Separation of Recombinant Human Erythropoietin (rhEPO) using the European Pharmacopoeia Method on the PA 800 *plus* Pharmaceutical Analysis System

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Introduction

Erythropoietin (EPO) is a naturally occurring hormone that stimulates red blood cell production and release from bone marrow. EPO was one of the first therapeutic recombinant glycoproteins commercialized for the treatment of anemia. It is known to have complex N- and O-linked glycosylation patterns and can exist as numerous protein isoforms that play a critical role in the bioavailability, activity, potency and stability of EPO. Proper characterization of EPO is extremely important in order to ensure the comparability or efficacy of a biologic preparation.

Until 1999, gel-based isoelectric focusing (IEF) separation of EPO was the standard method adopted by the European Pharmacopeia (EuPh). However, this method lacked adequate quantitation and reproducibility. Because of this, a collaborative study initiated by the European Directorate for the Quality of Medicine (EDQM) was performed. The results of this study established Capillary Zone Electrophoresis (CZE) as the method of choice for the quantitation of EPO isoforms.[1] Additional collaborative studies by pharmaceutical companies, government agencies and academic institutions have refined this method and developed a suitability standard.[2]

This application note provides guidance for the preparation of an EPO suitability standard and separation buffer. Instrument configuration and set up of the EuPh method parameters for the analysis of EPO in 32 Karat software using the PA 800 *plus* Pharmaceutical Analysis System are also described.

Experimental Design

Sample Preparation:

The EPO BRP3 – EuPh Reference Standard (LGC Promochem, Middlesex – UK) was used in this work. Two hundred and fifty milliliters of double distilled and deionized (ddi) water was added to reconstitute the lyophilized EPO standard. The resulting solution was desalted using a Millipore Microcon YM-3 (or equivalent) as described below.

Step 1 YM-3 – Membrane Wash

- Assemble the YM-3 sample reservoir into the centrifuge tube provided according to instructions provided by Millipore
- Dispense 250 µL of ddi water into the sample reservoir
- Centrifuge at 13,000 *g* for 10 minutes. Discard retentate and eluent

Step 2 – Sample Concentration

- Dispense 250 µL of reconstituted EPO to the washed sample reservoir
- Centrifuge at 13,000 *g* for 10 minutes

Discard only the eluent Step 3 – Sample Desalting

(this step must be repeated 4 times)

- Dispense 250 µL of ddi water onto the sample reservoir
- Centrifuge at 13,000 *g* for 10 minutes
- Discard only the eluent

Final step – Desalted Sample Recovery

- Carefully place sample reservoir upside down into a new vial (provided by Millipore for this purpose)
- Centrifuge at 2,000 *g* for 2 minutes
- The sample was stored at 4° C. The recovered volume was approximately 125 µL

Determination of Sample Concentration

The protein content was measured spectrophotometrically at 280 nm as per the procedure also described in the EuPh of EPO using a DU 800 Spectrophotometer (Beckman Coulter, Inc.). The absorptivity of a 1% solution of EPO was 7.43. The concentration of desalted EPO used in this work was 0.17 mg/mL.

Preparation of CZE Buffer Concentrate

The buffer preparation was performed as described in the European Pharmacopoeia method. The following components were added to a 100 mL volumetric flask: 0.582 g of NaCl (S1249 - Spectrum), 1.793 g of Tricine (part no. T5816 – Sigma), 0.820 g of sodium acetate (part no. 3470-01 - J.T. Baker), ddi water was added up to 100 mL. This solution was filtered through a 0.2 µm membrane using a Nalgene filter unit (MF75) and stored at 4º C.

Preparation of 1M Putrescine Solution

A bottle of putrescine (part no. 3279 - Sigma) was placed in a water bath at 60ºC and the contents were allowed to melt. This process took approximately 20-30 minutes. Using a disposable transfer pipette, 0.882 g of putrescine was weighed directly into a 10 mL volumetric flask, and ddi water was added to complete the volume to 10 mL. The solution was mixed and separated into 500 µL aliquots and stored at 4º C.

