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Chromatographic separation of recombinant human erythropoietin isoforms

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Abstract

Erythropoietin (EPO) is the main regulator of erythropoiesis. The human glycoprotein hormone is heterogeneous when analyzed by isoelectric focusing (IEF). We investigated the possibility of fractionating EPO isoforms using different chromatographic methods. A recombinant human EPO (rhEPO) was obtained from the culture supernatants of a human B-lymphoblastoid cell line transfected by the human EPO gene. Highly purified rhEPO preparations were obtained by immunoaffinity purification. More than fourteen isoforms were observed after IEF. Among the different methods developed for isoform fractionation, the most reproducible results were obtained by DEAE–Sephacel chromatography. Seven fractions of decreasing isoelectric point (p*I*) were obtained. The specific activity of these fractions measured by an immunoradiometric assay was not equally distributed.

Keywords: Erythropoietin

1. Introduction

Erythropoietin (EPO) is the main regulator of human erythropoiesis [1–3]. The protein exerts its activity on erythroid progenitor cells by binding to specific cell surface receptors. Clinical trials in anemic patients with end-stage renal disease have revealed that recombinant human EPO (rhEPO) is a very effective agent capable of relieving this anemia [4]. Human EPO consists of a 165 amino acid polypeptide chain heavily glycosylated (one O-linked and three N-linked carbohydrate chains corresponding to 40% of the molecular mass) [5,6]. The gene of hEPO has been cloned and expressed in several eukaryotic cell lines [7,8]. We were able to introduce the EPO gene in a B-lymphoblastoid cell line of human origin [9], then to select a clone for its high expression level and growth capacity and finally to purify this protein from culture supernatants by immunoaffinity [10,11].

The heterogeneity of highly purified urinary human EPO or of rhEPO produced in a Chinese hamster ovary (CHO) cell line was observed with the help of isoelectric focusing (IEF) [12,13]. The same phenomenon was also observed in unprocessed samples of serum with the help of zone electrophoresis [14] and by IEF [15]. Similar results were described following capillary electrophoresis (CE) of purified EPO [16,17].

Like several glycoprotein hormones, EPO exists as a mixture of isoforms [18]. In fact, its microheterogeneity is related to the charged carbohydrate moiety

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of the protein [19]. The carbohydrate structure of EPO produced in CHO cells was studied in several papers (in particular [6,20,21]). Results from these studies demonstrated that this microheterogeneity is associated with the presence or the absence of terminal N-acetyl neuraminic acid residues with varying amounts of acetylation and the presence or the absence of N-acetyl lactosamine extensions. Therefore the degree of sialylation of polysaccharidic chains strongly influences the electrophoretic mobility and the isoelectric point (p*I*) of the molecule.

The carbohydrate moiety is of great importance for the biological activity of EPO. Modification or removal of carbohydrate chains result in modifications of the ''in vivo'' and ''in vitro'' activity of the cytokine [19,22,23]. In addition, interactions between antibodies and EPO are influenced by the carbohydrate moiety of EPO [24]. It therefore seems probable that immunoassay systems differ in their selectivity for different hormone isoforms [13]. To evaluate this possibility, one must compare the specific activity of purified preparations of such isoforms.

Until recently, no method achieving the separation of appreciable quantities of the EPO isoforms has been described. In this study, using a purified preparation of rhEPO as starting material, we used preparative chromatographic methods to separate isoforms into several fractions according to their p*I*. The specific activity of each fraction was measured by an immunoradiometric assay

2. Experimental

2.1. rhEPO

rhEPO was obtained from the human B-lymphoblastoid cell line, RPMI 1788 (ATCC No. CCL156) which had been transfected with the expression vector pTS39 [9]. This vector contained the human EPO gene placed under the control of the enhancer and the major late promoter of Adenovirus 5. The selection of stable transformants was carried out in a selective medium for the dominant XGPRT marker introduced in the expression vector. Subclone 5B7 was selected for its high expression level and growth capacity. Supernatants containing 1350 IU/ml measured by an immunoradiometric assay (as described in Section 2.11) of rhEPO were obtained after 7 days of culture in fetal calf serum and Iscove's modified Dulbecco's medium (Gibco, New York, USA).

2.2. ^{125}I rhEPO

Purified rhEPO was labeled with ^{125}I as previously described [10,11]. Commercial preparations of [^{125}I]-rhEPO were purchased from Amersham (Les Ulis, France) as control protein.

