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Improved capillary isoelectric focusing method for recombinant erythropoietin analysis

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Abstract

Human erythropoietin (EPO) is an endogenously produced glycoprotein, which plays a key role in the erythropoiesis process. Production of erythropoietin by recombinant DNA techniques has made possible its therapeutical use besides its misuse in sport competitions. The link between glycosylated form and protein activity makes necessary a method to analyze the glycoforms' distribution in the recombinant products. In this work, a capillary isoelectric focusing (cIEF) method is presented that allows the analysis of erythropoietin glycoforms. Besides, the cIEF method can be easily implemented in different laboratories. In order to get a feasible and precise cIEF method the following factors have been studied and optimized: (i) neutral coated capillaries, 27 cm long are employed, (ii) ampholytes in the pH range 2 to 10 are used, (iii) bovine β-lactoglobulin A is chosen as internal standard, (iv) anolyte consisting of 91 m*M* H_3PO_4 in cIEF gel is made by weight and catholyte is prepared by titrating 20 m*M* NaOH with H_3PO_4 to pH 11.85–11.90, (v) sample is completely depleted of excipients and sodium chloride 10 m*M* final concentration is added, and (v) $t_n/t_{I.S.}$ and $(A_n - A_{I.S.})/A_{I.S.}$, n being the recombinant EPO glycoform considered and I.S. the internal standard, are chosen as indexes to express migration time and area. As a result, a precise method to analyze erythropoietin by capillary isoelectric focusing is achieved with intra-assay RSD$\leq$0.5% for index time and $\leq$1.5% for index area and inter-sample, inter-anolyte, and inter-catholyte precision better than 3.4% for index time and RSD lower than 2.2% for index area.

Keywords: Isoelectric focusing; Erythropoietin; Glycoproteins; Proteins

1. Introduction

Erythropoietin (EPO) is a glycoprotein of key importance in the erythropoiesis process. Its production by recombinant DNA techniques has made it one of the therapeutical proteins with higher economic interest in the USA [1].

It is known that the sialic acid content of EPO plays an important role in its biological activity [2]. Since the host cell line used for EPO production influences its glycosylation, the carbohydrate structure of natural human EPO may be different from that of recombinant EPO (rEPO) produced in different cell lines. Besides, differences may be also due to

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changes in purification methods and to inter-batch variations [3]. Thus, a method able to differentiate these small variations is needed. As each sialic acid group contributes a negative charge to the electric charge of the protein, methods based on separation of molecules with different isoelectric points (p*I* values) could be valid to study the relative proportion of glycoforms in the rEPO samples.

Capillary isoelectric focusing has been shown to be the method of choice to analyze molecules with very close p*I* values. This technique has the resolution power of the isoelectric focusing technique performed in slab gels, but avoids its drawbacks.

A previously published method showed the applicability of capillary isoelectric focusing for the analysis of rEPO, which enabled differentiation and quantitation of at least seven bands of rEPO glycoforms in 12 min [4]. However, some factors of that method meant it was not straightforward to implement it in different laboratories.

In this work modifications are made to obtain a precise capillary isoelectric focusing (cIEF) method. To do this, the effects of different parameters on the feasibility and precision of the cIEF separation are studied. Namely, the use of coated capillaries of different types, capillary lengths, range of pH of the ampholytes, internal standard selected, pH and composition of electrolytes, salt content of sample, and indexes used to express migration time and area, are studied.

2. Experimental

2.1. Samples

Standard samples of rEPO produced in a Chinese hamster ovary (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 $Na_2HPO_4 \cdot H_2O$, and it was adjusted to pH 7.0 with NaOH [5]. 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 as indicated in Ref. [4]. Briefly, samples were passed through a Microcon-10 cartridge (Amicon, Beverly, MA, USA) for 65 min at 2671 *g* and the retentate was washed three times with 0.2 ml water for 40 min under the same *g* value. The filtrates from each of the centrifugation steps were numbered as F1 to F4 and stored at 4 °C. The retentate was recovered from the cartridge by centrifugation at 353 *g* for 5 min and Milli-Q water was added to make up to 0.250 ml.

