

Comparison of Different Methods Used for the Determination of Isoform Distribution in rHuEPO

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Abstract

The biological activity of rHuEPO in vivo is influenced by the sialic acid content. Higher isoforms (more sialic acid) are longer acting in vivo. Thus, monitoring the isoform distribution during product characterization is an important part of evaluating product comparability. In this study, the sum of isoforms 12+13, was used as a basis for comparing compounds. In this report we compare the ability of six different techniques to provide information about the isoform distribution of rHuEPO. These techniques include two capillary zone electrophoresis methods (CZE using putrescine buffer and CZE using phosphate/urea buffer), two capillary based isoelectric focusing methods (imagined CE₂₈₀ and CE-IEF) and two isoelectric focusing gel methods (vertical mini-gels and thin layer polyacrylamide flatbed gels).

All six techniques exhibit similar trends in the isoform distribution among the nine preparations of Epoetin alfa tested. While the values determined for isoform 12+13 by these methods are comparable but not identical. Therefore, a direct comparison of samples should be limited to the same method. The choice of method is dependent on the availability of resources and the type of information desired.

Introduction

Isoelectric focusing in a polyacrylamide gel followed by Coomassie staining and densitometry measurement has been traditionally used to determine the isoform distribution of rHuEPO preparations. Recently, capillary electrophoresis including CZE and CE-IEF have been developed as alternative methods. In this study, the relative isoform distribution and isoform 12+13 were used as a basis of comparison for six different methods.

Test Samples

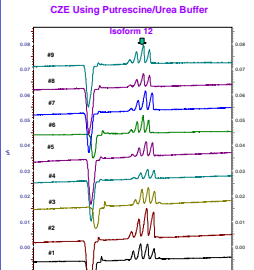
Test samples	Products	Lot s
#1	Sample A	NA
#2	Sample B	NA
#3	Sample C	NA
#4	Sample B	#1
#5	Sample B	#2
#6	Sample B	#3
#7	Sample B	#4
#8	Sample B	#5
#9	Sample B	#6

Methods, Equipments and Materials

Methods	Equipment	Major Materials
Putrescine CZE	Beckman MDC or PA-800	A bare (uncoated) silica-fused capillary, effective length 30 cm, 50 µm I.D. Buffer solution containing 5mM putrescine and 7M urea at pH 5.7-5.8 Separation in normal polarity mode UV detection at 214nm
PCZE/Urea	Beckman MDC or PA-800	A bare (uncoated) silica-fused capillary, effective length 50 cm, 50 µm I.D. Buffer solution containing 200 mM sodium phosphate and 6M urea UV detection at 200nm
CE-IEF	Beckman MDC	BioCap LFA capillary, effective length 20 cm, 50 µm I.D. Carrier: 750 µL of 7 M Urea, 200µL of 1% HPNC, 45 µL of Pharmalyte 2.5-5 and 5 µL of Servalyt 3-5 Test sample: 70 µL of carrier + 30 µL of de-salted protein + 2µL of 10% TEMED Focusing: 15KV for 8 mins Mobilization: 30KV for 22 mins UV detection at 280nm
ICE ₂₈₀	Convergent Prince Autosampler	Fc (FluoroCarbon) coated capillary Carrier: 650 µL of 8M Urea, 300 µL of 1% HPNC, 45 µL of Pharmalyte 2.5-5 and 5 µL of Servalyt 3-5 Test sample: 80 µL of carrier + 20 µL of de-salted protein + 2 µL mixture of pI marker (pI 3.7, 5 & 5.30) Focusing: 3000 V for 7 mins UV detection at 280nm
VEF	Electrophoresis cell Programmable Power Supply Orbital Shaker Platform Bio-Rad GS-800 densitometer	IEF Gel: 5M Urea, Bis-acrylamide, Acrylamide, Servalyt 3-10, Servalyt 3-5 and Potassium Persulfate Anode solution: 0.1 M Sulfuric acid Sample buffer: 2x Novex IEF buffer with bromophenol blue Separation: 100 volts for 15 mins + 200 volts for 15 mins + 500 volts for 90 mins Stain: Coomassie Brilliant Blue
IEF	Multiphor II IEF Unit pH surface electrode Orbital Shaker Platform Cooling apparatus Power supply Bio-Rad GS-800 densitometer	IEF Gel: 5M Urea, Acrylamide, Bis-acrylamide, Servalyt 3-10, Servalyt 3-5 and Potassium Persulfate Anode solution: 0.1 M Sulfuric acid Pre-focusing (20-40 min at 10 Watts) and Focusing (~2.5 hrs at 10 Watts) as specified in A01106 Electrofocusing strips Stain: Coomassie Brilliant Blue R (A0112)

