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Short communication

Optimization and qualification of capillary zone electrophoresis method for glycoprotein isoform distribution of erythropoietin for quality control laboratory

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ABSTRACT

The European Pharmacopoeia (Ph. Eur.) monograph for Erythropoietin Concentrated Solution describes a capillary zone electrophoresis method for identification of recombinant human erythropoietin. However, this method has shown poor reproducibility due to inadequate capillary conditioning. We have modified the Ph. Eur. method to make it more robust and suitable for the quality control laboratory for the analysis of epoetin alfa and epoetin alfa after formulation with polysorbate 80. This study qualified the modified method by showing improved robustness and reproducibility. The study also characterized and qualified a secondary standard of epoetin alfa as a substitute for the primary standard, Ph. Eur. erythropoietin Biological Reference Preparation, which is available in limited supply. Four sets of analyses were performed to assess repeatability, intermediate precision, and the secondary standard. The results showed that the modified method is suitable for its intended purpose to test epoetin alfa and formulated epoetin alfa samples. The epoetin alfa secondary standard is a suitable substitute for the primary standard. Further, we developed a procedure for the removal of polysorbate 80 from formulated epoetin alfa, allowing the material to be analyzed by the modified Ph. Eur. method.

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1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone that regulates erythropoiesis, or red blood cell production [1]. EPO is produced by the liver and kidney [2]. Capillary electrophoresis (CE) has been one of the widely used tools for the direct analysis of glycoform distribution of glycoprotein [3]. Several CE methods have been developed for the separation of EPO glycoforms [3–15]. Watson and Yao developed the first capillary zone electrophoresis (CZE) method using a fused-silica capillary with weakly acidic electrolyte solution containing 1,4-diaminobutane (DAB) [5]. After the re-examination of this method by Kinoshita et al., the method was found to be troublesome due to significant loss on resolution after several injections, and the life of the capillary was limited [3]. Lopez-Soto-Yarritu et al. proposed a higher DAB concentration, and the migration time was controlled, but the zone broadening was significantly increased, and the peak areas varied due to protein adsorption onto the capillary wall [9]. European Pharmacopoeia (Ph. Eur.) has published a monograph describing the separation of glycoforms of EPO by CZE [4]. However, the Ph. Eur. method was prone to poor reproducibility [11]. Sanz-Nebot et al. optimized the Ph. Eur. method by adjusting the pH value of separation electrolyte and capillary length in order to achieve better resolution for the separation of erythropoiesis-stimulating protein. Although the intraday reproducibility was improved, the inter-day reproducibility was relatively high, and not all the isoforms were completely baseline resolved [12]. Benavente et al. made further improvements on the CZE method and proposed a multivariate calibration method using partial least-squares (PLS) in order to characterize binary mixtures of two types of recombinant human erythropoietin (epoetin alfa and beta) [14]. Although the reproducibility was improved, the %RSD of migration times for all the peaks still failed to meet the system suitability acceptance criterion of <2% required by Ph. Eur. Other CE techniques such as isoelectric focusing (cIEF) method have also been developed for EPO analysis [16] but it is not required by Ph. Eur. [4]. Therefore, it was not studied and compared in this work. After the European Department for the Quality of Medicine first published the specifications for the CZE method in the 2002 European Pharmacopoeia, the EPO manufacturers have been required to adopt it as a quality control (QC) method for product release.

Abbreviations: BRP, Biological Reference Preparation; DAB, 1,4-diaminobutane; EDL, electrical double layer; EPO, erythropoietin; NID, non-ionic detergent; Ph. Eur., European Pharmacopoeia; QC, quality control.

