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Analysis of recombinant human erythropoietin in drug formulations by high-performance capillary electrophoresis

Henri P. Bietlot, Michel Girard*

Bureau of Drug Research, Health Canada, Sir. F.G. Banting Research Centre, Tunney's Pasture, Ottawa, Ont. K1A 0L2, Canada

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Abstract

A high-performance capillary electrophoresis (HPCE) method was developed for the analysis of recombinant human erythropoietin (rhEPO) in final drug preparations. All products examined were formulated with large amounts of human serum albumin (HSA) which is used as a protein excipient. Due to their similar physical characteristics in solution, rhEPO and HSA could not be resolved under HPCE conditions previously developed for the separation of bulk rhEPO. Addition of 1 m*M* nickel chloride to the electrophoretic buffer allowed complete separation of the two proteins as well as separation of rhEPO into several glycoform populations. The method was linear over the concentration range of 0.03–1.92 mg/ml, with limits of detection and of quantitation of 0.01 and 0.03 mg/ml, respectively. The precision of the method was evaluated from intra- and inter-day replicate injections of both rhEPO standard solution and formulation. Components of within- and between-batch variances were consistently below 5%, which constituted an acceptable level of variation. Products from two manufacturers were analyzed and showed little qualitative but appreciable quantitative lot-to-lot variations for rhEPO content when expressed in terms of units of biological activity. The method also revealed qualitative differences between the two products.

Keywords: Pharmaceutical analysis; Erythropoietin; Proteins; Recombinant proteins; Albumin; Glycoproteins

1. Introduction

Recombinant DNA technology has been successfully applied to the cloning and expression of human genes coding for proteins of pharmaceutical interest. As their number is expected to increase significantly over the next few years, methods for the direct chemical analysis of these proteins in final preparations will be needed. Developing such methods can be difficult because the amounts of active protein required for the therapeutic dose are often small compared to the large amounts of additives and/or excipients added either for protection during the manufacturing process or to increase stability during storage [1,2]. Particular difficulties are encountered when the excipients are also proteins, such as human serum albumin (HSA), which is usually obtained from large pools of plasma and cannot be considered chemically homogeneous [3].

Erythropoietin (EPO) is a glycoprotein produced primarily by the kidney and is the main factor regulating red blood cell production [4]. The mature protein has three N-linked and one O-linked glycosylation sites with a total molecular mass of $34–39 \cdot 10^3$ [5]. EPO has been expressed using recombinant DNA technology and pharmaceutical

*Corresponding author.

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preparations of recombinant human EPO (rhEPO) are now available from several manufacturers around the world. The heterogeneity of rhEPO [6,7] and urinary EPO (uEPO) [8] has been characterized and shown to be due to the extent of glycosylation [9] as well as to the variable number of sialic acid residues [10]. Wide and Bengtsson [11] have demonstrated that serum EPO can be present in an estimated 20–30 different forms in the blood of anaemic patients. The separation of intact rhEPO into distinct glycoform populations has recently been achieved by free solution HPCE [12–14].

Under current Canadian regulatory guidelines, manufacturers should assess final preparations for identity and stability. Because of their high degree of selectivity and reproducibility, physico-chemical methods are well suited to providing the required information. However, few methods have been reported for the direct determination of active proteins in formulations. HPCE has become a powerful analytical tool for resolving and quantifying complex protein mixtures as well as different forms of the same protein (for a recent review see Li [15]). The aim of the work presented was to develop a sensitive, selective and reproducible method for the characterization and quantification of rhEPO glycoforms in final formulations. We present herein a rapid one-step HPCE method that highlights differences between formulation batches and allows quantitative estimation of glycoform populations.

2. Experimental

2.1. Materials

Ampoules of formulated rhEPO were supplied by manufacturers to the Health Protection Branch (Bureau of Biologics, Health Canada). Purified bulk rhEPO was donated by the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ, USA). High purity HSA was obtained from Miles (Diagnostics Division, Kankakee, IL, USA). Molecular biology-grade reagents were purchased from Sigma (Toronto, Canada). All other reagents were of the highest purity available from commercial sources.

Bio-Sil TSK-125 (300×7.5 mm I.D.) size-exclusion HPLC columns were obtained from Bio-Rad (Mississauga, Canada). Polyclonal anti(rhEPO) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). All other reagents for Western blotting analysis were supplied by Promega (Montreal, Canada).

eCAP Amine capillaries and reagents were purchased from Beckman Instruments (Fullerton, CA, USA). The wash and conditioning procedures used were those recommended by the manufacturer.