Technical Comparison

CZE/Putrescine



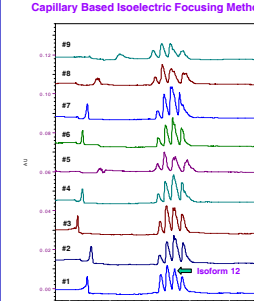
Advantages:

- A modified method from an official CZE method described in European Pharmacopoeia (EP)
- Produce close to baseline resolution between isoforms 9-14
- Has been fully qualified for general method parameters as well as robustness and ruggedness

Disadvantages:

- Capillary preparation is crucial for method performance and relatively time consuming (12 – 15 hours)
- Identification of isoforms can be complicated by shifts in elution times

Capillary Based Isoelectric Focusing Method CE-IEF

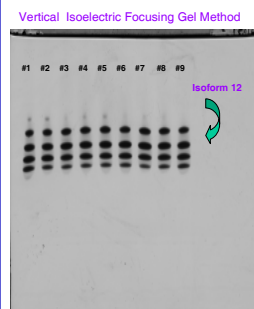


Method: "Capillary Electrophoretic Separation of EPO by Isoelectric Focusing using Beckman MDC"

Disadvantages:

- Relatively time-consuming (8 mins pre-rinsing, 8 mins focusing and 22 mins mobilization)
- Sample precipitation may occur over extended periods of time after mixing with ampholyte solution
- Noisy baselines and changes in peak shape
- Less reproducible than other methods

Vertical Isoelectric Focusing Gel Method vIEF



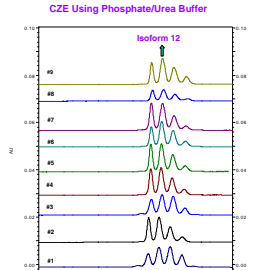
Advantages:

- Qualitatively reliable
- Equipment is small and inexpensive
- Very little sample preparation
- More tolerant of differences in sample composition
- Suitable for monitoring of in-process samples

Disadvantages:

- Gel preparation is an important step for reproducible results
- Densitometry parameters and staining effect isoform quantitation

CZE Using Phosphate/Urea Buffer CZEP/Urea



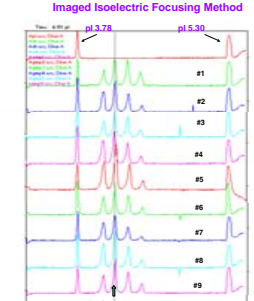
Advantages:

- Simple, straightforward, less time consuming than Putrescine CZE method
- Short capillary preparation time (1-2 hours)
- Less sample preparation
- Reproducible

Disadvantages:

- Relatively less resolution between isoforms 9-14
- Identification of isoforms can be complicated by shifts in elution times

Imaged Isoelectric Focusing Method iCE₂₈₀



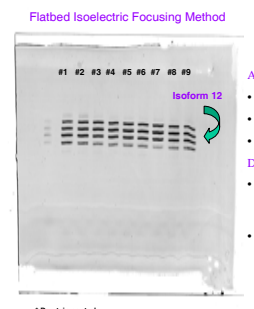
Advantages:

- High throughput (< 20 mins)
- Provides best resolution among techniques examined in this study
- Measure both of pI value and relative isoform distribution from a single injection
- Reproducible

Disadvantage:

- Slight differences in pI values when different capillaries, reagents, ampholytes and pI markers are used. However, the pI values are quite consistent within a single sequence (pI differences <= 0.03)

Flatbed Isoelectric Focusing Method fIEF



Advantages:

- Equipment is small and inexpensive
- Very little sample preparation
- Qualitatively reliable