2.3. Preparation and purification of anti-EPO mAbs

As described in a previous paper [10], three stable clones E14, E73 and D7 were selected for their production of high affinity antibodies against rhEPO. Immunoglobulins were purified from the ascitic fluids obtained from these clones by immunoaffinity on protein G Sepharose (Pharmacia, Saint Quentin, France) as follows: 1 ml of ascitic fluid was diluted in 4 ml 0.01 *M* phosphate buffer pH 7.0 and 5 ml water. The mixture was pumped onto a 5 ml column at a flow-rate of 30 ml/h. After extensive washing of the column with the same buffer, IgG was eluted with 0.1 *M* glycine–HCl at pH 2.7 and a flow-rate adjusted to 60 ml/h. The eluted fraction was rapidly brought to neutrality by the addition of 1 *M* Tris pH 9. Between 5 and 12 mg of monoclonal antibodies were obtained from 1 ml of ascitic fluid.

2.4. Preparation of an immunoaffinity column

Preparation was performed as previously described [11]. Briefly, 80 mg of purified IgG from E14 ascitic fluid was dialyzed overnight at 4°C against a hydrogen–carbonate buffer (0.1 *M* $NaHCO_3$ 0.5 *M* NaCl pH 8.3). A 4 g amount of CNBr-activated Sepharose 4B (Pharmacia) was extensively washed with 1 m*M* HCl (about 15 ml of swollen gel). The gel was poured into a column and equilibrated with the same buffer just before the addition of E14 mAb. The coupling of E14 mAb to the Sepharose 4B gel was performed overnight at 4°C with mild stirring. After several washings, residual active groups were neutralized for 3 h at room temperature with 1 *M* ethanolamine pH 8.8 with rotative shaking. The gel was finally washed 3 times with 30 ml carbonate

buffer and then once with 30 ml 0.1 *M* glycine–HCl pH 2.2. Glycine–HCl was replaced by PBS containing 0.2% NaN_3 and the column was kept at 4°C.

2.5. Immunopurification of rhEPO

Aliquots of 500 ml of supernatant from the lymphoblastoid cells containing on average 1350 IU/ml of rhEPO, were centrifuged for 30 min at 3000 *g* before being filtered through GF/B and GF/D Whatman glass microfiber filters (Maidstone, UK). The immunosorbent column (80 mg of mAb E14 immobilised on 15 ml of CNBr activated Sepharose 4B) was pre-equilibrated with 0.01 *M* Tris buffer 0.15 *M* NaCl pH 7.5. Each aliquot was pumped into the column overnight at 4°C with a flow-rate of approximately 30 ml/h. The immunosorbent column was successively and extensively washed with the Tris–NaCl buffer and a 0.1 *M* acetate buffer pH 4.5 at a flow-rate of 60 ml/h. Elution of rhEPO was obtained with 30 ml 0.1 *M* glycine–HCl buffer pH 2.2. The eluted material was rapidly brought to pH 7.5 by the addition of 1 *M* Tris pH 9 before being concentrated on PM10 membranes (Amicon, Danvers, Ireland). About 14% of rhEPO loaded did not bind to the antibody; it was recovered in the flow-through fraction or eluted at pH 4.5. The remaining 86% were eluted at pH 2.2. The rhEPO in this fraction had a specific activity of 156 000 IU/mg protein (Table 1). In some experiments, elution of rhEPO was obtained with a pH gradient (from 4.5 to 2.2). Four fractions were collected: (fraction 1: from pH 4.5 to pH 4, fraction 2: from pH 4 to pH 3.75, fraction 3: from pH 3.75 to pH 3.5 and fraction 4: from pH 3.5 to pH 2.5). The maximal optical density was observed in fraction 3. The fractions were brought to neutrality and concentrated by ultrafiltration.

2.6. Separation of rhEPO isoforms by DEAE–Sephacel chromatography and elution by a stepwise gradient of NaCl

The material of one immunopurification cycle (corresponding to 3.7 mg of EPO) was dialyzed overnight at 4°C against acetate buffer (0.04 *M* acetic acid, 0.0025 *M* $CaCl_2$ adjusted to pH 4.5 with 1 *M* NaOH). 7 ml of pre-swollen DEAE–Sephacel gel (Pharmacia) was equilibrated with the same buffer and poured onto a 1 cm diameter column. The dialyzate was loaded onto the column at 30 ml/h and room temperature. After absorption, rhEPO was eluted from DEAE with the acetate buffer to which 0.015, 0.03, 0.06, 0.15, 0.35 and 1 *M* NaCl were successively added (Fig. 1). These seven fractions were brought to pH 7.5 by the addition of 1 *M* Tris and concentrated by ultra-filtration on PM10 Amicon membranes.

