

Physico-Chemical Characterization and Biological Evaluation of Recombinant Human Erythropoietin in Pharmaceutical Products

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SUMMARY. Recombinant human erythropoietin (rhEPO) from different manufacturers was analysed by non-reducing polyacrylamide gel electrophoresis and by immunodetection with a specific antiEPO anti-serum, revealing a single broad diffuse band in the molecular weight range 30 - 40 kDa. The isoform compositions revealed by isoelectric focusing (IEF) after immunoblotting and lectin blotting showed extensive heterogeneity, with 5 - 8 isoforms evident over the isoelectric point (pI) range 4.4 - 5.2 and with the proportions and the position of the bands differing significantly between preparations. The biological activity of the pharmaceutical preparations of rhEPO was assessed by an *in vivo* bioassay using normocytthaemic mice. The IEF and lectin blotting profiles generally correlated with the bioassay results, indicating that the products containing the greater proportion of acidic isoforms had the greater biopotencies. The degree of aggregation of preparations formulated without human serum albumin shown to vary between 1.4 to 13.6% of the total EPO content.

RESUMEN. "Caracterización Fisicoquímica e Evaluación Biológica de Eritropoyetina Humana Recombinante en Productos Farmacéuticos". El objetivo de este trabajo es efectuar la identificación de eritropoyetina (EPO) humana recombinante en productos farmacéuticos comerciales por electroforesis en gel de poliacrilamida y su detección con anticuerpos específicos, demostrándose típica banda larga con peso molecular entre 30 - 40 kDa. La composición de las isoformas detectadas por isoelectrofoque (IEF) después de imundetección y de ligación a las lectinas presentaron heterogeneidad con 5 - 8 isoformas distribuidas en la banda de punto isoelectrónico (pI) de 4,4 - 5,2 y bandas con proporciones y posiciones significativamente diferentes entre las preparaciones. La actividad biológica fue evaluada por ensayos con ratones normocítémicos. Haciéndose una correlación entre los resultados de los bioensayos con los perfiles de IEF y su ligación a las lectinas, puede observarse que los productos con isoformas ácidas presentan mayor actividad biológica. Se evaluarán los agregados de las preparaciones sin seroalbúmina humana por cromatografía líquida a alta presión obteniendo valores entre 1,4 y 13,6 % del contenido total de EPO.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone produced primarily by the kidney and is the main factor regulating red blood cell production. The mature protein consist of a 165 amino acid polypeptide chain heavily glycosylated at three N-linked and one O-linked glycosylation sites yielding a total molecular mass of 30-34 kDa. About 40% of the fully glycosylated EPO molecule consists of carbohydrate. The N-

linked carbohydrate chains exhibit a wide variety of forms, ranging from mono- to multibranch structures with varying degrees of terminal sialylation¹.

The human gene of EPO has been cloned and expressed in mammalian cells and pharmaceutical preparations of recombinant human EPO (rhEPO) have been available for several years². Epoetin alpha (EPO- α) and beta (EPO- β) are the two forms of Chinese hamster ovary

KEYWORDS: Bioactivity, Isoelectric focusing, Isoforms, Lectins, Recombinant erythropoietin.

PALABRAS CLAVE: Bioactividad, Eritropoyetina recombinante, Isoelectrofoque, Isoformas, Lectinas.

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(CHO) cell-derived recombinant DNA-derived erythropoietin (rhEPO) that are used clinically, particularly in the treatment of anaemia in renal failure or renal insufficiency where it is used to restore the haematocrit and reduce transfusion dependency, and in the treatment of anaemias associated with severe infection such as HIV. There have also been reports of illicit use of the drug by athletes involved in endurance sports in order to increase the oxygen-carrying capacity of blood³.

As with several other glycoprotein hormones, EPO exists as a mixture of isoforms⁴. The extent of the microheterogeneity of the EPO molecule is mostly dependent on the charged carbohydrate moieties of the protein and it has been shown that both rhEPO and urinary EPO can be separated into at least 5–8 major components by isoelectric focusing⁵. The electrophoretic mobility and the isoelectric point (pI) of the various components of the protein mixture can be related to the extent of glycosylation, the number of sialic acid residues (with varying amounts of acetylation) and the presence or absence of N-acetyl lactosamine extensions within each glycan⁶. The dependence of the *in vivo* activity of EPO upon the number of terminal sialic acid residues on the glycan chains has been recognized and serves as an example of the relationship existing between pI and *in vivo* bioactivity which has been reported for some glycoproteins and emphasised for rhEPO^{7,8}.