2.2. Size-exclusion chromatography and Western blot analysis

HPSEC analyses of formulated rhEPO were carried out on a Waters chromatographic system using the Maxima software for data analysis. Running conditions were 100 m*M* ammonium bicarbonate, 1 *M* NaCl, pH 7.5, with a flow-rate of 1 ml/min at room temperature. Elution was monitored at 280 nm and peaks were collected. After extensive dialysis, protein identification was accomplished by Western blot analysis carried out using the protocols supplied by the manufacturers.

2.3. High-performance capillary electrophoresis

HPCE was carried out on a P/ACE 5500 (Beckman Instruments). Samples were hydrodynamically injected into a 47-cm (40 cm to the detector)×50 μm I.D. capillary using 0.5 p.s.i. (1 p.s.i.=6894.76 Pa) of nitrogen for 8 s. Peak migration was monitored at 200 nm by on-column UV detection. The instrument was operated under reversed polarity with detection at the anode. Data were collected and analyzed using the P/ACE Gold data analysis software version 8.1. The following three sets of experimental conditions were used: condition A was adapted from Ref. [14] and consisted of a 200 m*M* sodium phosphate buffer, pH 4.0, with electrophoresis carried out at 15 kV and run at a constant temperature of 25°C; condition B used the same phosphate buffer as that of condition A with the electrophoresis run at 8 kV at a constant temperature of 20°C – specific additives to the buffer are indicated in the figure legends; condition C consisted of adding 1 m*M* nickel chloride hexahydrate to the electrophoretic buffer used in condition A with electrophoretic conditions as in condition B.

2.4. Solutions

The rhEPO stock solution was that received from the manufacturer at a nominal concentration of 1.92 mg/ml and was kept frozen as individual aliquots (260 μl) at −20°C until analysis. rhEPO standard solutions were prepared in Milli-Q water by serial dilution of the stock solution to give concentrations of 0.96, 0.48, 0.24, 0.12, 0.06 and 0.03 mg/ml. Lyophilyzed rhEPO formulations were reconstituted with 1 ml of Milli-Q water and kept at −4°C until analysis. Fresh HSA stock solutions were prepared immediately prior to analysis in Milli-Q water at a nominal concentration of 2.0 mg/ml from high purity HSA.

3. Results and discussion

3.1. Method development

3.1.1. Bulk substance

Bulk rhEPO expressed from Chinese hamster ovary (CHO) cells was used to establish conditions for the separation of glycoforms. Optimization of the conditions described by Watson et al. [14] to the present system led to the development of two sets of conditions, (A) and (B), differing mainly in the voltage used (see Section 2). Both sets of conditions allowed resolution of rhEPO into four major and a number of unresolved minor components, as shown in Fig. 1 obtained with condition A. However, the conditions described for (B) were generally used in the remainder of the study as they led to better glycoform resolution. The migration pattern and relative proportions of the component peaks were comparable to published results [13,14]. Under these conditions, the polyamine-coated inner capillary walls are positively charged, effectively reversing the electroosmotic flow towards the anode, and glycoforms are expected to migrate in order of decreasing number of sialic acid residues and proportional molecular mass. In light of the considerable heterogeneity demonstrated in the carbohydrate chains of rhEPO [7], individual peaks are likely to correspond to multiple glycoforms with the same overall charge-to-mass ratio.

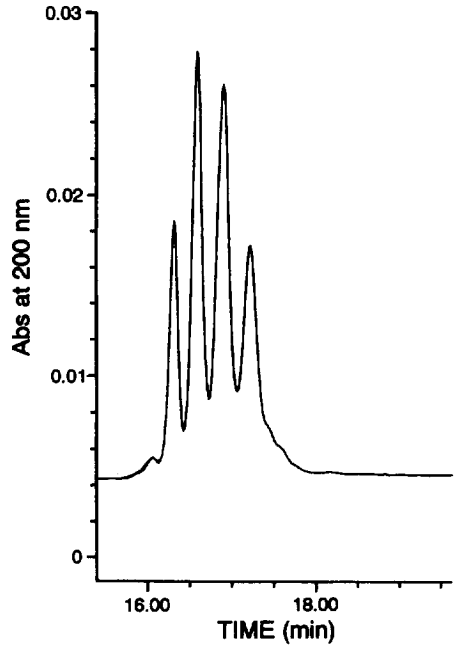

Fig. 1. Electropherogram of bulk rhEPO under HPCE condition A.

