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Capillary isoelectric focusing of erythropoietin glycoforms and its comparison with flat-bed isoelectric focusing and capillary zone electrophoresis[1]

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Abstract

The influence of several operation conditions on separation of recombinant human erythropoietin glycoforms by capillary isoelectric focusing (cIEF) is explored. From this study it is deduced that in order to separate several glycoforms of erythropoietin, urea has to be added to sample, which should not be completely depleted of the excipients used in its formulation. On-line desalting does not provide separation enhancement for samples with high content of salt. Better resolution is obtained using a mixture of a broad and a narrow pH-range carrier ampholytes than with either one used separately. Under the experimental conditions, focusing voltages of 25 kV improve separation compared to lower and higher electric fields. Focusing times shorter than the time necessary for electric current to reach a minimum provide similar separations than longer focusing times at which a minimum value of the current has already been achieved. The optimized method allows the separation and quantitation in 12 min of at least seven bands containing glycoforms of recombinant erythropoietin with apparent isoelectric points in the range 3.78–4.69. Compared to flat-bed isoelectric focusing, cIEF provides better separation of bands of glycoforms in a shorter time, and allows quantitative determination. Capillary zone electrophoresis (CZE) gives rise to resolution of erythropoietin glycoforms similar to that obtained by cIEF. Although CZE requires a longer analysis time, its reproducibility in terms of peak area of glycoforms is better than in cIEF. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Isoelectric focusing (IEF) has been in many cases the technique of choice to analyze proteins. Its high resolution power provides separation of complex mixtures and allows to estimate the isoelectric points (p*I*) of the constituents. However, some drawbacks are inherent to this technique when performed in slab gels: (i) it is a time-consuming, labor-intensive method, (ii) data obtained from the separation (i.e., p*I* and quantitation) are only approximated, (iii) the amount of ampholytes needed to carry out analysis is

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quite high, and (iv) the lack of reproducibility of gels from batch to batch can jeopardize the reproducibility of the analytical technique. These limitations can be overcome when IEF is performed in a capillary electrophoresis format. Capillary isoelectric focusing (cIEF), introduced by Hjertén and Zhu [1], combines the advantages of IEF and capillary electrophoresis (CE) being a technique with high resolution, short analysis time, automated operation, capable of precise quantitative analysis, and able to give an estimation of p*I* values of proteins using minute amounts of samples, ampholytes and electrolytes. Despite these advantages, the use of cIEF as a routine analytical tool has been limited due to the many factors which can influence its performance.

Recombinant DNA techniques have allowed the production at industrial level of therapeutical proteins. The development of these techniques is accompanied by the need of analysis methods to characterize these biologicals at different stages of the production. In control analysis several advantages, such as reduced animal requirements and testing time, are seen in the replacements of bioassays with physicochemical methodologies [2].

One of the therapeutical proteins with higher economic interest in the USA is recombinant human erythropoietin (rhEPO) [3]. EPO regulates erythropoiesis, the process which controls the production of erythrocytes in mammals [4]. Besides the possible misuse of EPO by athletes to increase the oxygen carrying capacity of blood [5], this pharmaceutical protein can be used to correct renal anemia and as transfusion substitute [6,7].

EPO produced in Chinese hamster ovary (CHO) cells is a glycoprotein with a molecular mass of 30 400 and a carbohydrate content of 39.5%, which shows a protein conformation apparently identical with the natural product isolated from human urine [8]. However, since glycosilation depends, among other factors, on the host cell line used for its production, the carbohydrate structure of natural and recombinant EPO may be different [5], being known that the sialic acid content of EPO plays an important role in its biological activity [9]. The negative charge, contributed by each sialic acid group to the electric charge of the whole protein, allows the study of the relative proportion of glycoforms in the rhEPO samples by separation methods based on either the different p*I* or the different charge-to-mass ratio of the proteins. The relationship existing between the p*I* and the ''in vivo'' bioactivity for some glycoproteins has been reported [10].

In this paper, the effect of several experimental conditions which can affect the performance of cIEF separation of rhEPO glycoforms is explored. The analysis achieved using cIEF is compared with those obtained by flat-bed IEF and by capillary zone electrophoresis (CZE).

2. Experimental

2.1. Samples

Standard samples of rhEPO produced in a CHO cell line were provided by the European Pharmacopoeia as Biological Reference Product (BRP). Each sample vial contained about 250 μg of EPO, 0.1 mg of Tween 20, 30 mg of trehalose, 3 mg of arginine, 4.5 mg of NaCl, and 3.54 mg of $Na_2HPO_4 \cdot H_20$, and it was adjusted to pH 7.0 with NaOH [11]. The content of each vial was dissolved in 0.250 ml of Milli-Q water (Millipore, Molsheim, France) and the excipients of low-molecular-mass were eliminated by passage through a Microcon-10 cartridge (Amicon, Beverly, MA, USA) for 65 min at 2700 *g*. The retentate was washed three times with 0.2 ml of Milli-Q water for 40 min under the same centrifugal force. The filtrates from each of the four previous centrifugation steps were individually collected, successively numbered as F1 to F4, and stored at 4°C. These fractions are supposed to contain decreasing concentrations of the excipients used in EPO formulation. The retentate was recovered from the cartridge by centrifugation (5 min at 350 *g*) and Milli-Q water was added to complete 0.115 ml.

