Quantitation of Somatropin Charge Variants Impurities by Capillary Zone Electrophoresis (CZE)

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Introduction

Recombinant human growth hormone (Somatropin, hGH) is a 191 amino acid protein of 22 kDa molecular weight. It is used in the treatment of a number of childhood growth disorders and adult growth hormone deficiency. Control and release methods have focused on physical-chemical parameters including the charge variants. [1]

In January 2006, Eu. Ph. replaced IEF with CZE for the control of charge related impurities based on the conclusion of a interlaboratory collaborative study, which concluded that capillary zone electrophoresis (CZE) was more sensitive and reproducible.[1] In the following three years, the method performance was monitored and further optimized. In 2009, Scientific Notes (2009-1)[4] published an optimized CZE condition, which introduced a capillary deactivation step and equilibration steps before the results are used for quantitation. The publication also included a complete package of verification data.

Here we demonstrated quantitation of Somatropin charge variants impurities by CZE can be successfully executed on PA800 Plus Pharmaceutical Analysis System.

Key Features

- Detailed instructions on Eu. Ph. Somatropin charged variants analysis method execution on the PA800 Plus Pharmaceutical Analysis System (Figure 1)
- Tips to reduce separation time and improve method repeatability

Figure 1. The PA800 Plus Pharmaceutical Analysis System

Materials and Method:

Instrument and Software: All experiments were performed on a PA800 Plus Pharmaceutical Analysis System equipped with UV detector and a 200 nm filter. Instrument control and data analysis were carried out with 32 Karat version 10.2.

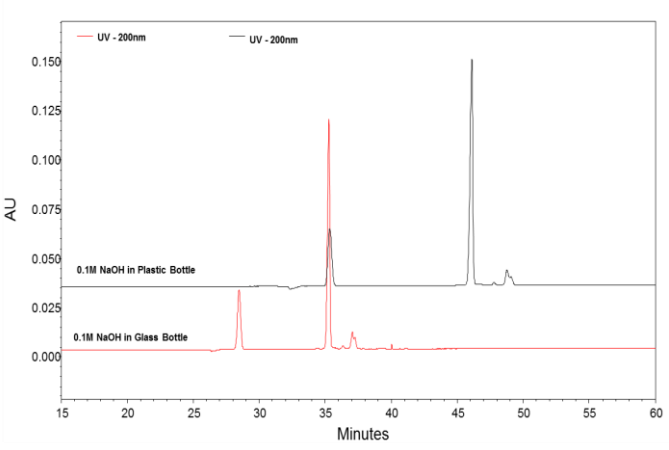

Figure 2. Electropherograms of Somatropin/desamidosomatropin resolution mixture CRS1 generated when rinsed with NaOH solution stored in plastic bottle only (black trace) and that exposed to borosilicate glass vial for 2 hours before transferred to plastic bottle for long term storage (red trace).

Sample Preparations: Eu. Ph. Ref. Std. Somatropin CRS3, Somatropin/desamidosomatropin resolution mixture CRS1 (LGC Standards, USA)[5] and USP Ref. Std. Somatropin (Sigma, USA)

were used in the studies. According to corresponding product leaflet instructions, 3.86 mL double distilled (DDI) water were added to reconstitute both Somatropin standards to a final concentration of 1 mg/mL. 0.85 mL of 0.05 M Tris buffer (pH 7.5) was added to reconstitute the resolution mixture to a final concentration of 2 mg/mL. Eu. Ph. Ref. Std. Somatropin and USP Ref. Std. Somatropin solution were analyzed without further treatment. Eu. Ph. resolution mixture standard was further diluted (1:1) to 1 mg/mL with sample buffer before subjected to CZE analysis. [1-3]

Reagent Buffer Preparation: The CZE buffer was prepared by dissolving 13.2 g of dibasic ammonium phosphate ($(NH_4)_2HPO_4$, Sigma, USA) in 800 mL DDI water, titrating to pH 6.0 using phosphoric acid (H_3PO_4, Sigma, USA) and QS to final volume of 1000 mL in a graduated cylinder. Sample buffer was prepared by diluting CZE buffer to 10 % V/V concentration with DDI water. 0.1 M NaOH solution was prepared by diluting 1 M NaOH solution (EMD Millipore, USA) to 10 % V/V with DDI water. 0.1 M HNO_3 was prepared by dissolving 473 μL of nitric acid, fuming (> 90%, Mallinckrodt chemicals, USA) into 100 mL of DDI water. All solutions used were filtered through 0.2 μm of syringe filter (Pall, USA).