2.2. Equipment, procedures, and reagents

Separations were carried out in a PACE 5000 CE instrument (Beckman Instruments, Fullerton, CA, USA). Two types of capillaries were used. Type one corresponded to laboratory-made polyacrylamide-coated capillaries [6], 27 cm (20 cm effective length)×50 μm I.D. Capillaries of type two were obtained from Beckman (eCAP Neutral Capillary). I.D. was 50 μm and two different total lengths (*L*), 47 and 27 cm (with effective lengths of 40 and 20 cm, respectively) were tried. In all cases temperature was set at 20 °C.

Initially anolyte was prepared by volume and it consisted of 91 m*M* H_3PO_4 in a poly(ethylene oxide) solution (with trade name cIEF gel, from Beckman [7]). Development of the method led to prepare it by weight and to control the apparent pH as will be indicated below.

Catholyte was first made as 20 m*M* NaOH in water. Method improvement led to the addition of H_3PO_4 until a defined pH, as will be shown below.

Ampholytes were added to the cIEF gel followed by urea (Sigma, St. Louis, MO, USA) to get a urea final concentration of 7 *M* and the mixture was filtered (0.5 μm, Millex-LCR, Millipore, Bedford, MA, USA). Different combinations of ampholytes were compared. The ampholyte solution of pH range 3–10 was from Beckman. The pH 2.5–5 Pharmalyte was from Pharmacia Biotech (Uppsala, Sweden). Servalyte solutions of ranges 2–4 and 4–6 were from Serva (Heidelberg, Germany). Ampholytes were mixed with cIEF gel in different ratios.

The usefulness of several substances with different p*I* values as internal standard was studied.

Samples were diluted with water or with different solutions, as it will be indicated below. NaCl and $Na_2HPO_4 \cdot 2H_2O$ (p.a.) used to this end were from Merck (Darmstadt, Germany). Five μl of diluted

EPO sample were added to 25 μl of cIEF gel containing urea and ampholytes. Internal standard was added. The capillary was filled with this mixture by applying N_2 pressure (20 p.s.i., 1 p.s.i. = 6894.76 Pa) at the anodic end for 1 min.

Separation was performed at 25 kV. Under this voltage, initially a focusing step took place. The effect of different length times for the focusing step was studied. Focusing was followed by a mobilization step performed by applying N_2 pressure (0.5 p.s.i.) at the anodic end while voltage was maintained.

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

Between injections the 27 cm capillaries were rinsed (N_2 pressure 20 p.s.i.) with 10 m*M* H_3PO_4 for 1 min followed by a 2-min rinse with water. For 47 cm capillaries, filling and rinsing times were three times longer.

At the end of the day capillaries were rinsed (N_2 pressure 20 p.s.i.) for 10 min with water and for 5 min with cIEF gel. The capillaries filled with gel and with the extremes immersed in the same gel were kept at 4 °C overnight. First injection of the day was preceded by a 5-min rinse with water under the same pressure.

3. Results and discussion

3.1. Selection of nature and concentration of ampholytes

In the present study, best results with the laboratory-made capillaries [6] were obtained with the (1:2) mixture of ampholytes (3–10) plus (2.5–5), in good agreement with our previous results [4]. However, when the eCAP Neutral Capillary (i.e. a commercial type of capillary) was used, change to a (1:2) mixture of (3–10) and (2–4) ampholytes was necessary to get the most acidic bands of EPO resolved and separated from the anolyte front.

This mixture of ampholytes was mixed with cIEF gel in (1:50) and (1:5) ratios. Although the higher concentrations of ampholytes led to a better focusing of the EPO glycoforms' peaks, the low ampholytes:gel ratio was chosen for further experiments to avoid a high UV noise.

3.2. Effect of the capillary length

Results comparing two lengths ($L = 47$ and 27 cm) for the eCAP column showed a better focusing in the shorter capillary and, as expected, a shorter analysis time. According to Righetti [8], the difference in p*I* of two resolved peaks [Δ(p*I*)] is provided by the following expression

$$\Delta(\mathrm{p}I) = 3\left[\frac{D(\mathrm{dpH}/\mathrm{d}x)}{E(-\mathrm{d}\mu/\mathrm{dpH})}\right]^{1/2}$$

in which *D* is the diffusion coefficient of the analyte, dpH/d*x* is the rate of change of pH with the distance, *E* is the applied electric field, and $-\mathrm{d}\mu/\mathrm{dpH}$ is the change in mobility with pH. This predicts that better p*I* resolution [lower Δ(p*I*)] would be achieved with longer capillaries by decreasing the slope of the pH gradient while keeping the rest of the variables constant. However, *E* was lower for the longer capillary as the applied voltage for the shorter column was close to the maximum allowed by the instrument, which explains the better results obtained with the shorter capillary.