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The objective of this study was to modify the current Ph. Eur. method to make it suitable for routine QC testing of EPO drug substance. The root cause for poor reproducibility of the CZE method was studied and identified. The study also qualified a secondary standard as a substitute for primary standard, which is available in limited supply from Ph. Eur. In addition, the study evaluated a strategy for the removal of polysorbate 80 from EPO formulated with polysorbate 80. Formulations containing polysorbate 80 interferes with the analysis of isoforms of EPO in CE separation, and the desalting procedure using molecular mass cut-off filter [4] is unable to remove polysorbate 80.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were of ACS reagent or electrophoresis grade. Acetic acid (HAc) (glacial), anhydrous sodium acetate (NaAc), 1,4-diaminobutane (DAB), sodium chloride (NaCl), sodium citrate dihydrate, citric acid, tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), tris-(hydroxymethyl)aminomethane (Tris-base), and N-tris-(hydroxymethyl)methylglycine (tricine) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide solutions (1 M and 0.1 M) and hydrochloric acid (0.1 M) were provided by Agilent Technologies (Wilmington, DE, USA). Urea was supplied by BioRad Laboratories (Hercules, CA, USA). Water with a conductivity value lower than 0.05 mS/cm was obtained using a Milli-Q water purification system from Millipore (Billerica, MA, USA). The running buffer was prepared by dissolving appropriate amounts of tricine, NaCl, NaAc, DAB, and urea in water to obtain the desired concentration of 10 mM tricine, 10 mM NaCl, 10 mM NaAc, 2.5 mM DAB, and 7 M urea. The pH was adjusted to 5.55 ± 0.02 with 10% acetic acid. The 20 mM citrate/250 mM NaCl buffer solution was prepared by dissolving appropriate amount of citrate, citric acid, and NaCl in water. The 10 mM Tris–HCl was prepared by dissolving appropriate amounts of Tris–HCl in water and the pH was adjusted to neutral with 10% Tris-base. The above solutions were filtered through a 0.22 μm nylon filter.

2.2. Sample preparation

Primary standard samples of recombinant human EPO produced in a Chinese Hamster Ovary (CHO) cell line were provided by Ph. Eur. as Biological Reference Preparation (BRP Batch 1). Epoetin alfa samples were obtained from Johnson & Johnson Ortho Biotech (Raritan, NJ, USA). The BRP and EPO samples were prepared according to the procedures described in the Ph. Eur monograph [4]. The epoetin alfa samples and the BRP sample were stored at −20 °C when not in use.

Epoetin alfa was formulated with polysorbate 80 to contain 2000 (2k) and 40,000 (40k) International Units (IU), which are equivalent to 16.8 μg/mL and 336.0 μg/mL epoetin alfa, respectively. The removal of polysorbate 80 from formulated epoetin alfa was achieved using a non-ionic detergent (NID) trap cartridge from Michrom BioResources (Auburn, CA, USA). The NID trap cartridge was connected to different sizes of syringes for conditioning cartridge, loading sample, washing cartridge, and eluting sample. The syringe with cartridge connected was placed on an electronic infusion pump from kd Scientific (Holliston, MA, USA). The electronic infusion pump is programmed for different syringe sizes and flow rates, which enable the process to be automated, yielding high throughput, accuracy, and precision. The NID trap cartridge was first conditioned by passage of 900 μL of 20 mM citrate/250 mM NaCl solution to remove any residual substance that may co-elute with epoetin alfa followed by 900 μL of 10 mM Tris–HCl buffer solution. The EPO was eluted with 200 μL of 20 mM citrate/250 mM NaCl. The sample was then taken through the desalting procedure as described in the Ph. Eur monograph. The formulated epoetin alfa samples were stored at 4 °C when not in use.

2.3. CZE

The CZE experiments were performed on an Agilent 3D-CE instrument equipped with a photo-diode array detector and ChemStation software from Agilent Technologies (Wilmington, DE, USA). Bare fused-silica capillary columns (104 cm × 50 μm id) were supplied by Agilent Technologies (Wilmington, DE, USA). The detection window was placed at 8.5 cm from the outlet of the capillary. The separation conditions for running buffer, capillary temperature, voltage, and UV detection were as described in the Ph. Eur. method [4]. The capillary temperature was maintained at 25 °C for all experiments. A voltage of 15.4 kV was applied during electrophoretic separations. Samples were injected hydrodynamically at 50 mbar for 30 s followed by a water injection at 5 mbar for 3 s. Detection was performed at 214 nm. Data were collected at a sampling rate of 5 Hz. The current was also monitored, and was typically 4–5 μA, or a Joule heating level of 0.07–0.08 W/m, about 1.5 orders of magnitude below the upper limit in an Ohm's law plot.

