric hindrance and in particular, the presence of a bisecting GlcNAc-residues slows down cleavage rates for other β-linked GlcNAc-residues. Specificity of *Steptococcus pneumoniae* β-N-acetylglucosaminidase for N-acetylgalactosamine (GalNAc) residues is controversially discussed in literature. While cleavage of simple β-GalNAc substrates (e.g. 4-Nitrophenyl-N-acetyl-beta-D-galactosamine, pNP-β-GalNAc)[54] occurs at *ca* sevenfold slower rate than for the corresponding β-GlcNAc substrates (information provided by the enzyme suppliers), generally no significant activity is assumed for β-GalNAc residues in complex glycans.

The results obtained on SDS-PAGE after RAAM digestion of uhEPO and rhEPO with all three enzymes working together (α-sialidase, β-galactosidase, β-N-acetylglucosaminidase) are shown in Figure 1 (lanes 7 and 8). While the apparent molecular masses of both EPOs continued to decrease, the decrease was more pronounced for rhEPO. This behaviour can be explained by

the presence of N-acetyllactosamine repeating units or a higer degree of them on the N-glycans of the recombinant protein.[47] What cannot be explained with this model is that the mass of rhEPO decreased to such an extent that it became lower than the corresponding mass of uhEPO. According to the published glycan structures of uhEPO and rhEPO, the main difference between these two glycoproteins lies in the distribution of bi-, tri- and tetraantennary N-glycans.[14] If this observation was correct, then uhEPO and rhEPO should have arrived at identical molecular mass positions on SDS-PAGE after digestion with the three exoglycosidases. Hence, it is assumed that uhEPO contains residues on its glycans, which partly block the activity of *Steptococcus pneumoniae* β-N-acetylglucosaminidase. One of these structures might be a bisecting β(1–4)-linked GlcNAc residue on the trimannosyl core. What is known is that glycosyltransferases are tissue-specifically expressed and that N-acetylglucosaminyl transferase-III (GlcNAcT-III), the enzyme responsible for generating bisecting GlcNAc-linkages, is highly expressed in human kidney and brain but not in human liver.[55] By mass spectrometric glycan profiling of tissues it was further shown that bisecting glycans are indeed present on N-glycans of human kidney but not for example, of human liver. Additionally, GlcNAcT-III is not expressed in CHO- and BHK-cells, the cell lines most frequently used for the production of recombinant erythropoietins. However, the existence of a bisecting GlcNAc residue on N-glycans of uhEPO could be not demonstrated by NMR spectroscopy or mass spectrometry so far and also cannot be deducted from the obtained SDS-PAGE results alone.

Further evidence for the partial blocking effect of uhEPO on *Steptococcus pneumoniae* β-N-acetylglucosaminidase was provided by the combined deglycosylation with with *Arthrobacter ureafaciens* α-sialidase, *Steptococcus pneumoniae* β-galactosidase, *Steptococcus pneumoniae* β-N-acetylglucosaminidase, and *Jack bean* α-mannosidase (Figure 1, lanes 9 and 10). *Jack bean* α-mannosidase is a broadly specific enzyme, which cleaves α(1–2,3,6)-linked mannoses.[31] When applied to the pentasaccharide core structure, the end product of the reaction is the [Man - β(1,4)-GlcNAc - β(1,4)-GlcNAc] core trisaccharide. Since kinetics are slower for cleavage of the α(1–6)-bond, all RAAM incubations with *Jack bean* α-mannosidase were performed overnight. While the majority of the glycoforms of uhEPO was not further degraded by α-mannosidase, rhEPO continued to be cleaved by this enzyme. Consequently, the apparent molecular mass of rhEPO further decreased on SDS-PAGE, while the median mass of uhEPO stayed at approximately the same level as obtained after cleavage with only the first three glycanases.

Upon application of the fifth exoglycosidase, β-D-mannosidase from *Helix pomatia*, the glycans of rhEPO could be further degraded while no reduction in molecular mass occured again for uhEPO (Figure 2, lanes 3 and 4). The enzyme cleaves terminal non-reducing mannose residues, preferably of the β(1–4)-linkage type, and to a much lesser extent β(1–2)- and β(1–6)-linkages, why it is frequently used in glycan sequencing studies for removing the mannose of the core trisaccharide.[56] RAAM digests of mixtures of uhEPO and rhEPO are shown in Figure 5 (*vide infra*).