3.3. Selection of internal standard

In order to study the feasibility of their use as internal standards (I.S.) in the rEPO glycoforms' analysis, the following compounds were assayed under the same electrophoretical conditions used for rEPO analysis: pepsinogen (p*I* 2.8), amyloglucosidase (p*I* 3.6), methyl red (p*I* 3.8), soybean trypsin inhibitor (p*I* 4.6), bovine β-lactoglobulin A (p*I* 5.15), glycin-tryptophan (p*I* 5.52), glycin-phenylalanine (p*I* 5.52), and carbonic anhydrase II (p*I* 5.9). Among all of them, bovine β-lactoglobulin A presented the best characteristics as I.S.

3.4. Selection of electrolytes

Lack of repeatability in the electrophoretic behavior of the I.S. and the glycoforms of EPO was observed among different days using different bat-

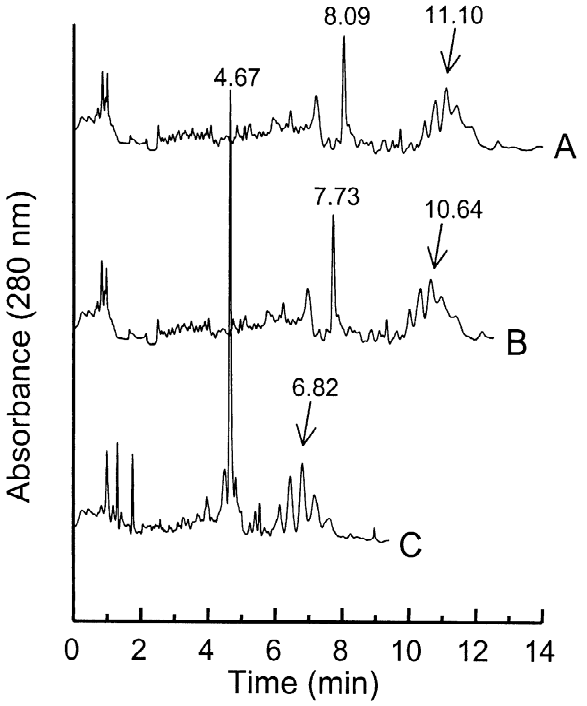

Fig. 1. cIEF electropherograms of rEPO performed on an eCAP neutral capillary 27 cm (20 cm effective length)×50 μm I.D. Sample with F2 was mixed with cIEF gel, 7 *M* urea, and ampholytes with pH ranges 3–10 and 2–4 (1:2 ratio). Focusing was at 25 kV for 1 min. Anolyte, 91 m*M* H_3PO_4 in cIEF gel, pH 1.52. Catholyte (A) 15 m*M* NaOH, pH 12.27; (B) 4 m*M* NaOH, pH 11.85; and (C) 16 m*M* NaOH titrated to pH 11.85 with H_3PO_4.

ches of anolyte and catholyte with theoretically exact composition.

By keeping constant the anolyte used, it was observed that the similarity of the electrophoretic behavior of the I.S. and EPO bands was much improved when the pH of the different batches of catholyte were adjusted to 11.90 by addition of 1 *M* H_3PO_4. Moreover, resolution was better than with any of the unadjusted catholytes.

By comparing the effect of NaOH solutions of different concentrations (4, 10, 13, and 15 m*M*) and pH values (11.85, 12.10, 12.22, and 12.27, respectively) as catholytes, it was observed that the higher the pH, the larger the migration times (Fig. 1A and B). This behavior could be related to cathodic drift, that is the progressive loss of the basic end of a pH gradient [9–11]. Lower cathodic drift, related to higher NaOH concentration, would imply slower electroosmotic flow and thus, larger migration times of EPO bands. Good resolution of EPO bands was not achieved in any case.