2.2. Procedures and reagents

2.2.1. Capillary isoelectric focusing

Separations were carried out in a PACE 5000 CE instrument (Beckman Instruments, Fullerton, CA, USA). Laboratory-made polyacrylamide-coated capillaries [12] [27 cm (effective length 20 cm)×50 μm I.D.] were used. Temperature was set at 20°C.

Anolyte, catholyte and rinse buffer were prepared from the reagents supplied in the cIEF kit (Beckman). Anolyte consisted of 91 m*M* H_3PO_4 in cIEF

gel. Catholyte was 20 m*M* NaOH in water. An aqueous solution of 10 m*M* H_3PO_4 was employed to rinse the capillaries between analysis using N_2 pressure (20 p.s.i., 1 p.s.i.=6894.76 Pa) for 1 min.

Two solutions of ampholytes were used. The ampholyte of pH range 3–10 was provided in the cIEF kit from Beckman. The pH 2.5–5 Pharmalyte was from Pharmacia Biotech (Uppsala, Sweden). Ampholytes were mixed with the cIEF gel in a ratio 1:50 (v/v). When 7 *M* urea (Sigma, St. Louis, MO, USA) was employed it was added to the cIEF gel and the mixture was filtered (0.5 μm, Millex-LCR, Millipore, Bedford, MA, USA) prior to the addition of ampholytes. Ampholyte solutions were used either individually or in a mixture (1:2, v/v) of the 3–10 and the 2.5–5 pH ranges. The total amount of ampholytes was kept constant.

Samples were diluted (1:1, v/v) with water or with the different filtrates collected from the centrifugation steps (F1 to F4, see Section 2.1). Five μl of diluted EPO sample was added to 25 μl of cIEF gel containing ampholytes. Capillary was filled by injection of this mixture at the anodic end applying N_2 pressure (20 p.s.i.) for 1 min. Introduction of the sample was preceded by a 1-min rinse with water under the same pressure.

After injection of the sample and ampholytes, separation was initiated. In those cases in which an on-line desalting step was included, it consisted in a ramp of voltage along a given time keeping the final value of voltage for 2 min before starting the focusing step. The influence of the focusing step was studied by performing it at different voltage values and for different lengths of time. After focusing, a mobilization step took place by applying N_2 pressure (0.5 p.s.i.) at the anodic end while the same voltage used for focalization was maintained.

Detection was performed at 280 nm. Data were collected and analyzed using the System Gold software (version 8.1) from Beckman.

2.2.2. Polyacrylamide gel isoelectric focusing (PAGIF)

2.2.2.1. PAGIF with conventional equipment

PAGIF was performed using a method similar to that described in the draft monograph for Erythropoietin Concentrated Solution of the European Pharmacopoeia [13]. Separation gels were prepared by mixing 9 g of urea, 6 ml of a solution of 30% (w/v) of acrylamide–bisacrylamide (36.5:1, w/w), 1.05 ml of ampholytes of pH range 3–5 (Serva, Heidelberg, Germany), 0.45 ml of pH 3–10 ampholytes (Serva), and 13.5 ml of Milli-Q water. The solution was degassed using a water pump. The gel was polymerized using 0.3 ml of 10% (w/v) ammonium persulfate and 15 μl of *N,N,N′,N′*-tetramethylethylenediamine (TEMED) (both from Sigma) on gelbond PAG film (Pharmacia, Uppsala, Sweden) in a 24×11×0.05 cm polymerization cassette (Ulttromould, Pharmacia). A solution of 0.1 *M* β-alanine was used as catholyte and a mixture of 0.1 *M* glutamic acid and 0.1 *M* phosphoric acid was used as anolyte.

Prefocusing of the gel took place at 10 W for 60 min, maximum voltage 2000 V and maximum current 100 mA. After prefocusing, 20 μl of the EPO solution were applied to the gel. The samples were focused for 60 min at 10 W, 2000 V maximum voltage and 100 mA maximum current.

Staining of the gels was carried out using Coomassie blue R-250 prepared according to the protocol provided by the European Pharmacopoeia [13]. After destaining, the gels were dried at room temperature.

The gels were scanned and the images analyzed using an Omni Media Scanner XRS12 CC provided with a 1-D manager software, version 1.6 (TDI, Madrid, Spain).

2.2.2.2. PAGIF with automatic equipment

A PhastSystem (Pharmacia) electrophoresis equipment was used. Separation was carried out using PhastGel dry IEF gels (T=5% and C=3%, Pharmacia)[2], rehydrated for half an hour with an aqueous solution containing carrier ampholytes, pH ranges 3–10 and 3–5 (both from Serva) in a ratio 1:2 (v/v). Samples (1 μl) were directly placed in each well of the Sample Applicator 8/1 (Pharmacia). Running conditions were as described in Ref. [14]. IEF Coomassie staining procedure was as described in PhastGel Coomassie Kit (Pharmacia). A Pharmacia Fine Chemicals low p*I* IEF calibration kit was used as marker.