2.7. Rechromatography of the fractions obtained after absorption on DEAE–Sephacel and elution by a stepwise gradient of NaCl

The fractions previously eluted with 0.06 *M* NaCl and 0.15 *M* NaCl (about 700 μg of rhEPO per fraction) were dialyzed against acetate buffer pH 4.5,

Table 1
Immunoaffinity purification of rhEPO produced by transfected lymphoblastoid cells

Fraction	Supernatant of rhEPO producing cells	Fraction eluted at pH 2.2
Total protein (mg, BCA)	2783	3.71
Total IU of rhEPO	675 000	581 000
Specific activity (IU EPO/mg of protein)	241	156 000
Purification factor	1	647
Yield (%)	100	86

EPO concentration was measured by a radioimmunometric assay and protein concentration with the bicinchoninic reagent (BCA).

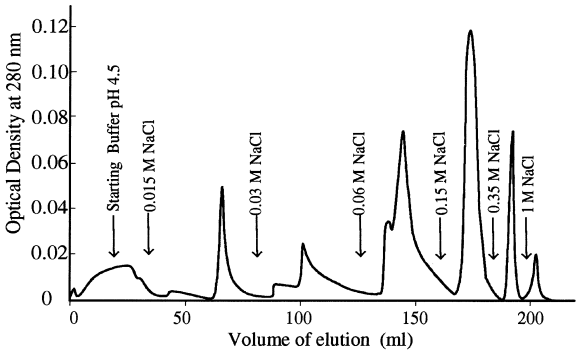

Fig. 1. Elution profile of rhEPO obtained with a stepwise gradient of NaCl (from 0.015 *M* to 1 *M*) after absorption on DEAE–Sephacel.

then absorbed onto DEAE–Sephacel as indicated in Section 2.6. Elution was performed with 0.03, 0.06, 0.15, 0.35 and 1 *M* NaCl. The elution profiles obtained by the rechromatography of these two fractions are shown in Fig. 2A,B. The main peaks were concentrated and dialyzed before analysis.

2.8. Separation of rhEPO isoforms by DEAE–Sephacel chromatography and elution by a continuous gradient of NaCl

We used the procedure described in Section 2.6 with the following modifications: elution was performed by a linear gradient of NaCl (from 0.01 *M* to 0.12 *M*) at a flow-rate of 18 ml/h and at room temperature. After 90 ml of this gradient, the remaining rhEPO was eluted by 1 *M* NaCl in acetate buffer pH 4.5. Seven fractions were collected (see Fig. 3). These fractions were concentrated and dialyzed before analysis.

2.9. Separation of rhEPO isoforms by high-performance liquid chromatography (HPLC) and elution by a continuous gradient of NaCl

A 600 μg amount of rhEPO dialyzed against acetate buffer pH 5.0 was applied on a TSK 3SW DEAE column (15×0.75 cm) equilibrated with the same buffer. rhEPO was loaded onto the column at a flow-rate of 30 ml/h and at room temperature. Elution was performed by a first linear gradient of NaCl (from 0.01 *M* to 0. 2 *M*) followed by second

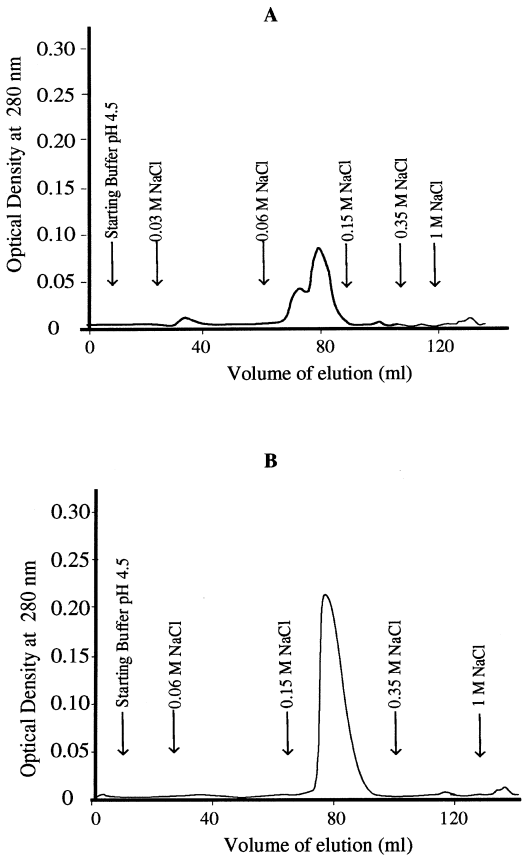

Fig. 2. Rechromatography of (A) 0.06 *M* NaCl fraction; (B) 0.15 *M* NaCl fraction (for conditions see Fig. 1).

