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Structural Features of EPO and Analogues: Glycosylation Profiles
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STRUCTURAL FEATURES OF EPO AND ANALOGUES: GLYCOSYLATION PROFILES

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INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that is secreted primarily by adult kidneys in response to tissue hypoxia, and it is involved in the regulation of the level of red blood cells. EPO molecule contains 3 N-linked (Asn-24, 38, 83) and 1 O-linked (Ser-126) oligosaccharide chains representing about 40% of the total mass (29.4 kDa). The recombinant analogue (rEPO), available since 1986 has found widespread use in the treatment of anaemia, renal failure, cancer, AIDS, etc. Furthermore, rEPO is illicitly used by athletes to boost the delivery of oxygen to the tissues and enhance their performance in endurance sports. For this reason, the IOC banned the use of rEPO as ergogenic agent in 1990. In 2001 a novel erythropoiesis-stimulating protein (NESP)[1] was synthesised. NESP possesses two additional N-glycans (Asn 30, 88) compared to endogenous or recombinant EPO. The number and composition of the N-glycans is clearly important in the metabolism of this glycoprotein because the carbohydrate content determines the ½-life time. Current tests to differentiate between urinary endogenous (uEPO) and recombinant analogues (rEPO, NESP) are based on differences in their isoelectric focussing (IEF) profiles. It is well known that both, the natural and recombinant EPO have identical peptide backbones. However, differences arising from post-translational modifications have been reported and we hypothesised that the observed IEF profiles[2] are originated by cell-type specific glycosylation. Here we describe the meticulous structural characterisation of both endogenous and exogenous EPO's and analogues. The particular structural features of each glycoprotein encountered may pave the way to the unambiguous detection of rEPO/NESP abuse and the developed methodology may be employed in a clinical-diagnostic setting.

EXPERIMENTAL

Materials - Human urinary EPO (2^{nd} international reference preparation 1971) was purchased from National Institute for Biological Standards and Control (NIBSC, Hertfordshire, United Kingdom). Recombinant human EPO was obtained from European Pharmacopoeia Commission (Strasburg/France). NESP (darbepoetin α) was acquired from Amgen. Monoclonal anti-human EPO antibody (clone: AE75A) was from R&D Systems. CNBr-activated Sepharose 4B was from Amersham Biosciences. Centricon plus-20™ and YM-30™ centrifugal filters were purchased from Millipore. Dextran T70 was from Pharmacia Biotech. Recombinant peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase F (PNGase F, EC 3.1.27.5), recombinant β-1,4-galactosidase (EC 3.2.1.23), recombinant endo-α-N-acetylglucosaminidase (EC 3.2.1.97), and recombinant α-2,3,6,8-neuraminidase (EC 3.2.1.18) were purchased from Calbiochem. Carbograph graphitised carbon ultra-clean columns (150 mg in 4 ml) were purchased from Alltech. 2,5-dihydroxybenzoic acid (DHB), bovine serum albumin (BSA), bovine fetuin and phosphate-buffered saline (PBS) were purchased from Sigma. 2-aminobenzamide (2AB), sodium cyanoborohydride ($NaCNBH_3$), dimethylsulfoxide (DMSO) were from Fluka. GELoader Tips were purchased from Eppendorf. Normal phase TSK gel Amide-80 column (4.6 x 250 mm) that was obtained from TOSOH BIOSEP. Weak anion exchange VYDAC 301 VHP column (7.5 mm ID x 50 mm) was from VYDAC. All other chemicals were of highest purity commercially available.

Sample preparation - Human urinary EPO was subjected to affinity-chromatography. Aliquots of ~ 3.5 ng were loaded column containing monoclonal anti-human EPO antibody covalently linked to CNBr-activated Sepharose 4B, previously equilibrated with 2 ml Tris-HCl 50 mM (pH 7.4) containing 1% BSA at a flow rate: 1 ml/min. Flow-through were re-applied onto the column at a flow rate of 50 µl/min. Then, the column was washed with 2 ml of equilibration buffer. Bound uEPO was eluted with 0.1 M Glycine-HCl (pH 2.2) and collected in 400 µl 1M Tris-HCl (pH 9.0) containing 1% BSA (uEPO-F1). Two subsequent elution fractions were collected (uEPO-F2 and uEPO-F3). Immuno-purified uEPO samples, NESP and rEPO dissolved in water, were desalted and concentrated by filtration using 30 kDa filters and lyophilised.