Glycosylation is essentially a post-translational process and it is well documented that the isoforms (glycosylation variants) of recombinant DNA-derived material may differ according to the cell line and culture conditions used⁹. Isoelectric focusing (IEF) has been in many cases the technique of choice to analyse proteins. Its high-resolution power allows separation of complex mixtures and provides an estimation of the pI of the components. The isoelectric patterns of the two recombinant EPO- α and - β forms are very similar (both have a pI in the range 4.4–5.1) although EPO- β has a greater proportion of more basic isoforms^{10,11}. Although differences in isoform composition have usually been investigated using such charge-based separation methods, the carbohydrate-binding properties of lectins, specific for different areas of the glycan chains, can also be used to discriminate between them^{12,13}.

Many manufacturers now produce therapeutic preparations of rhEPO. Isoform heterogeneity due to variations in the production process may

be expressed as differences in bioactivity and may introduce different pharmacokinetic and immunogenic characteristics¹⁴. It is therefore essential to have well-developed analytical methods to apply in the quality control of these materials. Consequently, the aim of this work was to evaluate the physico-chemical characteristics of rhEPO preparations by SDS-PAGE and IEF, using immunoblotting and lectin-blotting to identify the position of the isoforms, and to relate the findings to the biological activity of the preparations as assessed in the normocytahemic-mouse bioassay.

MATERIALS AND METHODS

Materials

The European Pharmacopoeia Biological Reference Preparation (Ph. Eur. BRP) for Erythropoietin (250 µg/32 500 IU/vial) was obtained from the European Department for the Quality of Medicines (EDQM), Strasbourg, France. Twelve batches of commercial preparations of recombinant DNA-derived erythropoietin, expressed in CHO cells, formulated at 1 000 IU/ampoule, 3 000 IU/ampoule and 4 000 IU/ampoule, were obtained from six manufacturers. Samples 1, 2, 3 and 6 were from manufacturer A; 4, 5, 8 and 9 from manufacturer B; 7, 10, 11, 12, from manufacturers C, D, E and F respectively. Samples A1, 2, 3, 6 and C7 were formulated without HSA and were used in SE-HPLC studies to determine the extent of aggregation. All preparations were within their shelf-life period. Goat anti-EPO polyclonal antiserum, G151/2, for immunoblotting was donated by the National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK. Anti-goat IgG (whole molecule-alkaline phosphatase conjugate) was obtained from Sigma, USA. The biotin-labelled lectin *Maackia amurensis* lectin II was from Vector, USA, and *Lycopersicon esculentum* and *Canavalia ensiformis* (Concanavalin A, Con A) were from Sigma, USA. All other reagents were of the highest purity available from commercial sources.

Methods

Polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was carried out on a vertical slab-gel electrophoresis apparatus using 1.5 mm thick polyacrylamide slab-gel (17x13cm). SDS and bromophenol blue were added to the samples, with final concentrations generally of 1% (w/v) and 0.1% (w/v) mg/ml, respectively. Samples 1 to 11 (0.33 µg) and 12

(0.20 µg) were left to migrate for about 10 h under 340 V, 20 mA and 50 W at 4 °C. Proteins were stained with silver nitrate.

Western blotting

After SDS-PAGE, the separated components were transferred to a nitrocellulose membrane (Bio-Rad) by electrophoresis at a constant voltage of 24 V in 192 mmol/l glycine + 25 mmol/l Tris, containing methanol (1:4), for 1 h using a Trans-blot apparatus (Bio-Rad). The membrane was washed for 1.5 h at room temperature in 50mmol/l sodium phosphate + 150 mmol/l sodium chloride pH 7, containing 5% (w/v) dried skimmed milk powder and then reacted for 14 h at room temperature with goat anti-rhEPO serum (1:1000), in the milk-containing phosphate-buffered saline as above. Antibody-binding to the membrane was detected by reaction with an anti-goat IgG whole molecule-alkaline phosphatase conjugate 1:5000, using a phosphatase substrate solution, containing (1:2:18) 5 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DMF, 1 mg/ml of nitro-blue tetrazolium (NAT) in water, and alkaline phosphatase buffer (0.1M Tris + 0.1M NaCl + 5 mmol MgCl₂, pH 9.5), to develop the colour.