linear gradient of NaCl (from 0.2 *M* to 1 *M*) both in acetate buffer pH 5.0 at a flow-rate of 30 ml/h and detection at 280 nm. Thirty fractions of 1 ml were

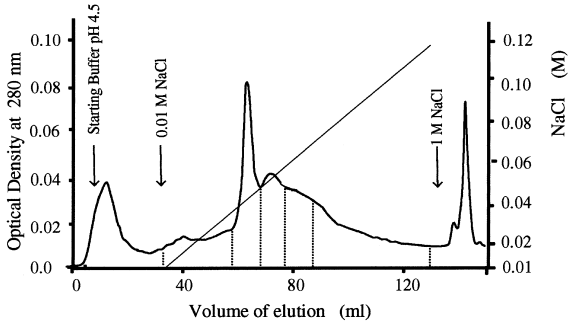

Fig. 3. Elution profile of rhEPO obtained with a linear gradient of NaCl (from 0.01 *M* to 0.12 *M*) after absorption on DEAE–Sephacel.

collected. These fractions were concentrated and dialyzed before analysis.

2.10. Separation of rhEPO isoforms by chromatofocusing

A 2.3 mg amount of rhEPO diluted in 2.5 ml of 0.025 *M* histidine–HCl buffer pH 6.2 was applied on a PBE-74 column (30×1.2 cm) (Pharmacia) equilibrated with the same buffer. Elution was performed with 0.025 *M* histidine–HCl buffer pH 3 containing 10% Polybuffer 74 and 4% Pharmalytes (pH 3 to 10) (Pharmacia) at a flow-rate of 20 ml/h and detection at 280 nm. Ten fractions were collected, ultrafiltrated (YM 10 membrane, Amicon), lyophilized and finally dissolved in 400 μl of water for further analysis.

2.11. Immunochemical assays for rhEPO

rhEPO was measured by an immunoradiometric assay (^{125}I EPO COATRIA) (BioMérieux, Marcy l'Etoile, France). In brief, the technique used two anti-EPO Mabs with high affinities for the EPO molecule. The first Mab was coated on the surface of plastic tubes. Samples containing EPO (in triplicate) were introduced into the tubes simultaneously with the second Mab labeled with ^{125}I. The mixture was incubated for 3 h at room temperature. After 2 washes with distilled water, the radioactivity bound to the tubes was counted. The EPO concentration of each sample was determined by interpolation from a calibration curve with seven EPO standards ranging from 0 to 800 mIU/ml. The accuracy (R.S.D.) was estimated to be 4% (intra-assay) and 8% (inter-assay) for a pool of sera with an EPO concentration of 17 mIU/ml [25] and unpublished observations.

rhEPO was also measured by a one site radioimmunoassay (RIA). The method is based on a competitive inhibition method after [26]. rhEPO labeled with ^{125}I was obtained as described above. A 100 μl volume of a sample dilution (in duplicate) was incubated in a microtiter well with 10 μl of polyvalent anti-EPO rabbit serum (diluted 1:800) at room temperature for 5 min under stirring. For the standards, rhEPO with a concentration range of 10 to 1280 mIU/ml prepared in PBS with 1% BSA was used. 10 μl of ^{125}I-labeled EPO was then added to each well (5000 to 10 000 cpm/well). The mixture was incubated at room temperature for 5 min under stirring. 100 μl of Tachisorb R (Calbiochem, San Diego, CA, USA) was then added to each well and incubated for 2 h at room temperature under stirring. The immune precipitates immobilized on Tachisorb R were collected by filtration on glass fiber membranes with a cell harvester (Skatron, Tranby, Norway). After 6 washings in PBS, the radioactivity of each membrane was counted with a γ-counter. Non-specific binding was determined using the dilution buffer. The EPO concentration of each sample was determined by interpolation from a calibration curve with 8 EPO standards ranging from 10 to 1280 mIU/ml. The R.S.D. was estimated to be 10% (inter-assay) for a preparation with an EPO concentration of 200 mIU/ml (unpublished observations).

