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Stereoselective determination of *R*-(−)- and *S*-(+)-prilocaine in human serum by capillary electrophoresis using a derivatized cyclodextrin and ultraviolet detection

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Abstract

A capillary electrophoresis (CE) method for the quantification of *R*-(−)- and *S*-(+)-prilocaine in human serum was developed and validated. Stereoselective resolution was accomplished using 15 m*M* heptakis(2,6-di-methyl)-β-cyclodextrin and 0.03 m*M* hexadecyltrimethylammonium bromide (HTAB) contained in 100 m*M* phosphate buffer, pH 2.5. Solid-phase extraction was used as a sample preparation technique to remove endogenous interferences. A 72-cm uncoated fused-silica capillary at a voltage of 25 kV and 30°C was used for the analysis. The detection limits for *R*-(−)- and *S*-(+)-prilocaine were 38 ng/ml using 1 ml of human serum and the limits of quantitation were 45 ng/ml. The calibration curve was linear over the range of 45–750 ng/ml with procainamide as the internal standard. Precision and accuracy of the method were 2.86–8.50% and 3.29–7.40%, respectively, for *R*-(−)-prilocaine, and 3.94–9.17% and 2.0–6.73%, respectively, for *S*-(+)-prilocaine. The CE method was compared to an existing chiral HPLC method in terms of sensitivity and selectivity for the routine analysis of the drug.

Keywords: Enantiomer separation; Prilocaine

1. Introduction

Prilocaine is a local anesthetic of the amide type. It is marketed as a racemic mixture of *R*-(−)- and *S*-(+)-prilocaine and is used for IV regional, nerve block and topical anesthesia [1]. Both enantiomers have similar biological activity, but the *S*-(+)-enantiomer is slowly hydrolyzed, while the *R*-(−)-enantiomer is quickly hydrolysed to toluidine, causing methemoglobinemia [2,3]. A chiral high-performance liquid chromatographic (HPLC) assay for the separation of *R*-(−)- and *S*-(+)-prilocaine was reported by this laboratory and others using a chiral stationary phase [2,4].

The most common analytical techniques for monitoring drugs in biological fluids are HPLC, gas chromatography (GC) and immunoassays. Analysis of biological fluids using capillary electrophoresis (CE) is less common and very few reports have been published [5]. One of the reasons most commonly cited against the widespread use of CE is the lack of sensitivity in comparison to HPLC. Various techniques have been developed to overcome the sensitivity problem by improving detection techniques, sample pretreatment or on-column sample concentration. Due to these recent developments, there has

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been a tremendous increase in the use of CE for pharmaceutical analysis and for therapeutic drug monitoring [6–10].

Resolution of enantiomers in CE has been performed using various chiral selectors. Native and derivatized cyclodextrins, crown ethers, chiral ligand exchange, chiral micelles, proteins, carbohydrates and macrocyclic antibiotics are some of the chiral selectors that have been used [11–23]. Cyclodextrins have the advantage of being commercially available, transparent to UV light, available in a wide range of size and charge, and they are inexpensive.

In this study, a CE method for the quantification of *R*-(−)- and *S*-(+)-enantiomers of prilocaine in human serum is reported using heptakis(2,6-di-O-methyl)-β-cyclodextrin as the chiral selector. The method is linear in the range of 45–750 ng/ml and provides the required selectivity and sensitivity for monitoring levels of *R*-(−)- and *S*-(+)-prilocaine in human serum after an effective dose of 525 mg.

2. Experimental

2.1. Reagents and chemicals

Racemic prilocaine hydrochloride and procainamide were obtained from Sigma (St. Louis, MO, USA). *R*-(−)- and *S*-(+)−prilocaine hydrochloride were gifts from Astra (Westborough, MA, USA). Hexadecyltrimethylammonium bromide (HTAB) was obtained from Sigma. Phosphoric acid (85%) and sodium dihydrogenphosphate monohydrate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Native α-, β- and γ-cyclodextrins, heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) and heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) were also obtained from Sigma. Hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-α-cyclodextrin (HP-α-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were obtained from Aldrich (Milwaukee, WI, USA). Hydroxyethyl-β-cyclodextrin (HE-β-CD), carboxymethyl-β-cyclodextrin (CM-β-CD) and amino-β-cyclodextrin were obtained from Advanced Separation Technologies (Whippany, NJ, USA). Sulfated-β-cyclodextrin was a gift from American Maize Products (Hammond, IN, USA). Drug-free human serum was obtained from Biological Specialty (Colmar, PA, USA). All solutions were filtered through a 0.2-μm nylon filter (Acrodisc 13, Gelman Sciences, Ann Arbor, MI, USA).