Isoelectric focusing (IEF)

IEF in the pH range 2.5-6.5 was carried out following the method of Storring et al, 1998¹⁰. Briefly, 1.5 mm thick slab gels (17x13 cm) were prepared by polymerization from a solution which contained 7.33% (w/v) acrylamide, 0.23% (w/v) N,N-methylene-bisacrylamide, 2.5% (v/v) ampholyte (Amersham Pharmacia Biotech, Uppsala, Sweden.), 22.5% (w/v) sucrose (Life Technologies, USA), 0.0002% (w/v) riboflavin, 0.026% (v/v) N,N,N¹,N¹-tetramethyl-ethylenediamine. Samples (0.5 to 1.7 µg) containing 20% (w/v) sucrose were applied to the top of the vertical gel, which was then subjected to a constant voltage of 340 V for 18 h at 4 °C in a Vertical Gel Electrophoresis System (Life Technologies), using an upper cathodal solution of 20 mmol/l sodium hydroxide and a lower anodal solution of 10 mmol/l phosphoric acid. After IEF, the EPO components were blotted onto a nitrocellulose membrane and visualized by immunodetection using the same procedures as described in the Western blotting technique.

Lectin Blotting

EPO samples (0.5 to 1.7µg) were run on an IEF gel and transferred to a nitrocellulose mem-

brane, using the same conditions as described for Western blotting. The membrane was boiled for 5 min in TBS buffer (50 mM Tris + 0.15 M NaCl, pH 7.5) and then incubated for 8 h in blocking buffer containing 3% HSA and 0.3% Tween 20 in TBS buffer. For each membrane the appropriate biotinylated lectin was diluted in blocking buffer (1:100) and incubated with the membrane for 6 h on a shaker at room temperature. The membrane was washed 3 times with TBS and incubated for 1 h with peroxidase-conjugated Avidin (Dako, Denmark) in the same buffer (1:5000). After washing 3 times in TBS buffer, the EPO components were visualized by incubating the membrane in the colorimetric substrate, containing 10 mg of chloronaphthol (Sigma, USA), 5.5 ml of methanol, 17 µl 30% H₂O₂ and 27.9 ml of TBS buffer.

Normocytthaemic-mouse bioassay

The development and optimization of the parameters for this assay has been previously described^{15,16}. Briefly, 8-week old mice were fully randomized and distributed into sample and standard treatment groups, using 6 to 8 animals per group for each assay. The samples were diluted to the appropriate concentration with phosphate-buffered saline (pH 7.2) containing 0.1% bovine serum albumin. A single dose of 10, 30 and 90 IU/0.5 ml/mouse was injected subcutaneously into the respective animal on day 1. On day 5, a blood sample was taken from the orbital venous sinus of each mouse, using a glass capillary tube with 5% EDTA as anticoagulant. All injections, blood sampling and the reticulocyte counting were carried out between 8.00 a.m. and 11.00 a.m. Blood samples of 130 µl were aspirated into an automated flow cytometer counter (ABX Diagnostics, France) and a maximum of 32 000 red cells was analysed. Customized gating for each sample allowed separation of the mature red cells, reticulocytes, white blood cells, and, on the lower threshold, platelets. Results reported as a percentage of the reticulocyte number, were submitted to statistical analyses by parallel line methods using SAS 6.1 for Windows (SAS Institute Inc., USA).

Dimer and higher molecular mass components

In order to assess the purity of monomeric EPO, 4 µg rhEPO were applied to a TSK G 2000 SW column (60 x 0.75 cm). The column was run isocratically with a mobile phase of 1.5 mM Na₂HPO₄, 8.1 mM KH₂PO₄ in 0.4 M NaCl buffer pH 7.4 at a flow-rate of 0.5 ml/min and a detec-

tion wavelength of 214 nm¹⁷. Peaks at 23-25 min (peak 1: high molecular weight component), 25-27 min (peak 2: possibly dimer) and 27-32 min (peak 3: monomer), were integrated, peak areas obtained and the percentage purity of the monomer material was calculated.

