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Application Note

Human Erythropoietin (EPO) - Protein, CZE

Analysis of Recombinant Human Erythropoietin on deltaDOT's CE platform

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Recombinant Erythropoietin (EPO) has uses in legitimate treatment of disease such as anaemia, but has become notorious for its use in blood doping in endurance sports. The glycoform fingerprint obtained by this analysis, using the Peregrine High Performance Capillary Electrophoresis system and deltaDOT's proprietary LFII® technology, gives high quality reproducible data in terms of peak mobility (<2% RSD) and peak area (<2% RSD). This can potentially be used to identify individual vendors' formulations as well as misuse.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone which is involved in stimulating the production of new red blood cells. Recombinant human EPO is the standard of care in the treatment of certain types of anaemia.

EPO has a molecular weight of about 30.6 kDa and a pI of ~ 4.4. Approximately 40% of the molecular mass of a mature molecule is made up of carbohydrate. Glycosylation of EPO has been shown to be essential for its biological activity. It has 4 glycosylation sites, three N-linked and one O-linked. These contain up to a maximum of 14 sialic acid residues. Consequently, EPO presents a mixture of glycoforms which differ in the structure, composition and charge of their isoforms. These glycoform populations have been shown to be both species- and tissue-specific.

Recombinant EPO (rEPO) has been available as a drug since 1988. It has been established that the electrophoretic profiles of these recombinant forms differ from natural, purified urinary EPO. Due to the illicit use of rEPO by athletes to boost their performance in endurance sports, the International Olympic Committee banned its misuse in 1989. Doping control to assess for its presence may be carried out by various indirect means by monitoring certain haematological parameters. However, unambiguous confirmation of the presence of rEPO in a physiological fluid is preferred. Currently, the established capillary electrophoresis (CE) protocol that allows baseline resolution of 8 EPO isoforms is the European Pharmacopoeia (EurPh) methodology.

This report demonstrates the use of the LFII® Peregrine HPCE instrument for the label free analysis of EPO sialoforms using capillary zone electrophoresis (CZE). The separation parameters and buffers are based on the protocol established by the EurPh with improved separation and analysis times.

MATERIALS AND METHODS

The separation of erythropoietin isoforms was performed on the deltaDOT CE platform, Peregrine. Separations were carried out in bare fused silica capillaries of 50 μm internal diameter and a separation length of 72 cm. Total length of capillary was 84 cm. The capillary was initially conditioned with a proprietary methodology and then with separation buffer for 30 minutes. The capillary was then held at 20 kV for 15 minutes. Between runs the capillary was rinsed with a proprietary rinse for 5 minutes followed by run buffer for 20 minutes, after which an automated Ohm's Law Plot was performed and the voltage held at 18 kV for 15 minutes.

Lyophilized rEPO standard was prepared as detailed in the European Pharmacopoeia. A proprietary separation buffer was also prepared. Prior to injection the samples were desalted on desalting columns (PIERCE). All samples were injected hydrodynamically at 1 psi for 15 seconds and run at a voltage of 15 kV. The capillary was held at 35 °C during the separation. Detection was carried out at 214 nm.

RESULTS

The rapid and efficient separation of the EPO glycoforms was performed using a deltaDOT Peregrine system and data was analysed using both deltaDOT's Equiphase Vertexing Algorithm (EVA) and General Separation Transform (GST) algorithm. GST is a method of combining the data from the 512 pixels in a natural way which preserves the peak shape information of the electropherograms while at the same time maximising the signal-to-noise ratio. We typically observe a 10-fold increase in signal-to-noise using GST as compared to a standard single pixel electropherogram. In addition, the absorbance A can be read directly off the GST plot peak height V as a function of time t ($A = 0.434 \times V(t)$).

EVA is an advanced pattern-recognition tool which maximizes the system resolution. In EVA the electropherograms are first analyzed to find local peaks. These are used first to perform vertexing (determine the point of origin of the bands) and then to produce a signal output.

The raw electropherogram and GST processed data for the EurPh standard EPO sample is detailed in Figure 1 below. Nine isoform peaks were observed with baseline resolution between each peak. Separation of the sample was complete within 45 minutes, as compared to an hour in the established EurPh methodology. The large front migrating in front of the glycoprotein (Figure 1 panel A and Figure 2) is a solvent front.

