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Stereoselective determination of *R*(−)- and *S*(+)-prilocaine in human serum using a brush-type chiral stationary phase, solid-phase extraction and UV detection

M. Siluveru, J.T. Stewart*

Department of Medicinal Chemistry, College of Pharmacy, University of Georgia, Athens, GA 30602-2352, USA

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Abstract

A chiral HPLC method was developed for the quantitation of *R*(−)- and *S*(+)-prilocaine in human serum. The method involves sensitive and selective detection of *R*(−)- and *S*(+)-prilocaine using normal-phase chiral HPLC on a pirkle-type naphthyl ethylamine stationary phase (Sumichiral OA-4700, 250 mm × 4 mm i.d.) at ambient temperature with a flow rate of 0.8 ml min^{-1}. A sample clean-up procedure was used for isolation of the analytes of interest from human serum using Bond-Elut C_{18} columns with high recovery and selectivity. The detection limits were 4 ng ml^{-1} for *R*-prilocaine and 5 ng ml^{-1} for *S*-prilocaine. The limits of quantitation were 10 ng ml^{-1} for both enantiomers. Linear calibration curves in the 10–1000 ng ml^{-1} range showed good coefficients of determination >0.999 ($n = 3$). Precision and accuracy of the method were within 4–5.8% and 1.5–4.8% respectively for *R*(−)-prilocaine, and 2.8–5.7% and 3.2–5.2% respectively for *S*(+)-prilocaine.

Keywords: Chiral chromatography; Human serum; Naphthyl ethylamine stationary phase; Solid-phase extraction

1. Introduction

Prilocaine is a local anesthetic frequently used for regional, nerve block and topical anesthesia [1]. Both enantiomers have equal biological activity, but the *S*(+)-enantiomer is slowly hydrolysed, while the *R*(−)-enantiomer is rapidly hydrolysed forming toluidine, which causes methemoglobinemia [2]. Prilocaine is clinically administered as a racemate. Separation of *R*(−)- and *S*(+)-prilocaine was performed using an α-AGP column, but there is only one method for the determination of *R*- and *S*-prilocaine in biological fluids [2–4]. This method lacks the necessary sensitivity for the detection of low levels of the *R*(−) isomer, uses a tedious liquid–liquid extraction for sample clean-up, and the quantitation is performed without the use of an internal standard. This paper describes a chiral HPLC method using a naphthyl ethylamine stationary phase coupled with solid-phase extraction (SPE) to determine nanogram per milliliter concentrations of *R*(−)- and *S*(+)-prilocaine with good sensitivity and selectivity. The method is linear up

* Corresponding author. Tel.: (+1) 706-542-4410; Fax: (+1) 706-542-5358.

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to 1000 ng ml^{-1} for each enantiomer. The assay possesses the required sensitivity for monitoring individual isomers of prilocaine in human serum.

2. Experimental

2.1. Reagents

Prilocaine and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO). *R*- and *S*-prilocaine and *S*-bupivacaine were gifts from Astra (Westborough, MA). Blank human serum was obtained from the Instrumentation Laboratory Company (Lexington, MA). The solvents used were all HPLC grade. Absolute methanol, hexane, dichloroethane and sodium dihydrogen phosphate monohydrate were obtained from J.T. Baker (Phillipsburg, NJ). All chromatographic solutions were filtered through a 0.45 μm filter (Alltech Associates, Deerfield, IL).

2.2. Preparation of stock and standard solutions

Three stock solutions were prepared in absolute methanol to give concentrations of 200 $\mu g\ ml^{-1}$ for *R*- and *S*-prilocaine and 2 mg ml^{-1} for *S*-bupivacaine (internal standard). Appropriate volumes of the *R*- and *S*-prilocaine stock solutions were pipetted into 2 ml volumetric flasks and evaporated. Then 1 ml of serum was added to each flask to give final serum concentrations of 10, 50, 250, 500 and 1000 ng ml^{-1} of each analyte. To each serum sample, 0.5 ml of 100 mM phosphate buffer (pH 4.5) and 4 μl of the internal standard solution were added and mixed well.