3.1.2. rhEPO formulations

HSA is generally added to rhEPO formulations as a protein excipient. Attempts at separating the two proteins by HPSEC were unsuccessful, as both proteins eluted with similar apparent molecular masses of around $66 \cdot 10^3$ (data not shown). Such similarity in hydrodynamic volumes has been observed previously [16]. A comparison of their respective physico-chemical properties (Table 1) also suggested that, due to their close p*I* values [17,18], separation of the two proteins by HPCE may be problematic. In fact, when HSA was subjected to the HPCE separation conditions (B), its migration time coincided with that of rhEPO glycoforms (Fig. 2a). Analysis of mixtures of the two proteins, as well as analysis of HSA-containing rhEPO formulations, confirmed that, in all cases, HSA co-migrated with rhEPO. Various attempts at separating the two proteins included changing the buffer pH, concentration or ionic strength, varying column temperature, adding organic modifiers (methanol, acetonitrile and ethanol) or zwitterions (betaine and taurine), or altering the nature of buffer ions [e.g., alkaline (Li, K, Na) and alkaline earth (Ca and Mg) chlorides, and potassium halides (I, Br and Cl)]. Most attempts

Table 1
Physical and dynamic characteristics of human serum albumin (HSA) and recombinant human erythropoietin (rhEPO)

HSA	rhEPO
M_r 66.8·10^3 [3]	M_r 34–39·10^3 [5]
Single polypeptide chain [3]	Single polypeptide chain [5]
One free S–H group [3]	No free S–H group
p*I* 4.5 [3,17]	p*I* 4.2–4.4 [12–14,18]
Globular [16]	Non-globular
Not glycosylated [3]	3 N-Glycosidation 1 O-Glycosidation [5,9,10]
Glycated [3]	No glycation reported
Bound to small molecules [3]	No binding to small molecules reported

proved unsuccessful as separation could only be slightly improved in a few cases, as shown in Fig. 2b,c, which were obtained after addition of sodium chloride and magnesium chloride, respectively.

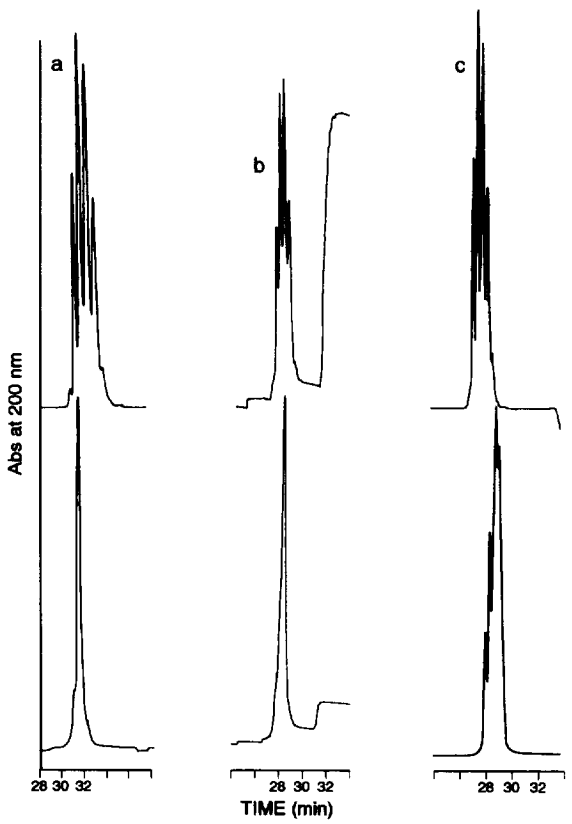

Fig. 2. Effect of buffer additives on the resolution of HSA (bottom) and rhEPO (top). (a) Condition B; (b) condition B+1 m*M* sodium chloride; (c) condition B+10 m*M* magnesium chloride.

While it is apparent in these examples that rhEPO glycoforms migrated slightly ahead of HSA, the resolution obtained was insufficient to allow detection of glycoforms in the presence of large amounts of HSA, such as that found in formulations.

