

# Simultaneous Determination of Ephedrine and Pseudoephedrine in Mice Plasma by Capillary Zone Electrophoresis

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## Abstract

A low cost, accurate, rapid and sensitive capillary zone electrophoresis (CZE) quantitative method, using thiamine hydrochloride as internal standard (IS), was developed for the simultaneous analysis of ephedrine and pseudoephedrine in mice plasma. Analytes were extracted from plasma samples by n-hexane/dichloromethane/isopropanol (2:1:0.1, v/v/v) after alkalization, and stripped with 1.5% hydrochloric acid solution. Separation was performed by CZE using 50mM borax-20mM threonine (pH 9.27) as running buffer, with voltage of +10 kV applied and UV detection wavelength at 210 nm. The total running time was within 10 min. Calibration curves for plasma samples were linear over the concentration range of 0.075-5.0 µg/mL for ephedrine and 0.0375-2.5 µg/mL for pseudoephedrine. The method had a limit of quantitation of 17.80 ng/mL for ephedrine and 22.00 ng/mL for pseudoephedrine, respectively. The method was reliable and feasible for the evaluation of pharmacokinetic profiles of drug products containing ephedrine and pseudoephedrine.

**Keywords:** Ephedrine, Pseudoephedrine, Capillary Zone Electrophoresis

## 1. Introduction

Ephedrine (E) and Pseudoephedrine (PE) are pairs of diastereoisomeric sympathomimetic amines that have human central nervous system stimulating properties, and they also can relieve the spasm of the bronchial smooth muscle (Lou Hong-gang, 2010, PP.682-688, Kang Yong, 2001, PP.37-39). Several methods have been employed for the detection of the individual component in plasma, including HPLC (Aymard G. 2000, P.25, Roman MC. 2004, PP.15-24), LC-MS/MS (Beyer J. 2007, PP.150-160, Yi Feng, 2008, P.572), GC-MS (Gunn J. 2010, PP.37-43, Nakano M. 2000, PP.583-586) and CE (Wei F. 2007, PP.38-44, Liu Gang, 2006, P.617). Recently, a GC-MS/SIM method was reported for the simultaneous determination of E and PE in human plasma through liquid-liquid extraction and derivatization by trifluoroacetic anhydride (TFA) with a lower limit of quantitation (LOQ) of 2 and 1ng/mL for E and PE, respectively (Shen Qun, 2002). Although the method achieved sufficient detection sensitivity in short run time, it suffered from high cost and time-consuming extraction procedure. Therefore, a highly sensitive and simple CZE method was developed and validated for the simultaneous determination of E and PE in mice plasma. This developed method offered smaller sample volume requirement and shorter run time, especially lower cost. This method can be flexibly applied to a bioequivalence study.

## 2. Experimental

### 2.1 Materials and reagents

E was purchased from national institute for the control of pharmaceutical and biological products (Beijing, China), and PE was obtained from national narcotics laboratories (Beijing, China). Thiamine hydrochloride manufactured by the Medical Chemical Reagent (Tianjin, China) was used as the internal standard (IS). HPLC grade methanol was purchased from Kermel Chemical (Tianjin, China). Borax and all the other reagents used in the experiment were all of analytical grade and obtained from Huaxin Chemical Reagent (Baoding, China). Blank mice plasma was obtained from the experimental animals center of Hebei Medical University (Shijiazhuang, China). Ultrapure water was obtained from AK water treatment system (Taiwan, China).

### 2.2 Instrumentation

High Performance Capillary Electrophoresis system (Cailu Co. Ltd, Beijing, China) is with a 0-30 kV high voltage power supply, and a variable wavelength UV detector near the column end. Data acquisition and procession were performed using HW-2000 software (Qianpu Co. Ltd, Shanghai, China). Capillaries with 50  $\mu\text{m}$  I.D. and 375  $\mu\text{m}$  O.D. (Yongnian Ruifeng Photoconductive Fiber Factory, Hebei, China). Buffer pH was measured with a PHS-3C pH meter (Shanghai, China). A vortex mixer (QL-901, Haimen Medical Instrument, Jiangsu, China) was used to extract the analytes and a centrifuge (L500, Xiangyi Centrifuge Instrument Co. Ltd, Changsha, China) was used to accelerate the separation of organic and inorganic phases.

### 2.3 CZE conditions

Before the experiment, the capillary column should be pretreated. Firstly, the capillary column was rinsed with 1.0 M NaOH solution for 30 min and then with water until the pH value of the outlet solution reached 7.0. Secondly, the capillary was rinsed with 1.0 M HCl solution for 30 min and then with water until the pH value of the outlet solution reached 7.0.

Then, the conditions of the experiment were optimized with a fused-silica capillary of 50 cm $\times$ 40  $\mu\text{m}$  (40 cm effective length) in a running buffer of 50 mM borax-20 mM threonine (the pH value was adjusted to 9.27 with 1 M NaOH solution) and an applied voltage of +10 kV at room temperature. Samples were introduced by hydrodynamic injection (15 cm $\times$ 30 s) and the detection wavelength was 210 nm.