Preparation of CZE Running Buffer

The following components were added to a 50 mL polypropylene tube: 21 g of urea (part no. U1250 – Sigma), 5 mL of CZE Buffer Concentrate, 125 µL of 1 M Putrescine and 25 mL of ddi water. The pH was adjusted to 5.5 at 30º C with 2 N HAc (prepared from glacial acetic acid, part no. A6283 – Sigma). This solution was filtered through a 0.2 µm membrane using a Nalgene filter unit MF75 and stored at 4º C. This buffer is stable for one week.

Capillary

A bare fused silica capillary, 50 µm i.d., 110 cm total length with 100 cm effective length was used for this separation (Beckman Coulter part no. 338472).

Instrument Set-up Details

The instrument used to perform these separations was the PA 800 *plus* Pharmaceutical Analysis System equipped with UV/vis detection configured with a 214 nm filter. System control was by 32 Karat v. 9.0 software. The cartridge and sample storage temperatures were set at 35º C and 4º C, respectively. The UV detector was set with a data rate of 0.5 Hz, the detector filter was set to normal and the filter peak width points were set to 16-25. These settings were used for all three methods in this work: equilibration, separation and shutdown which will be described later on this section.

Reagent Vial Volumes

For all methods used in this work, the reagent vials contained 1.5 mL of 0.1 N NaOH, ddi water and CZE running buffer. Both reagent and waste vials were incremented every 10 separations to avoid ionic depletion of run buffers and a decrease in volume of rinsing solutions.

Note: The waste vials must always contain 1.0 mL of ddi water to prevent crystallization and carryover of urea.

Capillary Equilibration Method

The capillary was rinsed with 0.1 N NaOH at 20 psi for 60 minutes followed by CZE running buffer at 20 psi for 60 minutes, and voltage equilibration performed at field strength of 181.8 V/cm for 720 minutes.

EPO CE Separation Method

The capillary was rinsed with ddi water at 20 psi for 10 minutes followed by 0.1 N NaOH at 20 psi for 5 minutes, CZE running buffer at 20 psi for 10 minutes and then sample injection at 0.7 psi for 20 s. Separation was performed at field strength of 143 V/cm. The total separation run time was 70 minutes. Auto-zero was performed 5 minutes after the start of the separation.

Important Note: For improved migration time, peak area and peak shape reproducibility, the use of 0.1 N NaOH solution which has been stored in borosilicate glass bottles may be needed. It was found that exposure of the 0.1 N NaOH rinse solution to borosilicate glass for a minimum of 24 hours improved the reproducibility of migration time for EPO. Studies indicated that using NaOH stored in a plastic container accounted for variation in EOF of 18.6 % (n=50) as compared to 3.4% (n=50) for NaOH stored in a borosilicate glass bottle for a period of 24 hours. For more information, see the poster, "Separation of Recombinant Human Erthyropoietin (rhEPO) using the European Pharmacopoeia Method on the PA 800 plus," on Beckman Coulter's web site.

Shutdown Method

The capillary was rinsed with ddi water at 20 psi for 10 minutes followed by 0.1 N NaOH at 20 psi for 5 minutes, CZE running buffer at 20 psi for 10 minutes. At the end of this method, the lamp was turned off and the ends of the capillary were stored in water.

Typical electropherogram obtained of EPO BRP batch 3 (EuPh standard) is shown in Figure 1. The current throughout the separation is around 6+/- 0.5 µA.