2.12. HPLC–size-exclusion chromatography

A 100 μg amount of rhEPO were applied on a TSK G 2000 SW column (60×0.75 cm) (Pharmacia) with a mobile phase of 0.15 *M* NaCl in 0.02 *M* HEPES–NaOH buffer pH 7.4 at a flow-rate of 30 ml/h and detection at 280 nm.

2.13. Protein assay

The protein concentration was determined by the bicinchoninic acid assay (BCA) (Pierce, Interchim, Montlucon, France) using bovine serum albumin (BSA) as reference [27].

2.14. Electrophoretic techniques

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed according to the instructions of the manufacturer (Excel-gel, Pharmacia) in thin layer polyacrylamide gels. SDS and bromophenol blue were added to the samples, with final concentrations of 1% and 0.1 mg/ml, respectively. The samples were left to migrate for about 75 min under 600 V, 50 mA and 30 W at 10°C. Proteins were stained with Coomassie blue R 250 or with silver [28].

2.14.1. IEF

rhEPO and p*I* markers were submitted to polyacrylamide gel isoelectric focusing (PAGIF) in a pH

3–10 gradient. The gels were polymerized with 7% acrylamide, 0.14% bisacrylamide, 9 *M* urea and 2.6% ampholines pH 3–10 (Servalyt) (Serva Feinbiochemica, Heidelberg, Germany). Electrophoresis was performed with a Multiphor II electrophoresis unit (Pharmacia) under 2000 V for 4 h at 12°C with a 30 min prerun under 500 V (proteins were stained with silver as above). Nine p*I* markers (from 3.5 to 8.65) (Pharmacia) were used to determine the shape of the pH gradient.

3. Results

3.1. rhEPO immunopurification

The EPO fractions resulting from the immunopurification step were analyzed by SDS–PAGE. It consisted of a single protein zone with an apparent M_r of 36 000 (Fig. 4). The purity of the protein estimated by gel scanning (Sebia Densitometer, Issy les Moulinaux, France) was greater than 98%. The same purity was observed when EPO fractions were analyzed by pore size-exclusion chromatography (SEC) (results not shown).

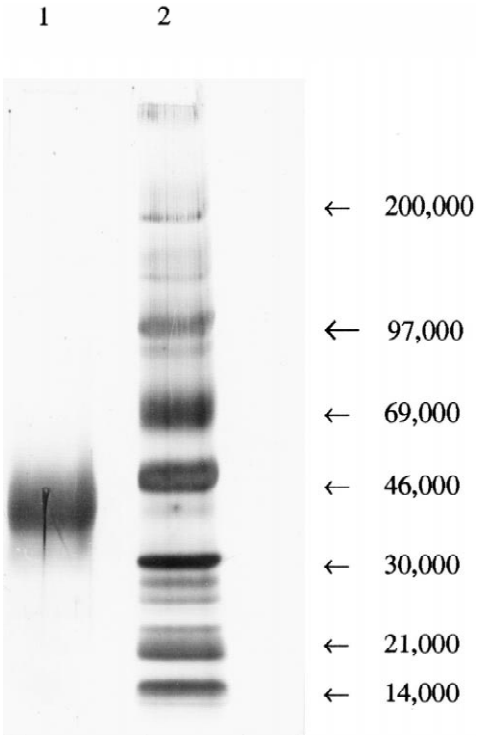

Fig. 4. SDS–PAGE followed by Coomassie blue staining. (1) Immunopurified rhEPO. (2) Molecular mass standards.

The protein concentration, the EPO concentration (measured by an immunoassay) and the resulting specific activity in the starting material and in the eluted fraction are indicated in Table 1 as well as purification factor and yield.

When analyzed by PAGIF, the purified rhEPO showed a micro-heterogeneity. About sixteen components with apparent p*I* between 7 and 3 were observed (Fig. 5, lane 1).

3.2. Separation of rhEPO isoforms by ion-exchange chromatography

3.2.1. DEAE–Sephacel with stepwise elution

The seven fractions separated by DEAE chromatography with a discontinuous gradient of NaCl (see Section 2.6) were analyzed by PAGIF. Each of them consisted of several bands (from 3 to 7). The mean value of their apparent p*I* decreased from 5.8 to 3.7 (Fig. 5, lane 2 to 8 and Table 2).

The same fractions were also analyzed by SDS–PAGE. In contrast to the heterogeneity observed after PAGIF, a component with an apparent M_r of 36 000 (similar to the one of the unchromatographied fraction) was observed in all cases (results not shown).