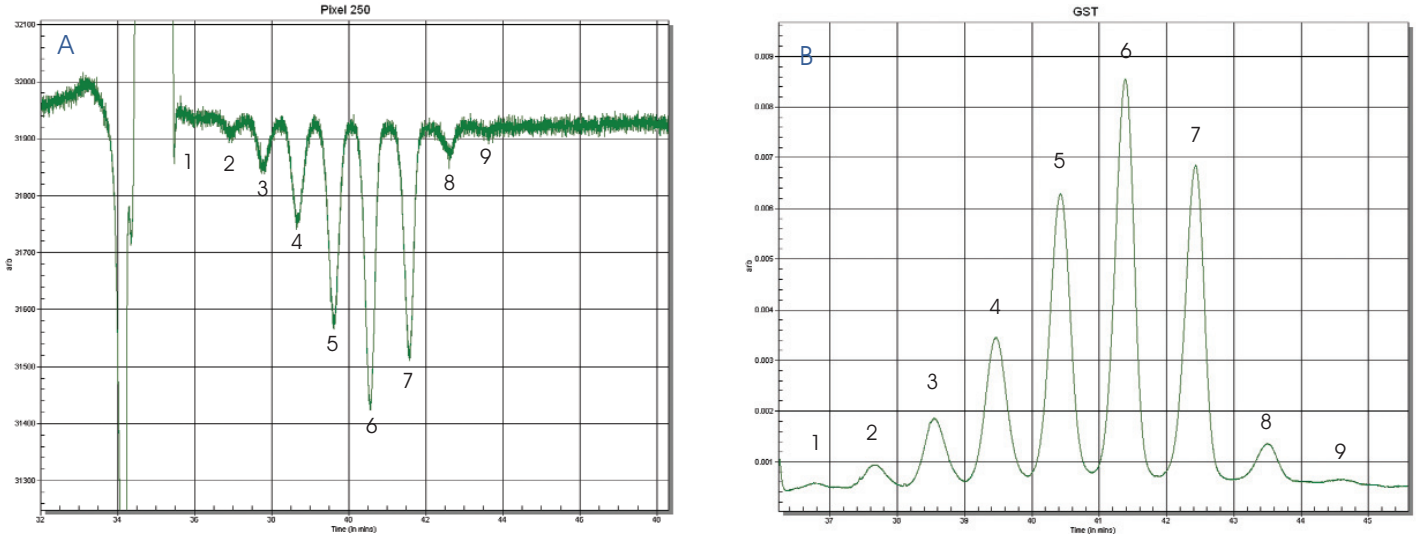

Figure 1: **Panels A and B detail the raw electropherogram and GST processed data respectively, for the recombinant human EPO. The isoform peaks have been labelled. Isoform 1 and 9 are seen reproducibly but are present in trace amounts.**

The recombinant EPO sample analysed is similar, but not identical to the European Pharmacopoeia EPO standard, some variation between the glycoform profiles was observed. The percentage content of each isoform was determined from its GST peak area (Table 1). These values compared well with the range established by the EurPh. All the isoforms fall within the range except for isoform 7. Glycosylation patterns are influenced by variation in the specific cell line used during sample manufacture, variable incubation culture conditions and the purification procedure used.