Gel analysis and image analysis were performed as indicated for PAGIF in conventional equipment.

[2] T=[g acrylamide+g *N,N′*-methylenebisacrylamide (Bis)]/100 ml solution; C=g Bis/% T.

2.2.3. Capillary zone electrophoresis

Separations were carried out in a PACE 5000 CE instrument (Beckman) using a fused-silica uncoated capillary [107 cm (effective length 100 cm)×50 μm I.D.] from Polymicro Technologies (Composite Metal Services, Worcester, UK). The separation temperature was 35°C and the detection was carried out at 214 nm. Separations were performed at 15.4 kV.

The separation buffer used was a mixture of 0.01 *M* tricine (Sigma), 0.01 *M* NaCl (Merck), 0.01 *M* sodium acetate (Merck), 7 *M* urea, and putrescine (Sigma) at different concentrations. The mixture was adjusted to pH 5.5 with 2 *M* acetic acid (Merck) and filtered through a 0.5-μm filter (Millipore).

Samples were injected at the anodic end using N_2 pressure (0.5 p.s.i.) for 25 s. Data acquisition and analysis were performed with the SystemGold software.

3. Results and discussion

3.1. Optimization of cIEF separation

It is known that the presence of salts in the sample may have a detrimental effect on cIEF separations [15,16], and it is usually recommended that protein samples be desalted prior to separation. Salts from the EPO sample were eliminated using a Microcon system with a membrane of nominal molecular mass (M_r) cut-off 10 000. When analyzing the desalted sample diluted 1:1 (v/v) with water, very low current (5.4 μA) was produced at the start of the focusing step performed at 25 kV, indicating the effective depletion of salts from the sample. However, under these conditions separation of EPO glycoforms could not be achieved (Fig. 1A). Afterwards, an aliquot of desalted EPO was diluted (1:1, v/v) with the filtrate F3 (obtained in the second washing of the retentate) to keep some of the excipients initially present in the EPO standard. In this case the initial current increased to 23.8 μA (at focusing voltage of 25 kV) and several peaks (at least 7) of the glycoforms of erythropoietin were separated (Fig. 1B). A higher concentration of excipients in the sample was obtained by diluting (1:1, v/v) desalted EPO with the filtrate obtained in the first washing of the retentate

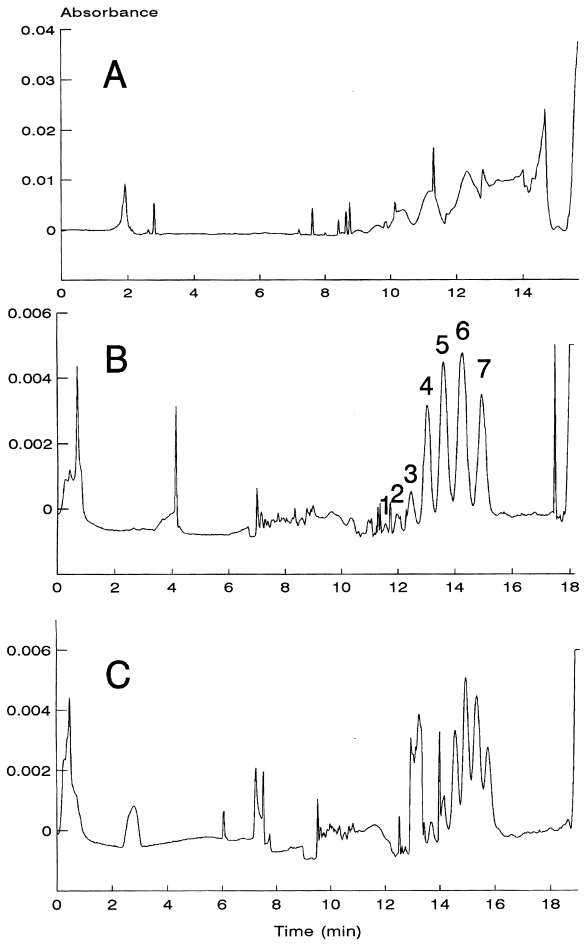

Fig. 1. cIEF electropherograms of desalted rhEPO diluted (1:1, v/v) with (A) water, (B) filtrate 3 and (C) filtrate 2. Polyacrylamide-coated capillary 27 cm (effective length 20 cm)×50 μm I.D. Focusing at 25 kV for 6 min. Sample mixed with cIEF gel, 7 *M* urea and ampholytes. Carrier ampholytes mixture (1:2, v/v) of pH 3–10 and 2.5–5 ranges. Detection at 280 nm.

(F2). The initial current (51.4 μA) at the same focusing voltage indicated a high content of salt, which leads to a loss of resolution (Fig. 1C).