RESULTS

Polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE of the twelve samples followed by transference to a nitrocellulose membrane and immunodetection with a EPO-specific anti-serum revealed the same general pattern for all samples (Fig. 1), including the Ph. Eur. Reference Preparation for EPO (Std.), that is, a single diffuse band, typical of a glycoprotein, in the molecular weight range 30-40 kDa. No dimeric or oligomeric bands were apparent, within the detection limits of the system, indicating the absence of covalent (disulfide-linked) associations.

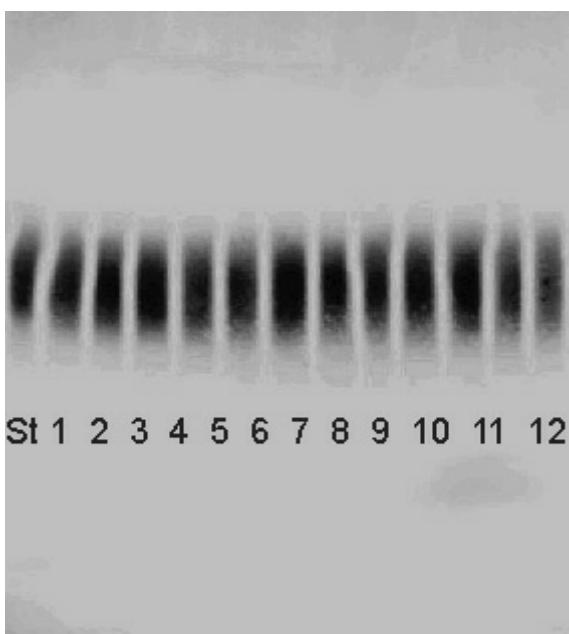


Figure 1. Single broad bands obtained with commercial rhEPO preparations after SDS-PAGE, transference to nitrocellulose membrane, immunodetection with EPO-specific antiserum and development of signal with alkaline phosphatase. Lane: Std. - Ph. Eur. BRP of rhEPO (0.33 µg), 1-12 pharmaceutical products, respectively, 1 to 11 (0.33 µg) and 12 (0.20 µg).

Isoelectric focusing IEF and lectin blotting

The isoform compositions revealed by IEF after immunoblotting (panel A) and lectin blotting (panels B-D) for the twelve samples are shown in Fig. 2 (samples 1-6) and Fig. 3 (samples 7-12). Where present in different preparations, the isoelectric points of the components

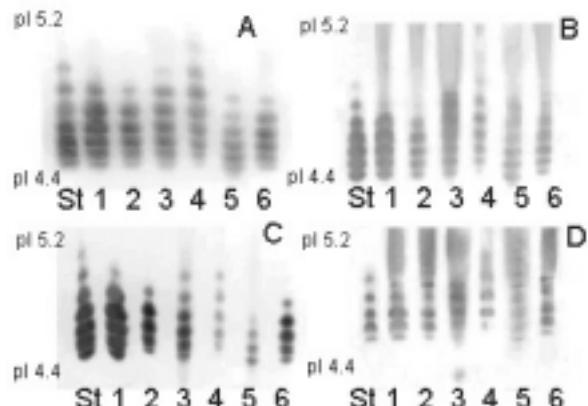


Figure 2. Isoelectric focusing of rhEPO preparations: (A) Immunodetection with EPO-specific polyclonal antiserum; and detection with labelled lectins using (B) *Maackia amurensis* lectin II; (C) *Lycopersicon esculentum* (tomato); (D) *Canavalia ensiformis* (Con A). Lane: Std. - Ph. Eur. BRP for EPO, 1.7 µg; Sample 1, 1.7 µg; Sample 2, 0.8 µg; Sample 3, 0.8 µg; Sample 4, 0.8 µg; Sample 5, 0.5 µg; and Sample 6, 0.8 µg.

were similar. However, the number of components (varying from 5 to 8), the proportion (intensity of staining) and the position of the isoforms (reflecting the basic/acidic nature of the material) differed significantly between preparations.

