Rapid CZE analysis of erythropoietin variants

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Introduction

This study provides a rapid-CZE based method with faster separation time and capillary condition time for rapid analysis of EPO variants. Compared with the CZE method recorded in the *European Pharmacopoeia*, the results of the quantity and relative content of EPO isoforms are more consistent.

Erythropoietin (EPO) is a heavily glycosylated glycoprotein hormone secreted by the kidney and liver. Its main function is to promote erythropoiesis. EPO has a higher amount of glycosylation modifications, resulting in a microheterogeneity of EPO molecules, which becomes a mixture carrying different glycan variants (isoforms). The isoform type and amount are important parameters for EPO product quality control. Capillary Zone Electrophoresis (CZE) is a basic mode of capillary electrophoresis, which uses the charge/volume difference of the substance to be tested for separation. The EPO isoforms are separated in CZE due to differences in glycosylation sites, quantity, and glycoforms. At present, the *European Pharmacopoeia* uses the capillary zone electrophoresis (EP-CZE) method to characterize EPO isoforms. However, this method has disadvantages such as long capillary activation and separation time along with poor repeatability of migration time.

In this study, we developed a rapid CZE method (Rapid-CZE) that enables rapid analysis of EPO isoforms and provides better repeatability. Compared with the CZE method recorded in the *European Pharmacopoeia*, the results of the quantity and relative content of EPO isoforms are more consistent.

Key features

- A rapid CZE method (Rapid-CZE), which can provide rapid analysis of EPO variants and provides better repeatability.
- The resolution of the 5th and 6th variants is 1.39, which meet the requirements of *European Pharmacopoeia*.

The PA800 Plus Pharmaceutical Analysis System.

- The Rapid-CZE method has good repeatability. The RSD of migration time of each variant is about 1%, and the RSD of the corrected peak area% of the main variants is less than 5%.
- The Rapid-CZE method has faster separation time and capillary condition time than the EP-CZE method, which is very appropriate for the rapid analysis of EPO variants.

Materials and methods

Instruments

Capillary electrophoresis apparatus (PA800 Plus Pharmaceutical Analysis System, UV detector, SCIEX); analytical balance (AL104, Mettler Toledo); centrifuge (Biofuge, Thermo); pH meter (FE20, Mettler Toledo).

Reagents and consumables

pH 4.0 buffer, pH 7.2 buffer and Initiator (NTMP kit, Analis); urea (Urea, U0631-500G, Sigma-Aldrich); sodium hydroxide (NaOH, 795429-500G, Sigma-Aldrich); twice-deionized water (Millipore); fused silica capillary (SCIEX PN 338472); Ultracel YM-10 membrane filter (Millipore PN A11530).

Samples and pretreatment

European Pharmacopoeia EPO standard samples (Eur, purchased from EDQM). Ultracel YM-10 membrane filter was used for ultrafiltration concentration treatment. The final concentration was 10 mg/mL.

CZE method

Capillary: Fused silica capillary, 50 µm internal diameter, 80/90 cm effective/total length; injection; 0.5 psi, 12 s; separation voltage: 30 kV; separation time: 40 minutes; background electrolyte: buffer pH 4.0: buffer pH 7.2 = 9:1, 7 M urea; sample chamber temperature: 10° C; capillary temperature: 25° C; detection wavelength: 214 nm.

New capillary preequilibrium: 0.1 mol/L NaOH, rinse for 5 minutes at 20 psi pressure; Initiator, rinse for 5 minutes at 20 psi pressure; background electrolyte, rinse for 10 minutes at 20 psi pressure; repeat Initiator with background electrolyte rinse twice. Rinse between each needle: 0.1 mol/L NaOH, rinse for 3 minutes at 20 psi pressure; Initiator, rinse for 3 minutes at 20 psi pressure; background electrolyte, rinse for 5 minutes at 20 psi pressure.

Results and discussion

Analysis of EPO isoforms by the Rapid-CZE method

The results of using the Rapid-CZE method to analyze standard EPO isoforms are shown in Figure 1. Eight isoforms from the standard EPO samples were baseline separated with migration times between 31-36 minutes. The separation degree between P5 and P6 was 1.39, which met the requirement in the *European Pharmacopoeia* that the separation degree should not be less than 1.

Observation of the repeatability of the Rapid-CZE method

The repeatability of the Rapid-CZE method was observed by consecutively running 3 needles of EPO standard samples. The RSD of migration time and corrected area percent (CAP, %) is shown in Table 1. The RSD of migration time of every isoform was about 1%, and the RSD of the corrected area percent of major isoforms (corrected area percent peak greater than 3%) was less than 5%.