Two batches of catholyte with the same pH (pH 11.85) but different composition were compared. The first batch was made of 4 m*M* NaOH (Fig. 1B). The second batch contained 16 m*M* NaOH plus the amount of 1 *M* H_3PO_4 needed to adjust the pH (Fig. 1C). Results shown in Fig. 1 indicated that the presence of the acid reduced the migration times and markedly enhanced the resolution of glycoforms. Table 1 shows the migration times of the I.S. and the major EPO glycoforms (named as 3 to 7) when three different catholytes prepared by adjusting the pH with H_3PO_4 were used. According to these data a larger ΔpH, which could be related to a higher amount of H_3PO_4 added, corresponded to a larger decrease in the migration times. The addition of phosphoric acid to the cathode could contribute in a combined way by complexing the remaining silanols in the capillary wall [12,13] and by collaborating in cathodic mobilization [10,14,15].

Variations in the anolyte batches on reproducibility were considered. Anolyte was initially prepared by adding the corresponding volume of 1 *M* H_3PO_4 to the needed volume of cIEF gel. Measurement of the apparent pH of several anolyte batches showed

Table 1
Effect of H_3PO_4 added to catholyte to adjust pH. Focusing voltage, 25 kV. Focusing time, 6 min. Ampholytes (3–10)+(2–4) in 1:2 ratio

pH_i	pH_f	ΔpH	$t_{I.S.}$ (min)	t_3 (min)	t_4 (min)	t_5 (min)	t_6 (min)	t_7 (min)
12.44	11.84	0.60	6.14	7.75	8.23	8.71	9.14	9.55
12.41	11.84	0.57	6.45	8.53	8.95	9.33	9.74	10.07
12.35	11.88	0.47	7.28	9.79	10.18	10.59	11.01	11.47

pH_i corresponds to the initial pH value of the catholyte made with NaOH. pH_f corresponds to the final pH value of the catholyte after addition of H_3PO_4. ΔpH corresponds to the difference $pH_i - pH_f$. $t_{I.S.}$ is the migration time of the internal standard. t_n for $n=3$ to 7 is the migration time of the bands of the major EPO glycoforms.

different values, with higher pH values corresponding to larger migration times of protein bands but with low effect on resolution. Anodic drift is thought as a possible cause for the differences in migration times [9,16]. As lack of reproducibility was probably related to the difficulty of pipetting the viscous cIEF gel, anolyte was prepared by weight, getting constant pH values in the range 1.5–1.6

3.5. Influence of the focusing time

Comparison of electropherograms obtained using the eCAP Neutral Capillaries with 27 cm of total length together with 1 min and 6 min focusing times indicated that mobilization can be started before the current reaches a constant value. These results show that focusing times shorter than needed to get a constant current value (indicative of complete focusing) can be used without loss of resolution [4]. Most probably the application of the same voltage value during the mobilization step as that used for focusing helps to reduce the length of the focusing step. Moreover, shorter focusing times would provide a lower contribution to anodic drift. Unless otherwise indicated, focusing time of 1 min was then used in the following experiments.

3.6. Effect of sample solvent

Samples of rEPO diluted 1:1 with water and with the solution F2 containing part of the original excipients and obtained as indicated in Section 2 were compared. Initial current under 25 kV was 8.7 μA for the aqueous sample and 30.7 μA for the sample containing F2. No resolution of the EPO bands was observed in the first case, while good resolution of glycoforms was obtained in the presence of excipients. The effect of different concentrations of the two salts contained in the excipients was studied. The best separation of the EPO bands was achieved when 0.60 mg/ml sodium chloride, corresponding to 10 m*M* final concentration, was added to the (cIEF gel + ampholytes + aqueous rEPO) mixture originating an initial current value of 23 μA. According to these results the presence of a carbohydrate and/or a detergent that are present in the commercial formulation of rEPO is not needed for separating several bands of glycoforms.

Salt could be needed to avoid protein denaturation. Moreover, the presence of a small amount of salt, namely sodium chloride, causing slight currents has been mentioned as a possible cause of improved focusing [17]. Furthermore, the adsorption of the Na^+ ions on uncoated silanols of the capillary wall reducing the interaction of EPO with the capillary wall cannot be ruled out [18].

This procedure for sample preparation makes the method easy to implement in different laboratories independently of the origin of the EPO sample under study (i.e. which can contain different types of excipients). The current practice only requires elimination of the original excipients in the rEPO and to add NaCl to get an initial current around 20 μA.

Under the optimized conditions, cIEF separation of the major glycoform bands of rEPO may be performed in less than 5 min (Fig. 2).

