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Separation of carbohydrate-mediated microheterogeneity of recombinant human erythropoietin by free solution capillary electrophoresis

Effects of pH, buffer type and organic additives

AN D. TRAN*, SUNGAE PARK and PETER J. LISI
BioAnalytical Research Department, R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation, Route 202, Raritan, NJ 08869 (U.S.A.)
and
OANH T. HUYNH, RALPH R. RYALL and PHILIP A. LANE
Analytical R & D Department, R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation, Route 202, Raritan, NJ 08869 (U.S.A.)

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ABSTRACT

Free solution capillary electrophoresis has been investigated as an alternative to isoelectric focusing for the separation of the glycoforms of recombinant human erythropoietin (r-HuEPO), a primary regulator of erythropoiesis. A systematic approach was used to study the effect of pH, buffer type and organic modifiers on the resolution of the microheterogeneity of erythropoietin. The main factors for improving the resolution were the regulation of the electroosmotic flow of the running buffer and the reduction of solute-wall interaction. The best resolution of the glycoforms of r-HuEPO was obtained with a mixed buffer pH 4.0 (100 m*M* acetate–phosphate, 10 h prcequilibration time).

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone which is produced primarily in the kidney of adult mammals and which acts on bone marrow erythroid progenitor cells to promote development into mature red blood cells [1]. The molecular mass of EPO is in the range of 34 000–38 000 with approximately 40% of its weight attributed to its carbohydrate structure [2]. The molecule has a peptidic backbone of 165 amino acids (p*I* 4.5–5.0) and contains two types of carbohydrates: three N-linked complex polysaccharide antennaries at the asparagine positions 24, 38 and 83, and one O-linked polysaccharide chain at the serine position 126 [2].

Since EPO is present at picomolar levels in the serum and urine, it was not purified until 1977 [3] and it has only recently become available as recombinant human EPO (r-HuEPO). Like the naturally occurring hormone, r-HuEPO exhibits microheterogeneity[a] in the charged carbohydrate moieties bound to the protein. As the only

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major charge-bearing component of the r-HuEPO carbohydrate, sialic acid has been shown to be the origin of different charge classes of r-HuEPO (Fig. 1). These classes differ from each other in the degree of sialylation of the polysaccharide chains [4], where sialic acid is linked to the terminal end of the polysaccharide chain through the hydroxy group located at position 2 of the pyran ring.

The critical role of sialic acid in the *in vivo* activity of r-HuEPO has been well established [5]. Therefore, it is of interest to examine the relative proportion of the glycoforms[b] contained in the purified r-HuEPO.

Currently, the technique of isoelectric focusing (IEF) offers an unrivaled method for screening the pattern of glycoforms [6]. Because of the labor involved in IEF using conventional slab gels and the semi-quantitative nature of the assay, capillary electrophoresis (CE) has been investigated as an alternative method for determining the ratio of EPO glycoforms. This technique is based on the same principles as traditional slab gel electrophoresis, but offers on-line injection and detection. Although spectacular results have been reported in the use of the IEF mode in capillary electrophoresis [7,8], the technique does require the use of a coated capillary column (*i.e.* polyacrylamide or methylcellulose). Currently, because of the short lifetime of these columns under severe operating conditions such as wide pH range and high operating voltage, the IEF mode of electrophoresis in capillary columns is not yet suitable for routine analysis. Among different CE modes, free solution capillary electrophoresis (FSCE) is the simplest and the most widely used. We report here the use of FSCE as an alternative method to IEF for the separation of the glycoforms of r-HuEPO. The effects of separation variables such as pH, buffer type and organic modifiers (methanol, acetonitrile, ethylene glycol, polyethylene glycol) were evaluated as part of this study.

MATERIALS AND METHODS

Apparatus

Separations were carried out on a capillary electropherograph System P/ACE

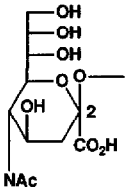

Fig. 1. Structure of sialic acid (N-acetyl neuraminic acid). Ac = Acetyl.

[a] Microheterogeneity, as used herein, is defined as variation in carbohydrate primary structure between molecules of a glycoprotein, such as variation in the number of terminal sialic acid residues with the same chain length or variation in the number of branches (*e.g.* di-, tri-, tetraantennary structures of N-linked chains).

[b] Glycoform, as used herein, is defined as a subset of glycoprotein molecules sharing an identical polypeptide backbone but differing in carbohydrate structure (*i.e.*, sequence or disposition).