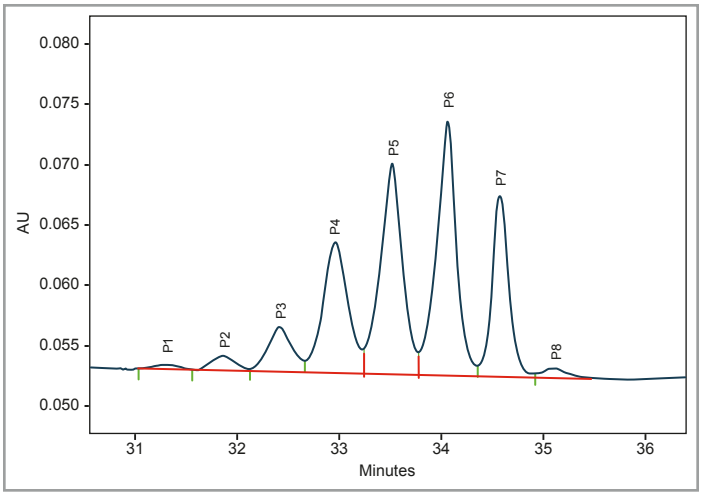

Figure 1. Electrophoretogram of EPO standard isoform analysis by the Rapid-CZE method. For electrophoresis conditions, please see section 3.

Pk#	Migration time (MT, min)				Corrected area percent (CAP, %)			
	Rep1	Rep2	Rep3	RSD	Rep1	Rep2	Rep3	RSD
P1	30.77	31.25	31.31	0.95%	0.56	0.54	0.68	12.76%
P2	31.32	31.85	31.95	1.07%	1.84	1.85	2.19	10.17%
P3	31.88	32.41	32.5	1.04%	5.96	6.05	6.35	3.34%
P4	32.43	32.98	33.05	1.03%	18.34	18.39	18.66	0.93%
P5	32.99	33.56	33.63	1.05%	26.82	26.73	26.71	0.22%
P6	33.53	34.11	34.18	1.05%	28.54	28.27	28.08	0.82%
P7	34.04	34.64	34.7	1.06%	16.61	16.83	16.38	1.35%
P8	34.58	35.21	35.24	1.06%	1.33	1.34	0.96	17.90%

Table 1. Observation of the repeatability of the migration time and corrected area percent (CAP, %) by the Rapid-CZE method (n=3).

Comparison of the analysis results of the Rapid-CZE method and the EP-CZE method

We separately analyzed the isoforms of the EPO standard samples using the Rapid-CZE method and the EP-CZE method. Eight isoforms could be separated by both methods. The migration time of the Rapid-CZE method was 31-36 minutes, and the migration time of the EP-CZE method was 55-68 minutes. The comparison results of the migration time and corrected area percent (CAP, %) of P1-P8 are shown in Table 2. The corrected area percent (CAP, %) of the eight isoforms are highly consistent between the two methods.

Pk#	Migration time (MT, min)		Corrected area percent (CAP, %)	
	Rapid-CZE	EP-CZE	Rapid-CZE	EP-CZE
P1	30.77	55.36	0.63	0.56
P2	31.32	57.11	1.92	1.65
P3	31.88	58.77	5.98	5.18
P4	32.43	60.52	18.46	18.27
P5	32.99	62.38	26.77	28.11
P6	33.53	64.27	28.47	28.59
P7	34.04	66.15	16.59	16.68
P8	34.58	67.83	1.19	0.96

Table 2. Comparison of the EPO isoforms analysis results using the Rapid-CZE method and the EP-CZE method.

Conclusions

This study shows the development of a rapid CZE method to analyze EPO isoforms and compared it to the EP-CZE method. Results show that:

1. The quantity and relative content of the isoforms separated by the Rapid-CZE method and the EP-CZE method were consistent.
2. The separation degree of the Rapid-CZE method for isoforms 5 and 6 was not less than 1, which met the requirements of the *European Pharmacopoeia*.
3. The repeatability of Rapid-CZE method is better. The RSD of migration time of every isoform was about 1%, and the RSD of the corrected area percent of major isoforms (corrected area percent peak greater than 3%) was less than 5%.
4. Separation time using the Rapid-CZE method is fast with the peak time between 31-36 minutes, whereas the peak time of the EP-CZE method is 55-68 minutes or longer.
5. The capillary equilibrium can be reached in 35 minutes using the Rapid-CZE method, which is very fast. Meanwhile, the time to reach capillary equilibrium using the EP-CZE method can be as long as a day.

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