RAAM analysis of pharmaceutical rhEPO preparations including biosimilar epoetins

In order to investigate whether the revealed structural differences between the reference standards for uhEPO (NIBSC) and rhEPO

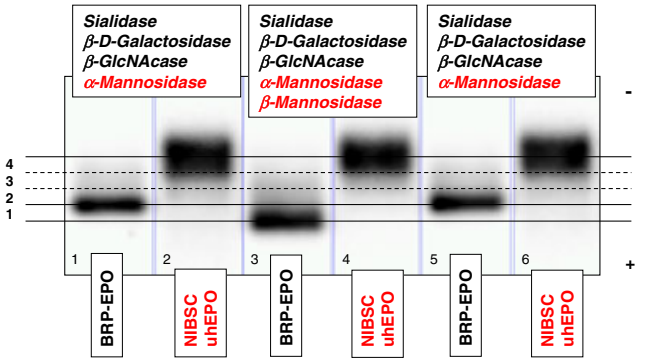

Figure 2. The action of β-mannosidase is also blocked by uhEPO in the five exoglycosidase RAAM experiment (lane 4). No blockage is observed for rhEPO (lane 3). The apparent difference in molecular mass on SDS-PAGE corresponds to approximately four monosaccharide units of each N-glycan.

(BRP) were generally observable for recombinant erythropoietins, a RAAM study of 28 pharmaceutical preparations of rhEPOs was performed. The study included pharmaceuticals of originator companies (e.g. Amgen/Erypo, Roche/NeoRecormon, Shire/Dynepo) as well as biosimilar epoetins of many different companies from around the world. Since some biosimilars behave differently on IEF- and SDS-PAGE than the originator substances, the detection of ESA-abuse for anti-doping purposes became more complicated. If human endogenous EPO indeed contains structural elements on its glycans, which are specific for the glycosylation machinery of the human kidneys (the main organ responsible for EPO synthesis in adults),[3,57] then *all recombinant erythropoietins* expressed in cell lines missing elements of this machinery (e.g. CHO and BHK cells) should be easily differentiable from endogenous EPO by the combined exoglycosidase digestion described above. SDS-PAGE analysis results of intact rhEPOs and rhEPOs digested by four glycanases (*Arthrobacter ureafaciens* α-sialidase, *Steptococcus pneumoniae* β-galactosidase, *Steptococcus pneumoniae* β-N-acetylglucosaminidase, and *Jack bean* α-mannosidase) are shown in Figures 3 and 4, respectively.

The recombinant epoetin pharmaceuticals behaved remarkably similar after RAAM deglycosylation. All of the tested rhEPOs could be cleaved down to the apparent molecular mass as observed for BRP-EPO on SDS-PAGE. Some pharmaceuticals showed an additional but weaker band slightly above the band of digested BRP-EPO (Dynepo, Hemax, Erythrostim, Eposure, Wepox, Zyrop), indicating incomplete digestion. Only Repotin, an rhEPO produced in a baby hamster kidney (BHK) cell line, was more difficult to cleave for the four enzymes and thus led to an additional strong third band with higher molecular mass than the two other bands but still lower in mass than the uhEPO band. Consequently, all of the 28 tested rhEPO pharmaceuticals were accessible to cleavage by the four exoglycosidases and did not block the action of β-N-acetylglucosaminidase and α-mannosidase the way uhEPO did.

RAAM analysis of EPO isolated from human serum

Since human endogenous erythropoietin is mainly produced by the kidneys (*vide supra*) human urinary and serum EPO should behave similar upon exoglycosidase digestion. Figure 5A shows the behavior of a mixture of rhEPO and uhEPO on SDS-PAGE after treatment with up to five exoglycosidases. After the action of four