Monosaccharide analysis - Monosaccharide analysis of purified glycoproteins was performed as described by Kamerling et al.[3]. The quantitative analyses were carried using a AT-1 column (30m x 0.25mm, Alltech).

Sialic acids analysis - Sialic acids were released by hydrolysis in 3 M acetic acid, 3 h, 80 °C and converted into fluorescent derivatives with 1,2-diamino-4,5-methylene-dioxybenzene (DMB) as reported by Hara et al.[4] Analysis of DMB labelled residues was performed by reversed phase HPLC on a Cosmosil 5C18-AR-II column (4.6 x 250 mm) using acetonitrile-methanol-water (9:7:84) as isocratic eluent at a flow rate of 1ml/min. Fluorescence detection was carried out at an excitation λ=373 and an emission λ=448 nm.[5]

Enzymatic digestions - In order to release N-linked oligosaccharides purified glycoproteins were dissolved in 50 mM sodium phosphate buffer (pH 7.3). PNGase F was added and the mixture incubated 16 h, 37 °C. Liberated oligosaccharides were separated from remaining detergent, protein and salts in a single step, on graphitised carbon columns as described by Packer et al.[6] 2-Amino benzamide (2AB) labelled N-glycans were submitted either individual or combined exoglycosidases digestions using α2-3,6,8,9 neuraminidase, β1-4 galactosidase, and β1-R *N*-acetylglucosaminidase in 50 mM sodium phosphate buffer (pH 6.0) 16 h, 37 °C. After digestion, samples were filtered over 5 kDa filters, lyophilised, re-suspended in 100 µl bidistilled water and profiled again.

N-linked glycosylation analysis - Purified oligosaccharide mixtures were derivatised with 2AB as described by Bigge et al.[7] Finally, samples were analysed by HPLC and MALDI-TOF.

HPLC profiling - Normal phase HPLC profiling was basically performed on a similar systems and employing identical conditions as described by Gutiérrez Gallego et al [8]. Weak anion exchange HPLC was performed essentially as described by Guile et al. [9].

MALDI-TOF - Purified carbohydrates (2AB labelled and non-labelled sugars) were dissolved in water at varying concentrations. An aliquot was mixed with 2,5-dihydroxybenzoic acid 10 mg/ml solution (MeCN:H_2O) and <1µl of this preparation applied to the target and analysed. Experiments were carried out on a Voyager-DE™ STR Biospectrometry workstation

(Applied Biosystems), equipped with a N_2 laser (337nm). Typically, spectra were acquired in linear mode for negative polarity and in reflectron mode for positive polarity. Mass scans were accumulated in the range between 900-5000 Da. External calibration of the spectrometer was performed using a mixture of glucose oligomers in positive ion mode and 2AB-derivatised fetuin *N*-glycans in negative mode. Recorded data were processed with Data Explorer™ Software (Applied Biosystems).

RESULTS AND DISCUSSION

Monosaccharide analysis- Monosaccharide analysis of fraction I of rEPO purified with Bio-Gel P-2 (Figure 1) revealed the presence of Fuc, Man, Gal, GalNAc, GlcNAc, Neu5Ac in the molar ratio of 1.0 : 3.0 : 4.7 : 0.2 : 0.2 : 6.1 : 4.9 with respect to the internal standard and setting the molar value of Man at 3. Neu5Ac represents the total sialic acid content.

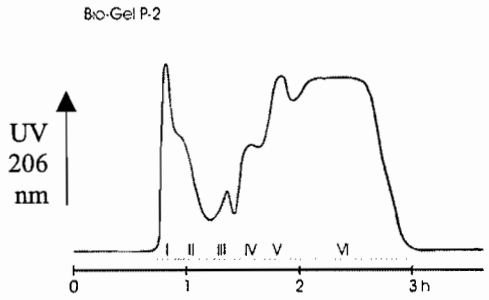


Figure 1: Elution profile of rEPO on Bio-Gel P-2. I – VI indicate the different fractions collected. Only fraction I and II contained glycoprotein.