These results indicate that salt should be removed from EPO samples prior to the focusing step, but not completely, so that some of the excipients remain in the sample. It has been mentioned [15] that probably small amount of salts remaining in the samples could be at the origin of increased resolution compared to samples completely depleted of salt, by causing slightly higher currents which would favor the

focusing process. However, there could be another explanation to the observed results dealing with denaturation of EPO. It could be possible that the molecule of EPO required a minimum amount of the excipients present in the original formulation in order to keep the native conformation. If the excipients were completely eliminated, the erythropoietin molecule would undergo a denaturation process. If the lack of separation observed with desalted EPO reflected a denaturation process, the results obtained would indicate that denaturation is a reversible process.

It has also been indicated the convenience of performing the desalting of samples by a step on-line, by applying a gradient of increasing voltage prior to the focusing step [15]. In this way, salts of much lower size than proteins migrates first while the slowly increasing voltage avoids an excessively high current. We tried to apply this on-line desalting method to a sample of EPO which had not been filtered through the Microcon system, and therefore contained all the original excipients. The on-line desalting step consisted on a ramp of gradient from 0 to 10 kV in 9 min. This voltage was kept for 2 additional min and then the focusing step at 25 kV took place. It was observed that even with this slowly increasing voltage, the current value was too high (45 μA at 5.4 min), what most probably accounted for the lack of separation observed in Fig. 2A. The on-line desalting technique, with ramp times of 3, 6 and 9 min in different experiments, was further applied to a sample with an intermediate content of excipients (desalted EPO diluted 1:1, v/v, with F2). In this case separation of glycoforms was observed (Fig. 2B), but no improvement in separation was achieved compared to the separation without on-line desalting (Fig. 2C). Thus, for samples with high salt content, off-line desalting before introducing the sample into the capillary is preferred to an on-line desalting step.

Separations shown up to this point have been performed by including 7 *M* urea in the mixture of sample, ampholytes and cIEF gel. Urea was included following the indications of a previous method [13] to perform separations of EPO glycoforms by slab gel IEF. Fig. 3 shows the analysis of rhEPO in the absence (Fig. 3A) and in the presence (Fig. 3B) of 7 *M* urea with the other conditions identical. When

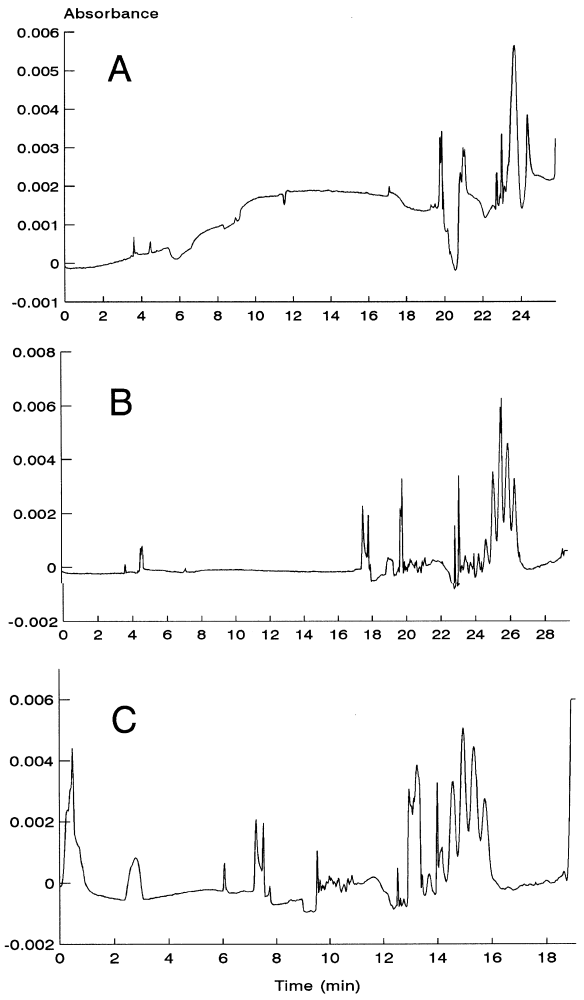

Fig. 2. Effect of on-line desalting on cIEF separations of rhEPO. (A) Sample with the total salt content of the original sample, (B and C) desalted sample diluted (1:1, v/v) with F2. On-line desalting step is performed in (A) and (B) but not in (C) by applying a voltage ramp from 0 to 10 kV in 9 min and keeping 10 kV for 2 extra minutes. Other conditions as in Fig. 1.

urea was excluded from the mixture, cIEF separation of isoforms failed. Urea is a common additive in protein analysis by cIEF as it can suppress protein aggregation by avoiding the formation of intermolecular hydrogen bonds [16]. In the analysis of EPO by CZE, deaggregation and disruption of hydrophobic and non-covalent interactions were named among the reasons for the effectiveness of 7 *M* urea in the separations [17].