Capillary Cartridge Preparation: A bare fused silica capillary (BEC PN 338472), 50 μm i.d., 80 cm total length with 70 cm effective length was used for separation. A 61.5 cm long coolant tube and a 100X800 (BEC PN 144711) aperture was used to build the cartridge.

Reagent Vial Preparation: For all methods used in this work, refer to Figure 3 for reagent vial layout. Reagent vials contained 1.5 mL of solutions in both inlet and outlet buffer trays. 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. The waste vials contain 1.0 mL of DDI water to prevent capillary clogging and carryover.

Initial Conditions Tab Settings: The sample storage temperature was set to 4 ºC. The cartridge temperature was set to 50 ºC during capillary deactivation and 30 ºC for all other methods. The UV detector was set with a data rate of 4 Hz, the detector filter setting was set to normal and the filter peak width points were set to 16-25. These detector settings were used for all methods in this work.

New Capillary Deactivation Time Program: When a new capillary is installed, the capillary deactivation procedure is performed. The capillary was rinsed with buffers listed below in the following order and conditions specified: acetonitrile at 50 psi for 10 min; water at 50 psi for 15 min; 0.1 M HNO_3 at 50 psi for 30 min; 0.1 M NaOH at 50 psi for 30 min; 0.1 M HNO_3 at 50 psi for 30 min; water at 50 psi for 15 min; CZE buffer at 50 psi for 60 min.

Capillary Condition Time Program: Capillary condition method is performed at the beginning of each sequence. The capillary was rinsed with the listed buffers following the order and the conditions specified: 1 M NaOH at 20 psi for 20 min; CZE buffer at 20 psi for 20 min; 0.1 M NaOH at 20 psi for 10 min; water at 20 psi for 3 min; CZE buffer at 20 psi for 6 min.

Shutdown Method Time Program: Shutdown method is performed at the end of each sequence. The capillary was rinsed with the listed buffers at following the order and the conditions specified: 0.1 M NaOH at 20 psi for 20 min; water at 20 psi for 20 min; CZE buffer at 20 psi for 20 min. Lamp was turned off at the end.

Separation Method: The capillary was first rinsed with 0.1 M NaOH at 20 psi for 10 min, water at 20 psi for 20 min and CZE buffer at 20 psi for 6 min. Sample was injected at 0.7 psi for 8 seconds. Then, a CZE buffer plug was injected after the sample at 0.1 psi for 1 second from a CZE buffer vial in the sample tray. Separation was performed at a field strength of 217 V/cm (17.5 kV). Auto-zero was performed 10 min after the start of the separation (Figure 4). A minimum of 3 equilibration injections need to be performed at the beginning of each sequence.

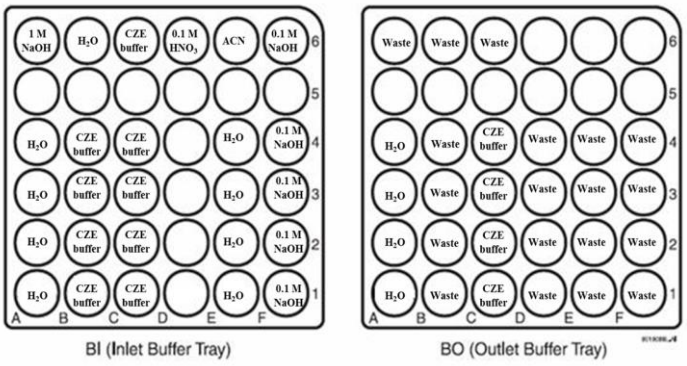

Figure 3. Schematic set up for Inlet (left) and Outlet (right) buffer trays. Note: Row 6 is dedicated for buffers used in capillary deactivation, capillary conditioning and shutdown method. Rows 1-5 are for buffers used in CZE separation. The reagent and waste vials were incremented every 10 injections and this buffer tray set up is enough for 50 continuous injections. Column A water vials are dedicated for capillary end dipping rinse.