Table I shows a normalised compilation of monosaccharide values for rEPO. Although at first glance these data are similar, significant structural feature can be deduced: i.e. the ratio 3:1 (Man:Fuc) in rEPO indicates a complete core fucosylation of the N-linked glycans as CHO cells are known to lack the α-1,3/4-fucosyl transferase. Similarly, the 3:6 ratio for Man:GlcNAc in combination with the ratio of 3:5 for Man and NeuAc points towards the presence of predominantly tetra-antennary complex-type N-glycans with a high degree of sialylation as well as the presence of *N*-acetyl lactosamine repeats. These trends are slightly different for uEPO indicating possible structural glycosylation differences.

Table I Monosaccharide composition of erythropoietin from different origins. Explanation of the abbreviations: rEPO, recombinant erythropoietin from Chinese hamster ovary (CHO) cells; rEPO-T, recombinant erythropoietin from Chinese hamster ovary (CHO) cells as produced by Teknika; rEPO BHK, recombinant erythropoietin from baby hamster kidney cells; uEPO, human urinary erythropoietin.

MOLAR RATIO (mannose taken as 3)	rEPO BRP Stnd*	uEPO[10]	rEPO-T[5]	uEPO[11]	rEPO BHK[11]
Fucose (Fuc)	1.0	0.9	1.0	1.0	1.0
Mannose (Man)	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**
Galactose (Gal)	4.7	4.2	4.3	4.9	4.7
***N*-acetyl galactosamine (GalNAc)**	0.2	0.3	traces	8.2	8.6
***N*-acetyl glucosamine (GlcNAc)**	6.1	5..3	6.0		
***N*-acetyl neuraminic acid (NeuAc)**	4.9	3.4	4.1	3.9	5.6

* Monossacharide analysis performed as a part of this study.

Sialic Acid analysis - Analysis of the sialic acid speciation of the different erythropoietin samples resulted in chromatograms depicted in figure 2A-C. The profile corresponding to the uEPO displayed , in addition to excess reagent peaks, a single sialic acid species that when compared to the reference compound (not shown) corresponded to Neu5Ac. The recombinant glycoproteins showed the presence of this sialic acid residue as the most abundant, yet not only, species.

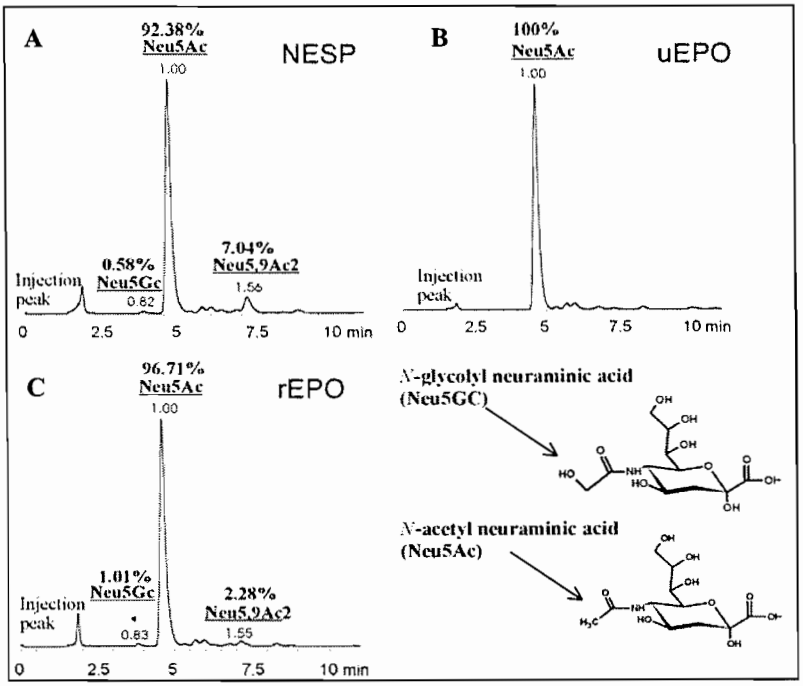
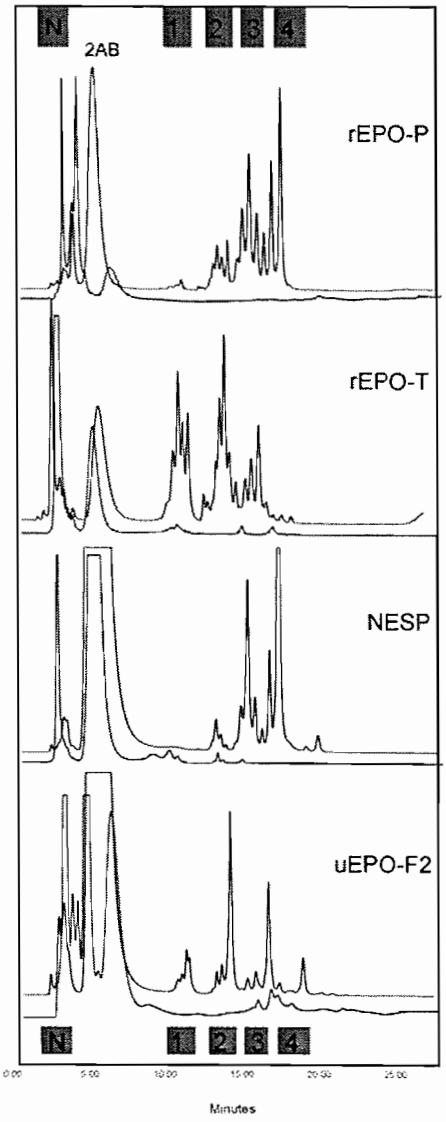