2000 (Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with a capillary cartridge, 75 μm I.D. × 375 μm O.D.; the total length of the capillary was 27 cm (20 cm effective length) for runs at low pH values (2.0, 3.0 and 4.0) and 57 cm (50 cm effective length) for runs at higher pH values (6.0, 7.0, 8.0 and 9.0). Prior to use the capillary was pretreated successively with 0.1 *M* HCl and 0.1 *M* NaOH for 10 min each, then rinsed with water and electrolyte. The column temperature was maintained at 25 ± 0.1°C by means of a fluorocarbon liquid continuously circulated through the cartridge. A deuterium light source with a 214-nm bandpass filter was used and absorbance was monitored at a range of 0.02 a.u.f.s. Injection was made by nitrogen pressure with sample concentrations ranging from 0.1–0.3 mg/ml. Data analysis and collection was accomplished using the Beckman System P/ACE 2000 software, version 1.0.

Materials

The phosphate buffers (100 m*M*, pH 2.0, 3.0 and 4.0) were prepared by titrating a solution of 0.1 *M* phosphoric acid (Baxter Health Care Corp., McGraw Park, IL, U.S.A.) with 0.1 *M* sodium hydroxide to the desired pH. The acetate buffer (100 m*M*, pH 4.0) was prepared by titrating a solution of 0.1 *M* of acetic acid (Mallinckrodt, Paris, KY, U.S.A.) with a solution of 0.1 *M* sodium acetate (Mallinckrodt) to a desired pH. The acetate–phosphate buffer (100 m*M*, pH 4.0) was prepared by titrating a solution of 0.1 *M* sodium acetate with a solution of 0.1 *M* phosphoric acid to the desired pH. The phosphate buffer (100 m*M*, pH 4.0) was prepared by titrating a solution of 0.1 *M* of phosphate monobasic with a solution of 0.1 *M* phosphoric acid. The 2-[N-morpholino]ethanesulfonic acid (MES) (50 m*M*, pH 6.0), bis[2-hydroxyethyl]imino–tris[hydroxymethyl]methane (Bis–Tris) (50 m*M*, pH 7.0), and tricine (50 m*M*, pH 8.0) were prepared by titrating a solution of 0.05 *M* of the corresponding product (Sigma, St. Louis, MO, U.S.A.) with a solution 1.0 *M* hydrochloric acid to the corresponding pH.

Viscosity measurements

The kinematic viscosities and densities of buffer solutions were measured using the Cannon-Ubbelohde viscometer and a Kimble pycnometer, respectively. The absolute viscosity of a solution is a product of the kinematic viscosity and density. All measurements were performed at 25 ± 0.05°C and are the mean of five independent measurements for each buffer solution.

RESULTS AND DISCUSSIONS

Effect of pH and organic modifiers in the separation of r-HuEPO glycoforms

In FSCE, ionic species are separated on the basis of the differential electrophoretic mobilities of the analytes. According to Jorgenson and Lukacs [9] the resolution (R_s) of two zones in FSCE can be given by eqn. 1:

$$R_s = 0.18\,\Delta u_{ep}[Vl/DL(u_{eo} + u_{ep})]^{1/2} \quad (1)$$

where u_{ep} is the electrophoretic mobility of an ionic solute, u_{eo} is the electroosmotic flow of the running buffer, V is the applied voltage, D is the diffusion coefficient of the

ionic solute in the running buffer system, l and L are the effective length of the capillary column between the inlet and the detector and the total length of the capillary respectively.

As indicated in eqn. 1, the resolution in FSCE could in principle be improved by increasing the difference in electrophoretic mobility of the two separated zones or by reducing the electroosmotic flow of the running buffer. To test this hypothesis, our initial attempts at separating the glycoforms of r-HuEPO were performed at pH 6.0, 7.0, 8.0 and 9.0 of the running buffer. At these pH values, r-HuEPO (pI 4.5–5.0) exists as a negative species and the interaction between solute and the capillary wall is minimized. Fig. 2 illustrates the overlayed electropherograms of r-HuEPO obtained at pH 6.0, 7.0, 8.0 and 9.0. At pH 9.0 where the osmotic flow is the fastest in the range of pH selected, r-HuEPO eluted as a sharp peak with no separation of glycoforms observed. Some sign of separation was observed at a lower pH of 8.0 or 7.0 and clearly improved at pH 6.0. At this pH, a combination of two effects, reduction in the electrophoretic flow and increase in differences in charge between glycoforms are presumably the origin for the separation observed.