Initial Conditions | UV Detector Initial Conditions | Time Program

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Rinse - Pressure	20.0 psi	10.00 min	BI:F1	BO:F1	forward, In / Out vial inc 10	0.1N NaOH rinse
2		Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	water dip
3		Rinse - Pressure	20.0 psi	3.00 min	BI:E1	BO:E1	forward, In / Out vial inc 10	water rinse
4		Rinse - Pressure	20.0 psi	6.00 min	BI:B1	BO:B1	forward, In / Out vial inc 10	CZE buffer rinse
5		Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	water dip
6		Inject - Pressure	0.7 psi	8.0 sec	SI:A1	BO:B1	Override, forward	sample injection
7		Wait		0.00 min	SI:D2	BO:B1		water dip after sample
8		Inject - Pressure	0.1 psi	1.0 sec	SI:D1	BO:B1	No override, forward	buffer injection for stacking
9		Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 10	water dip
10	0.00	Separate - Voltage	17.4 KV	60.00 min	BI:C1	BO:C1	0.50 Min ramp, normal polarity, In / Out vial inc 10	separation
11	10.00	Autozero						
12	60.00	Rinse - Pressure	20.0 psi	3.00 min	BI:E1	BO:E1	forward, In / Out vial inc 10	water rinse
13								

Figure 4. Time program for Somatropin separation method. Line 2 is an example to set up capillary dip rinsing between different solutions. Line 6-8 illustrate the injection of sample followed by the injection of CZE buffer from a dedicated vial in the buffer tray for temperature and ionic strength consistency with samples. Line 10 run time can be adjusted as necessary.

Results and Discussion:

Somatropin/desamidosomatropin resolution mixture CRS1 was first analyzed to demonstrate the method resolution on a PA800 Plus Pharmaceutical Analysis System. The electropherogram matches the description in the Eu. Ph. Monographs including: two peaks prior the surrogate main peak and two peaks after. Among them, P2 corresponds to cleaved form, P3 corresponds to Gln-18 mutation and P4 corresponds to the deamidated forms, eluting as a doublet and is the key impurity to monitor (Figure 2).

A previous publication indicated exposing the NaOH rinse solution to borosilicate glass vial can improve migration time and method repeatability[6]. Similar trends were observed here. Using NaOH solutions stored in borosilicate glass vial for 2 hours improved method repeatability and decreased the separation time by 10 minutes without changing the relative migration time and %corrected area for each critical peak (Figure 2, Table 1).

Note: Minimum 3 injections of the Ref. Std. were necessary to achieve a more stable migration time of all peaks.

The same method can be used to analyze both Eu. Ph. Ref. Std. Somatropin and USP Ref. Std. Somatropin. The distribution of charge variants between the two standards are slightly different, but relative migration time of all impurities are the same and fall in the range specified by Eu. Ph. indicating the identity of the impurities are consistent from the two sources (Figure 5). The amount of P5 in USP. Ref. Std. Somatropin is slightly elevated and better separated from the duplet deamidated peaks.

Table 1. Quantitation results of Somatropin/desamidosomatropin resolution mixture CRS1 from Figure 2.

	Exposed to Glass Bottle		Plastic Bottle Only	
	Relative MT	%CA	Relative MT	% CA
P1	*0.94*	*0.06*	*0.93*	*0.06*
P2	*0.97*	*0.31*	*0.98*	*0.34*
MP	*1.00*	*88.87*	*1.00*	*88.96*
P3	*1.03*	*1.30*	*1.04*	*1.15*
P4	*1.05*	*9.47*	*1.06*	*9.50*

Note: The results are average of triplicate injections from the same sample on the same capillary within the same day.

Repeatability of the method in analyzing both Eu. Ph. Ref. Std. Somatropin and USP Ref. Std. Somatropin was evaluated. Ten consecutive injections of each sample were performed. Five representative charge variant electropherograms of USP Ref. Std. Somatropin (Figure 6) and Eu. Ph. Ref. Std. Somatropin (Figure 7) were overlaid. Relative migration time of each impurity peak and the %CA for the main peak and the critical impurity desamidosomatropin were calculated and summarized in Table 2 and 3. In all cases, the %RSD for relative migration time is < 0.5%; the % RSD for %CA of Somatropin is < 0.5%, much lower than the Eu. Ph. Method repeatability requirement of 5 %; the %RSD for %CA of desamidosomatropin are also lower than the Eu. Ph. requirement of < 20 %.