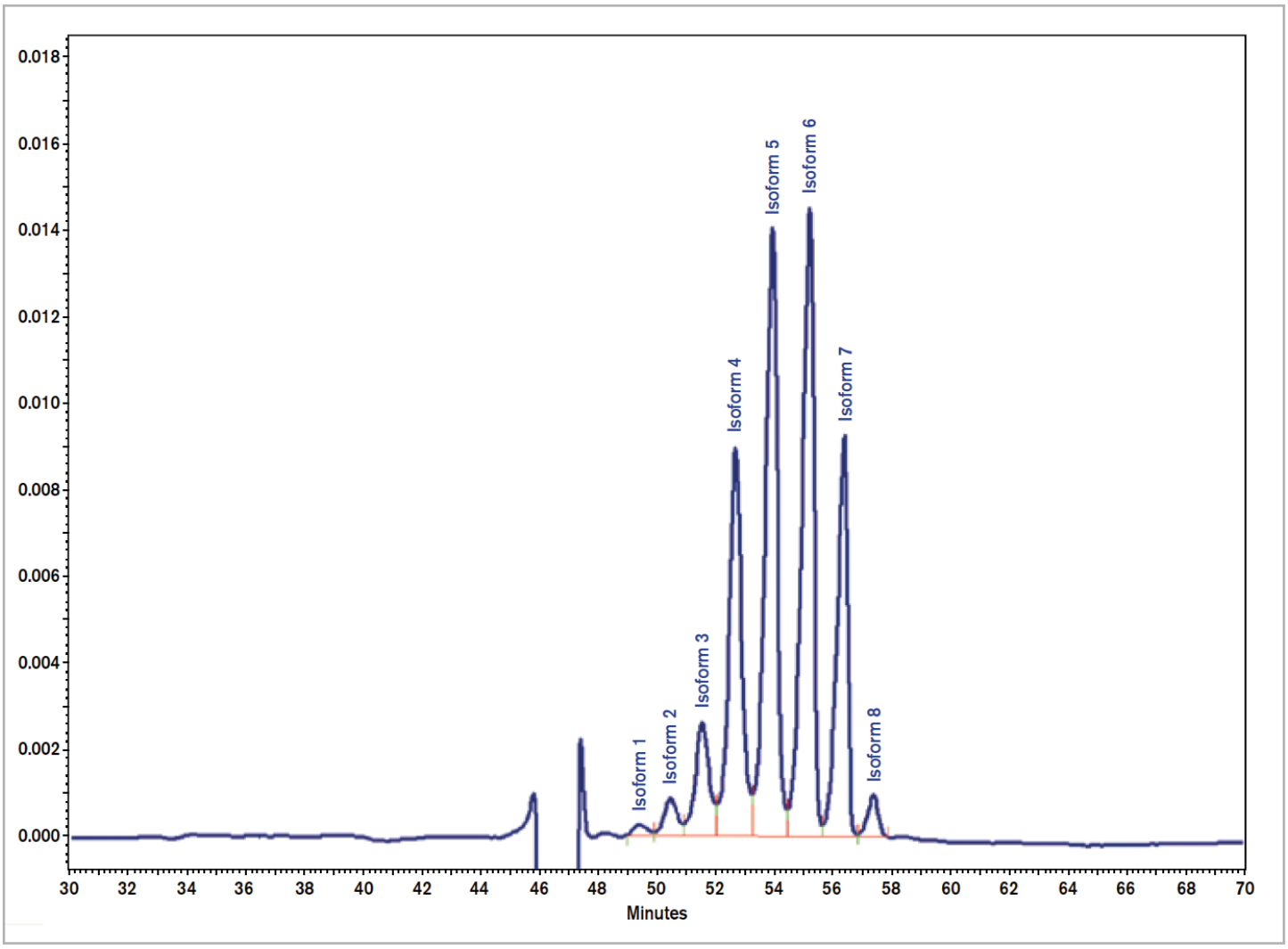

Figure 1. Typical electropherogram of EPO BRP batch 3.

Data Analysis Parameters and Peak ID Table Software Set Up

The screen shot below (Figure 2) shows the parameters used to integrate the electropherogram in Figure 1. These parameters may change depending on the performance of the separation.

#		Event	Start Time	Stop Time	Value
1	✓	Width	0.000	0.000	0.5
2	✓	Threshold	0.000	0.000	75
3	✓	Shoulder Sensitivity	0.000	0.000	100000
4	✓	Minimum Cluster Distance	0.000	0.000	25
5	✓	Integration Off	0.000	48.000	0

Figure 2. Integration events table.

Following proper integration, a peak table can be created for proper peak identification and for system suitability reporting. Figure 3 shows the Peak ID table used to name the peaks on the electropherogram in Figure 1. The numbers displayed for the migration time and MT Window are populated by the software.