### 2.4 Preparation of mixed stock and working solutions

The mixed stock solutions of E and PE were prepared together by dissolving accurately weighed reference standards in methanol to result in a final concentration of 1mg/mL for E and 0.5 mg/mL for PE. Standard working solutions were prepared by diluting the mixed stock solutions with methanol to get six different concentrations in a range of 0.3-20  $\mu\text{g}/\text{mL}$  for E and 0.15-10  $\mu\text{g}/\text{mL}$  for PE. IS working solution was prepared with 1.5% hydrochloric acid solution to the nominal concentration of 10  $\mu\text{g}/\text{mL}$ .

### 2.5 Preparation of standard and quality control samples

100  $\mu\text{L}$  of each of the above different concentration working solutions was added to 400  $\mu\text{L}$  blank mice plasma to obtain the desired series plasma concentration of analytes. The plasma concentrations of calibration standards were 0.075, 0.125, 0.25, 0.5, 1.25, 5.0  $\mu\text{g}/\text{mL}$  for E and 0.0375, 0.0625, 0.125, 0.25, 0.625, 2.5  $\mu\text{g}/\text{mL}$  for PE, respectively. The quality control (QC) samples were prepared in the same way as the calibration standards to obtain plasma concentrations of 0.125, 0.5, 5.0  $\mu\text{g}/\text{mL}$  for E and 0.0625, 0.25, 2.5  $\mu\text{g}/\text{mL}$  for PE. All of these plasma samples were kept at -20  $^{\circ}\text{C}$  before use.

### 2.6 Sample preparation

#### (1) Liberation and organic solvent extraction

In 15 mL centrifuge tubes, a standard sample (mentioned above) was mixed with 0.5 mL, 2 M NaOH, and the analytes were liberated from plasma by vortex-mixing for 1 min. 4 mL mixed organic solutions of n-hexane/dichloromethane/isopropanol (2:1:0.1, v/v/v) was added and vortex-mixed for 3 min, then the analytes diverted into the organic phase. After centrifugation with 5000 rpm for 6 min, the organic phase was transferred to another centrifuge tube.

#### (2) Back extraction

100  $\mu\text{L}$  IS working solution was added and the mixture was back extracted by vortex-mixing for 3 min. Discarding the upper organic phase after centrifugation with 5000 rpm for 6 min. The lower aqueous phase was dried in 75  $^{\circ}\text{C}$  water bath, and then the residues was dissolve in 10  $\mu\text{L}$  ultrapure water to obtain the enriched analytes, which was analyzed by CZE subsequently.

### 3. Results and discussion

#### 3.1 Optimization of CZE conditions

The influence of the separation voltage on the separation efficiency was evaluated. The separation voltage at +20, +15 and +10 kV were tested. The electropherograms in fig. 1 obviously showed that optimum separation voltage was +10 kV.

With the increasing of the injection time, the intensity of the signals of analytes increased, generally to obtain the high sensitivity. A standard mixture was injected into the capillary at different time between 10 s and 40 s by hydrodynamic injection, keeping the sampling height constant at 15 cm. The results indicate that peak areas are proportional to the injection time up to 40 s. However, when an injection time was longer than 30 s, so did peak broadening and worse resolution presented. Thus, an injection time of 30 s was selected.

#### 3.2 Selection of sample preparation conditions

Due to the low concentration of E and PE in human plasma, the high extraction efficiency and sample enrichment is in need. The developed extraction method could avoid the interference of endogenous compounds and overcome the shortcoming of low recovery rate through the process of basification, extraction followed by acidification. Additionally, the investigation for the organic extractant indicated that the extraction efficiency with the mixture of *n*-hexane-dichloromethane-isopropanol as extractant was obviously higher than that with the cyclohexane-dichloromethane as extractant. Simultaneously, this work studied the effects of the different hydrochloric concentrations (0.3%, 0.5%, 1.0%, 1.5%, 2.0%) on the extraction efficiency for the analytes. The results indicated that the higher recoveries with the increase of the hydrochloric concentrations, but when 2.0% hydrochloric solution was used, extraction efficiency has no obvious change compared with 1.5% hydrochloric solution, so 1.5% was chosen.

#### 3.3 Method validation

##### 3.3.1 Separation and specificity

The CZE-based method in this paper was investigated by analyzing the blank plasma sample, spiked plasma samples and mice plasma sample, and their electropherograms were shown in Fig. 2, Fig. 3 and Fig. 4. Under the CZE conditions described above, the migration time was about 7.210 min for IS, 7.857 min for E and 9.292 min for PE, respectively. There were no interference peaks found at the migration times of the two analytes and IS.