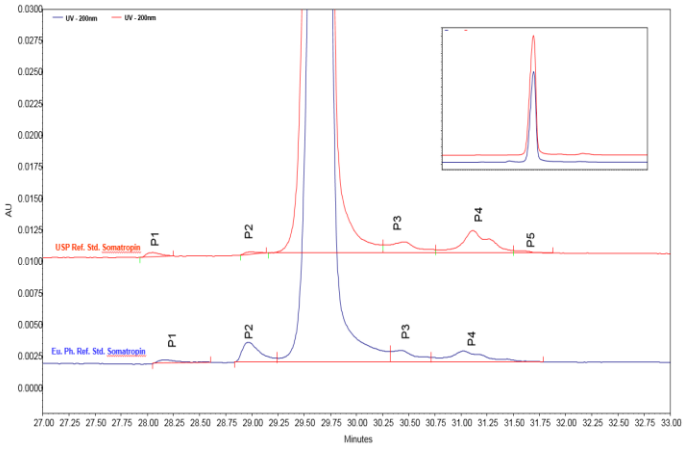

Figure 5. CZE electrophoretic profiles of USP Ref. Std. Somatropin (Red trace) and Eu. Ph. Ref. Std. Somatropin (blue trace) using NaOH solutions exposed to borosilicate glass. Zoomed in views with full views as insert.

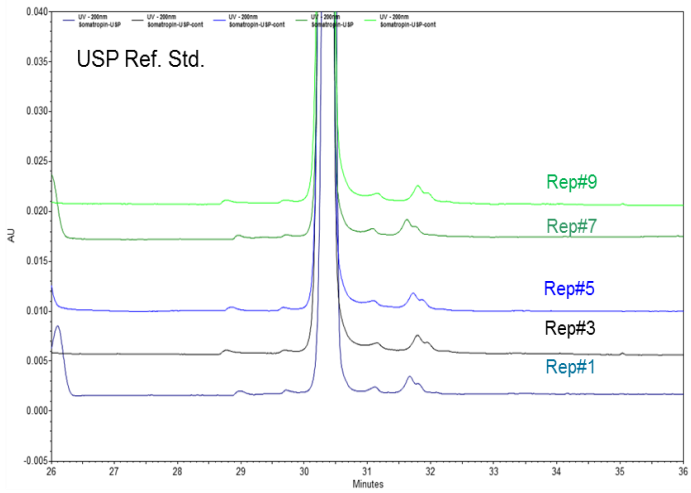

Figure 6. Enlarged representative electrophoretic profiles of USP. Ref. Std. Somatropin.

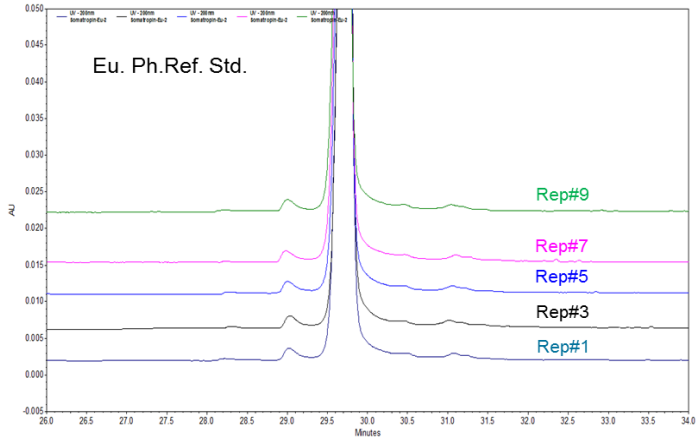

Figure 7. Enlarged representative electrophoretic profiles of Eu. Ph. Ref. Std. Somatropin.