To ascertain that these multiple peaks originate from the microheterogeneity of the product, r-HuEPO was incubated with neuraminidase, an enzyme known to selectively remove sialic acid from the polysaccharide backbone. The neuraminidase treated r-HuEPO converged into a single peak when subjected to capillary electrophoresis. These results strongly suggest that the multiple peaks observed at pH 6.0 originate from the microheterogeneity of r-HuEPO. To further improve the separation between glycoforms, the osmotic flow of the running buffer was reduced by increasing the viscosity of the solution. Several organic modifiers with different viscosities and dielectric constants were selected and are listed in Table I. The main consequences

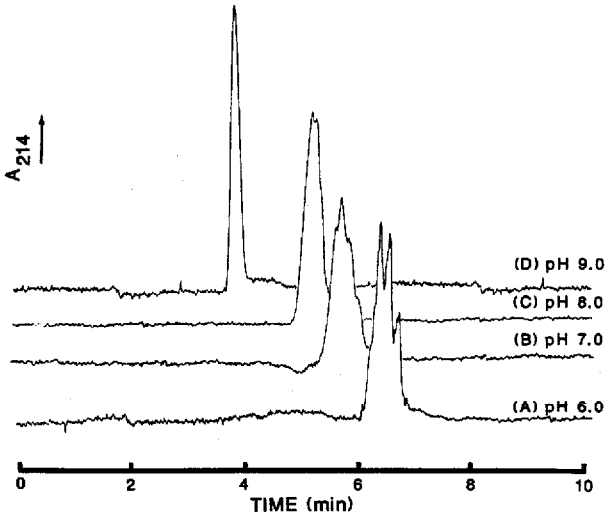

Fig. 2. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 50 cm × 75 μm I.D. (A) pH 6.0 (50 m*M* MES, 25 kV, 44 μA); (B) pH 7.0 (50 m*M* Bis-Tris, 25 kV, 15 μA); (C) pH 8.0 (50 m*M* tricine, 25 kV, 70 μA); (D) pH 9.0 (50 m*M* tricine, 25 kV, 85 μA).

TABLE I

LIST OF DIFFERENT ORGANIC MODIFIERS USED IN THE MANIPULATION OF VISCOSITY OF THE RUNNING BUFFER

Alcohol	Diols	Others
Methanol [11]	Ethylene glycol	Acetonitrile [12,13]
Ethanol	Glycerol	Sucrose
1-Propanol [12]	1,2-Butanediol	
2-Butanol	1,3-Butanediol	
	1,4-Butanediol	
	trans-Cyclohexane-1,2-diol	
	cis,trans-Cyclohexane-1,2-diol	
	PEG-200	
	PEG-400	

resulting from the addition of an organic modifier to a running buffer consisted of multiple changes, *i.e.* of the viscosity and the ionic strength of the buffer. However, an effect such as the binding of the organic modifier to the capillary wall, through hydrogen bonding or dipole interaction, could induce a drastic change of the osmotic flow caused by the change of the net charge of the capillary surface or the local viscosity of the double layer [10]. To simplify the interpretation of the observed results, the ionic strength of the organic–buffer solution was kept constant during the addition of the organic modifier. Therefore the change of the osmotic flow induced by the presence of organic modifier in a capillary column is mainly due to the change of the viscosity of the resulting buffer, and the change of the net charge of the capillary wall resulting from the binding of the organic molecule to the capillary wall. The selection of organic modifiers (*i.e.* positional isomers 1,2-, 1,3- and 1,4-butanediol or isomers of *cis,trans*-cyclohexane-1,2-diol) was intended to detect the effect of binding of diol compounds onto the capillary wall since the viscosities of the neat solvent of the corresponding isomer compounds are identical. Different binding constants of corresponding isomers onto the wall could induce different changes of the osmotic flow of the running buffer.