Named Peaks | Groups

#		Name	ID	Mig. Time	MT Window	Ref. ID #	ISTD. ID #
1	✓	Isoform 1	1	49.4	2.47	0	0
2	✓	Isoform 2	2	50.4333	2.52167	0	0
3	✓	Isoform 3	3	51.5333	2.57667	0	0
4	✓	Isoform 4	4	52.6667	2.63333	0	0
5	✓	Isoform 5	5	53.9333	2.69667	0	0
6	✓	Isoform 6	6	55.2	2.76	0	0
7	✓	Isoform 7	7	56.4	2.82	0	0
8	✓	Isoform 8	8	57.4	2.87	0	0

Figure 3. Peaks/Groups table.

System Suitability Parameters Set Up

According to EuPh the system suitability parameters are as follows.

Parameter System Suitability 1

The relative standard deviation of the migration time of the peak corresponding to isoform 2 is less than 2 %. In 32 Karat, the System Suitability screen should be set up as shown in Figure 4.

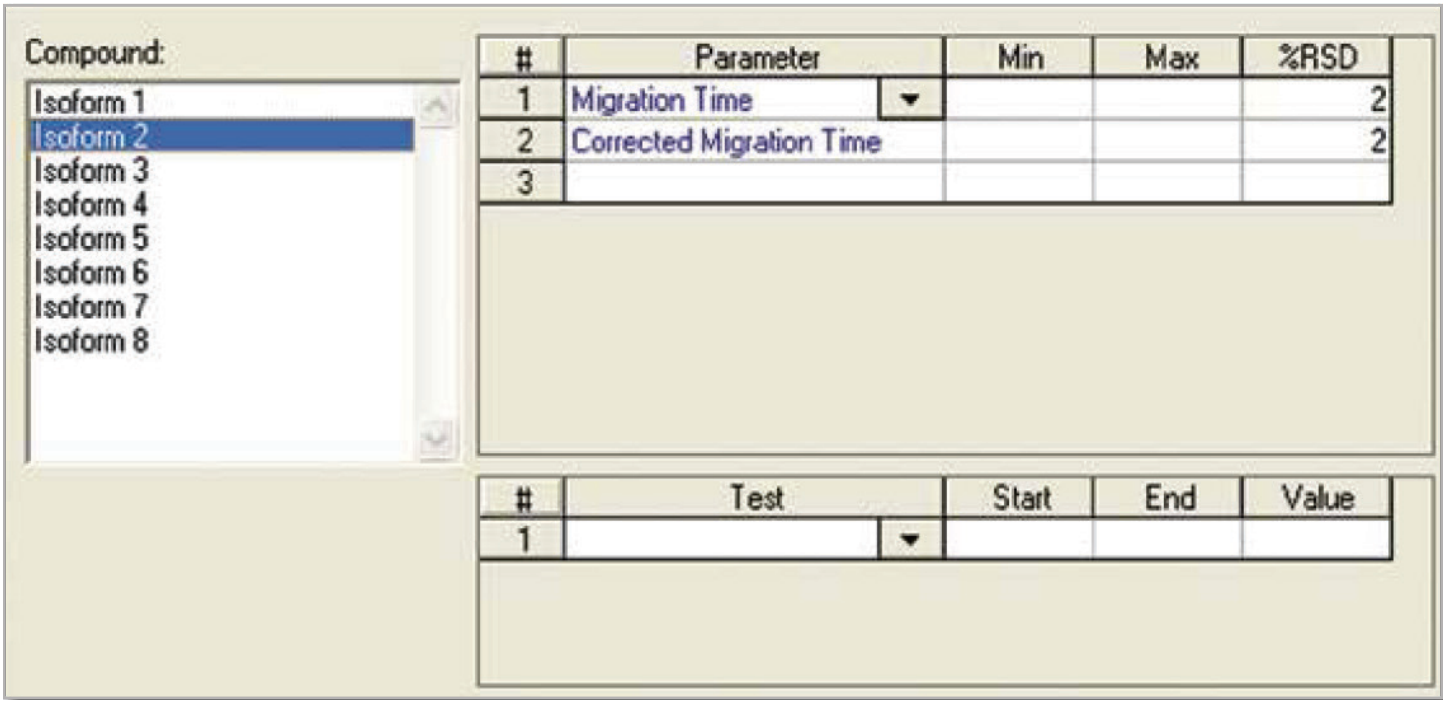

Figure 4. System suitability parameter for isoform 2.