Figure 2: Reversed phase HPLC analysis of fluorescently labelled (DMB) sialic acid residues from: A, NESP; B, uEPO; C: rEPO.

In addition, mono-O-acetylated Neu5,9Ac$_2$ was observed for NESP (~ 7%) and rEPO (~ 2%) and importantly, *N*-glycolyl neuraminic acid was found to be present in both (0.6 and 1.0%, respectively). This residue cannot be present on human glycoproteins as humans lack CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) that converts Neu5Ac into Neu5Gc. Therefore, this observation provide a handle for the unequivocal discrimination between endogenous and recombinant EPO.

Weak Anion Exchange (WAX) HPLC profiles (figure 3)- For the urinary preparation, four (flow-through, F1, F2, and F3) fractions were profiled and peaks of significance were only observed in the flow-through (FT) and F2. From a comparison between the profiles from FT (not shown) and elution (F2, figure 3) derived from immuno-purification of uEPO it could be observed that neutral oligosaccharides are almost absent in F2. In contrast, they were very abundant in FT. This can be explained because FT fraction contains, in addition to non-retained EPO other glycoproteins. The charge profiles for both rEPOs from the European Pharmacopoeia (rEPO-P) and Teknika (rEPO-T), respectively clearly demonstrated the post-expression purification of rEPO-P towards higher sialylated oligosaccharides with the complete absence of neutral oligosaccharides in the former and their presence in the latter. It should be noted that the chromatogram observed for rEPO (rEPO-P) shows a similar profile as previously reported for epoetin beta by Skibeli et al[12]. This similarity is corroborated in the normal phase HPLC profiles (*vide infra*).



In figure 3, the profiles from rEPO-P and NESP correspond in terms of structures, although significant differences are observed for the absolute amounts of particular structures. Also for NESP a complete absence of neutral structures is visualised. Significant differences can be appreciated between the charge profiles of NESP, rEPO-P and uEPO-F2 indicating absolute differences in glycosylation. Where the profiles of the recombinant molecules display increasing intensities for structures containing from 1 to 4 charges, respectively, and significant heterogeneity within uniformly charged structures, the charge distribution for uEPO-F2 appears to be more homogeneous with the most intense peak corresponding to di-charged structures. Nevertheless, the retention times of the latest eluting structures (higher charged) structures in uEPO-F2 appear to be longer than those corresponding to the latest eluting structures from the recombinant variants.