Metal ions are well known to interact with proteins and affect their properties and, in some cases, their conformation [19]. They have been used as mobile phase additives in chromatographic separations of proteins [20] or to selectively modify the electrophoretic mobility of metal binding proteins in HPCE separations [21]. The effect of metal ions in the electrophoretic buffer was investigated as a way to selectively alter the electrophoretic mobility of one of the formulation protein components. Electropherograms obtained following addition of 1 m*M* nickel chloride to condition B's electrophoretic buffer (condition C) are shown in Fig. 3. These conditions afforded complete separation of HSA and rhEPO (Fig. 3a,b, respectively), without affecting the glycoform resolution pattern. It appeared that the electrophoretic mobility of HSA was predominantly affected leading to longer migration times, whereas rhEPO was only marginally affected. The migration times and separation patterns of the component proteins from a HSA-containing rhEPO formulation (Fig. 3c) were in very good agreement with those observed for the individual components. However, significant variations of migration time and separation pattern were observed for HSA upon replicate same-day injections (Fig. 4). Typically, while the migration time for the main rhEPO glycoform peak was found to vary marginally, ranging from 30.11 to 30.78 min, the migration time for the main HSA peak steadily decreased as the number of injections

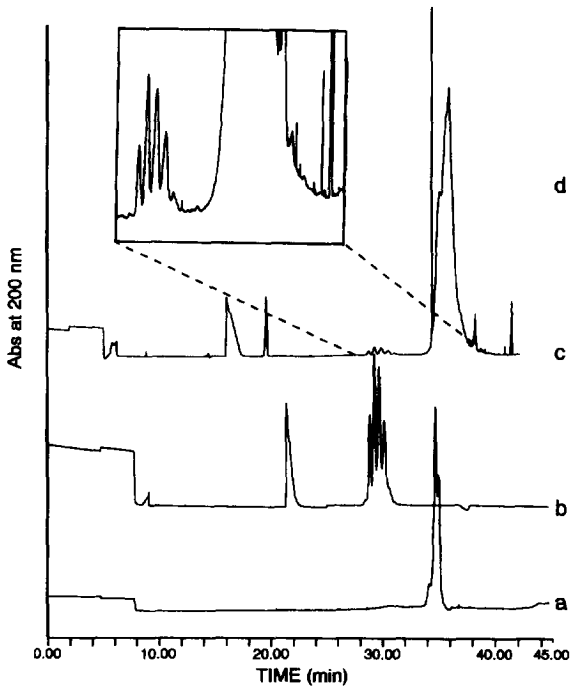

Fig. 3. Electropherograms of (a) HSA, (b) bulk rhEPO, (c) rhEPO formulation under condition C (condition B + 1 m*M* nickel chloride). Inset: expanded view of the region between 30 and 45 min in electropherogram (c), showing separation between HSA and rhEPO.

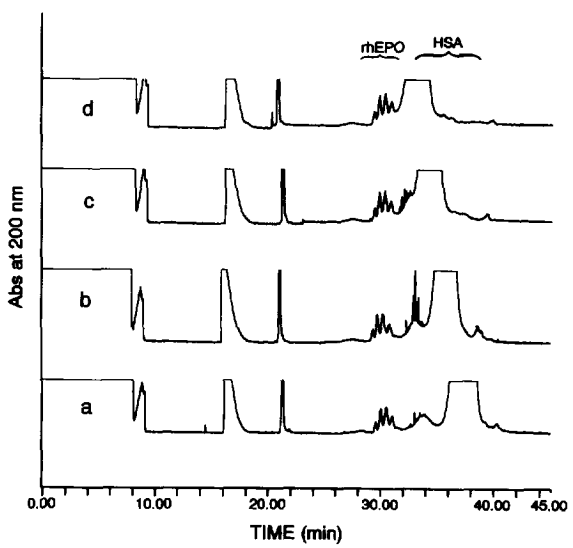

Fig. 4. Effect of consecutive, replicate injections on the mobilities of rhEPO and HSA after (a) run 1, (b) run 3, (c) run 6 and (d) run 9.

increased (Fig. 4a,c), leading to an eventual loss of complete resolution between HSA and rhEPO after nine runs (Fig. 4d). The initial resolution could be re-established by replacing the buffer at the electrodes with fresh buffer, indicating that ion depletion was the cause of the observed HSA shift.