Parameter System Suitability 2

The largest peak in the electropherogram is at least 50 times greater than baseline noise. On 32 Karat's system suitability table, parameters should be set up as described in Figure 5.

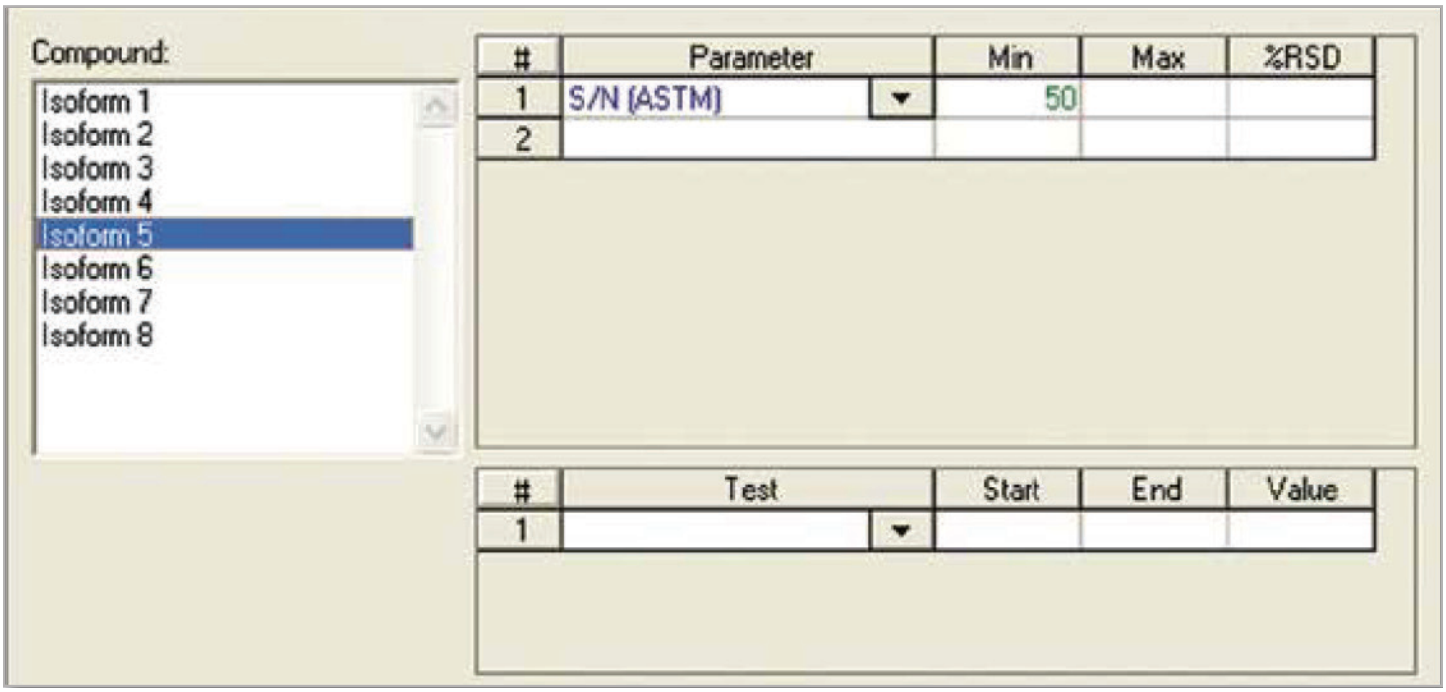

Figure 5. System suitability parameter for S/N ratio.

Parameter System Suitability 3

The resolution between isoforms 5 and 6 is not less than 1.
Figure 6 shows software set up.