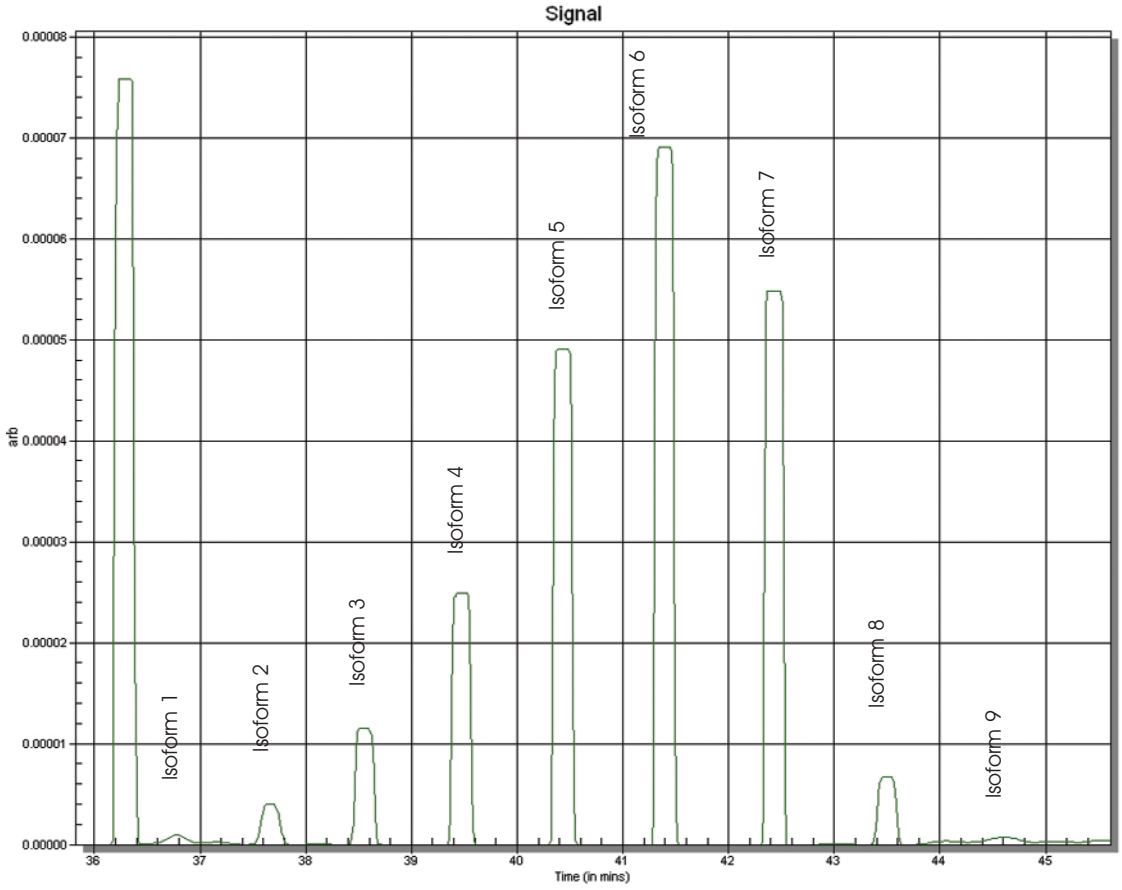

Figure 2: EVA processed data for the recombinant Human EPO sample. The isoform peaks are labelled.

Isoform	Percent content (as defined by European Pharmacopoeia)	Peregrine GST processed Area	Peregrine Content (%)
1	0 - 15	0.0492	0.5
2	0 - 15	0.1968	1.9
3	5 - 20	0.5788	5.5
4	10 - 35	1.3038	12
5	15 - 40	2.5532	23
6	10 - 35	3.46	30
7	0 - 20	2.7414	23.5
8	0 - 15	0.3417	2.9
9	Not defined	0.0402	~ 0.3

Table 1: Percentage content of each isoform determined from the corresponding peak area are detailed in the table above compared with range established by the European Pharmacopoeia.