3.7. Selection of migration times and area indexes for improved precision

Different relationships between migration time of EPO glycoforms and migration time of the internal standard β-lactoglobulin were considered. Similarly, different relationships between corrected areas (the peak areas calculated taking into account the migration time) of the I.S. and EPO bands were compared.

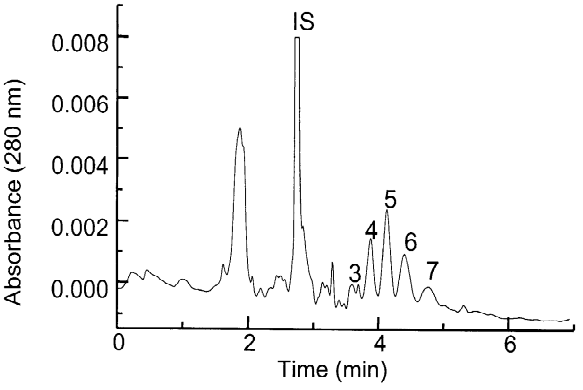

Fig. 2. cIEF electropherogram of rEPO. NaCl to a final concentration 10 m*M* was added to rEPO depleted of excipients. Anolyte, 91 m*M* H_3PO_4 in cIEF gel, pH 1.58 prepared by weight. Catholyte, 20 m*M* NaOH titrated to pH 11.86 with H_3PO_4. Other conditions as in Fig. 1.

The aim was to obtain parameters that are unaffected by small undesired changes in the analysis.

To select the best migration time and area indexes, five groups of experiments were performed. In each group different parameters were kept constant while small variations in other parameters were introduced.

3.7.1. Intra-assay precision

The first set of experiments was performed to calculate the precision of the assay using the same vial of sample, the same vial of anolyte (pH 1.58), and the same vial of catholyte (pH 11.86) for four analyses. Values of migration times and areas and different ratios between the values obtained for the major bands of the glycoforms of EPO and those obtained for the internal standard are shown with superscript e in Table 2.

It can be observed that the lowest relative standard deviation (RSD) for migration times is obtained when the $t_n/t_{I.S.}$ ratio, t_n being the migration time of each EPO band and $t_{I.S.}$ the migration time of β-lactoglobulin, is considered. For corrected areas the best precision is obtained for the $(A_n - A_{I.S.})/A_{I.S.}$ relationship.

Repeatability obtained in this way, with RSD≤ 0.5% and 1.5% for time and area indexes, respectively, compares favorably with values found in the literature for cIEF analysis. Values of RSD for migration time index $[(t_n - t_{I.S.1})/(t_{I.S.2} - t_n)]$ ≤ 4.7% [19], for migration time ≤0.9% [20,21], ≤1% [22,23], and ≤1.4% [24], and for peak area ≤2.1% [24], ≤5.4% [25], ≤6.0% [21], and ≤7.5% [22,23] have been reported.

3.7.2. Inter-sample preparation, inter-anolyte, and inter-catholyte precision

To estimate variations due to sample preparation, anolyte, and catholyte, experiments shown with superscripts f to h, respectively, in Table 2 were performed. It can be observed that in every case the best relationship of migration times corresponds to the $t_n/t_{I.S.}$ ratio, while the relationship $(A_n - A_{I.S.})/A_{I.S.}$ is always the best choice.

3.7.3. Usefulness of the migration time and area indexes for inter-assay comparison

The feasibility of using the selected migration times and area indexes for comparing results obtained under different conditions was studied. As data from different laboratories and different instruments were not available, three samples from different vials of the same batch of rEPO were analyzed. Samples were depleted of excipients independently. F2 was added to the samples to provide conditions conferring more variability than addition of a known concentration of NaCl. The three samples were prepared and analyzed more than 2 months apart in the same capillary. For this assay the focusing time was 6 min. Results are shown with superscript i in Table 2.

This part of the study is aimed to estimate the feasibility of comparing data of bands of glycoforms of EPO obtained from different samples with different anolyte and catholyte and in a capillary column that has been used for a long time even when some parameters of the method did not correspond to optimized values. It can be seen that differences in RSD for migration times were decreased from the range 24.9–30.5% to 0.7–1.8% when the index $t_n/t_{I.S.}$ instead of the absolute t_n values were considered. The usefulness of this index can be graphically seen in Fig. 3. For corrected areas, precision was also very much improved, with RSD 0.7 to 11.7% instead of 8.9 to 51.2% when $(A_n - A_{I.S.})/A_{I.S.}$ instead of A_n values were considered.