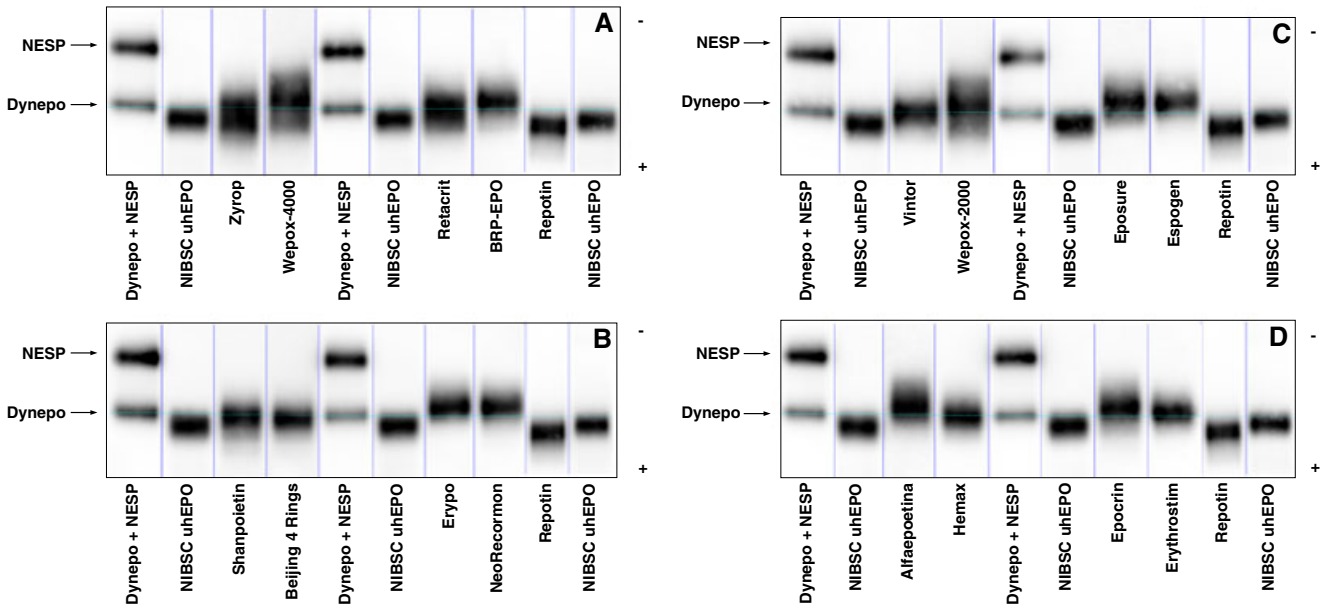

Figure 3. Performance characteristics of various intact recombinant erythropoietins after SDS-PAGE and Western double-blot. Note the differences in glycoform distribution as indicated by the vertical band shape.