2.3. Instrumentation

A spectroflow Model 400 solvent delivery system (Kratos Analytical Instruments, Ramsey, NJ) and a Model 728 autosampler (Micromeritics Instruments Corporation, Norcross, GA) equipped with a 50 μl loop were used for the analysis. The stationary phase was naphthyl ethylamine (Sumichiral OA-4700, 5 μm, 250 mm × 4 mm i.d.; YMC Inc, Wilmington, NC). The mobile phase consisted of hexane–ethylenedichloride–absolute methanol–TFA (85:10:5:0.1 v/v/v/v). The flow rate was 0.8 ml min^{-1} and the column was at ambient temperature (22 ± 1°C). A Model 481 Lambda-Max LC spectrophotometer (Waters, Milford, MA) at 220 nm was used to monitor the eluent. A Spectra-Physics Model 4290 integrator (Spectra Physics, San Jose, CA) was used to record each chromatogram and peak area responses.

2.4. Assay procedure

The SPE was performed using a reported method with minor changes [5]. To 1 ml human serum samples containing *R*- and *S*-prilocaine were added 0.5 ml of phosphate buffer and 4 μl of the internal standard. The samples were vortexed and then passed through a 1 ml C_{18} Bond-Elut SPE column attached to a vacuum manifold (Vac-Elut, Varian, Harbor City, CA). The column was previously conditioned with 1 ml of 0.1 M HCl followed by 1 ml of methanol and 1 ml of water. The column was washed with 4 × 0.5 ml of water and 1 × 0.5 ml of acetonitrile and allowed to air-dry for 1 min between each wash. The analytes of interest were eluted with 4 × 0.25 ml of acidified methanol (2% HCl in methanol). The tubes were evaporated under a nitrogen stream and the samples reconstituted in 1 ml of hexane after which 50 μl was injected into the chromatograph. Linear regression analysis of D/IS peak-area ratios vs. concentration gave slope and intercept data for each analyte, which were used to calculate the concentration of each analyte in the serum samples. For absolute recovery experi-

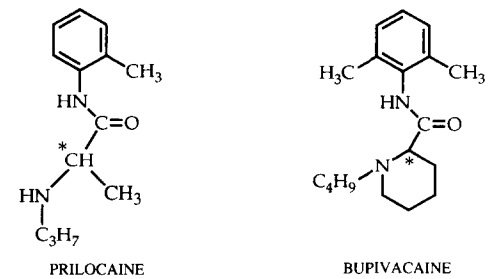

Fig. 1. Chemical structures of prilocaine and bupivacaine.

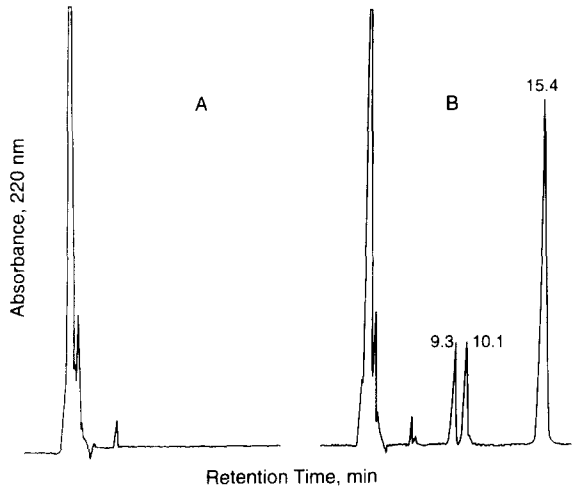

Fig. 2. (A) Representative chromatogram of serum blank. (B) Representative chromatogram of spiked human serum sample containing *R*-prilocaine (25 ng ml^{-1}; 9.3 min), *S*-prilocaine (25 ng ml^{-1}; 10.1 min) and *S*-bupivacaine (internal standard, 8 μg ml^{-1}; 15.4 min).

ments, spiked samples were compared with unextracted stock solutions which had been injected directly into the chromatograph. Drug peak-area ratios were used to calculate the absolute recoveries of each analyte.