The effect of the pH of the carrier ampholytes

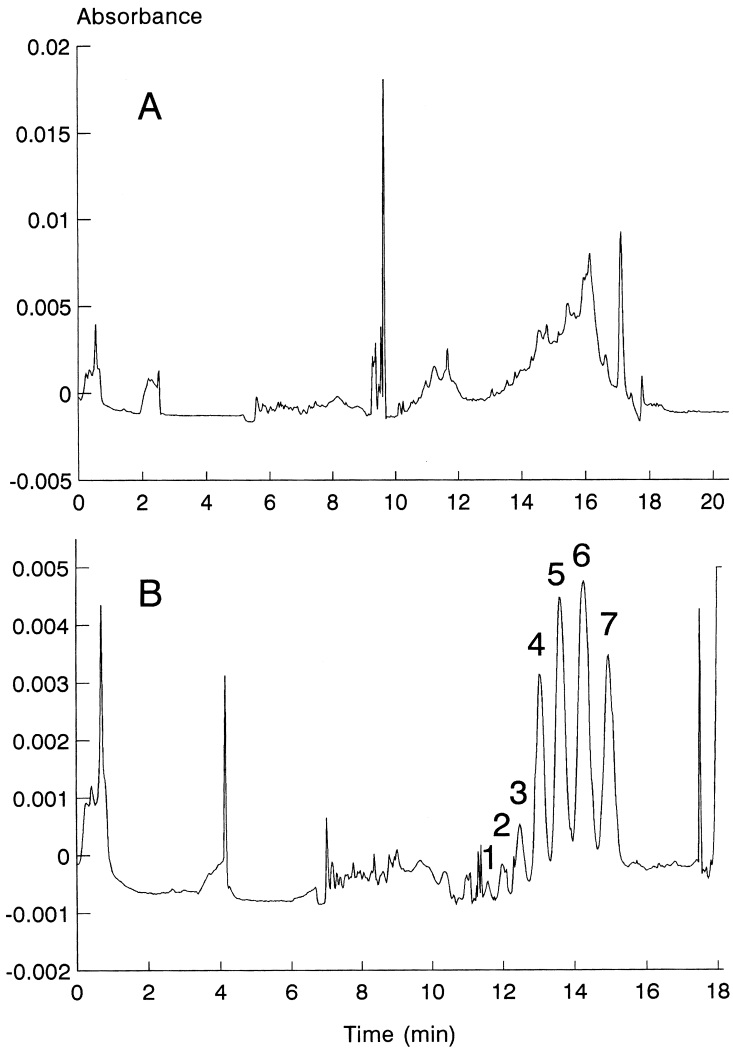

Fig. 3. Effect of urea on cIEF separations of rhEPO diluted (1:1, v/v) with F3. (A) Without urea in the sample mixture, and (B) with 7 *M* urea. Other conditions as in Fig. 1.

employed on the separation of isoforms was the next factor studied. When broad pH range ampholytes (pH 3–10) were used, resolution of the glycoforms of higher p*I* was acceptable but separation of the latest detected glycoforms of EPO was limited (Fig. 4A). Data from literature [8,18] indicate p*I* values for EPO glycoforms in the range 4.2 to 5.0. However, the use of ampholytes of narrow pH range (2.5–5) which would cover the whole range of EPO isoforms p*I*, did not provide better resolution (Fig. 4B), probably due to the non-homogeneous pH gradient formed. The best results were obtained with a mixture of carrier ampholytes in a ratio similar to that normally recommended in flat-bed IEF. Fig. 4C gives the separation achieved with ampholytes of ranges 3–10 and 2.5–5 mixed in a ratio 1:2 (v/v). Similar results have been obtained in the cIEF analysis of human hemoglobin variants [19].

The focusing step is of major importance in a process such as cIEF, in which separation is based on the simultaneous migration and concentration of each protein at the zone of the capillary at which the