Figs. 3 and 4 illustrate the effect of organic additives on the viscosity and the electroosmotic flow of the running buffer tricine buffer (50 m*M*, pH 8.0). In the series of organic additives selected (Table I), acetonitrile is the least efficient in modifying the viscosity of the running buffer. Acetonitrile could be added up to 30% (v/v) without inducing a significant change in the viscosity of the running buffer while a different pattern was observed for the osmotic flow (Fig. 4). With 5% (v/v) of acetonitrile added to the running buffer, a 10% change was observed for the osmotic flow which stayed constant within the range of 5–25% of acetonitrile added. The change of osmotic flow observed with 5% of acetonitrile could originate from the dipole interaction of acetonitrile with the capillary wall causing a change in the net charge and the local viscosity of the double layer. The binding of acetonitrile to the wall seems to be saturated at the 5% level of organic solvent added since the osmotic flow remained constant within the range of 5–25% of acetonitrile added. A significant reduction of the osmotic flow up to 40% was observed when the level of added acetonitrile reached 30% and the phenomena still remined unclear. These results are in agreement with the

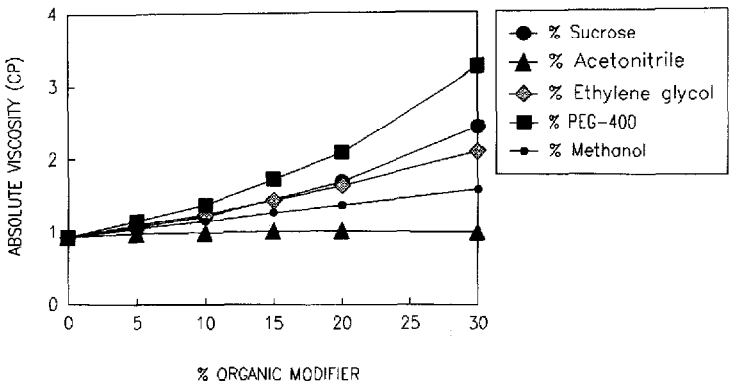

Fig. 3. Absolute viscosity as a function of the percentage of organic modifier. For conditions see Experimental.

recently reported results by VanOrman *et al.* [10]. On the other hand, methanol was shown to be clearly more efficient in increasing the viscosity and decreasing the electroosmotic flow of the buffer when compared to acetonitrile (Figs. 3 and 4). The dependency of the electroosmotic flow on the chain length of the alcohol follows the order: methanol < ethanol < propanol < butanol. Diol compounds are more efficient than alcohol in increasing the viscosity or decreasing the electroosmotic flow of the running buffer. As indicated by the viscosities of the neat solvent, the efficacy of all diol compounds selected increased from ethylene glycol < glycerol < 1,2-butanediol, 1,3- or 1,4-butanediol < *trans* or *cis,trans*-cyclohexane-1,2-diol < PEG-200 < PEG-400 and are clearly more efficient than methanol. The geometry or the location of the diol groups (*i.e.* 1,2-, 1,3- and 1,4-butanediol and *cis,trans*-cyclohexane-1,2-diol) does not play an important role in the binding to the capillary wall as indicated by their effect on the osmotic flow.

When acetonitrile was used as the organic modifier, the separation of the glycoforms of r-HuEPO was evident as illustrated in Fig. 5. At a level of 5%

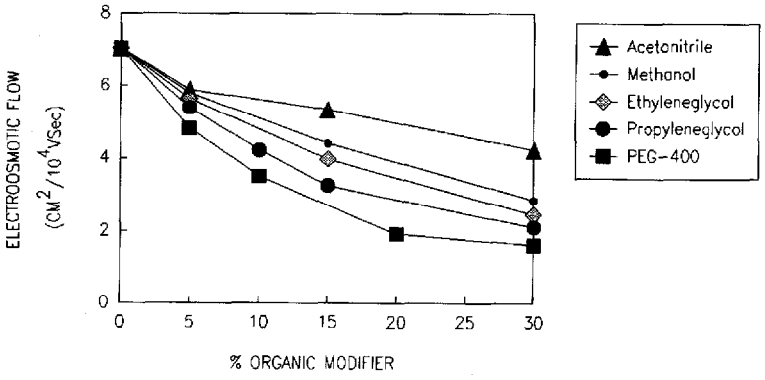

Fig. 4. Effect of organic modifier on the electroosmotic flow, u_{eo}, of the running buffer. Conditions: fused-silica capillary column 50 cm × 75 μm I.D.; voltage 25 kV.

acetonitrile in tricine buffer (50 m*M*, pH 8.0), the product eluted as a multiplet, however the resolution deteriorated when more organic modifier was added.