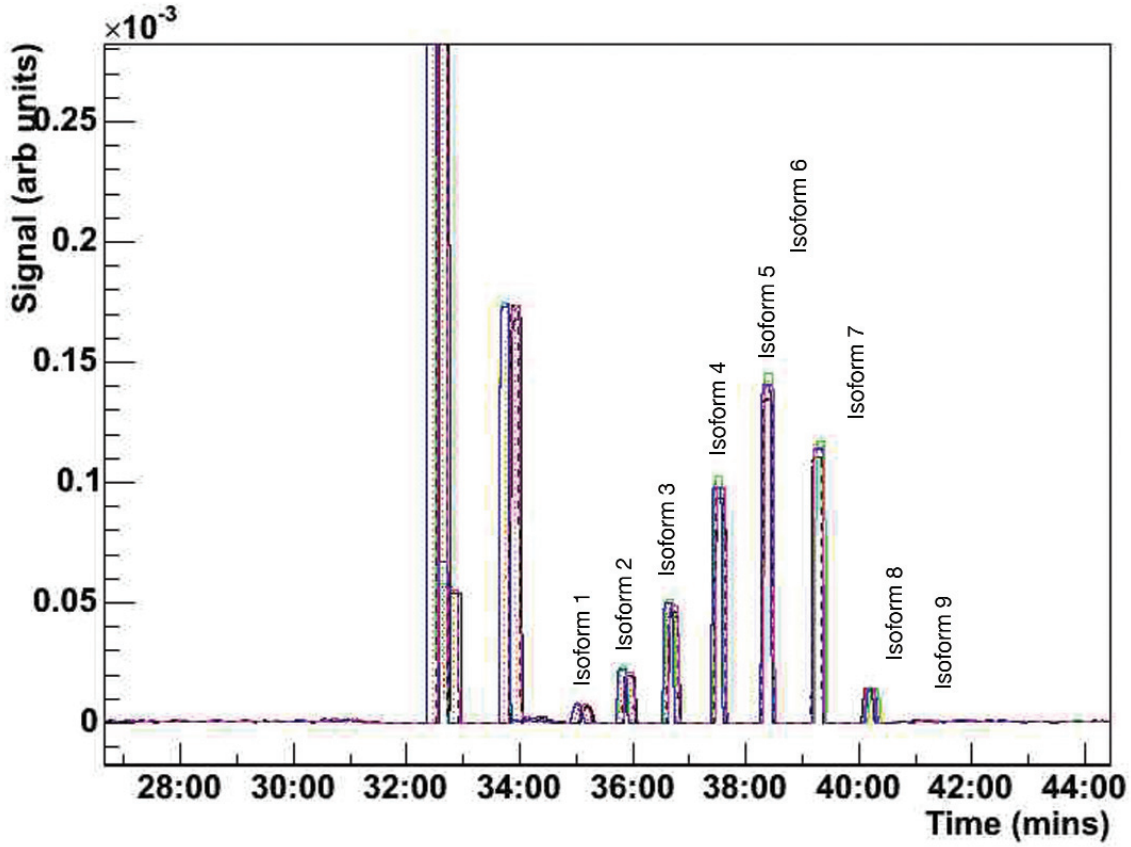

Figure 3: Overlay of EVA processed data for recombinant human EPO (N=10). The peaks migrating in front of the EPO peaks are solvent fronts associated with the buffer.

Reproducibility of the Peregrine HPCE with LFII® system was demonstrated for each of the nine isoform peaks. Reproducibility was calculated from EVA data, based upon peak migration times and peak areas, as shown in Table 2 and Table 3 respectively.

EPO Isoform No.	Peak Migration time (in minutes) Average	St. Dev	Percentage RSD
1	35.2152	0.4740	1.3461
2	36.0109	0.5128	1.4240
3	36.6607	0.4210	1.1485
4	38.0270	0.6650	1.7488
5	39.0444	0.5380	1.3779
6	39.9386	0.5709	1.4294
7	41.3253	0.4575	1.1071
8	41.9922	0.7302	1.7390
9	42.1821	0.8110	1.9225

Table 2: EVA reproducibility for peak migration time for 9 rEPO isoform peaks observed (N=10).

EPO Isoform No.	Peak Area Average	St. Dev	Percentage RSD
1	0.0002	1.92E-05	7.1528
2	0.0008	1.71E-05	1.9925
3	0.0024	4.08E-05	1.6512
4	0.0054	8.25E-05	1.5261
5	0.0107	18.62E-05	1.7428
6	0.0159	25.66E-05	1.6160
7	0.0126	22.76E-05	1.8078
8	0.0016	2.17E-05	1.3616

Table 3: EVA reproducibility for Peak Area for rEPO isoforms peaks 1 to 8 (N=10). Peak 9 was observed consistently but its area varied significantly between separations. Large variations in area were observed for peaks 1 and 9 because of their small size.

CONCLUSION

It has been shown that recombinant EPO glycoforms may be reproducibly analysed using the deltaDOT Peregrine LFII® HPCE system. Modification of the European Pharmacopoeia protocol gave similar or better results with shorter capillary conditioning and sample analysis times. According to the established EurPh protocol the capillary should be initially conditioned for 12 hours at 20 kV, while the modified protocol recommends a 15 minute condition at 20 kV. This has allowed same day separations with reproducible results; variation in peak migration time for isoforms 1-9 over 10 runs was below 2 percent and the peak area variation for isoforms 2-8 was the same.

A robust analytical technique may have important applications not only in doping control but also in the pharmaceutical industry, where it may be applied to quality control of recombinant human EPO and its analogues. New products may be qualitatively and quantitatively compared to a standard (EurPh) to look for impurities and variations in glycoform content.

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