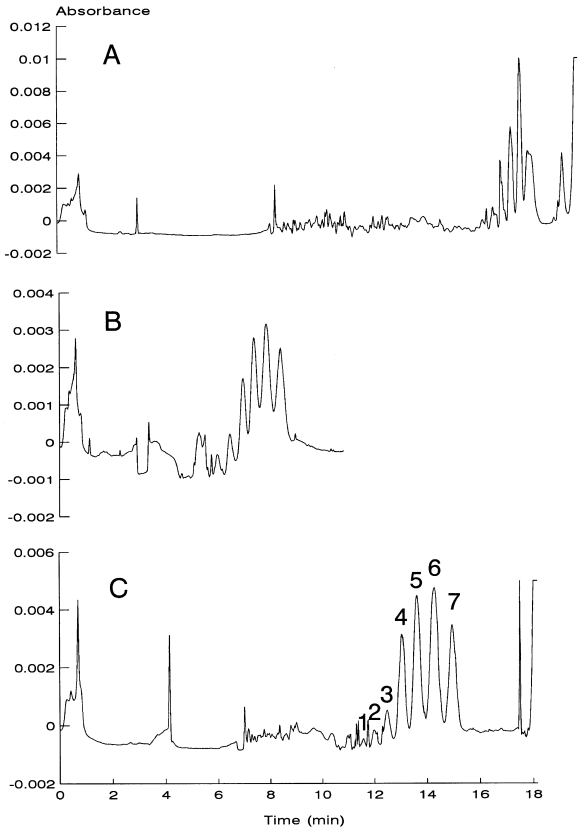

Fig. 4. Effect of ampholytes on cIEF separations of rhEPO diluted (1:1, v/v) with F3. (A) With carrier ampholytes in the pH range 3–10, (B) with carrier ampholytes in the pH range 2.5–5, and (C) with a mixture (1:2, v/v) of carrier ampholytes in the pH ranges 3–10 and 2.5–5. Other conditions as in Fig. 1.

pH of the ampholytes equals to the protein p*I*. It has been noticed that incomplete focusing at the time of detection is responsible for reduced resolution [19]. Two parameters control the focusing step: the applied voltage and the time length. In a first experiment, focusing time was kept constant (6 min before starting the mobilization step) while the effect of focusing voltage was studied. Constant voltages of 15, 20, 25 and 30 kV were assayed. Slightly better resolution was obtained with increasing voltages up to a 25 kV value (data not shown). At 30 kV a small loss of resolution was observed, probably due to an excess of Joule heating. As for the focusing time, Clarke et al. [15] have established as focusing length for a given sample the time at which the current decreases below 10% of the initial value and stabilizes. In our case, it was observed in the previous experiment that at the selected voltage (25 kV) the initial current (23.8 μA) decreased to achieve its 10% value at 1.75 min and got practically stabilized at 1.93 μA in 2 min. This drop to a constant value of current is interpreted as the completion of the focusing step [20]. In this way, the time at which focusing was achieved could be identified and the mobilization step could be started. According to our previous experiment, the 6 min initially assayed as focusing time seems too long. As expected, a shorter focusing time, 4 min, lead to the same resolution than the longer one. Surprisingly, a loss in resolution was not observed when time of focusing was shorter (1 min and 0.5 min) than that needed to get a low and constant value of current. Fig. 5 shows the separation of EPO glycoforms obtained with a focusing step of 25 kV for half a minute. At 0.5 min, when mobilization step begins, the current value is 16.5 μA, 69% of the initial value. This result can only be understood if it is thought that during the mobilization step, at which the same value of voltage is maintained together with a positive N_2 pressure, focusing continues taking place. Actually, Schwer [21] has mentioned that acidic proteins are additionally focused during the mobilization step.

After focusing, separated bands have to be mobilized so that they can be monitored at the detection window. Mobilization was carried out by applying N_2 pressure at the anodic end of the capillary. It has been reported that the higher is the mobilization pressure, the lower is the resolution [22]. Since our instrument has only two available pressure rates (0.5 and 20 p.s.i.), we chose 0.5 p.s.i. for the mobilization step, which is very close to the best value obtained by other authors for capillaries of the same dimensions than ours [22]. However, mobilization by pressure gives rise to parabolic flow displacement of the liquid contained in the capillary, which could disturb the separation of the bands. To make the rate of refocusing faster than the rate of diffusion [20] the same voltage applied during the focusing step was maintained during mobilization, and a solution of high viscosity (the cIEF gel provided by the manufacturer), was introduced into the capillary together with the sample and added to the anolyte solution.

Under the above mentioned conditions the analysis time for the seven bands of glycoforms of the rhEPO