2.4. Modified capillary conditioning

The capillary was conditioned between every 10 injections (one water and nine samples) by flushing the capillary (50 mbar) with the following sequence of solutions for the specified periods: water for 30 min, 1 M sodium hydroxide for 60 min, water for 60 min, 0.1 M hydrochloric acid for 30 min, water for 30 min, 0.1 M sodium hydroxide for 45 min, water for 30 min, CZE running buffer for 15 min. If the capillary was new or had been stored in air, a voltage of 20 kV was applied to the capillary filled with running buffer for 120 min. Between injections, the capillary was rinsed with water for 10 min and with running buffer for 10 min.

3. Results and discussion

3.1. Restoration of the fused-silica capillary surface

Fused silica possesses different surface silanol groups—isolated, vicinal, and geminal—that are responsible for the pH-dependent charge on the capillary surface [17]. At pH > 3, the silanol groups will ionize, resulting in a negative charge on the capillary wall. In solutions containing ions, cations will migrate to the negatively charged wall forming an electrical double layer (EDL) described by the Stern–Gouy–Chapman model [18]. When an electrical potential is applied to the capillary, the cations and solvent molecules in the EDL migrate towards the cathode; this is the origin of electroosmotic flow (EOF). EOF can be affected by pH, the chemical nature of the capillary wall, the composition of the background electrolyte (BGE), the ionic strength, and the temperature.

The separation of proteins is complicated due to their adsorption onto the negatively charged surface of fused-silica capillaries [19]. The most common approach to minimize adsorption is to coat the capillary wall [20]. Coatings can be broadly categorized as: covalently linked polymeric coatings; physically adsorbed polymer coatings; or small molecule additives [21]. Coating agents (additives) that are positively charged and have more than one bonding center can effectively interact with the capillary wall, changing the capillary surface charge and, in consequence, change the magnitude of the EOF or even its direction. The Ph. Eur. method uses 2.5 mM DAB as a small molecule amine additive to dynamically coat the capillary wall. The binding of amine cations to the silanol sites on the