3.2.2. Rechromatography of the fractions obtained after absorption on DEAE–Sephacel and elution by a stepwise gradient of NaCl

About 75% of the fraction eluted with 0.06 *M* NaCl in the first chromatography was eluted with the same molarity of NaCl during the second chromatography (Fig. 2A). About 85% of the fraction eluted with 0.15 *M* NaCl in the first chromatography was eluted with 0.15 *M* NaCl during the second chromatography (Fig. 2B). When analyzed by PAGIF (Fig. 6) the fractions corresponding to the second chromatography showed a pattern similar to that obtained by the first chromatography.

3.2.3. DEAE–Sephacel and elution with a continuous gradient of NaCl

Seven fractions were collected (see Fig. 3). Their analysis by PAGIF revealed a similar pattern to that

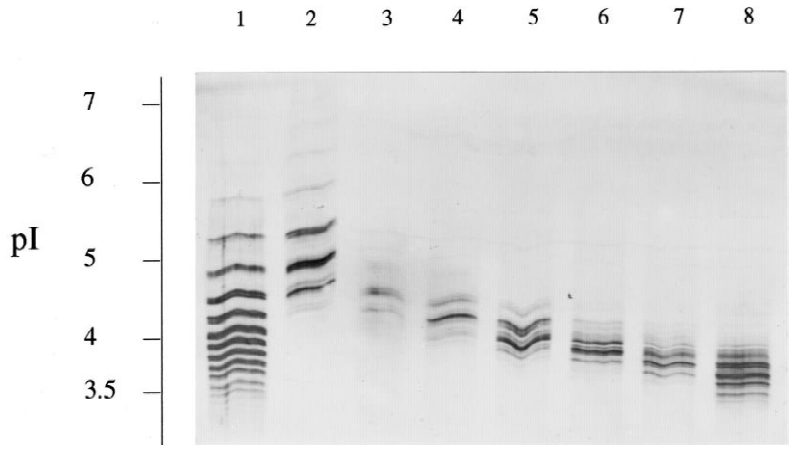

Fig. 5. PAGIF of rhEPO. Fractions obtained by DEAE–Sephacel chromatography: (1) starting material; (2) unabsorbed material; (3) material eluted with 0.015 *M*; (4) 0.03 *M*; (5) 0.06 *M*; (6) 0.15 *M*; (7) 0.35 *M* and (8) 1 *M* NaCl.

Table 2
Separation of rhEPO isoforms by DEAE chromatography with a discontinuous gradient of NaCl

Fraction	NaCl	p*I* (mean value)	Protein (μg)	EPO IU	EPO IU/mg	EPO IU (%)
Starting material		4.9	3717	581 424	156 423	100
F1	0	5.85	160	11 752	73 450	2
F2	0.015 *M*	4.8	249	38 038	152 765	6.5
F3	0.03 *M*	4.5	320	54 104	169 075	9.3
F4	0.06 *M*	4.35	780	145 845	186 980	25.1
F5	0.15 *M*	4.15	650	114 250	175 581	19.7
F6	0.35 *M*	3.9	56	6069	108 774	1.04
F7	1 *M*	3.75	16	8.93	55 800	0.15

EPO concentration was measured by a radioimmunometric assay and protein concentration with the bicinchoninic reagent (BCA).

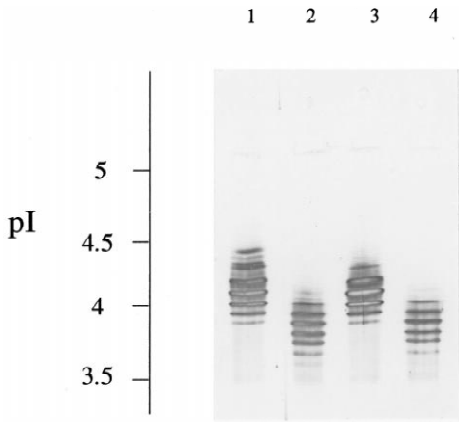

Fig. 6. PAGIF of rhEPO. (1) Fraction eluted with 0.06 *M* NaCl (first chromatography); (2) fraction eluted with 0.15 *M* NaCl (first chromatography); (3) fraction eluted with 0.06 *M* NaCl (second chromatography); (4) fraction eluted with 0.15 *M* NaCl (second chromatography).

obtained with a stepwise elution of NaCl (results not shown).