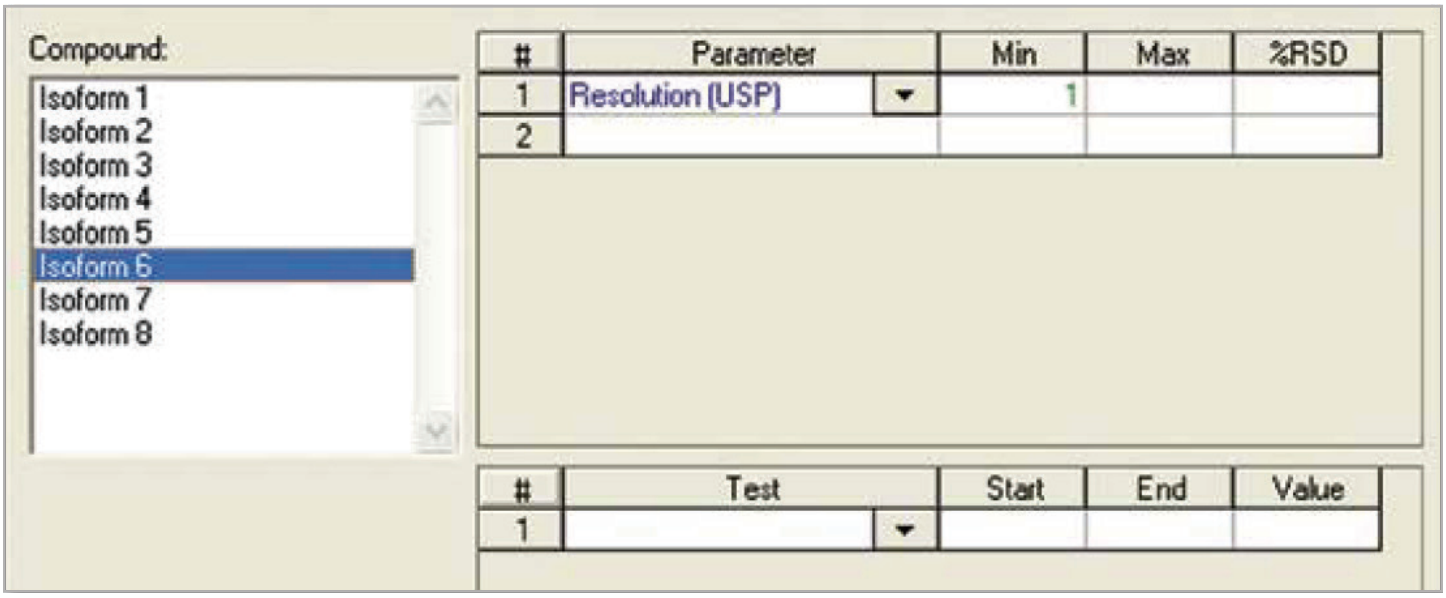

Figure 6. System suitability parameter for S/N ratio.

It is a requirement from the EuPh to perform 3 consecutive system suitability separations prior to each sequence table.

For more information on how to access these screens for software set up please consult 32 Karat online help.

Limit of the Assay

The EuPh has also established the limits for isoform content based on the peak area percent composition. The peaks corresponding to isoforms 1-8 must have a percentage composition in peak area according the ranges below:

Peak Name	Content Allowed Range (Peak Area Percent)
Isoform 1	0-15
Isoform 2	0-15
Isoform 3	5-20
Isoform 4	10-35
Isoform 5	15-40
Isoform 6	10-35
Isoform 7	0-20
Isoform 8	0-15

Conclusion

This work demonstrates preparation, from reconstitution to desalting, of an EPO suitability sample. In addition, important parameters describing method and instrument set up, capillary conditioning and successful separation of the EPO isoforms by CZE including software parameters for data analysis and system suitability are described.

References

1. A. Bristow and E. Charton. 1999. Assessment of the suitability of a Capillary Zone Electrophoresis method for determining Isoform Distribution of Erythropoietin. *Pharmeuropa.* Vol. 11-2: page 290.
2. Behr-Gross ME, Daas A, Burns C, Bristow AF. 2007. Collaborative Study for the Establishment of Erythropoietin BRP Batch 3. *Pharmeuropa Bio & Scientific Notes.* Vol. 1: page 49.

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