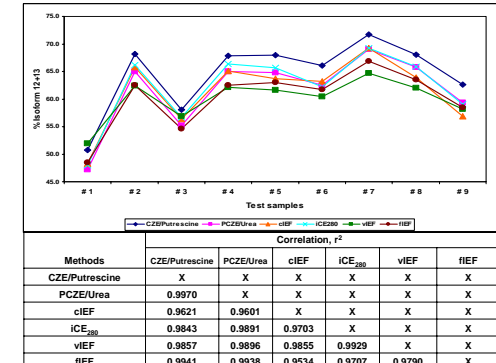
Disadvantages:

- Gel preparation, sample loading technique and positioning of the anode and cathode strips impact the band separation
- Densitometry parameters and staining effect isoform quantitation

Comparison of Analytical Results

No.	Samples	Isoforms 12+13						Min Avg(%)	Min %RSD (%)
		CZE/Putrescine	PCZE/Urea	cIEF	ICE ₂₈₀	vIEF	fIEF		
#1	Sample A	50.7	47.2	48.3	48.0	51.9	48.5		
#2	Sample B	68.2	65.0	65.8	66.2	62.4	62.5	65.0	3.5
#3	Sample C	58.1	55.2	56.4	56.7	54.9	54.9		
#4	Sample B Lot#1	67.8	65.0	65.1	66.4	62.1	62.5	64.8	3.4
#5	Sample B Lot#2	68.0	64.8	63.7	65.7	61.6	63.0	64.5	3.5
#6	Sample B Lot#3	66.1	65.5	63.2	62.2	60.4	61.7	62.7	3.1
#7	Sample B Lot#4	71.7	69.1	69.3	64.7	66.9	68.5	68.5	3.5
#8	Sample B Lot#5	68.1	65.8	63.9	65.9	62.0	63.5	64.9	3.2
#9	Sample B Lot#6	65.6	64.4	65.9	69.1	65.2	65.5	69.1	3.3
Sample B Lot-to-Lot Average (%)		67.5	64.5	64.9	65.0	61.6	62.7		
Sample B Lot-to-Lot %RSD (%)		4.1	4.6	5.4	5.1	3.2	4.0		

The percent isoform distribution of isoforms 9-14 for nine test samples were measured in each of six methods. The sums of isoform 12+13 value were summarized and used as a basis for comparison. The Relative Standard Deviation (RSDs) of % Isoform 12+13 for the six different methods (pink) and for different lots (Sample B) (blue) are compared.



The correlations between methods were derived by plotting isoform 12+13 values of nine test samples in one method (x-axis) against the isoform 12+13 values in another method (y-axis). The coefficient of determination (r²) based on a linear regression was determined for each comparison. A correlation of r² (0.9970) indicates good agreement between Putrescine CZE and PCZE/Urea methods, while r² (0.9534) indicates the least agreement between cIEF and fIEF methods.

Conclusions

- All six methods studied in this report provide comparable, but not equivalent, results for % Isoform 12+13.
- The putrescine CZE method trends slightly higher in isoform 12+13 than other techniques, while the vertical IEF gels trends slightly lower.
- The average coefficient of variation (RSD) for the method-to-method comparison (3.4%) is less than the lot-to-lot comparison using the same method (4.5%).
- Direct comparison of samples should be limited to the same method when possible.
- The choice of method is dependent on the equipment available, analyst expertise and the type of information desired.

Recommendations

- Comparability**
IEF gel electrophoresis is probably the most accessible method in the majority of laboratories. The equipment is small and inexpensive. This method requires less sample preparation and is recommended for a qualitative assay or monitoring in-process samples. Of the two gel methods (vertical IEF or horizontal flat bed IEF), vIEF tends to be more tolerant of differences in sample composition.
- Quantification:**
CZE methods provide reliable quantification of isoform distribution. Resolution of isoforms is slightly better in putrescine CZE but the relative ease of capillary preparation and greater tolerance of differences in sample composition favor the use of PCZE/Urea. For both CZE methods, identification of individual isoforms can be complicated by differences in sample composition since there are no ampholytes to stabilize migration.
- Characterization:**
Capillary isoelectric focusing method (iCE₂₈₀) is theoretically the most accurate since it actually focuses the samples in a pH gradient. This method is reliable in quantification of isoform distribution and the resolution of the isoforms is superior to cIEF and the other methods in this study. The method can also provide an isoelectric point aiding in identification of the individual isoforms based on comparison to internal standards, i.e. pI marker. All of these advantages make this the method of choice for characterization.