In order to gain some preliminary information regarding the interactions involving HSA in the presence of nickel ions, rhEPO formulation samples were preincubated with up to 10 m*M* $NiCl_2$ for up to 12 h and analyzed by HPCE condition B (nickel ions not present in the buffer). In all cases, rhEPO and HSA were not resolved from each other. This suggests that nickel ions are unlikely to form strongly bound complexes with HSA and that the interactions are likely to be reversible, a situation that would require the presence of nickel ions in the buffer. Alternatively, nickel ions may interact with active sites on the column coating, or facilitate interactions between HSA and active sites. It is apparent that this type of interaction does not occur with rhEPO and that it selectively decreases the electrophoretic mobility of the HSA, allowing for the resolution of the two proteins. Further studies are underway to elucidate the interaction mechanism.

The homogeneity of the purchased high purity HSA used throughout this study was greater than 99%, when evaluated by conventional sodium dodecyl sulfate–polyacrylamide gel electrophoresis. However, the enhanced efficiency of HPCE allowed resolution of HSA into multiple components, as illustrated in Figs. 2–4. Although the number of components observed varied with the composition of the buffer, the relative amounts of the individual components were generally greater than the combined rhEPO glycoforms present in formulations. Guzman et al. [22] and Yowell et al. [23] also observed HSA variants in HSA-containing pharmaceutical preparations of recombinant cytokines. Additional components were observed in the analysis of preparations that did not appear when analyzing the purified proteins individually. The nature of these components is not known at this time.

3.2. Method validation

Usual test parameters were evaluated for validation of the nickel-containing HPCE method. Al-

though rhEPO was resolved into several glycoform populations, the lack of complete resolution (baseline resolution) precluded using data from individual peaks. In fact, Meyer [24] has recently shown that integration of partially resolved peaks can lead to substantial errors in the determination of peak heights and peak areas. Thus, data for rhEPO were obtained by measuring glycoform peaks as a single peak using standard integration procedures.

3.2.1. Linearity and range

Standard curves were established from serial dilutions of the rhEPO stock solution, at six concentrations ranging from 0.03 to 1.92 mg/ml. Plots of area counts measured at 200 nm versus the amount of rhEPO injected indicated that the detector response was linear over the entire range of concentrations assayed, as shown in Fig. 5. Coefficients of determination, R^2, were greater than 0.998 and the R.S.D. of the slopes was 0.7% ($n=3$). This range of concentrations encompassed that of all available formulations, with the exception of the lowest one with a reported label potency of 2000 IU/ampoule. In this case, the signal was detectable but was found to be smaller than that of the lowest standard on the curve. The reported potency of the product could be estimated at approximately 0.02 mg/ml using an average specific activity for rhEPO of 100 000 IU/mg [25] after reconstitution. The linearity and the total peak areas of solutions spiked with large amounts of HSA were unchanged when compared to those of unspiked solutions. This indicated that the presence of HSA did not produce a matrix effect.

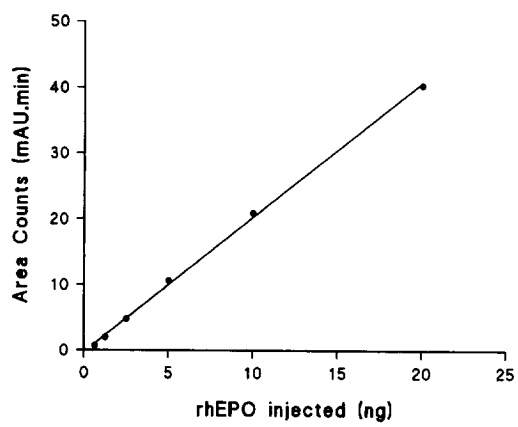

Fig. 5. Plot of area counts vs. rhEPO injected, from electropherograms recorded under HPCE condition C at 200 nm.

3.2.2. Limit of detection and limit of quantitation

The limit of detection (LOD) was assessed as the lowest concentration giving a signal-to-noise ratio of about 3:1 and the limit of quantitation (LOQ) was measured as the lowest concentration giving a R.S.D. $<10\%$. Both were determined by measuring the detector response for replicate injections of standard solutions prepared at progressively lower concentrations. The LOD and LOQ were set at 0.010 and 0.030 mg/ml, respectively. These results suggested that most rhEPO formulations would be quantifiable.