The large variability observed for the area index of one of the bands could be related to the fact that the third analysis was performed when the capillary had been used for more than 400 analyses. Actually, by considering only the first two injections, RSD values of $(A_n - A_{I.S.})/A_{I.S.}$ decreased markedly, being in the range 0.4 to 4.0%. Hundreds of runs in a given capillary had been reported, but limiting the pH of ampholytes to 8.5 or less was required [26], while in our case ampholytes in the range 2–10 were used.

4. Conclusions

The use of commercially available capillaries, the simplicity of sample preparation, the knowledge of the effect of electrolyte variations and how to control them, and the employment of relative indexes make this cIEF method precise and easy to implement for rEPO analysis in any laboratory.

Table 2
Precision (RSD, %) of the different indexes considered for migration time (min) and corrected area (arbitrary units)

	Mean[e]	RSD[e] (%)	Mean[f]	RSD[f] (%)	Mean[g]	RSD[g] (%)	Mean[h]	RSD[h] (%)	Mean[i]	RSD[i] (%)
$t_{I.S.}$[a]	2.76	2.0	2.87	1.1	2.89	0.8	2.71	5.4	6.19	26.2
t_3[b]	3.61	1.8	3.73	1.5	3.75	0.9	3.49	6.2	8.18	26.9
t_4	3.90	1.8	4.00	1.8	4.01	1.2	3.72	6.7	8.65	25.9
t_5	4.14	1.7	4.29	1.8	4.29	1.2	3.98	7.1	8.02	30.5
t_6	4.41	1.6	4.57	2.5	4.57	1.3	4.20	7.7	9.32	26.0
t_7	4.76	2.3	4.95	2.2	4.97	1.3	4.56	8.6	9.74	24.9
$t_3/t_{I.S.}$	1.31	0.2	1.30	0.5	1.30	0.5	1.29	0.8	1.32	0.9
$t_4/t_{I.S.}$	1.41	0.2	1.39	0.8	1.39	0.5	1.37	1.4	1.40	1.4
$t_5/t_{I.S.}$	1.50	0.3	1.49	0.8	1.49	0.5	1.46	1.7	1.45	0.9
$t_6/t_{I.S.}$	1.60	0.4	1.59	1.7	1.58	0.5	1.55	2.4	1.51	0.7
$t_7/t_{I.S.}$	1.73	0.5	1.73	1.2	1.72	0.6	1.68	3.4	1.58	1.8
$(t_3 - t_{I.S.})/t_{I.S.}$	0.31	0.9	0.30	2.1	0.30	2.4	0.29	3.4	0.32	3.9
$(t_4 - t_{I.S.})/t_{I.S.}$	0.41	0.8	0.39	2.7	0.39	1.9	0.37	5.0	0.40	4.8
$(t_5 - t_{I.S.})/t_{I.S.}$	0.50	1.0	0.49	2.4	0.49	1.4	0.46	5.3	0.45	2.9
$(t_6 - t_{I.S.})/t_{I.S.}$	0.60	1.1	0.59	4.7	0.58	1.5	0.55	6.8	0.51	2.0
$(t_7 - t_{I.S.})/t_{I.S.}$	0.73	1.1	0.73	2.9	0.72	1.5	0.68	8.4	0.58	4.9
$A_{I.S.}$[c]	11.70	5.3	13.60	7.6	13.60	5.3	14.30	6.7	6.50	18.7
A_3[d]	0.46	9.1	0.61	11.0	0.61	14.7	0.66	9.6	0.74	25.7
A_4	1.48	2.3	1.76	1.7	1.76	4.0	1.96	5.2	1.19	23.7
A_5	2.09	2.1	2.46	3.8	2.46	5.4	2.83	3.1	1.41	8.9
A_6	1.42	2.8	2.05	4.3	2.05	8.2	2.09	4.9	1.42	51.2
A_7	0.63	10.5	0.95	1.4	0.95	7.9	0.99	6.7	0.39	14.3
$A_3/A_{I.S.}$	0.04	13.6	0.04	17.1	0.04	15.9	0.05	9.7	0.11	14.7
$A_4/A_{I.S.}$	0.13	7.2	0.13	7.7	0.13	8.1	0.14	10.9	0.18	8.3
$A_5/A_{I.S.}$	0.18	6.8	0.18	7.0	0.18	9.7	0.20	8.4	0.22	12.9
$A_6/A_{I.S.}$	0.12	6.9	0.15	9.7	0.15	12.1	0.15	8.5	0.22	42.3
$A_7/A_{I.S.}$	0.05	9.4	0.07	6.7	0.07	11.1	0.07	5.6	0.06	10.3
$(A_3 - A_{I.S.})/A_{I.S.}$	−0.96	0.6	−0.96	0.5	−0.96	0.7	−0.95	0.5	−0.89	1.9
$(A_4 - A_{I.S.})/A_{I.S.}$	−0.87	1.0	−0.87	1.1	−0.87	1.2	−0.86	1.7	−0.82	1.9
$(A_5 - A_{I.S.})/A_{I.S.}$	−0.82	1.5	−0.82	1.5	−0.82	2.1	−0.80	2.1	−0.78	3.7
$(A_6 - A_{I.S.})/A_{I.S.}$	−0.88	1.0	−0.85	1.7	−0.85	2.2	−0.85	1.5	−0.78	11.7
$(A_7 - A_{I.S.})/A_{I.S.}$	−0.95	0.5	−0.93	0.5	−0.93	0.8	−0.93	0.4	−0.94	0.7