2.2. Preparation of stock solutions

Individual stock solutions were prepared in absolute methanol to give concentration of 100 μg/ml of *R*-(−)- and *S*-(+)-prilocaine and 10 μg/ml of procainamide (internal standard). Appropriate volumes of the individual *R*-(−)- and *S*-(+) prilocaine stock solutions for the standard curve were pipetted, to give concentrations of 45, 100, 250, 500 and 750 ng/ml, into 2 ml volumetric flasks and evaporated under a stream of nitrogen. Then 1 ml of drug-free serum followed by 200 μl of phosphate buffer and 40 μl of internal standard were added to the tubes and vortex-mixed.

A stock solution of 100 m*M* sodium dihydrogenphosphate was prepared by dissolving 13.1 g of sodium dihydrogen phosphate monohydrate in 1 l of double-distilled, deionized water and the pH was adjusted to 2.5 using 100 m*M* phosphoric acid.

2.3. Instrumentation

All CE experiments were performed using an ABI model 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with an UV detector. An uncoated fused-silica capillary (L=72 cm, l=50 cm, 50 μm I.D., Polymicro Technologies, Phoenix, AZ, USA) was used for the analysis. A 0.5-cm detection window was created by stripping the polyimide coating of the capillary. The sample injection was towards the anodic end and the detection was towards the cathodic end.

2.4. Electrophoretic conditions

The background electrolyte consisted of 100 m*M* sodium dihydrogen phosphate, pH 2.5 (adjusted with 100 m*M* phosphoric acid) containing 15 m*M* heptakis(2,6-di-O-methyl)-β-cyclodextrin and 0.03 m*M* HTAB. All of the analytes were monitored at 215 nm. New capillaries were conditioned by rinsing them with 1 *M* sodium hydroxide, water and back-

ground electrolyte for 15 min each. The sample was introduced using a vacuum injection (5′ Hg) for 24 s. The typical running currents were 71 μA. Prior to each analysis, the capillary was washed with 0.1 *M* NaOH for 1.8 min and with background electrolyte for 2.2 min.

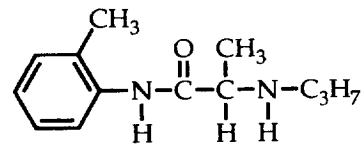
2.5. Assay procedure

Sample pretreatment was performed using a solid-phase extraction (SPE) method, as previously reported by this laboratory [4]. To 1 ml of human serum containing *R*-(−)- and *S*-(+)-prilocaine were added 200 μl of phosphate buffer and 40 μl of internal standard. The samples were vortex-mixed and passed through a 1-ml C_{18} Bond-Elut SPE column attached to a Vacuum manifold (Vac-Elut, Harbor City, CA, USA). The C_{18} column was preconditioned with 1 ml of 0.1 *M* HCl, followed by 1 ml each of methanol and water. The column was washed with 5×0.5 ml of water and with 1×0.5 ml of acetonitrile. The cartridge was allowed to dry between each wash. Finally, the analytes were eluted using 4×0.25 ml of acidified methanol (2% HCl in methanol). The eluate was filtered through a 0.2-μm syringe disc filter, followed by evaporation, and the residue was reconstituted in 45 μl of water, after which it was vacuum injected (5′ Hg) into the capillary for 24 s.

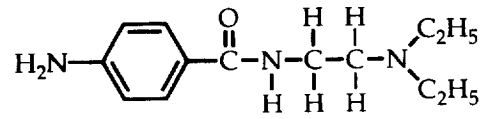
Absolute recovery of each analyte by SPE was conducted by comparing spiked samples to unextracted stock solutions that were injected directly onto the capillary. Drug peak-area ratios were used to calculate the absolute recoveries of each analyte. Calibration curves were constructed in the range of 45–750 ng/ml using 45, 100, 250, 500 and 750 ng/ml as the points. Linear regression analysis of normalized D/I.S. peak-area ratios versus concentration gave slope and intercept data for each analyte, which were used to calculate the concentration of each analyte in the serum sample.

3. Results and discussion

The chemical structures of *R*-(−)- and *S*-(+)-prilocaine and procainamide are shown in Fig. 1. To develop a reliable and sensitive CE method for the quantification of *R*-(−)- and *S*-(+)-prilocaine in human serum, an analytical method was developed and validated with respect to the resolution of *R*-(−)- and *S*-(+)-prilocaine, the use of SPE to remove endogenous interferences and for concentrating the analytes, optimization of loading capacity and, finally, accuracy and precision of the CE method.



PRILOCAINE



PROCAINAMIDE

Fig. 1. Chemical structures of prilocaine and procainamide (internal standard).

Various native and derivatized cyclodextrins were investigated for their ability to separate prilocaine enantiomers. The neutral cyclodextrins investigated were the native α-, β- and γ-, DM-β-CD, TM-β-CD, HP-β-CD, HP-α-CD, HP-γ-CD and HE-β-CD. The charged cyclodextrins investigated were CM-β-CD, amino β-cyclodextrin and sulfated-β-cyclodextrin.