Probing of the IEF blots with biotinylated lectins to detect glycosylation variants (panels B-D) showed a glycoform pattern similar to the protein pattern seen with immunoprobining. Most isoforms of CHO cell-derived EPO contain terminal sialic acid α 2-3-linked to the glycan chain and this was clearly detected with the biotinylated lectin from *Maackia amurensis* (panels B). The presence of repeating N-acetylglucosamine oligomers in the N-glycan chains was readily detected with *Lycopersicon esculentum* lectin (panels C). The biantennary N-glycan content of the preparations was shown by binding to Con A (panels D) which recognises α -linked mannose residues in that particular conformation. With all four signalling systems, it was particularly evident that samples 8, 9, 10 and 12 contained the most basic isoforms and fewer acidic components. Visual inspection showed that these samples had a lower number of sialic acid-containing isoforms, staining less densely, (Fig. 3B) and a greater proportion of biantennary glycan forms relative to the more acidic samples. Figures 2 and 3 show results from single experiments but are representative of several experiments showing similar results. However, it was noted that the intensity of staining, particularly with the lectins, did show some day-to-day variation.

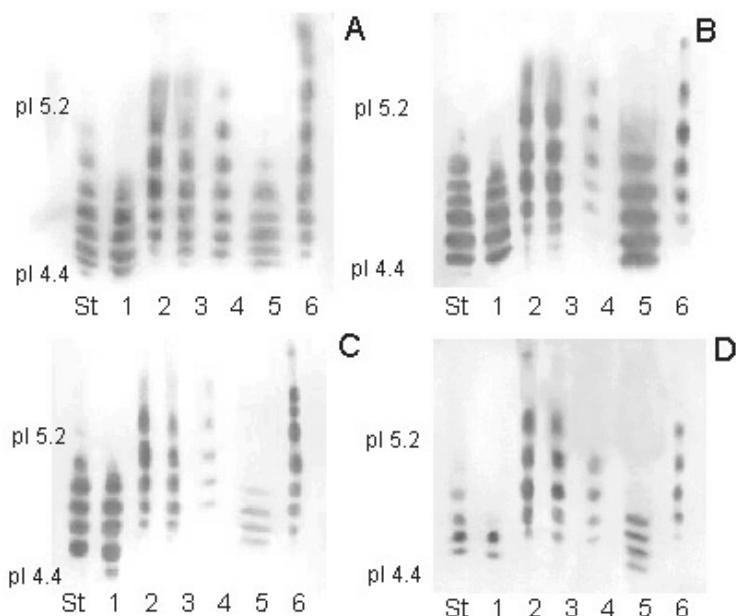


Figure 3. Isoelectric focusing of rhEPO:
(A) Immunodetection with EPO-specific polyclonal antisera; and detection with labelled lectins using
(B) *Maackia amurensis* lectin II;
(C) *Lycopersicon esculentum* (tomato);
(D) *Canavalia ensiformis* (Con A).
Lane: Std. - Ph. Eur. B.R.P for EPO, 1.7 µg; Sample 7, 1.7 µg; Sample 8, 0.8 µg; Sample 9, 0.8 µg; Sample 10, 0.8 µg; Sample 11, 0.8 µg; and Sample 12, 0.5 µg.

rhEPO potency evaluation

The biological potencies of the pharmaceutical preparations were assessed *in vivo* by the normocytthaemic-mouse bioassay (Table 1) and the percentage recovery calculated relative to the stated dose. The agreement between the two values was within the acceptable limits established by E.P., that is, between 80 - 125% for some of the samples but samples 8, 9, 10 and 12 showed very low potencies relative to the

stated potency and fell outside the required limits. There was general agreement between the lower biopotencies and the lower number of acidic bands and lower proportions (staining density) in the test for α 2-3-linked sialic acid, and between the lower biopotencies and the higher number of basic bands and higher proportions of biantennary-containing forms seen with Con A (Table 1).

Sample	Stated IU/vial	Potency		IEF					
		Found		MA			Con A		
		IU/vial	%	Fiducial limits (95%)	Number of bands	pI range	Number of bands	pI range	
1***	3 000	3 461.40	115.38	94.36-141.10	5	4.5-5.0	4	4.7-5.0	
2***	4 000	4 244.80	106.12	89.16-126.31	5	4.5-5.0	4	4.7-5.0	
3**	4 000	4 006.40	100.16	77.03-130.21	6	4.6-5.1	4	4.7-5.0	
4**	4 000	3 340.80	83.52	65.65-106.26	5	4.7-5.1	3	4.8-5.1	
5***	4 000	4 098.80	102.47	79.48-132.12	6	4.4-5.0	4	4.6-5.0	
6***	3 000	3 479.10	115.97	93.26-144.21	5	4.5-5.0	4	4.7-5.0	
7**	3 000	3 072.30	102.41	74.11-131.08	5	4.5-5.0	2	4.6-4.7	
8***	4 000	2 854.00	71.35	55.89-91.07	5	4.7-5.2	4	4.8-5.2	
9**	4 000	2 692.00	67.30	52.93-85.57	6	4.6-5.2	5	4.7-5.2	
10**	4 000	2 704.00	67.60	51.90-88.10	5	4.8-5.3	4	4.7-5.1	
11**	4 000	3 272.80	81.82	65.11-102.83	5	4.5-5.0	4	4.5-4.8	
12**	1 000	707.00	70.70	54.09-92.36	5	4.8-5.3	4	4.8-5.2	