3.3. Separation of rhEPO isoforms by chromatofocusing and TSK–DEAE chromatography

Fractions obtained by this method were analyzed by PAGIF. The resolution obtained using these methods was according to our personal experience inferior to the one obtained using DEAE–Sephacel chromatography (results not shown).

3.4. Specific activity of the fractions obtained after DEAE chromatography

The mean p*I*, the protein concentration, the EPO concentration (measured by an immunoradiometric

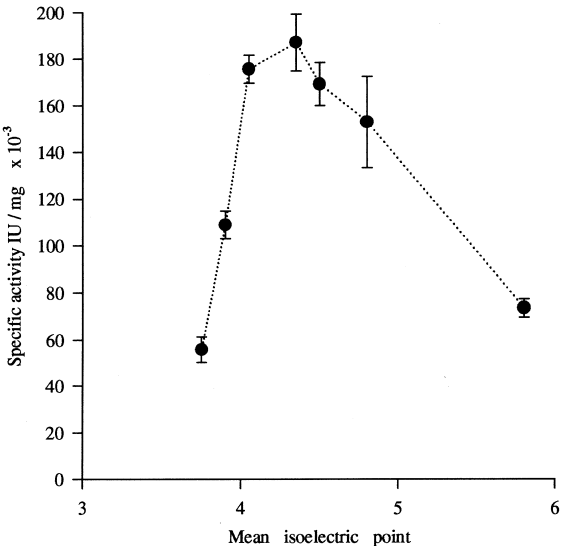

Fig. 7. Specific activity (and S.D.) of rhEPO isoforms, fractionated by DEAE–Sephacel chromatography, measured by an immunoradiometric assay.

assay), the specific activity and the respective percentages of each fraction are indicated in Table 2. On the whole, 64% of the rhEPO activity loaded on the column was recovered in the eluted fractions.

The resulting specific activity measured by an immunoradiometric assay was not evenly distributed in these seven fractions: a minimum was observed with the more basic and the more acidic isoforms (about 75 000 IU/mg protein) whereas fractions of an intermediate p*I* (4.3) had the highest values (about 180 000 IU/mg protein) (Fig. 7). Similar results were obtained when the measures were performed by the RIA competitive method (results not shown).

4. Discussion

4.1. Purification of EPO and electrophoretic analysis of the starting material

A monoclonal antibody (E14) obtained by immunization of mice with SDS-treated EPO, was used to purify rhEPO [10]. This mAb had a high affinity for native EPO. According to the measurements performed by radioimmuno-precipitation followed by Scatchard analysis, its dissociation constant was about 0.3 n*M*. In spite of this high affinity, elution was possible under non-denaturing conditions and with a satisfactory yield (86%). The specific activity of rhEPO after this step was increased by approximately 647-fold and was found to be of 156 000 IU/mg protein or 196 000 IU/A 280 nm. This value is similar to that obtained with other purified preparations of rhEPO secreted by CHO cells [29]. The specific bioactivity (both ''in vivo'' and ''in vitro'') of rhEPO of lymphoblastoid origin was also similar to that of rhEPO from CHO and of urinary EPO [10].

The resulting protein was homogeneous when examined by SDS–PAGE. The percentage of impurities estimated by scanning the gel after Coomassie staining was less than 2%.

When this material was examined by PAGIF in the presence of 9 *M* urea, about sixteen isoforms with p*I* values between 3 and 7 were observed. Such a large number of isoforms is not surprising since fifty-two different N-linked carbohydrate structures were identified [21] in rhEPO secreted by CHO cells. In addition, the number of EPO isoforms was estimated to be between 20 and 30 in a single unprocessed serum specimen [14]. The number of isoforms detected in [13] was limited from five to eight components in a pH range from 2.5 to 7.0. However, these forms were indirectly detected by western blotting after PAGIF. The resolution of CE was of the same order [16,17]. It appears that resolution of PAGIF is improved not only by the utilization of limited range of the pH gradient, but also by the presence of 9 *M* urea in the gel as it is the case in this report.