Separation of the prilocaine enantiomers was not achieved with any of the cyclodextrins, except for DM-β-CD. To establish the optimum resolution conditions, the effects of buffer and chiral selector concentration and pH on resolution and migration time were studied. The effect of buffer concentration was investigated at 25, 50, 75 and 100 m*M* phosphate buffer and it was found that higher buffer concentrations gave good peak shapes with no appreciable increase in migration times. An increase in chiral selector concentration led to an increase in migration time with no significant increase in resolution. The effect of buffer pH was very significant. As the pH increased above 5.5, there was a complete loss of resolution with a concurrent decrease in

migration time. Based upon these investigations of various electrophoretic parameters, 15 m*M* DM-β-CD in 100 m*M* sodium phosphate buffer, pH 2.5, containing 0.03 m*M* HTAB was chosen as the background electrolyte. Cationic detergents are often added to background electrolyte to reduce the non-specific interaction of endogenous substances in serum with the silanol groups [24,25]. In this separation, a 0.03 m*M* HTAB concentration was added to the background electrolyte and was shown to provide stable migration times and symmetrical peaks. The addition of HTAB as a background electrolyte additive increased the lifetime of the capillary by decreasing the non-specific adsorption of endogenous substances on to the silica wall. Typical electropherograms of blank human serum and serum spiked with *R*-(−)- and *S*-(+)-prilocaine and the internal standard procainamide are shown in Fig. 2A,B. The elution order was *R*-(−)- followed by *S*-(+)-prilocaine.

SPE using an octadecylsilane cartridge gave absolute recoveries greater than 84% for both *R*-(−)- and *S*-(+)-prilocaine, with good sample clean-up. Octylsilane and ethylsilane SPE cartridges gave recoveries of less than 80% for both *R*-(−)- and *S*-(+)-prilocaine. The mean absolute recoveries using the octadecylsilane cartridge were 85.2±3.9% for *R*-(−)-prilocaine, 84.9±4.8% for *S*-(+)-prilocaine and 69.8±4.7% for procainamide (n=8).

To increase the detection limits, sample loading onto the capillary was increased to achieve a better signal-to-noise ratio, without sacrificing resolution and theoretical plates. Test runs were performed with injection times from 5 to 40 s. The best signal-to-noise ratio was obtained at 24 s without any baseline humps and with a good plate count. The sample was reconstituted in water to achieve a field amplification and sample concentration effect.

To obtain excellent reproducibility, the effects of different modes of injection and different procedures were investigated for capillary washing and their influence on reproducibility. It was found that vacuum injection was more reliable than electrokinetic injection in terms of reproducibility, and washing with 0.1 *M* NaOH and buffer before each run was an absolute necessity. The migration time reproducibility was very good with R.S.D.s of less than 0.5% for all three peaks. Normalized peak-area values were

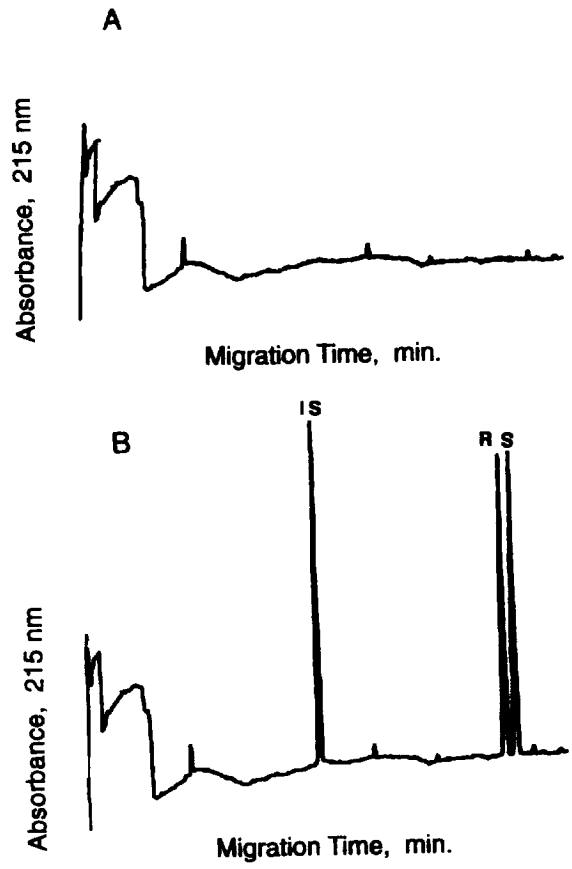


Fig. 2. Electropherograms of (A) serum blank and (B) serum spiked with *R*-(−)-prilocaine (400 ng/ml, 16.0 min), *S*-(+)-prilocaine (400 ng/ml, 16.4 min) and procainamide (internal standard, 400 ng/ml, 8.9 min). Electrophoretic conditions: uncoated fused-silica capillary (50 μm I.D., 72 cm total length, 50 cm effective length), the background electrolyte consisted of an aqueous solution of 100 m*M* sodium dihydrogen phosphate, pH 2.5 (adjusted with 100 m*M* phosphoric acid), containing 15 m*M* heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) and 0.03 m*M* HTAB; voltage, 25 kV; temperature, 30°C; vacuum injection, (5″ Hg), 24 s, and detection was at cathodic end.

obtained by dividing the corresponding peak areas by the migration times [26]. These normalized values were used in calculating the unknown concentrations in serum samples.