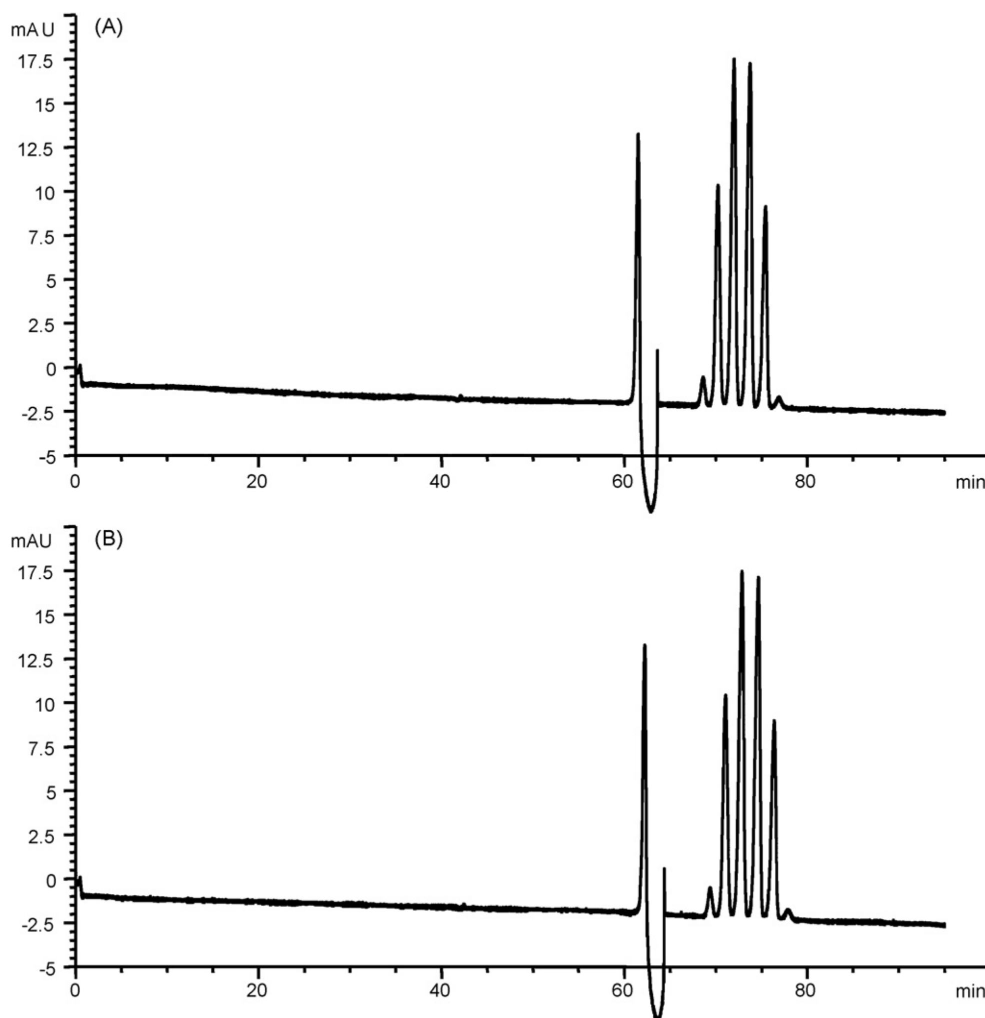
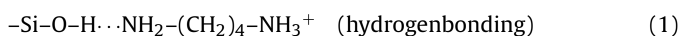


Fig. 1. Demonstration of capillary restoration. The peak area and migration time appear consistent after 36th injection. (A) 1st EPO sample injection and (B) 36th EPO sample injection.

capillary wall slows the EOF, which is necessary for the separation of the isoforms of the glycoprotein. Coating of the capillary surface with the amine cations in the BGE was via a dynamic equilibrium [22]. At pH 5.5 used in the Ph. Eur. running buffer, all the isoforms are negatively charged, as are some of the surface silanols. The available silanol groups for DAB to bind depend on the pH and DAB concentration. The interactions between DAB and the silanol groups are through hydrogen bonding, ionic interaction, or hydrophobic interactions, the first two of which are depicted below and depend on the ionization of DAB (pKa1 = 10.68) and the silanol groups:



$$-Si–O–H\cdots NH_2–(CH_2)_4–NH_3^+ \quad \text{(hydrogenbonding)} \tag{1}$$

$$-Si–O^- \, {}^+H_3N–(CH_2)_4–NH_3^+ \quad \text{(ionic interaction)} \tag{2}$$

The DAB concentration and the pH and ionic strength of the running buffer as described in the Ph. Eur. method were experimentally proven to be adequate for the separation of all the isoforms [11].

The remaining question is how to regenerate the capillary conditions after a certain number of injections so that the run can be continued without changing the capillary. During the separation, a portion of the isoform anion (A^-) is associated via electrostatic interactions with the DAB that is anchored to the silica surface through hydrogen bonding, ionic, and/or hydrophobic interactions (vide supra). The DAB–isoform association reduces the net electrophoretic migration of the isoforms, resulting in a slower net migration velocity and a longer retention time [22]. This association may be described as the following equilibrium:

$$-R–NH_3^+ + A^- = -R–NH_3^+A^- \tag{3}$$

where –R refers to all substituents in Eqs. (1) and (2) above except for the $–NH_3^+$ group facing away from the surface (towards the bulk solution). The equilibrium constant is

$$K = \frac{[-R–NH_3^+A^-]}{[-R–NH_3^+][A^-]} \tag{4}$$

The ratio of $[-R–NH_3^+A^-]$ to $[A^-]$ in Eq. (3), and hence the degree with which the net migration of the sample anion is slowed, is seen to be a function of both K and $[-R–NH_3^+]$, the concentration of the surface-immobilized pairing cation [22]. The value of K will be different for each isoform anion, thus providing an additional parameter for the resolution of anion mixtures by CZE.