In contrast, when methanol or ethylene glycol was used as the organic modifier, the separation of glycoforms improved between 5% and 30% of organic modifiers (Figs. 6 and 7) and began to decrease at higher concentrations. When the percentage of methanol and ethylene glycol reached the 50% level, the resolution clearly deteriorated. These results were observed for all linear diols or polydiols selected (Table I). The same results were obtained at pH 6.0 (50 m*M*, MES).

Since the electrophoretic mobility is dependent on the osmotic flow of the running buffer (eqn. 2), a plot of the product of viscosity and the electrophoretic mobility *versus* the percent of organic solvent illustrates the effect of organic additives on the ionic state and shape of r-HuEPO during electrophoresis.

$$u_{ep} = v/E = q/6\pi r\eta \tag{2}$$

where u_{ep} is the electrophoretic mobility, v is the migration velocity, E is the electric field strength, q is the net charge on the molecule, η the solvent viscosity and r is the apparent Stokes radius of the molecule.

Fig. 8 shows the plot of $u_{ep} \cdot \eta$ *vs.* the percent of organic additives. The origin of a negative slope observed for acetonitrile may be due to the change in ionic state of r-HuEPO caused by the low dielectric constant of the solvent used or the change in conformation of the product. Measurement of the UV and UV second derivatives spectra of r-HuEPO in the presence of 30% of acetonitrile in 50 m*M* tricine buffer (pH 8.0) corroborate this hypothesis. A shift to the far UV was observed when the UV spectrum of r-HuEPO was recorded in 30% acetonitrile in tricine buffer (50 m*M*, pH 8.0).

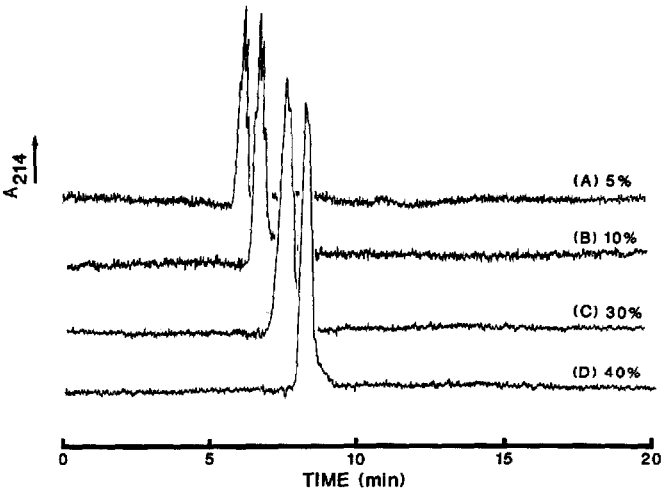

Fig. 5. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 50 cm × 75 μm I.D. (A) 5% Acetonitrile in 50 m*M* tricine buffer pH 8.0 (25 kV; 18 μA); (B) 10% acetonitrile in 50 m*M* tricine buffer pH 8.0 (25 kV; 16 μA); (C) 30% acetonitrile in 50 m*M* tricine buffer pH 8.0 (25 kV; 14 μA), (D) 40% acetonitrile in 50 m*M* tricine buffer pH 8.0 (25 kV; 12 μA).

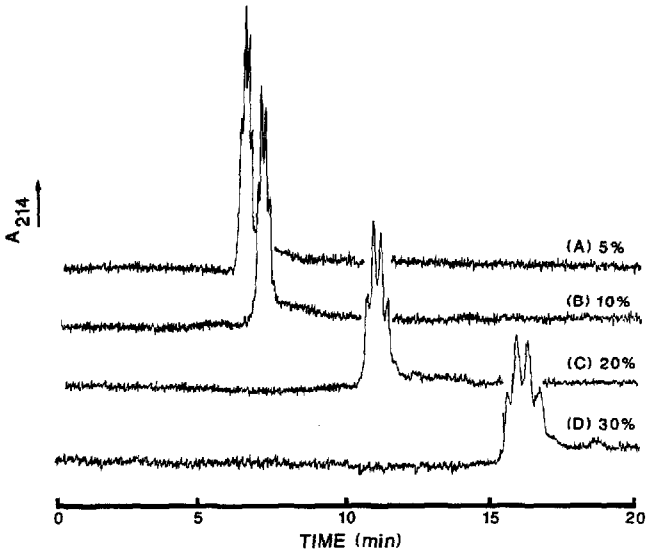

Fig. 6. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 50 cm × 75 μm I.D. (A) 5% methanol in 50 m*M* tricine buffer pH 8.0 (25 kV; 20 μA); (B) 10% methanol in 50 m*M* tricine buffer pH 8.0 (25 kV; 18 μA); (C) 30% methanol in 50 m*M* tricine pH 8.0 (25 kV; 15 μA), (D) 30% methanol in 50 m*M* tricine buffer pH 8.0 (25 kV; 13 μA).