[a] $t_{I.S.}$, migration time of the internal standard (β-lactoglobulin A).
[b] t_n, migration time of the major bands of EPO.
[c] $A_{I.S.}$, corrected area of β-lactoglobulin A.
[d] A_n, corrected area of the major bands of EPO.
[e] Same sample, same catholyte (pH 11.86), and same anolyte (pH 1.58) are used for all injections ($n=4$). Focusing time, 1 min.
[f] Same catholyte (pH 11.85) and anolyte (pH 1.52) are used for all injections ($n=4$). Sample vial is changed for every injection. Focusing time, 1 min.
[g] Same catholyte (pH 11.85) and sample vial are used for all injections ($n=5$). Anolyte is changed for every injection (pH values: 1.52, 1.53, 1.53, 1.57, and 1.60). Focusing time, 1 min.
[h] Same anolyte (pH 1.53) and sample vial are used for all injections ($n=5$). Catholyte is changed for every injection (pH values 11.77, 11.88, 11.85, 11.88, and 11.89). Focusing time, 1 min.
[i] Anolyte, catholyte, and sample vial are changed for every injection ($n=3$). Focusing time, 6 min.

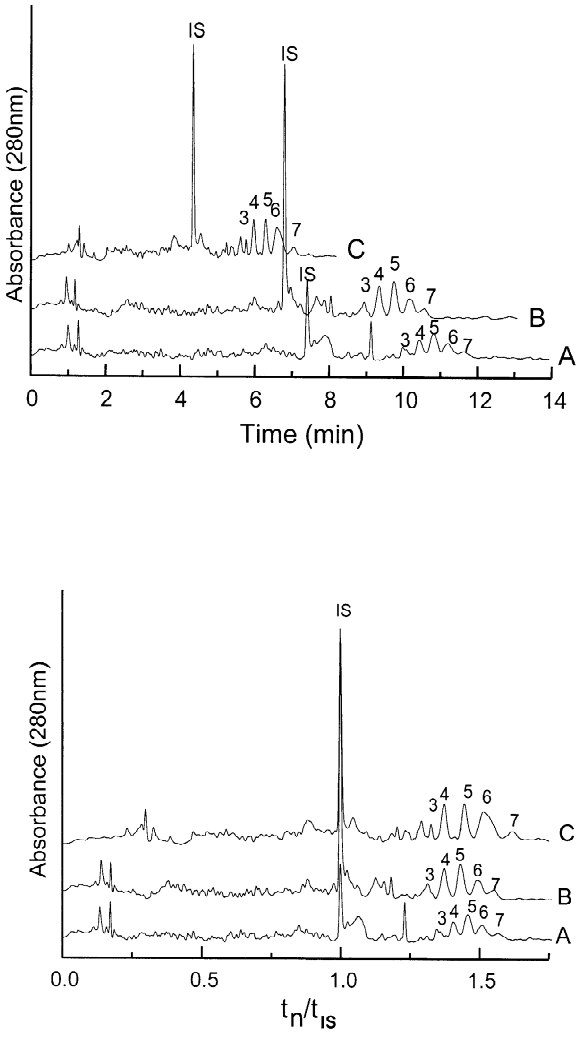

Fig. 3. cIEF electropherograms of rEPO. Upper part of the figure corresponds to representation considering absolute migration times. Lower part of the figure corresponds to representation considering relative index of migration time. Focusing time was 6 min. Anolytes (91 m*M* H_3PO_4 in cIEF gel) with pH 1.47, 1.48, and 1.53 and catholytes (20 m*M* NaOH titrated with H_3PO_4) with pH 11.91, 11.90, and 11.90 were used in (A), (B), and (C), respectively. (A) and (B) correspond to analyses performed within 1 week; (C) was performed 2 months later. Other conditions as in Fig. 1.

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References

[1] R.E. Shamel, M. Keough, Gen. Eng. News 15 (1995) 6.
[2] M.N. Fukuda, H. Sasaki, L. Lopez, M. Fukuda, Blood 73 (1989) 84.
[3] P.L. Storring, R.E. Gaines Das, J. Endocrinol. 134 (1992) 459.