Taking this into consideration, the complete removal of the complex from the silanol groups and the re-establishment of pH are crucial to restore the surface of the fused-silica capillary. The proposed capillary conditioning procedure using the combination of base, acid, water, and buffer flushes over an extended period of time was able to both (i) effectively remove all the isoform complexes from the capillary wall and (ii) provide approximately the same number of available silanol groups for DAB to bind again. As a result, reproducible results for both migration times and peak areas performed on the same capillary were obtained. Fig. 1 shows a comparison of the 1st and 36th injection on the same capillary after

Table 1
Precision of relative peak areas (PA) and relative migration times (RMTs) for two sets of analyses (Runs 1 and 2) based on triplicate injections of each preparation.

		Iso-1[a]		Iso-2		Iso-3		Iso-4		Iso-5		Iso-6		Iso-7		Iso-8	
		PA	RMT	PA	RMT	PA	RMT	PA	RMT	PA	RMT	PA	RMT	PA	RMT	PA	RMT
Run-1	BRP[b]	19.5	0.1	4.7	0.2	1.3	0.2	0.6	0.3	0.8	0.4	0.8	0.4	0.1	0.4	18.4	0.5
	EPO-1[c]	ND[d]	NA[e]	ND	NA	1.1	0.3	0.3	0.3	0.5	0.4	0.1	0.4	0.3	0.4	10.9	0.5
	EPO-2[f]	ND	NA	ND	NA	2.5	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.4	0.2	1.4	0.2
Run-2	BRP	7.2	0.1	2.4	0.1	0.7	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.3	0.0	2.4	0.1
	EPO-1	ND	NA	ND	NA	1.9	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.1	8.1	0.0
	EPO-2	ND	NA	ND	NA	3.8	0.0	0.5	0.0	0.3	0.0	0.2	0.0	0.5	0.0	11.8	0.0

[a] Isoform.
[b] Biological Reference Preparation (Batch 1).
[c] First preparation of epoetin alfa secondary standard.
[d] Not detected.
[e] Not applicable.
[f] Second preparation of epoetin alfa secondary standard.

using the proposed conditioning procedure. The number of injections beyond 36 was not examined only due to the limited number of samples available within one run. More injections on one capillary are likely to be successful if needed due to the thoroughness of the capillary re-conditioning.

3.2. Characterization and qualification of epoetin alfa secondary standard

Two sets of analyses designated as Run-1 and Run-2 were performed to characterize and qualify a secondary standard. In each of the electropherograms, there was a negative peak that appeared before the isoform peaks. This negative peak also appeared when water alone was injected. After injecting a neutral marker, benzyl alcohol, a large positive peak appeared at the same migration time as the negative peak. This confirmed that the negative peak was caused by water. As such, the negative peak migrates only due to electroosmotic flow and is therefore a suitable reference point for calculating a relative migration time (RMT). The RMT was calculated as follows:

$$\text{RMT} = \frac{\text{absolute sample migration time}}{\text{corresponding negative peak migration Time}} \quad (5)$$

Each run included triplicate injections of one BRP primary standard and triplicate injections of two preparations of epoetin alfa from a batch that was designated as a secondary standard. Although the precision (%RSD) of absolute migration time corresponding to isoform 2 obtained was 1.8%, which passed the Ph. Eur. acceptance criterion of less than 2%, the RMT had a much lower RSD of 0.2%, therefore, using RMT is a better approach to identify the isoforms.