Table 1. Combined bioassay potency estimates and 95% fiducial limits of 12 rhEPO pharmaceutical products, and the corresponding number of isoform bands and pI ranges seen after IEF, transference and probing with biotinylated *M. amurensis* (MA) and *C. ensiformis* (Con A). * = number of independent assays.

Detection of higher molecular weight components

The presence and content of dimeric and higher molecular weight components in EPO preparations formulated without human serum albumin (HSA) were assessed by high-performance size-exclusion chromatography (SE-HPLC) (Fig 4). In the five batches examined, areas under the peaks, expressed as a percentage of the total area, showed varying amounts of aggregate material, ranging from 1.4-13.6% (Table 2) although the oligomers have a greater tendency to bind to the column matrix than does the monomer. The presence of relatively large amounts of HSA often found in EPO therapeutic preparations usually precludes analysis by SE-HPLC.

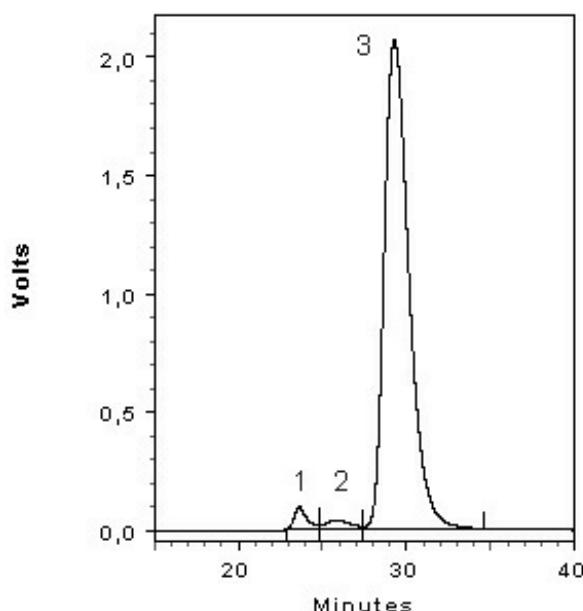


Figure 4. Typical chromatogram from isocratic high-performance size-exclusion chromatography using TSK G 2000 SW column (60 x 0.75 cm), with an injection load of 4 µg of the EPO product A3 (without HSA). Peak 1 - rhEPO, higher molecular mass; peak 2 - rhEPO, possibly dimer; peak 3 - rhEPO monomer.

DISCUSSION

After probing the membranes with an EPO-specific antiserum, the electrophoretic profiles revealed the presence of a single broad band of immunoreactive material (typical of a glycoprotein run under non-reducing conditions), in the molecular weight range 30 - 40 kDa, similar to that seen with the Eur. Ph. BRP of rhEPO in this study and in the collaborative study that established the Eur. Ph. BRP of rhEPO^{1,15}. The ab-

Batch	% Total area	
	Aggregates*	Monomer*
A1	1.40	98.60
A2	5.50	94.50
A3	3.69	96.31
A6	13.60	86.40
C7	8.69	91.31

Table 2. Monomeric and aggregate (dimeric and higher molecular mass material) content of batches of rhEPO pharmaceutical preparations formulated without human serum albumin from manufacturers A and C, evaluated by high-performance size-exclusion chromatography. * n=3

sence of any detectable higher molecular weight components probably excludes the presence of significant amounts of covalently linked aggregates.