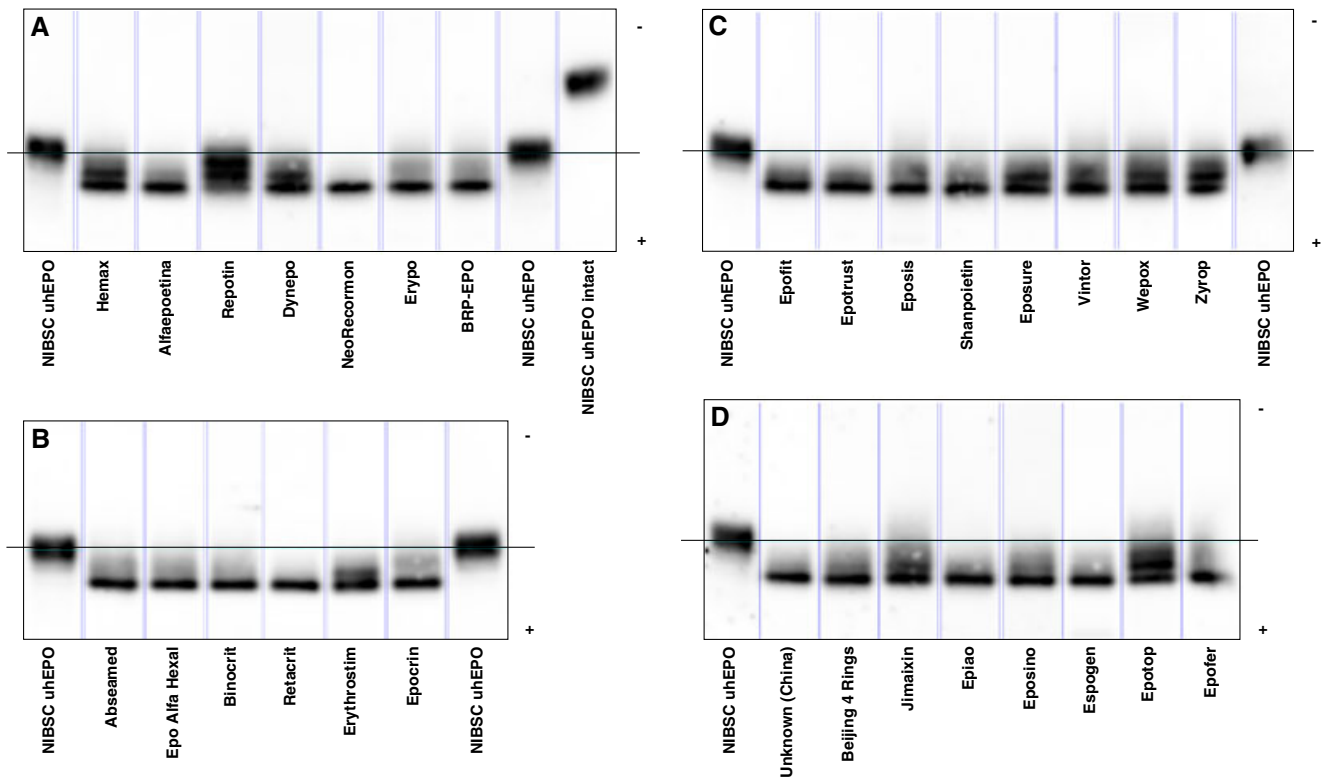

Figure 4. Treatment with four exoglycosidases and subsequent SDS-PAGE separation of rhEPOs in comparison to the reference standards for rhEPO (BRP) and uhEPO (NIBSC). In contrast to uhEPO, all rhEPOs are cleaved by the four enzymes.

enzymes (Figure 5A, lanes 3 and 4) the two epoetins were already much better separated than in intact form (Figure 5B, lanes 1 and 2). As expected, RAAM deglycosylated shEPO performed similarly on SDS-PAGE as uhEPO (Figure 5B, lanes 3 and 5) and with the most intense band exhibiting the same apparent molecular mass as the RAAM degraded uhEPO.

IEF-PAGE analyses of exoglycosidase digested endogenous and recombinant human EPOs

As shown by IEF-PAGE, the isoform distribution of recombinant human erythropoietins significantly differs from the isoform distribution of uhEPO.[18,58] In general, rhEPO isoforms are on

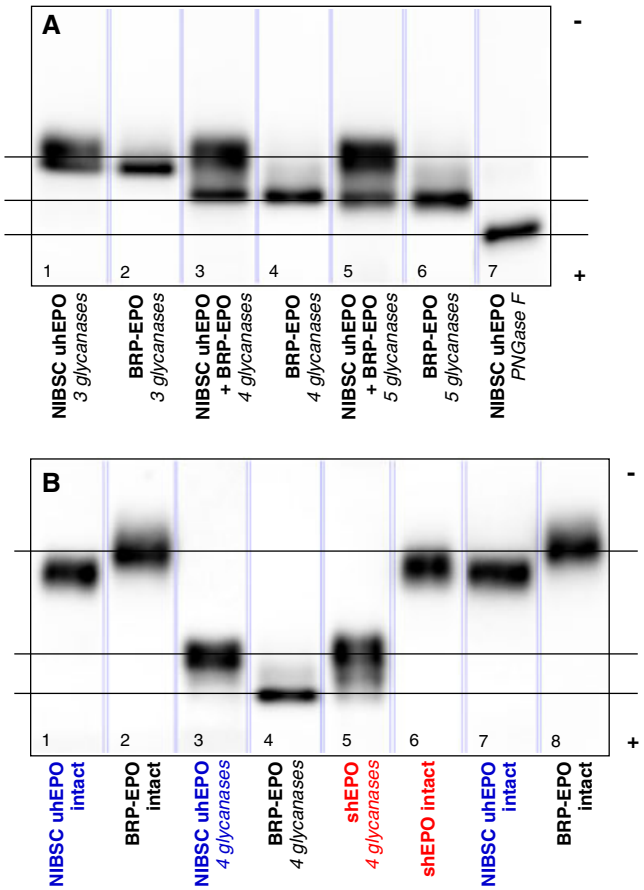

Figure 5. Exoglycosidase degradation of a mixture of uhEPO and rhEPO (A). Removal of four types of monosaccharides already results in an enhanced separation between the two erythropoietins on SDS-PAGE (lanes 3 and 4). A similar blockage of *Steptococcus pneumoniae* β-GlcNAcase as for uhEPO is seen for shEPO (B, lane 5), demonstrating close structural relationship between the two enodogenous epoetins. Hence, the observed difference to rhEPO is independent of the kidney passage of uhEPO. SDS-PAGE analysis with Western double-blot.