Figure 3: WAX HPLC profiles of different erythropoietins before (upper trace) and after sialidase digestions (lower trace). rEPO-P: from European pharmacopoeia (NIBSC); rEPO-T from Teknika; NESP from Amgen.; uEPO-F2: elution fraction from the IAC purification of endogenous uEPO. Explanation: N, neutral structures; 1, mono-charged; 2, di-charged; 3, tri-charged;

From the WAX profiles after de-sialylation it becomes evident that the charges present in the N-linked carbohydrate chains from both rEPO and NESP correspond solely to sialic acids as the profiles show no peaks of significance in the region of charged structures. However, the uEPO-F2 profile after the same treatment still shows charged structures. As all digestions were performed on similar amounts of material and the enzyme used has a broad specificity, it must be concluded that the endogenous uEPO contains additional charges that reside in part, or in whole, in the glycosylation and are different from sialic acids. As such we hypothesised that these could be sulfate substituents at galactose or *N*-acetyl glucosamine residues.[13]

Normal Phase HPLC profiles - The same N-linked samples were analysed by normal phase HPLC (figure 4A-D). The overall glycosylation profiles (1st profile of each panel) represent the structural heterogeneity contained in the N-glycosylation.

From a comparison between the different EPO's it became evident that both rEPO-P and NESP contain relatively large structures, possibly indicating the presence of *N*-acetyl lactosamine repeating units. In contrast the uEPO-F2 profile showed peaks with shorter retention times indicating less structural heterogeneity.

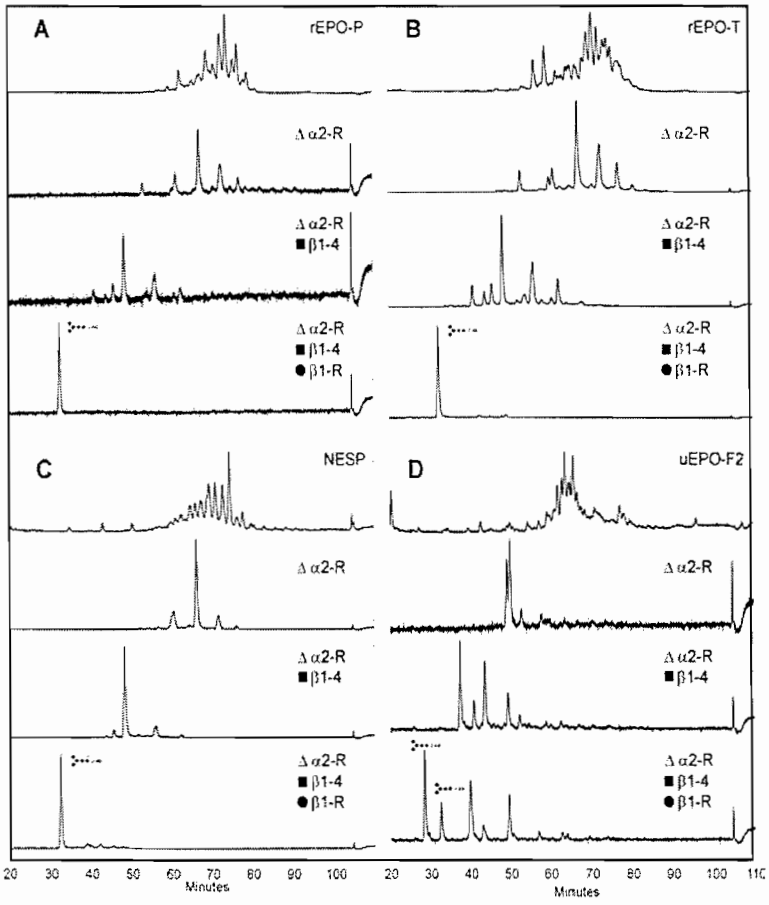


Figure 4: Normal phase HPLC profiles of different erythropoietins before (top panels) and after RAAM. **A**: rEPO-P from European pharmacopoeia (NIBSC); **B**: rEPO-T from Teknika; **C**: NESP from Amgen.; **D**: uEPO-F2 elution fraction from the IAC purification of endogenous uEPO. Enzymatic digestions: Δα2-R, sialidase; ■β1-4, galactosidase; ●β1-R, N-acetyl glucosaminidase.