The high-resolution capability of IEF is an essential tool in the physico-chemical characterization of glycoprotein products. It is a reproducible, semi-quantitative technique that produces similar results to capillary electrophoresis and with a more robust performance than other physico-chemical analytical methods^{8,20,21}. This is particularly true when IEF is used in conjunction with immunodetection, which allows specific probing of the separated species from complex mixtures. In the present study, IEF/immunoblotting showed the presence of 5-8 isoforms with a range of basic and acidic values in preparations of rEPO obtained from different manufacturers. The results are similar to those described by others who found 5-7 isoforms for EPO- α and EPO- β with pI ranges from 4.4 - 5.1^{6,10,11,15}. The IEF profile has been used to predict the bioactivity of preparations of recombinant glycoprotein hormones²². Analysis of isoforms of rFSH showed a relationship between the pI and the *in vivo* bioactivity, with isohormones of pI 3.5 having 100-200-fold greater potency than isohormones with a pI of 5.5-6.0. Although bioassay of specific isoforms was not carried out in the present study with EPO, it is evident (Table 1) that a general relationship exists between pI and bioactivity, since the products containing the greater proportion of acidic isoforms have the greater biopotencies.

The type and extent of glycosylation of the EPO molecule are very important for its biological activity, particularly *in vivo*, since this ap-

ars to be directly related to the degree of terminal sialylation of the glycan chains^{7,17,23,24}. Although aglycosylated rEPO (*E. coli*-derived) or deglycosylated native EPO exhibit full activity *in vitro*, glycosylation is necessary for full expression of activity *in vivo*²⁵. The presence of terminal sialic acid on the glycan chains is required to prevent rapid clearance from the circulation and to maintain the pharmacokinetic properties of the EPO molecule. However, not only the extent of sialylation seems to be important but also the branching pattern of the glycan chains. Predominantly tetra-antennary branched EPO shows full bioactivity *in vivo*, but predominantly bi-antennary branched EPO shows only 15% of normal *in vivo* activity²⁶. Using IEF and probing the separated components with lectins specific for different areas or different conformations of the glycan chains has provided new information on the quality of the recombinant products. The results obtained with *M. amurensis* lectin, which recognises terminal α2,3-linked sialic acid, showed the variations in numbers and proportions of sialic residues among the isoforms within each preparation and the often large differences between preparations. A similar pattern was obtained with *L. esculentum* lectin, which revealed the presence of repeating N-acetylglucosamine oligomers in the N-glycan chains. Con A, which shows a preference for binding to biantennary glycans, gave a good discrimination between the predominantly basic isoform-containing preparations and the predominantly acidic isoform-containing preparations which correlated with the lower *in vivo* bioactivity of the former. This is probably a function of the lower number of sialic acid residues in the biantennary oligosaccharides.

SE-HPLC was used to determine the presence and content of dimeric and higher molecular weight aggregates of EPO in those preparations that were formulated without human serum albumin. In the five preparations examined, amounts of aggregated material varied between 1.4-13.6%. It is to be noted that the European Pharmacopoeia suggests a limit of 2% of higher mass related substances relative to the main peak to comply with the test¹⁷. The presence of

significant amounts of aggregated material may have clinical implications since it is has been shown that dimeric and oligomeric material have increased biological activity because of their significantly longer half-lives in the circulation and may even cause an immunogenic response²⁷.

Preparations of EPO for therapeutic use are produced in many commercial biotechnology laboratories around the world. The significant influence of factors such as the cell culture conditions, the clone used, post-translational glycosylation and the purification process on product quality is demonstrated by the large degree of heterogeneity seen in the materials examined. It is these differences in the isoform composition of the various EPO preparations that can give rise to differences in their biological activities and immunoreactivities^{5,12}. It may also be the case that differences arising from variations in any one of these factors could be responsible for the development of anti-EPO antibodies and the resulting pure red cell aplasia reported recently in patients treated with recombinant EPO²⁸.

This is the first paper to characterize the biological and physicochemical properties of batches of rEPO from several manufacturers and provides significant evidence to show the large differences that can exist when comparing preparations of the final product. It is insufficient to rely on a single parameter such as biopotency because protein integrity and consistency of glycosylation are also important determinants of quality and therapeutic efficacy, particularly when dealing with a complex glycoprotein such as EPO. The IEF profiles provide new data on heterogeneity among widely sourced EPO preparations and, in conjunction with the lectin-probe procedure, offer a novel method to show the general relationship between charge distribution and specific sugar structure and biopotency which may provide an alternative to *in vivo* bioassay in the future.

Acknowledgements. The authors wish to thank Dr. Brian Rafferty for reviewing the manuscript and providing many insightful comments regarding this work; and CNPq and CAPES for financial support.

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