The validation of the CE assay was performed by evaluating the limit of detection, the limit of quantitation, the linearity of the detector response, method precision and accuracy. The limits of detection (LOD) were 38 ng/ml, and the limits of quantitation (LOQ) were 45 ng/ml for each enantiomer (n=3).

Table 1
Accuracy and precision of serum samples with added *R*-(−)- and *S*-(+)-prilocaine

	Concentration added (ng/ml)	Concentration found (ng/ml)[a,b]	R.S.D. (%)	Error (%)
Intra-day[a]				
R	50	58.56±3.64	6.2	6.5
	730	700.3±20.01	2.9	4.1
S	50	57.49±3.14	5.5	4.5
	730	752.70±29.69	3.9	3.1
Inter-day[b]				
R	50	59.07±5.00	8.5	7.4
	730	706.0±36.72	5.2	3.3
S	50	58.70±5.39	9.2	6.7
	730	715.70±38.69	5.4	2.0

[a] Based on $n=4$, for intra-day assay.
[b] Based on $n=16$, for inter-day assay.

The linear calibration curves showed good linearity in the range of 45–750 ng/ml for *R*-(−)- and *S*-(+)-prilocaine. The coefficients of determination were greater than 0.999 ($n=4$). Representative linear regression equations obtained for *R*-(−)- and *S*-(+)-prilocaine were $y=0.001976x-0.00689$ (standard error=0.000012) and $y=0.001946x-0.00293$ (standard error=0.000010) respectively, where y and x are the normalized drug-to-internal standard peak-area ratios and the concentration of each analyte, respectively. The intra-day precision and accuracy ($n=4$), as expressed by R.S.D. (%) and error (%) were 2.86–6.22 and 4.07–6.47%, respectively, for *R*-(−)-prilocaine, and 3.94–5.50 and 3.10–4.53% for *S*-(+)-prilocaine, respectively. The inter-day precision and accuracy ($n=16$, over four days), expressed by R.S.D. (%) and error (%) were 5.20–8.50 and 3.29–7.40%, respectively, for *R*-(−)-prilocaine, and 5.40–9.17 and 2.00–6.73%, respectively, for *S*-(+)-prilocaine. Detailed data are listed in Table 1.

The CE method developed herein provides good sensitivity, accuracy and selectivity. Table 2 shows a comparison to a chiral HPLC assay recently reported by our laboratory [4]. The method involves a normal phase chiral HPLC on a pirkle-type naphthyl ethylamine stationary phase (Sumichiral OA-4700, 250× 4 mm I.D.) at ambient temperature with a flow-rate of 0.8 ml/min. The mobile phase consisted of hexane–ethylenedichloride–absolute methanol–TFA (85:10:5:0.1, v/v). The high efficiency of CE is seen in the enormous plate count compared to HPLC. The selectivity (α) in HPLC was calculated as k'_2/k'_1 and, in CE, it was calculated as the ratio of mobilities of *R*-(−)- and *S*-(+)-prilocaine. The CE

Table 2
Comparison of CE and chiral HPLC assays for *R*-(−)- and *S*-(+)-prilocaine in human serum

Prilocaine	CE		HPLC[a]	
	R	*S*	*R*	*S*
Selectivity (α)	1.03		1.13	
Resolution (R_s)	1.82		1.95	
Theoretical plates (N)	84 624	88 912	9101	8866
Migration times (t_m, min) or retention times (t_r, min)	16.0	16.4	9.3	10.1
Linear concentration range (ng/ml)	45–750		10–1000	
r^2	>0.999 ($n=4$)		>0.999 ($n=3$)	
Limit of detection (ng/ml)	38	38	4	5
Limit of quantitation (ng/ml)	45	45	10	10

[a] See reference [4] for method.

detection limits could be further improved by using a Z-shaped capillary or by using a bubble-shaped detection window.

In summary, the CE assay described herein provides a selective and reliable method for the quantification of *R*-(−)- and *S*-(+)-prilocaine in human serum. The assay method is sensitive to 45 ng/ml of each enantiomer, with a total run time of less than 17 min. This assay is a useful alternative to existing HPLC methods for the routine analysis of prilocaine in human serum.

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