3.2.3. Precision

Intra- and inter-day variations of peak area responses for triplicate injections of rhEPO standard solutions and formulations were studied over four batches. For a 0.5 mg/ml standard solution, components of within- and between-batch variance, reported as R.S.D.s were 2.86 and 4.57%, respectively. Similar experiments using a 4000 IU formulation gave essentially similar results over three batches, with values of 3.68 and 1.94% for within- and between-batch components, respectively. The expected total variation in analyzing a sample once over several batches would be 2.88%. Within- and between-batch variances were analyzed by one-way analysis of variance (ANOVA). Migration time variations were usually smaller than those for peak area, with R.S.D. values of below 2% for both standard solutions and formulations. The overall results indicated that the method had a high degree of precision and could be used adequately for quantitative measurement of rhEPO in formulations.

3.3. Analysis of formulations

Drug formulations from two manufacturers were examined using the nickel-containing HPCE method and were assessed in terms of the distribution of glycoform populations and the consistency of manufacture by comparing different lots from the same manufacturer. Qualitatively both products showed little lot-to-lot variations in their glycoform patterns, indicating good reproducibility of the manufacturing process. On the other hand, a quantitative evaluation

Table 2
rhEPO content in formulations measured by HPCE condition C for products from two manufacturers

Manufacturer	rhEPO area counts/1000 IU [standard error(%)]
A	
Lot 1	0.1402 (2.6)
Lot 2	0.1593 (0.9)
Lot 3	0.1925 (2.3)
B	
Lot 1	0.1698 (2.3)
Lot 2	0.2087 (3.3)
Lot 3	0.2332 (3.4)

revealed significant variability (Table 2). Results are presented as mean values ($n=3$) and are expressed as rhEPO area counts per 1000 IU of the reported potency on the product label. While standard errors from the means were consistently below 4%, lot-to-lot variations of 25 and 33% in rhEPO area counts per 1000 IU were observed for products from manufacturers A and B, respectively. It is not possible at this time to determine the major cause for such discrepancies. However, a number of factors may be involved, such as irreversible adsorption of the active ingredient on the walls of the container, overfilling, variability of the biological assay, or other factors.

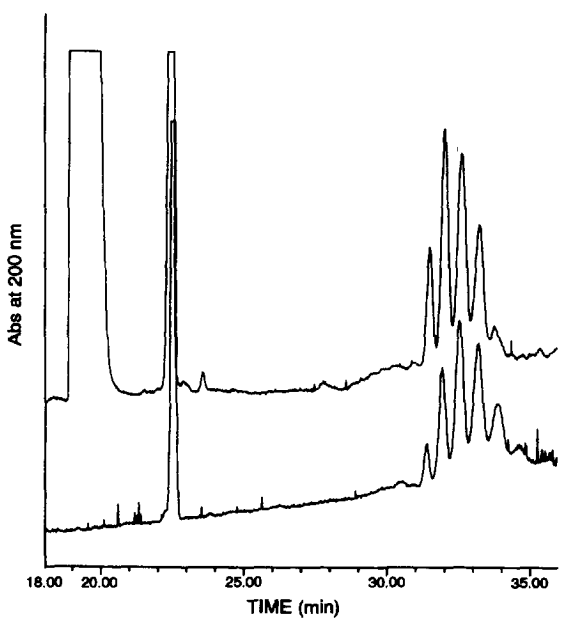

Fig. 6. Comparison of the electrophoretic profile of rhEPO formulations from manufacturers A (bottom) and B (top) obtained under condition C.

A direct quantitative comparison of the two products was not possible due to possible differences in standardization used by manufacturers. However, appreciable qualitative differences in glycoform patterns were observed (Fig. 6). The rhEPO migration times for both products were almost identical, but clear differences emerged in the number and relative amounts of the resolved glycoforms. The product from manufacturer A consisted of five major and one minor peaks (Fig. 6, bottom) while that from manufacturer B showed four major and one minor tailing peaks (Fig. 6, top). Clear differences were also observed when comparing peak shape and relative proportions of the four largest peaks of both products. These differences were not further characterized and may reflect variations in manufacturing processes or actual differences in post-translational modifications.

4. Conclusion

Difficulties in assessing protein drug products by physico-chemical methods are often related to the presence of large amounts of excipients that interfere with the detection and separation of the active ingredient. In the present HPCE method, nickel ions were used to interact selectively with HSA present in large amounts in recombinant erythropoietin preparations, leading to complete separation of the two proteins. The method was found to be useful for quantitative estimation of rhEPO in final drug preparations. The method was also capable of distinguishing products from different manufacturers.

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