In order to complete the structural analysis of the N-glycans a reagent array analysis method (RAAM) was carried out which consisted in successive and sequential exoglycosidase digestions. For standard complex-type N-glycans RAAM should result in the single penta- or hexasaccharide depicted in figure 5. Indeed the profiles corresponding to the recombinant molecules yielded a single peak corresponding to a single structure as demonstrated by mass spectrometry (*vide infra*). As such, the differences in glycosylation profiles observed for the recombinant molecules can be tracked down to the level of branching (i.e. bi-, tri -, and tetra-antennary complex type N-glycans) and presence of a different number of *N*-acetyl lactosamine repeating units. Strikingly, the comparative profiles of uEPO with respect to any of the recombinant analogues were completely different after each single exoglycosidase digestion. For this erythropoietin the triple-enzyme combination does not lead to a single structure but results in five major and even so many minor compounds. One of these co-elutes with the resulting structure in the recombinant analogues for which structural identity was assumed at this point. A single structure elutes at shorter time and assigned the same structure

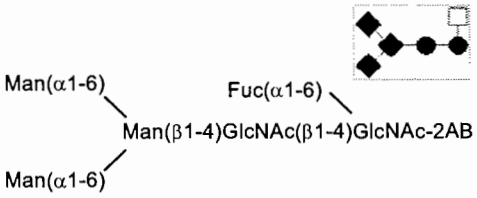
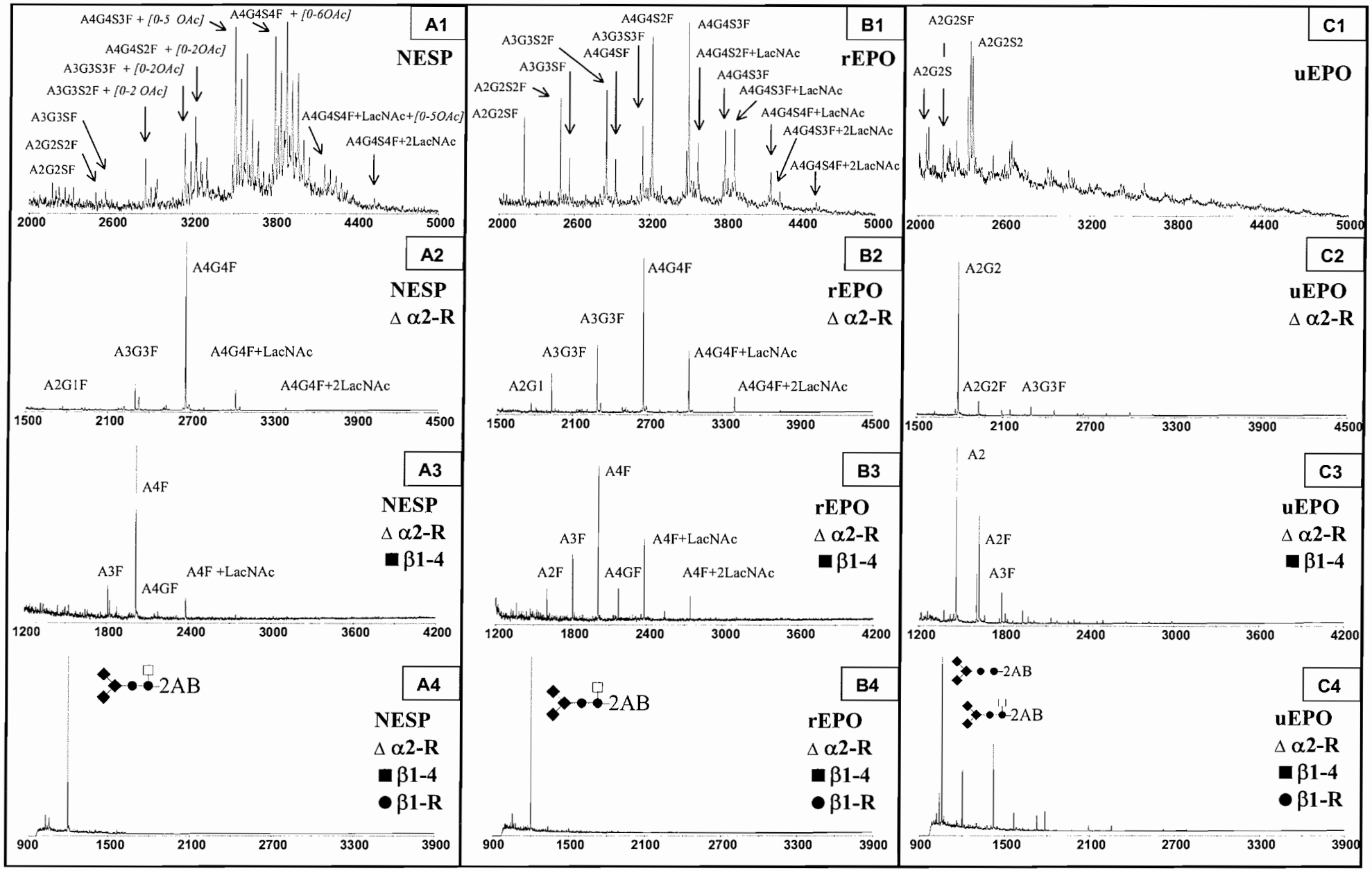