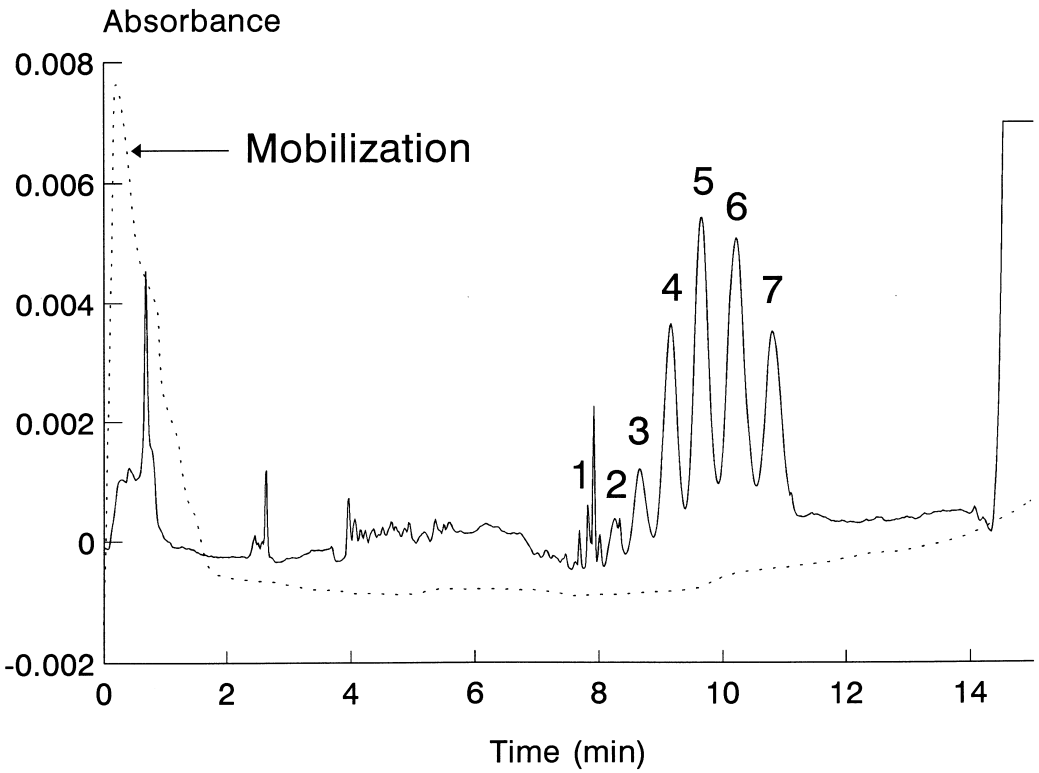

Fig. 5. cIEF electropherogram of rhEPO diluted (1:1, v/v) with F3. Focusing step 0.5 min at 6 kV. Dashed line corresponds to electric current profile. Other conditions as in Fig. 1.

are in the range 8 to 11 min (see for instance Fig. 5). This analysis time is substantially shorter than that obtained by other authors for similar resolution of a lower number of EPO bands [23]. It should be remarked that our analysis time corresponds to the values obtained in a capillary column which behaves reproducibly after several analysis of rhEPO have been performed with it. The analysis time which was obtained the first time that EPO was analyzed by cIEF in a brand new capillary was longer and resolution was slightly higher than in used capillaries. Some authors have mentioned that original time and resolution can be recovered by replacing the catholyte [22]. In our case the replacement with new solutions of anolyte, catholyte, rinsing buffer and sample mixture did not allow the recovery of the original resolution and analysis time achieved with the capillary. Most probably these modifications in the analysis time and resolution could be due to alterations of the capillary coating, as it has been hypothesized before [24,25]. These changes could be due to the transformation of the amide groups of polyacrylamide in acrylic acid at the high pH region of the ampholytes employed, which could degrade some sections of the coating along the capillary [26].

3.2. Estimation of pI values by cIEF

Isoelectric points of the seven bands of rhEPO glycoforms separated by cIEF were estimated using as standard soybean trypsin inhibitor (p*I* 4.55) and pepsin (p*I* 3.09) and assuming a linear behavior of p*I* in the zone. Several intermediate proteins of nominal intermediate p*I* and the kit of isoelectric point markers used in slab gel IEF could not be used as references as they originate multiple peaks. This difficulty of finding suitable standards to be used as p*I* markers had been already mentioned by other authors [16,22,27].

The calculated p*I* values for the seven bands

separated were 3.78, 4.02, 4.22, 4.37, 4.55, 4.65 and 4.69 [relative standard deviations (R.S.D.s)<0.4% except for peak number 7]. They should be regarded as estimated values because, besides of the assumed linearity of pH along the capillary, other factors could contribute to the inaccuracy of the p*I* value determined by this cIEF method, for instance, the necessary presence of urea in the sample mixture, which modifies the actual values of p*I*. It has to be also taken into consideration that p*I* values depend on the technique used for their determination, and in the case of electrophoretic techniques the experimental conditions also influence the obtained p*I* values [28].

3.2.1. Comparison of cIEF to flat bed IEF and CZE

Five bands (results not shown) were observed when analysis of rhEPO diluted with F3 was carried out by PAGIF with conventional equipment. Probably these bands correspond to the major components (peaks 3 to 7) obtained in cIEF. Analysis time, including gel casting, separation, and staining took about 8 h. In order to improve the method and to decrease analysis time, PAGIF separations were performed with automated PhastSystem equipment. First attempts were carried out on commercial gels including ampholytes of pH range 3.0–9.0. To improve band resolutions, separations were carried out using, as mentioned in cIEF, a mixture (1:2, v/v) of ampholytes of broad and narrow pH range. Under these conditions analysis time was reduced to 1 h and seven bands, with approximate p*I* values in the range 2.5–4.0 observed (results not shown).

Although seven bands could be observed using PAGIF with automated equipment, separation was rather poor and only semiquantitative results could be achieved. One advantage of flat-bed IEF in comparison with cIEF is that the former can analyze several samples in the same run. However, the optimized cIEF method described in this work takes about 15 min per analysis, including the injection and rinsing steps, which compares favorably with the PAGIF technique.