Table 2. CZE charge variants quantitation results of ten replicates of USP Ref. Std. Somatropin injections

	% MP	% P4	RMT P1	RMT P2	RMT P3	RMT P4	RMT P5
1	*97.65*	*1.42*	*0.95*	*0.98*	*1.03*	*1.04*	*1.05*
2	*97.47*	*1.47*	*0.95*	*0.98*	*1.02*	*1.04*	*1.06*
3	*97.13*	*1.52*	*0.95*	*0.98*	*1.02*	*1.04*	*1.06*
4	*97.23*	*1.44*	*0.95*	*0.98*	*1.02*	*1.04*	*1.06*
5	*96.59*	*1.76*	*0.95*	*0.98*	*1.02*	*1.05*	*1.06*
6	*96.79*	*1.71*	*0.95*	*0.98*	*1.03*	*1.05*	*1.06*
7	*96.69*	*1.74*	*0.95*	*0.98*	*1.03*	*1.05*	*1.06*
8	*97.00*	*1.74*	*0.95*	*0.98*	*1.03*	*1.05*	*1.06*
9	*97.21*	*1.53*	*0.95*	*0.98*	*1.03*	*1.05*	*1.06*
10	*96.69*	*1.68*	*0.95*	*0.98*	*1.03*	*1.05*	*1.06*
Average	***97.05***	***1.60***	***0.95***	***0.98***	***1.02***	***1.05***	***1.06***
%RSD	***0.37***	***8.57***	***0.24***	***0.08***	***0.10***	***0.18***	***0.38***

Note: The results listed are average of triplicate injections from the same sample on the same capillary. MP corresponds to somatropin and P4 corresponds to the critical attribute of deamidation species**.**

Table 3. CZE charge variants quantitation results of ten replicates of Eu. Ph. Ref. Std. Somatropin injections.

	% MP	% P4	RMT P1	RMT P2	RMT P3	RMT P4
1	*95.23*	*1.57*	*0.95*	*0.98*	*1.02*	*1.04*
2	*95.30*	*1.53*	*0.95*	*0.98*	*1.02*	*1.04*
3	*95.56*	*1.43*	*0.95*	*0.98*	*1.02*	*1.05*
4	*95.32*	*1.45*	*0.95*	*0.98*	*1.03*	*1.05*
5	*95.89*	*1.52*	*0.95*	*0.98*	*1.02*	*1.05*
6	*95.76*	*1.49*	*0.95*	*0.98*	*1.02*	*1.05*
7	*95.77*	*1.31*	*0.95*	*0.97*	*1.03*	*1.05*
8	*95.55*	*1.57*	*0.95*	*0.98*	*1.03*	*1.05*
9	*95.62*	*1.55*	*0.95*	*0.98*	*1.02*	*1.05*
10	*95.67*	*1.47*	*0.95*	*0.98*	*1.03*	*1.05*
Average	***95.57***	***1.49***	***0.95***	***0.98***	***1.02***	***1.05***
%RSD	***0.23***	***5.34***	***0.07***	***0.05***	***0.03***	***0.11***

Note: The results are average of triplicate injections from the same sample on the same capillary within the same day. MP corresponds to Somatropin and P4 corresponds to the critical attribute of deamidation species,

Conclusions:

- The Eu. Ph. CZE method for charge variant analysis of Somatropin can be successfully executed on a PA 800 Plus Pharmaceutical Analysis System
- The method successfully resolves and quantifies the different charge isoforms indicated by the European Pharmacopeia and the electropherogram are consistent with examples shown in the reference standard leaflet
- Method repeatability was demonstrated using both Eu. Ph. and USP Somatropin standard

References:

1. EUROPEAN PHARMACOPOEIA 6.0 Somatropin Monographs
2. Information Leaflet Eu. Ph. Reference Standard Somatropin CRS batch 3
3. Information Leaflet Eu. Ph. Reference Standard Somatropin/desamidosomatropin resolution mixture CRS batch 1
4. Pharmeuropa Scientific Notes 2009-1
5. Pharmeuropa Bio&SN, BSP 108, Jan 2013
6. Separation of Recombinant Human Erythropoietin (rhEPO) using the European Pharmacopoeia Method on the PA 800 Plus Pharmaceutical Analysis System (RUO-MKT-02-6597-A)

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