Figure 5: N-glycan core structure containing 3 Man (◆) 2 GlcNAc (●) and 1 Fuc (□). The latter is partially absent in uEPO.

devoid of the core-fucose. These observations were corroborated by mass spectrometric analyses.

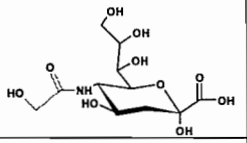
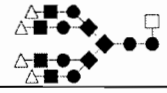
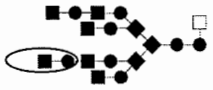
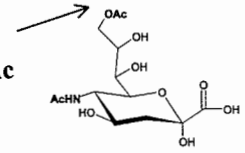
Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS)
The mass spectrum of the entire 2AB-labelled glycans pool obtained from rEPO showed around 20 different structures in the mass range between 2000 and 4500 Da (Figure 6-B1). Predominantly, tetra-antennary type N-glycans with a high degree of sialylation, complete core fucosylation and one or two *N*-acetyl lactosamine repeating units could be assigned. This observation is in complete agreement with the selective purification of this pharmaceutical. The equivalent mass spectrum of NESP (Figure 6-A1) presented similar structural heterogeneity, albeit a distinctive mass profile due to peak-clusters spaced by 42 Da. These clusters could be identified as O-acetylated *[OAc]* variants of a single structure. In case of IAC purified-uEPO the overall spectrum mass (Figure 6-C1) proved more difficult to interpret. Even though peaks were encountered throughout the entire mass range the most abundant signals corresponded to fairly simple biantennary type N-glycans, both with and without core fucosylation. Mass spectrometric analyses after RAAM at different stages (Figures 6-A-C panels 2-4) were performed and corroborated the earlier findings from HPLC. The structural heterogeneity of rEPO and NESP are solely due to different sialic acids present in both species as post-sialidiase mass spectra resulted nearly identical, both in mass values and peak intensities. As expected after the triple-enzyme treatment, the N-glycans of NESP and rEPO are reduced to a single structure corresponding in mass to the core-fucosylated (α1-6 linked to the first *N*-acetyl-glucosamine) hexasaccharide. However, this structure only accounts for approximately 30% in uEPO, the remaining 70% being non-fucosylated. This difference in core-fucosylation could be another potent tool in discriminating between urinary EPO and recombinant preparations.

Legend to figure 6 (next page): Mass spectra of different erythropoietins. **A**: NESP from Amgen.; **B**: rEPO-P from European pharmacopoeia (NIBSC); **C**: uEPO-F2 elution fraction from the IAC purification of endogenous uEPO. **A1-C1:** Negative ion MALDI mass spectra of the entire 2AB-labelled glycan pools. **A2-C4:** Positive ion MALDI mass spectra after different enzymatic digestions: $\Delta\alpha$2-R: sialidase; ■ β1-4: galactosidase; ● β1-R: N-acetyl glucosaminidase. Structure compositions are indicated with the following symbols:
e.g **A4G4S2F+LacNac+[0-5*OAc*]**
A: n° of antenna (4)
G: galactose (4)
S: sialic acid (2)
F: fucose (1)
LacNAc: *N*-acetyl-lactosamine repeats (1)
OAc: O-acetylation in sialic acid (from 0 to 5)