The precision of the relative peak areas (peak area percentages) and RMTs for isoforms 1–8 are shown in Table 1 for two sets of analyses (Runs 1 and 2). The electropherograms of BRP primary standard (Fig. 2A) appeared identical to the reference electropherogram provided in the Ph. Eur. monograph [4]. All the electropherograms showed well-separated peaks, low baseline noise, good resolution and detection, stable baseline, and relatively constant migration time.

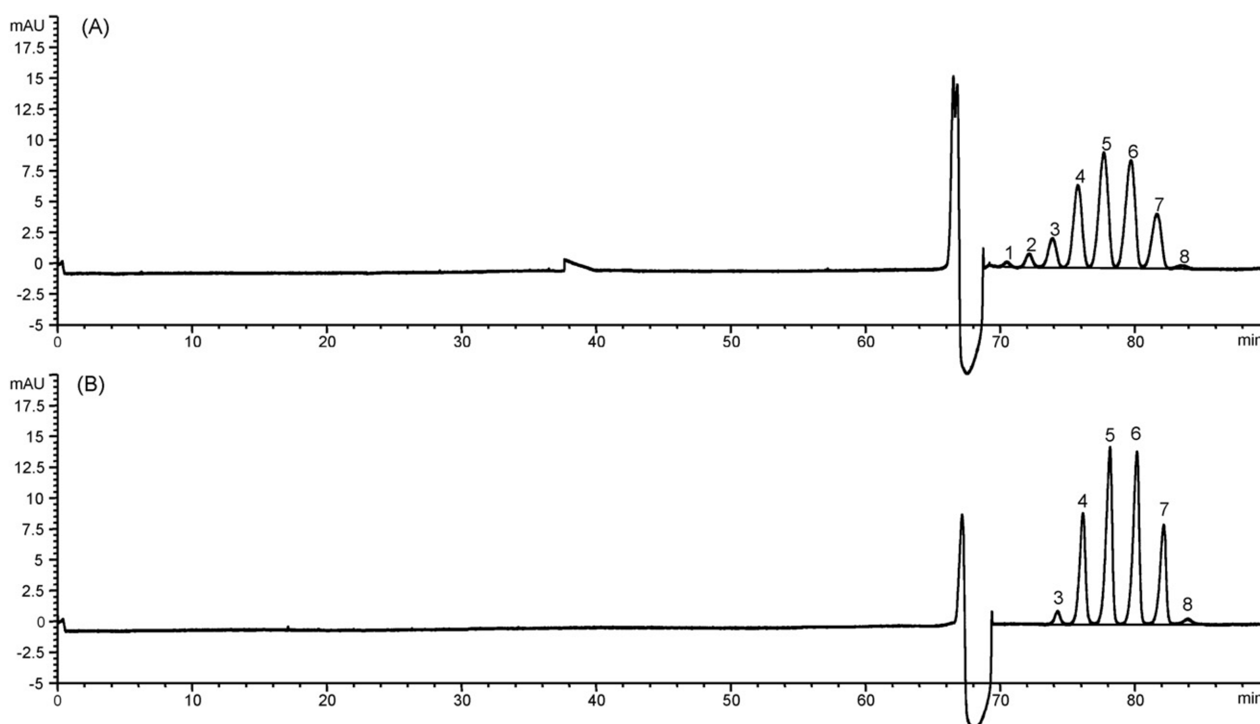


Fig. 2. Electropherograms of BRP primary standard (A) and epoetin alfa secondary standard (B). Isoforms 1–8 were very well separated. Isoforms 1 and 2 were not present in the epoetin alfa sample.

Table 2
Average relative areas (%) of the isoforms in all secondary standard preparations. Runs 1 and 2 include two preparations and Runs 3 and 4 include three preparations. Average relative areas (%) were calculated from three injections of each preparation. The intra-run mean ($N=2$) was calculated from the two preparations for Runs 1 and 2. The intra-run mean ($N=3$) was calculated from the three preparations for Runs 3 and 4.