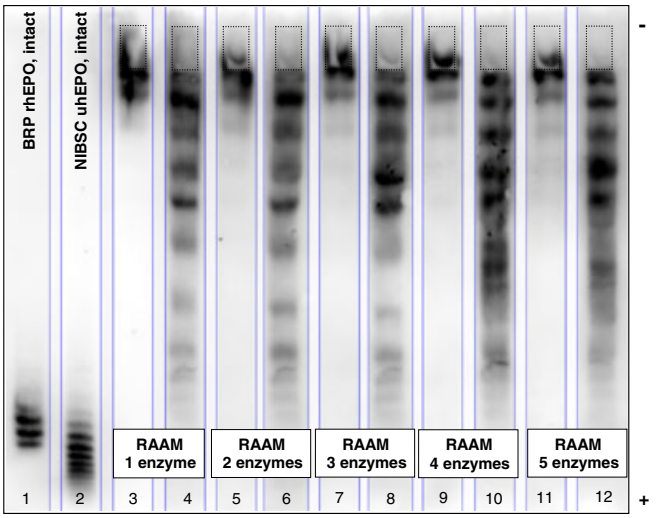

Figure 6. Separation of sequentially deglycosylated rhEPO and uhEPO on IEF-PAGE (pH 3–10). After *Arthrobacter ureafaciens* α-sialidase treatment additional basic isoforms are observed for uhEPO (lane 4), which are missing for rhEPO (lane 3). Most of these uhEPO isoforms resist further exoglycosidase digestion (lanes 6, 8, 10, 12), which is not the case for rhEPO (lanes 5, 7, 9, 11). Dotted rectangles indicate position of sample application (wells; cathode).

average more basic compared to the majority of uhEPO isoforms, with exception of NESP, a biotechnologically modified epoetin, which shows a more acidic isoform distribution.[59] So far, the reasons for this altered behavior of uhEPO have not been elucidated. Usually, the appearance of up to fourteen EPO isoforms has been ascribed to the presence of up to fourteen sialic acids, and a fifteenth isoform was attributed to desialylated EPO (asialo-EPO). Partial removal of sialic acids leads to a shift to more basic isoforms.[17,60] However, since sialic acids alone cannot explain the general acidification of uhEPO isoforms, it has been assumed that uhEPO contains additional negative charges (e.g. sulfate groups).[60]

In order to investigate the resulting isoform distribution after sequential deglycosylation with the five exoglycosidases (*vide supra*), IEF-PAGE analyses were performed within the pH-range of 3 to 10. According to theoretical calculations, the isoelectric point (pI) of the non-posttranslationally modified amino acid chain of EPO is around pH 8.2–8.4. Hence, an IEF-gel with broad pH range was selected. The results are shown in Figure 6. Removal of sialic acids by *Arthrobacter ureafaciens* α-sialidase led to the expected complete disappearance of most isoforms on rhEPO (lane 3). However, the same treatment resulted in an appearance of more than ten additional basic isoforms on uhEPO. The pattern remained unaltered after subsequent deglycosylation with *Steptococcus pneumoniae* β-galactosidase and β-N-acetylglucosaminidase, indicating that these charges must be either present on the core sugars or on the non-cleavable residues of the glycan arms (lanes 6 and 8). Additional cleavage with *Jack bean* α-mannosidase and *Helix pomatia* β-D-mannosidase introduced only minor changes to the pattern, which further strengthens the assumption that these charges must be either present on inner core residues or the few enzymatically resistant arm-sugars. The presence of these charges on arm residues might also explain the partial blocking of *Steptococcus pneumoniae* β-N-acetylglucosaminidase by uhEPO.

Conclusion

The presented data support the assumption that there indeed exists a profound structural difference between human endogenous erythropoietins isolated from urine and serum/plasma and *all pharmaceutical preparations of human recombinant erythropoietins*, regardless of whether they are produced by animal (CHO, BHK) or human (HT-1080) cell lines. The majority of endogenous EPO glycoforms are only partly accessible to *Steptococcus pneumoniae* β-N-acetylglucosaminidase digestion and largely block the subsequent action of α- and β-mannosidases in RAAM experiments. Recombinant human erythropoietins do not show this behaviour and are accessible to further cleavage after β-GlcNAcase treatment. The discovery of this for decades overlooked difference might also prove useful in sports drug testing, and in particular for enhancing the already established SDS- and SARCOSYL-PAGE methods for the detection of rhEPOs in urine and blood or the development of generally applicable mass spectrometric EPO-test. More comprehensive studies on the glycan structures of human endogenous erythropoietins are required in order to elucidate the exact structural differences responsible for these results. Such studies will also be of importance for the production of EPO pharmaceuticals with

closer structural carbohydrate identity to the endogenous human glycoforms.

Acknowledgement

The author wishes to thank following persons for providing biosimilar epoetins for this study: S. Jain, C. Donninger, R. Nicolich, J. A. Pascual, N. Robinson, G. Rodchenkov, M. Saugy, P. J. van der Merwe, M. Wu.

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