A1
NESP
A4G4S3F + [0-5 OAc]
A4G4S4F + [0-6OAc]
A4G4S2F + [0-2OAc]
A3G3S3F + [0-2OAc]
A3G3S2F + [0-2 OAc]
A3G3SF
A2G2S2F
A2G2SF
A4G4S4F+LacNAc+[0-5OAc]
A4G4S4F+2LacNAc
2000 2600 3200 3800 4400 5000
A2
NESP
Δ α2-R
A4G4F
A3G3F
A4G4F+LacNAc
A2G1F
A4G4F+2LacNAc
1500 2100 2700 3300 3900 4500
A3
NESP
Δ α2-R
■ β1-4
A4F
A3F
A4GF
A4F +LacNAc
1200 1800 2400 3000 3600 4200
A4
NESP
Δ α2-R
■ β1-4
● β1-R
2AB
900 1500 2100 2700 3300 3900
B1
rEPO
A4G4S2F
A4G4S3F
A3G3S2F
A3G3S3F
A4G4SF
A3G3SF
A2G2S2F
A2G2SF
A4G4S2F+LacNAc
A4G4S3F
A4G4S3F+LacNAc
A4G4S4F+LacNAc
A4G4S3F+2LacNAc
A4G4S4F+2LacNAc
2000 2600 3200 3800 4400 5000
B2
rEPO
Δ α2-R
A4G4F
A3G3F
A3G3F
A4G4F+LacNAc
A2G1
A4G4F+2LacNAc
1500 2100 2700 3300 3900 4500
B3
rEPO
Δ α2-R
■ β1-4
A4F
A3F
A4F+LacNAc
A2F
A4GF
A4F+2LacNAc
1200 1800 2400 3000 3600 4200
B4
rEPO
Δ α2-R
■ β1-4
● β1-R
2AB
900 1500 2100 2700 3300 3900
C1
uEPO
A2G2SF
A2G2S2
A2G2S
2000 2600 3200 3800 4400 5000
C2
uEPO
Δ α2-R
A2G2
A2G2F
A3G3F
1500 2100 2700 3300 3900 4500
C3
uEPO
Δ α2-R
■ β1-4
A2
A2F
A3F
1200 1800 2400 3000 3600 4200
C4
uEPO
Δ α2-R
■ β1-4
● β1-R
2AB
2AB
900 1500 2100 2700 3300 3900

CONCLUSIONS

A meticulous structural analysis of the N-linked carbohydrate chains of erythropoietin molecules from different origins has been carried out. In the case of endogenous EPO immuno affinity purification preceded structural analysis whereas this was not necessary for the recombinant variant. The most promising features are summarised in table II. At the monosaccharide level structural differences include the presence of Neu5Gc in a 1 mol % was in all recombinant EPO preparations. This sialic acid species cannot be produced by human, so its presence on erythropoietin provides an absolute evidence of the exogenous origin. Furthermore, different ratios between fucose and mannose were found. This was corroborated by both HPLC profiling of the N-glycans and mass spectrometry after full RAAM, with the observation of complete fucosylation of the core in rEPO and NESP, present only in ~ 30 % in uEPO. From WAX analyses, both before and after sialidase digestion, the presence of charged residues, other than sialic acids in uEPO became evident. These residues are apparently absent in recombinant species. This feature could be used for the development of a confirmatory method in EPO doping control. Even though it remains to be proven structurally, we hypothesise that these additional charges are due to sulfate groups present on galactose or *N*-acetyl glucosamine. Noteworthy is the presence of more complex and branched structures with presence of LacNAc repeats in recombinant EPOs as well as the existence of multiple O-acetylation of the sialic acid residues in NESP. As far as we know, this phenomenon has not been described for the pharmaceutical preparations and experiments are being conducted to clarify this point. At the moment, further studies are being performed to elucidate the structure of the different glycoforms of rEPO, uEPO and NESP, contained in uniformly charged isoforms after isoelectric focusing in order to enable an unambiguous discrimination between endogenous and exogenous EPO preparations.

TABLE II	NESP	rEPO	uEPO
Neu5Gc 	**~0.6%**	**~1.0%**	**0%**
Charges 	SIALIC ACIDS	SIALIC ACIDS	Sialic Acids **Additional Charges (SO_4?)**
Non-fucosylated Core 	**~0%**	**~0%**	**~70%**
LacNAc repeats 	1 and 2 repeats	1 and 2 repeats	None
Most abundant structures	Tetra-antennary	Tetra-antennary	Bi-antennary
NeuOAc 	**1 to 6***	-	-

* this number of O-acetyl groups is present in one N-glycan containing up to four sialic acid residues.

Acknowledgments

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