Similar to acetonitrile, the addition of methanol to the running buffer also induces a negative slope on the plot of the product of electrophoretic mobility and viscosity *versus* percent organic modifier (Fig. 8). However when compared to

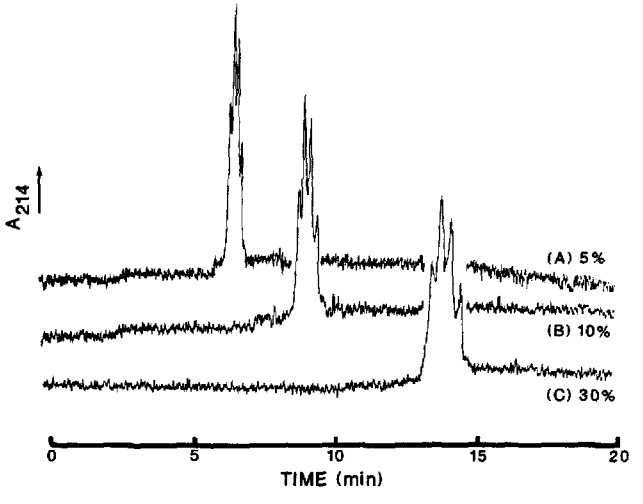

Fig. 7. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 50 cm × 75 μm I.D. (A) 5% ethylene glycol in 50 m*M* tricine buffer pH 8.0 (25 kV; 27 μA); (B) 10% ethylene glycol in 50 m*M* tricine buffer pH 8.0 (25 kV; 21 μA); (C) 30% ethylene glycol in 50 m*M* tricine buffer pH 8.0 (25 kV; 10 μA).

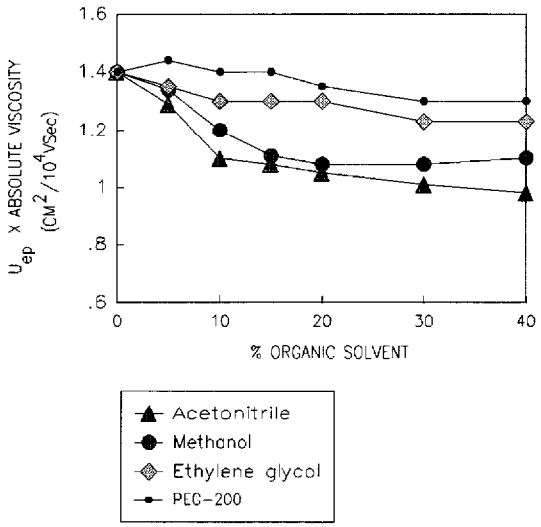

Fig. 8. Plot of the product of electroosmotic flow and viscosity of glycoforms of r-HuEPO as a function of the organic modifier. For conditions see Fig. 4.

acetonitrile, the slope is slightly less pronounced when compared to acetonitrile. A shift to the far UV was observed when the UV and its second derivative spectra of r-HuEPO were recorded in 30% methanol in tricine buffer (50 m*M*, pH 8.0). With polydiols, and especially polyethyleneglycol, the slope observed is clearly much less affected by increasing the concentration of organic modifier (Fig. 9). Unlike methanol and acetonitrile, the addition of PEG-200 to the running buffer does not cause a drastic change in the dielectric constant of the media, and provides less perturbation of the ionic state of the protein.

Effect of acetate, phosphate and sulphate ions in the separation of r-HuEPO at low pH

At low pH, the resolution in FSCE should in principle be improved since the electroosmotic flow of the bulk solution is drastically reduced [14,15]. Our initial attempts at separating the glycoforms of r-HuEPO were performed at pH 2.0, 3.0 and 4.0 of the running buffer. At these pH values r-HuEPO (p*I* 4.5–5.0) exists as a positive species. Fig. 9 shows an overlay of three electropherograms of r-HuEPO performed at pH 2.0 (100 m*M* phosphate buffer), pH 3.0 (100 m*M* phosphate buffer) and pH 4.0 (100 m*M* acetate buffer). At pH 2.0, where the osmotic flow is the slowest in the range of pH selected, r-HuEPO eluted as a sharp peak with some separation observed. The poor peak shape observed at pH 3.0 is not due to sample overload on the column but rather originates from the microheterogeneity of the product. A solute–capillary interaction is also possible since the operating pH is below the p*I* of the r-HuEPO.