	Isoform-3	Isoform-4	Isoform-5	Isoform-6	Isoform-7	Isoform-8
Run-1	2.1	18.8	30.4	30.2	17.2	1.3
	2.1	18.9	30.5	30.2	17.1	1.2
Intra-run mean ($N=2$)	2.1	18.9	30.5	30.2	17.2	1.3
%RSD	0.0	0.4	0.2	0.0	0.4	5.7
Run-2	2.2	19.0	30.6	30.2	17.0	1.1
	2.2	19.0	30.5	30.1	17.0	1.2
Intra-run mean ($N=2$)	2.2	19.0	30.6	30.2	17.0	1.2
%RSD	0.0	0.0	0.2	0.2	0.0	6.1
Run-3	2.2	18.6	30.3	30.3	17.3	1.3
	2.3	18.7	30.3	30.1	17.3	1.4
	2.2	18.8	30.3	30.2	17.2	1.3
Intra-run mean ($N=3$)	2.2	18.7	30.3	30.2	17.3	1.3
%RSD	2.6	0.5	0.0	0.3	0.3	4.3
Run-4	2.2	18.9	30.4	30.1	17.1	1.2
	2.0	18.8	30.4	30.2	17.3	1.2
	2.1	19.0	30.5	30.1	17.1	1.2
Intra-run mean ($N=3$)	2.1	18.9	30.4	30.2	17.2	1.2
%RSD	3.3	0.7	0.3	0.2	0.6	6.8
Inter-run mean ($N=10$)	2.1	18.9	30.4	30.2	17.2	1.2
%RSD	3.3	0.7	0.3	0.2	0.6	6.8

It was necessary to qualify a secondary standard because of insufficient supply of the BRP primary standard. The sample from the same lot of epoetin alfa secondary standard was prepared in duplicate and injected in triplicate in the two runs. The RMT, pattern of peaks, and relative area were used to identify the isoforms. The epoetin alfa secondary standard contains a subset of the isoforms that are present in the BRP primary standard because the BRP primary standard possesses both alfa and beta forms of EPO [23]. The RMT values confirmed that the isoforms in epoetin alfa secondary standard correspond to isoforms 3 through 8 in BRP primary standard. Fig. 2 shows a comparison of the BRP primary standard with epoetin alfa secondary standard. Figs. 1 and 2 were generated on different instruments using different capillaries and running buffers. The difference in absolute migration time between these two runs was due to the running buffer pH, which was adjusted according to a different pH meter. An investigation was conducted and the conclusion was confirmed. Nevertheless, the sample migration time lined up well with the standard migration time.

Table 3
Relative area (percentage) of each isoform of the epoetin alfa secondary standard (EPO Std), the 2000 (2k) IU[a]/mL F-EPO[b], and the 40,000 (40k) IU/mL F-EPO. The precision (%RSD) was determined from triplicate injections of each sample.

	Iso-3	Iso-4	Iso-5	Iso-6	Iso-7	Iso-8
Day 1						
EPO Std	2.3	19	30.2	29.9	17.2	1.4
2K-1	3.8	23.8	31.1	27.3	13.2	0.9
2K-2	3.7	23.9	31.2	27.3	13.1	0.9
2K-3	3.9	23.8	31	27.2	13.1	0.9
RSD (%)	2.6	0.2	0.3	0.2	0.4	0.0
EPO Std	2.1	19	30.6	30.2	17.2	1.4
40K-1	3.5	20.9	29.8	28.5	15.9	1.3
40K-2	3.5	20.8	29.9	28.5	16	1.3
40K-3	3.5	20.8	29.9	28.5	16	1.3
RSD (%)	0.0	0.3	0.2	0.0	0.4	0.0
Day 2						
EPO Std	2.2	18.9	30.3	30.1	17.2	1.3
2K-1	3.7	23.7	31.1	27.1	13.3	1.1
2K-2	3.6	23.8	31.4	27.1	13.1	1.1
2K-3	3.7	23.7	31.1	27.2	13.3	1
RSD (%)	1.6	0.2	0.6	0.2	0.9	5.4
EPO Std	2	18.9	30.7	30.2	17	1.2
40K-1	3.2	21.1	30	28.6	15.8	1.3
40K-2	3.3	20.7	30	28.8	15.9	1.3
40K-3	3.6	20.9	29.9	28.6	15.8	1.2
RSD (%)	6.2	1.0	0.2	0.4	0.4	4.6

[a] International Unit 2k IU/mL = 16.8 mg/mL epoetin alfa 40k IU/mL = 336.0 mg/mL epoetin alfa.