[4] A. Cifuentes, M.V. Moreno-Arribas, M. de Frutos, J.C. Diez-Masa, J. Chromatogr. A 830 (1999) 453.
[5] Pharmeuropa BIO, Vol. 2, 1997, p. 31.
[6] A. Cifuentes, P. Canalejas, A. Ortega, J.C. Diez-Masa, J. Chromatogr. A 823 (1998) 561.
[7] T.-L. Huang, M. Richards, J. Chromatogr. A 757 (1997) 247.
[8] P.G. Righetti, in: Isoelectric Focusing: Theory, Methodology, and Applications, Elsevier Biomedical, Amsterdam, 1989, p. 1.
[9] R.A. Mosher, W. Thormann, Electrophoresis 11 (1990) 717.
[10] S. Hjerten, in: P.D. Grossman, J.C. Colburn (Eds.), Capillary Electrophoresis—Theory and Practice, Academic Press, San Diego, CA, 1992, p. 191.
[11] J.R. Mazzeo, I.S. Krull, Anal. Chem. 63 (1991) 2852.
[12] R.M. McCormick, Anal. Chem. 60 (1988) 2322.
[13] B.A. Morrow, S.J. Lang, I.D. Gay, Langmuir 10 (1994) 756.
[14] T. Manabe, H. Miyamoto, A. Iwasaki, Electrophoresis 18 (1997) 92.
[15] J.R. Mazzeo, I.S. Krull, in: N.A. Guzman (Ed.), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, p. 795.
[16] J.R. Mazzeo, I.S. Krull, J. Chromatogr. 606 (1992) 291.
[17] N.G. Clarke, A.J. Tomlinson, G. Schomburg, S. Naylor, Anal. Chem. 69 (1997) 2786.
[18] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63.
[19] A.A. Mohammad, A.O. Okorodudu, M.G. Bissell, P. Dow, G. Reger, A. Meier, P. Guodagno, J.R. Petersen, Clin. Chem. 43 (1997) 1798.
[20] C. Joccheim, S. Novick, A. Balland, J. Mahan-Boyce, W.-C. Wang, A. Goetze, W. Gombotz, Chromatographia 53 (2001) S59.
[21] S. Tang, D.P. Nesta, L.R. Maneri, K.R. Anumula, J. Pharm. Biomed. Anal. 19 (1999) 569.
[22] N. Mario, B. Baudin, C. Aussel, J. Giboudeau, Clin. Chem. 43 (1997) 2137.
[23] N. Mario, B. Baudin, J. Giboudeau, J. Chromatogr. B 706 (1998) 123.
[24] G. Hunt, T. Hotaling, A.B. Chen, J. Chromatogr. A 800 (1998) 355.
[25] A.M. Lasdun, R.R. Kurumbail, N.K. Leimgruber, A.S. Rathore, J. Chromatogr. A 917 (2001) 147.
[26] R. Rodríguez-Díaz, M. Zhu, T. Wehr, J. Chromatogr. A 772 (1997) 145.