At pH 4.0, the product eluted as a broad single peak. Unlike the migration of r-HuEPO occurred at the pH of 2.0 and 3.0, the product eluted behind the neutral marker. Since the operating pH is closer to the p*I* of the product, the overall positive charge of the molecule is reduced and the friction between solute and the buffer is dominant.

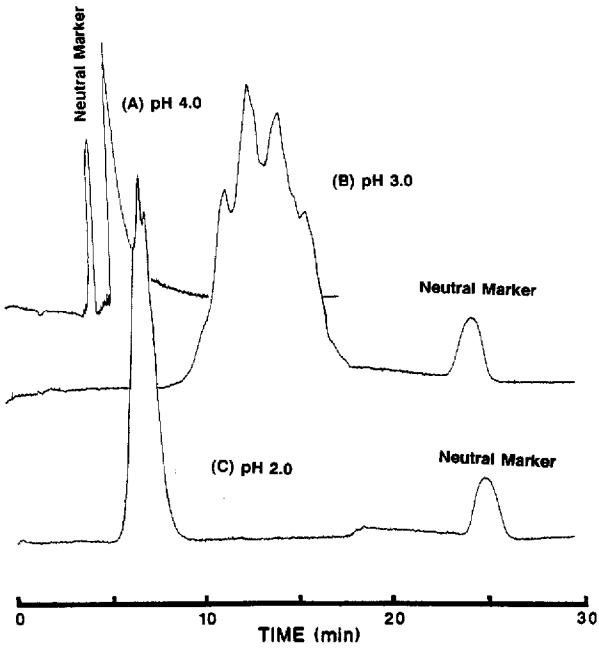

Fig. 9. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 20 cm × 75 μm I.D. (A) pH 2.0 (100 m*M* phosphate buffer, 10 kV, 65 μA); (B) pH 3.0 (100 m*M* H_3PO_4, 10 kV, 80 μA); (C) pH 4.0 (100 m*M* acetate buffer, 10 kV, 3 μA). Phenol was used as neutral marker.

The critical role of the phosphate ion in improving the separation was demonstrated when a mixed buffer (100 m*M* acetate–phosphate, pH 4.0) was used. The sample was resolved into four different major and one minor glycoforms (Fig. 10B). The presence of phosphate resulted in a better separation. A decrease in the migration time of the product in the mixed buffer, as compared to the migration time obtained with the acetate buffer (100 m*M*, pH 4.0) was due to a change in electroosmotic flow. These results may indicate, as suggested by McCormick [15], that phosphate binds strongly to the silica surface of the capillary wall. Such binding could convert the residual acidic silanols to a more easily protonated silica–phosphate complex at pH 4.0, thereby reducing surface charge on the capillary wall. To obtain good and reproducible results (*e.g.* electrophoretic mobility, area counts and electroosmotic flow relative standard deviation $<2\%$) the column must be equilibrated in the mixed buffer for 4 h before use. Reproducible results can be achieved for at least 30 injections as long as no washing step with NaOH and waiting period are included into the running sequence. If the column starts to lose its performance, the phosphate layer can be regenerated by washing the column with NaOH (0.1 *M*). The capillary column must be equilibrated in the mixed buffer (100 m*M* acetate–phosphate buffer, pH 4.0) for 4 h before use.

The migration time of the product and the electroosmotic flow of the running buffer was further decreased with the mixed buffer (100 m*M* acetate–sulphate, pH 4.0) (Fig. 10C); however resolution of the glycoforms is not as good as observed with the acetate–phosphate buffer.