To carry out the separation of EPO using CZE, a rhEPO standard sample was desalted, diluted 1:1 (v/v) with F3 and analyzed by CZE. In previous experiments uncoated capillaries, preconditioned with 0.1 *M* NaOH for 5 min before being used for the first time in EPO analysis, were employed. However, we observed that this washing procedure caused in the new capillaries very high electroosmotic flow (EOF), with the concomitant decrease in resolution for glycoform separations. According to the previous experience in our group, by washing the capillary with 1 *M* NaOH for 30 min, lower EOF values were obtained, which gave rise to higher resolution of EPO isoforms likely due to the increase of viscosity at the capillary–buffer interface as a result of the gel layer developed on the fused-silica surface [29].

The washing routine between injections initially tried in previous CZE experiments for EPO separation (rinsing with water for 10 min) only lead to good reproducibility when the capillary had been previously used to analyze several EPO samples. Some assays performed about this point indicated that good reproducibility could be obtained when capillaries were rinsed between injections with water (5 min) and with separation buffer (10 min).

The introduction of additives in the separation buffer has been shown to improve the resolution of rhEPO glycoforms by decreasing the value of EOF [17]. In previous experiments the use of 3.9 m*M* putrescin lead us to baseline resolution of EPO glycoforms. However, it could be observed that under these conditions, analysis of each sample took more than 70 min. In order to decrease the analysis time without decreasing substantially the precision in glycoforms quantitation, we studied the influence of putrescine concentration on migration time and quantitation. The results indicated that the use of this additive at concentration as low as 0.025 m*M* significantly reduced the analysis time providing enough resolution for the determination of the content of each band of glycoforms (Fig. 6). R.S.D. ($n=4$) for percentages of each band were in the range 0.10 to 9.40%, while for migration times the R.S.D. values were in the range 1.33 to 1.49%. Compared to cIEF analysis, slightly better reproducibility in analysis time (R.S.D. from 0.80 to 1.13%) was obtained by the latter technique, while R.S.D.s for relative peak areas ranged from 0.70 to 20.09%.

In both techniques the lowest reproducibility of percentages correspond to the smaller peaks as

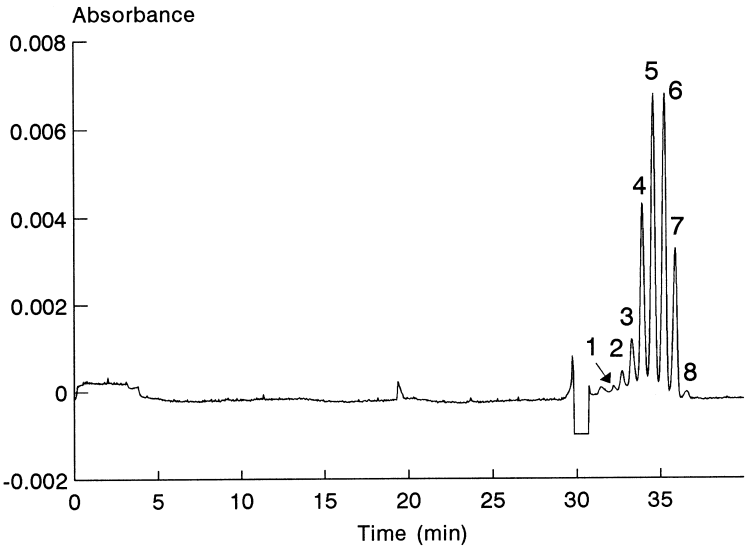

Fig. 6. CZE electropherogram of rhEPO. Uncoated capillary 107 cm (effective length 100 cm)×50 μm I.D. pretreated with 1 *M* NaOH for 30 min. Separation buffer: 0.01 *M* tricine, 0.01 *M* NaCl, 0.01 *M* sodium acetate, 7 *M* urea and 0.025 m*M* putrescine, pH 5.5. Temperature: 35°C. Separation voltage 15.4 kV. Injection by pressure: 0.5 p.s.i. for 25 s. Detection at 214 nm.

expected, and as observed by other authors [19]. It can be seen that from the point of view of reproducibility of quantitative analysis CZE should be the method of choice when strict controls are required. On the other hand, cIEF requires less than a half of the time than CZE to perform an analysis, what makes it the most convenient method when rapid quality control analysis have to be performed. Moreover, CZE employs uncoated capillaries while cIEF requires the use of a coated capillary to reduce or eliminate the EOF. One advantage frequently mentioned of cIEF methods is the increase in sensitivity provided by the concentration in narrow bands of proteins that initially fill the whole capillary [20,21]. However, the lack of ampholytes specifically designed for cIEF makes it necessary to use those usually employed in flat-bed IEF, which have a high absorbance at low UV wavelengths. In this regard, note that cIEF detection had to be performed at 280 nm [16] while in CZE, detection at 214 nm could be carried out. Actually both techniques showed similar sensitivity allowing the detection of the seven mentioned bands plus another minor one eluting later in the electropherograms. In some instances cIEF electropherograms presented two minor bands before the seven major ones, which may correspond to EPO isoforms. It has to be noticed that probably each of the bands separated by any of the techniques could correspond to multiple glycoforms with the same p*I* and charge-to-mass ratio [30]. One last point in favor of cIEF is that this technique provides information about one important characteristic of proteins, the isoelectric point.

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