##### 3.3.2 Linearity

Calibration curves for plasma were good linear over the concentration range of 0.075-5.0  $\mu\text{g/mL}$  for E and 0.0375-2.5  $\mu\text{g/mL}$  for PE. Typical equations of calibration curves were as follows: for E,  $C=2.688A+0.1999$  ( $r=0.9982$ ), and for PE,  $C=3.322A+0.1418$  ( $r=0.9988$ ), where  $A$ =peak-area ratio and  $C$ =concentration ( $\mu\text{g/mL}$ ).

##### 3.3.3 LOQ and LOD

The current assay had a LOQ calculated on the basis of a signal-to-noise (S/N) ratio of 10:1 were 17.18 ng/mL for E, and 22.00 ng/mL for PE. The LODs were 5.34 ng/mL for E and 6.60 ng/mL for PE (S/N=3). These limits are sufficient for clinical pharmacokinetic studies after oral administration of therapeutic dose.

##### 3.3.4 Intra-day and inter-day precisions

The precision of the method was evaluated by analyzing five replicates of QC samples at low, medium and high concentrations, respectively. Intra-day precision was assessed as the relative standard deviation (RSD) resulting from the same day. Inter-day assay precision was expressed by the RSD of the mean concentrations on three consecutive days. The summary of intra-day and inter-day precisions at QC concentrations was shown in Table 1. The results were less than 5.30% and 6.75% for the two analytes, which indicated good precision.

##### 3.3.5 Recovery

The recovery experiment was carried out to evaluate the accuracy of the method. In this study, the recovery was determined from QC samples of low, medium and high concentrations, and  $\text{recovery}\% = \frac{\text{measured concentration}}{\text{QC sample concentration}} \times 100\%$ . Table 2 summarized the results of recoveries of the two analytes, which were in range of 97.6-106.9% and RSD were in range of 2.46-8.29%. Thus, recoveries were within the acceptable criteria.

### 4. Conclusions

In our study, a low cost and accurate CZE method with high sensitivity was developed. The satisfactory results of the methodology validation indicated that the method can be applied to simultaneous determination of E and PE plasma concentrations in complex clinical samples, for example, human plasma and herbal medicine.

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Table 1. Intra-day and inter-day precision for E from plasma samples (n=5)

Concentration added (µg/mL)	Intra-day (n=5)		Intra-day (n=5)		
	Measured concentration (µg/mL)	Precision (RSD %)	Measured concentration (µg/mL)	Precision (RSD %)	
E	0.125	0.139±0.003	2.33	0.120±0.007	6.04
	0.5	0.447±0.016	3.50	0.478±0.021	4.30
	5.0	4.966±0.073	1.46	5.021±0.134	2.66
PE	0.0625	0.067±0.004	5.30	0.062±0.004	6.75
	0.25	0.232±0.010	4.48	0.261±0.011	4.40
	2.5	2.516±0.064	2.53	2.413±0.110	4.55

Table 2. Recoveries for E and PE for E from plasma samples (n=5)

Concentration Added (µg/mL)	Measured Concentration (µg/mL)	Recovery ±s (%)	RSD (%)	
E	0.125	0.134±0.009	106.9±6.94	6.50
	0.5	0.489±0.018	97.8±3.69	3.78
	5.0	5.063±0.183	101.3±3.66	3.61
PE	0.0625	0.061±0.005	97.6±8.08	8.29
	0.25	0.246±0.006	98.3±2.42	2.46
	2.5	2.520±0.131	100.8±5.24	5.20

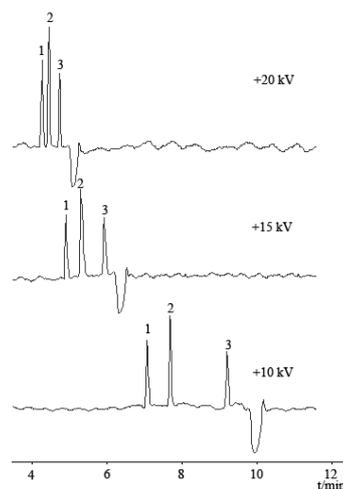


Figure 1. The eletropherograms of analytes were at different separation voltage  
Peak identification: (1) IS; (2) E; (3) PE

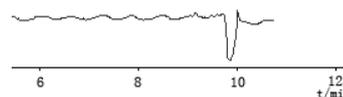


Figure 2. The eletropherogram of a blank plasma



Figure 3. The eletropherogram of a blank plasma sample was added with E, PE and IS  
Peak identification as in Fig. 1

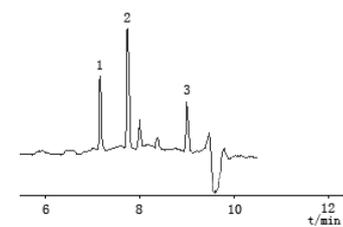


Figure 4. The eletropherograms of a plasma sample from the mice after  
2 h of *ig* Maxingshigan Decoction. Peak identification as in Fig. 1