4.2. Separation of isoforms

By ion-exchange chromatography (on DEAE–Sephacel), we were able to separate the rhEPO isoforms into seven fractions of basic, intermediate and acidic p*I*. Due to the large number of EPO isoforms in the immunopurified preparation, the resolution provided by this method was not sufficient enough to isolate an individual form of EPO. In fact, at least 3 to 5 components were observed in each

fraction. However, it was possible to obtain sufficient amounts of these fractions to estimate their purity and measure their specific activity. The global yield of this fractionation (measured by the protein concentration or by an immunoassay) was satisfactory since about 65% of EPO loaded on the ion exchanger was recovered following the different steps of the chromatography (absorption of EPO on the gel, elution with the different molarities of NaCl and concentration of the different fractions). The eventual presence of impurities in each fraction was estimated by SDS–PAGE. The percentage of impurities was in all samples at the limit of detection, i.e., about 2%. In addition, all the fractions had a similar M_r.

To check the reproducibility of this stepwise elution, the 2 main fractions (eluted with 0.06 and 0.15 *M* NaCl) were again absorbed onto DEAE–Sephacel. A new elution was performed with increasing molarities of NaCl according to the conditions used in the first chromatography. The main peak of these two fractions was also eluted with 0.06 and 0.15 *M* NaCl (see Fig. 2A,B). The p*I* of the isoforms collected during the first experiment was not significantly different from that obtained during the second experiment (see Fig. 6). Therefore the reproducibility of the separation can be considered as satisfactory.

When the elution was performed using a continuous gradient of NaCl on the same DEAE matrix, the elution profile did not allow to define clearly all the fractions (see Fig. 3). The aliquots were collected into seven fractions so as to compare the results obtained using this method to those obtained by a stepwise elution. A similar pattern (with the same shift of p*I* fractions from basic to acidic isoforms) was observed with increasing molarities of NaCl after PAGIF analysis.

Absorption of rhEPO on the TSK–DEAE column and elution by a linear gradient of NaCl provided a weaker resolution of rhEPO isoforms. The same was observed with the chromatofocusing technique. In addition the presence of polybuffer and ampholines in the eluates make the purification difficult.

The comparison of different matrix of anion exchangers and different methods of elution showed that the more reproducible results are obtained by DEAE–Sephacel absorption combined with stepwise elution.

4.3. Biological activity of isoforms

It has been previously shown that the sialic acid content of EPO strongly influences the ''in vivo'' biological activity [22]. Moreover, it was demonstrated [19] that partially and fully de-N-glycosylated EPO have a higher in vitro biological activity than intact EPO, this activity being dependent on the number of sialic acids. It was also shown [30] that the affinity of monoclonal antibodies (although specific for protein epitopes) could be influenced by the structure of the carbohydrates of the molecule.

A similar phenomenon was observed in the case of antibody E14 used for EPO purification. When rhEPO was eluted from the immunoaffinity column by a continuous gradient of pH instead of a single acidic buffer pH 2.2, a limited but reproducible difference between the mean p*I* of the four collected fractions was observed: the most rapidly eluted fractions having a lower p*I* than the most delayed fractions. One probable explanation of this phenomenon is that the antibody used for immunoaffinity purification has a higher affinity for the basic than for the acidic isoforms of EPO.

The measurement of specific activity of each fraction by a two-site immunoassay (immunoradiometric assay) or a one-site immunoassay (RIA) showed that the most acidic and the most basic fractions have a lower specific activity than the intermediate fractions. These differences are significant as the relative standard deviation on EPO concentration of the immunoradiometric assay used in this report is about 4% (intra-assay) and 8% (inter-assay). These results were confirmed by a RIA. In addition, it must be mentioned that the error on protein estimation (by BCA technique) is similar or inferior to these values. The presence of impurities in the most acidic or the most basic fractions cannot be considered to be the cause of a diminution of the specific activity in these fractions since the proportion of impurities measured by the scanning of the gels was of the same order as in the unfractionated rhEPO preparation (about 2%).

Although the epitopes recognized by these antibodies appear to consist only of peptide elements, one possible explanation for the differences in specific activity could be the influence of the bulky and highly charged carbohydrate structure of EPO.

Since immunoassays are the most convenient and the most accurate way to measure the in vitro activity of EPO, the selection of isoforms during the purification process of rhEPO may influence the specific activity.

5. Conclusions

It was possible to obtain a fraction containing more than 98% of rhEPO by immunoaffinity purification. DEAE–Sephacel chromatography of this preparation allowed us to separate EPO isoforms into seven fractions of decreasing p*I*. Due to the large number of EPO isoforms in EPO preparations, the resolution provided by this method was not sufficient to isolate a single isoform of EPO. However, it was possible to obtain adequate amounts of each fraction in order to analyze them. We observed that the specific activity of EPO isoforms was not homogeneous: the most acidic and the most basic fractions having a lower specific activity than the most commonly represented fractions.

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