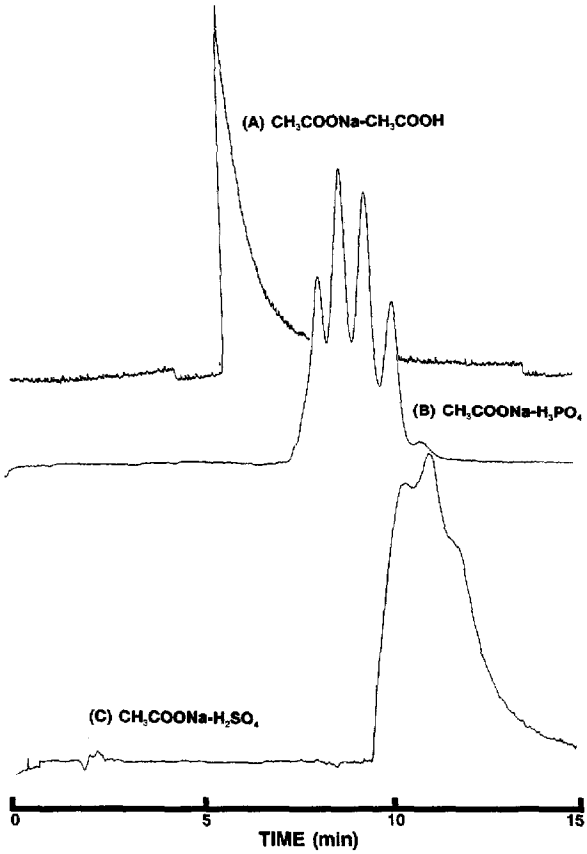

Fig. 10. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 20 cm × 75 μm I.D. (A) pH 4.0 (100 m*M* acetate buffer, 10 kV, 30 μA); (B) pH 4.0 (100 m*M* acetate–phosphate buffer, 10 kV, 120 μA); (C) pH 4.0 (100 m*M* acetate–sulfate buffer, 10 kV, 200 μA).

Better separation was obtained when equilibration time of the column is extended to 10 h (Fig. 11) prior to use.

The long period required for equilibration time of the column before use is due to the presence of the acetate ions in the buffer. The same resolution can be achieved with much shorter preequilibration time (30 min) when phosphate buffer pH 4.0 is used. Since this buffer did not have a good buffering capacity, the inlet and outlet reservoir of the running buffer needs to be replaced after every three runs to avoid the pH drop. Such change will cause a lack of reproducibility from run to run. Because of the short equilibration time required, the pH 4.0 phosphate buffer is useful for proteins which need to be washed with NaOH after each run.

CONCLUSIONS

This work provides very encouraging results for the separation of the glycoforms of r-HuEPO using FSCE with a fused silica at low pH. The best condition found was

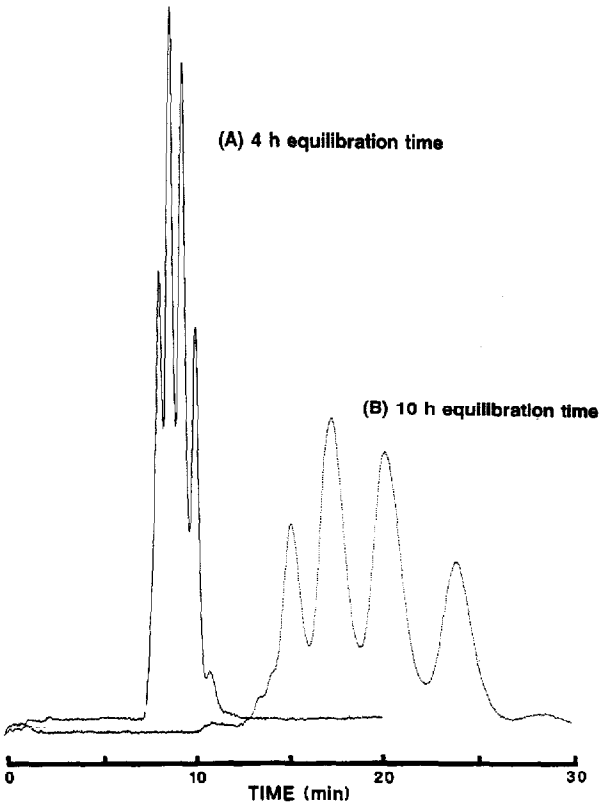

Fig. 11. High-performance capillary electrophoresis of r-HuEPO in free solution. The electropherograms were obtained using a fused-silica capillary tube 20 cm × 75 μm I.D. (A) pH 4.0 (100 m*M* acetate–phosphate buffer, 10 kV, 120 μA); the column was filled with the running buffer and equilibrated for 4 h prior to use. (B) same conditions as (A) with the column equilibrated in the running buffer for 10 h prior to use.

with buffer pH 4.0 (100 m*M* acetate–phosphate, 10 h preequilibration time). The resolution of glycoforms obtained by this technique is close to those obtained by conventional isoelectric focusing slab gel.

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