[b] Epoetin alfa secondary standard prepared in formulation buffer.

3.3. Precision

In addition to Runs 1 and 2, two more sets of analyses, Runs 3 and 4, were carried out to provide data for evaluation of repeatability and intermediate precision. Runs 3 and 4 included three independent preparations of epoetin alfa secondary standard. Taking all epoetin alfa secondary standard results from Runs 1–4 and using intra-run data as a measure of repeatability, RSD (%) of relative area ranged from 0.0% to 0.7% for isoforms 4–7, and 0.0% to 6.8% for isoforms 3 and 8 (Table 2). Higher RSD (%) for isoforms 3 and 8 are expected due to their low relative areas. Intermediate precision was evaluated with inter-run data by comparing relative area of all different preparations of epoetin alfa secondary standard from Run-1 through Run-4. The RSD (%) for isoforms 3–8 were 0.2–6.8% (Table 2). Based on these results, the precision and intermediate precision were considered acceptable.

3.4. Removal of polysorbate 80 from formulated epoetin alfa

Polysorbate 80, used in the formulation of drug product from epoetin alfa, interferes with the analysis of isoforms of EPO in CZE separation. Polysorbate 80 is an amphiphilic molecule containing hydrophilic and hydrophobic moieties whose removal has been a significant challenge for the formulation scientist due to its amphiphilic characteristics [24]. The desalting procedure using molecular mass cut-off filter as described in the Ph. Eur. monograph [4] is unable to remove polysorbate 80. The NID trap cartridge contains a mixed bed of weak anion and weak cation exchange packing. The NID trap is designed to bind proteins, while allowing non-ionic detergents such as polysorbate 80 to be eluted. The 2000 (2k) IU/mL and the 40,000 (40k) IU/mL formulated EPO samples (F-EPO) were prepared by spiking the formulation buffer, which contains polysorbate 80, with epoetin alfa secondary stan-

dard. As such, the F-EPO sample has a known amount of epoetin alfa. Polysorbate 80 was removed and desalted according to the proposed procedure as described in Section 2.2. The epoetin alfa secondary standard, the 2k IU/mL F-EPO, and the 40k IU/mL F-EPO samples were analyzed by CZE on two days. No extra or missing peaks were found in both 2k IU/mL F-EPO and 40k IU/mL F-EPO electropherograms when compared to that of the epoetin alfa secondary standard. The relative area of each isoform was recorded in Table 3. Precision ranged from 0.0% to 6.5%. The relative areas of each isoform for 2k IU/mL F-EPO and 40k IU/mL F-EPO differed from the epoetin alfa secondary standard by 0.7% or less, and were considered to be equivalent to that of the epoetin alfa standard.

Recovery of the protein from the polysorbate 80 removal procedure was evaluated with both 2k IU/mL F-EPO and 40k IU/mL F-EPO samples. The protein concentration was measured by UV absorbance at 280 nm. The recoveries for both 2k IU/mL and 40k IU/mL F-EPO samples were 95–98%.

4. Conclusions

The CZE method for Ph. Eur. monograph for erythropoietin concentrated solution has been modified. The modifications made to the Ph. Eur. method include the use of secondary standard in place of EPO BRP, the conditioning of the capillary, and the removal of polysorbate 80 for formulated epoetin alfa samples. Improved repeatability and intermediate precision were obtained. The method is suitable for use in quality control laboratories.

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