3. Results and discussion

The chemical structures of *R*- and *S*-prilocaine and *S*-bupivacaine (internal standard) are shown in Fig. 1. The internal standard *S*-bupivacaine was chosen because it is structurally similar to prilocaine and gave good recoveries from serum using the SPE method. Baseline separation of *R*- and *S*-prilocaine was achieved using a naphthyl ethylamine column with retention times of 9.1 and 9.9 min respectively, and $R_s > 2.0$. The internal standard *S*-bupivacaine gave a retention time of 15 min. The efficiency of the chiral column as expressed in terms of number of theoretical plates (N) was 9101, 8866 and 7319 for *R*- and *S*-prilocaine and *S*-bupivacaine respectively. Previous work in this laboratory in developing chiral separations on various types of chiral stationary phases and mobile phases showed that prilocaine enantiomers were easily separated using a brush-type naphthyl ethylamine stationary phase and a mobile phase of hexane–ethylenedichloride–absolute methanol–TFA (85:10:5:0.1 v/v/v/v). Brush-type stationary phases utilize π–π, H-bonding, steric hindrance and dipole interactions between prilocaine and the stationary phase in order to separate *R*- and *S*-prilocaine. It was found that ethylenedichloride could be replaced by methylene chloride, but the replacement of methanol with ethanol or propanol led to significant changes in chromatography. The stock solution of prilocaine in methanol was stable and did not show any evidence of inversion upon prolonged storage. When a blank human serum was spiked with the *R*-enantiomer there was no detectable peak for the *S*-enantiomer and vice versa. Fig. 2A shows the chromatogram of a serum blank and Fig. 2B shows a chromatogram of the analytes and internal standard spiked in serum. The order of elution for the enantiomers was determined to be *R*- then *S*-prilocaine. There were no interferences from endogenous substances and no late eluters were observed on continuous operation for 7 h.

To decrease the sample preparation time involved with liquid–liquid extraction, an SPE procedure was used for sample clean-up. The C_{18} sorbent allowed the elution of prilocaine and bupivacaine using acidified methanol, which was evaporated to dryness under a nitrogen stream and reconstituted with hexane. Absolute recoveries greater than 90% were obtained for all three analytes using 1.0 ml of methanol as eluent. The mean absolute recoveries using C_{18} SPE were $90.1 \pm 5.2\%$ for *R*-prilocaine, $89.3 \pm 4.3\%$ for *S*-prilocaine and $91.6 \pm 3.9\%$ for *S*-bupivacaine ($n = 3$). The limits of detection, based on a signal-to-noise ratio of 3, were 4 and 5 ng ml^{-1} for *R*- and *S*-prilocaine respectively ($n = 3$).

The calibration curves showed good linearity in the range 10–1000 ng ml^{-1} for *R*- and *S*-prilocaine. The coefficients of determination were more than 0.999 ($n = 3$). Representative linear regression equations obtained for *R*- and *S*-prilocaine were $y = 0.006930x - 0.03867$ and $y = 0.006817x + 0.021104$ respectively, where y and x are the drug-to-internal-standard peak area ratios and the concentration of each analyte respectively. The intra-day precision and accuracy ($n =$

Table 1
Accuracy and precision for determination of serum samples with added *R*- and *S*-prilocaine

Enantiomer	Conc. added (ng ml^{-1})	Conc. found (ng ml^{-1})[a,b]	Error (%)	RSD (%)
Intra-day	15	14.80 ± 0.82	1.5	5.5
R	975	957.96 ± 51.34	1.8	5.4
S	15	14.40 ± 0.73	4.0	5.1
	975	939.00 ± 45.61	3.7	4.8
Inter-day	15	15.70 ± 0.64	4.7	4.0
R	975	928.6 ± 54.08	4.8	5.8
S	15	14.53 ± 0.83	3.2	5.7
	975	924.0 ± 25.9	5.2	2.8

[a] Based on $n = 3$, for intra-day assay.
[b] Based on $n = 9$, for inter-day assay.

3) as expressed by %RSD and % error were 5.5–5.4% and 1.5–1.8% respectively for *R*-prilocaine, and 4.8–5.1% and 3.7–4.0% respectively for *S*-prilocaine. The inter-day precision and accuracy ($n = 9$, over three days) expresed by %RSD and % error were 4–5.8% and 4.7–4.8% respectively for *R*-prilocaine, and 2.8–5.7% and 3.2–5.2% respectively for *S*-prilocaine. The detailed data are listed in Table 1.

In summary, a sensitive and selective isocratic chiral HPLC method employing SPE clean-up has been developed for the quantitation of *R*- and *S*-prilocaine in human serum. The method is sensitive to 10 ng ml^{-1} of each isomer and the total chromatographic run time is 15 min.

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