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Journal of Chromatography B, 719 (1998) 135–140

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic separation and nanogram quantitation of bupivacaine enantiomers in blood

Xiao Qing Gu, Bronwyn Fryirs, Laurence E. Mather*

Department of Anaesthesia and Pain Management, University of Sydney, Royal North Shore Hospital, St. Leonards, NSW 2065, Australia

Received 3 March 1998; received in revised form 24 July 1998; accepted 4 August 1998

Abstract

Chiral separation of *rac*-bupivacaine extracted from blood was achieved with similar limits of detection but using a much simpler sample preparation than reported previously. The simple one-step sample preparation devised was highly robust and efficient and allowed a very high throughput of samples. The high-performance liquid chromatography (HPLC) conditions used gave baseline separation of the enantiomers with high sensitivity. *R*-(+)-bupivacaine and *S*-(−)-bupivacaine blood concentrations were determined using a chiral stationary phase (AGP, ChromTech) with diode array detection at 220 nm; this wavelength produced a stable baseline allowing semi-automated analysis. Sample preparation involved addition of internal standard (diphenhydramine), basification of blood, extraction with *n*-hexane, concentration of the extract to dryness and reconstitution in 0.002 *M* phosphoric acid. At *rac*-bupivacaine concentrations of 0.5, 5 and 50 μg/ml in blood, assay accuracy as estimated by coefficients of variation (C.V.s), were 3.3, 1.4, and 1.6%, respectively, for *R*-(+)-bupivacaine and 3.7, 2.0 and 1.5%, respectively, for *S*-(−)-bupivacaine. Using 0.6-ml samples, the estimated limits of detection for *R*-(+)-bupivacaine and *S*-(−)-bupivacaine were both 15 ng/ml of blood. Calibration curves ($n=188$) were linear from 0.1 to 50 μg/ml with all correlation coefficients being greater than 0.99. This semi-automated method was applied to studies involving whole body pharmacokinetics with intravenous doses ranging from 12.5 to 350 mg and regional myocardial pharmacokinetics with coronary arterial doses ranging from 2.5 to 12.5 mg. These studies generated approximately 12 000 blood samples.

Keywords: Enantiomer separation; Bupivacaine

1. Introduction

Bupivacaine, (±)-1-butyl-*N*-(2,6-dimethylphenyl)-2-piperidinecarboxamide (Fig. 1a), a local anaesthetic used since the 1960s for its long duration of effects, is synthesised and used as the racemate. Bupivacaine enantiomers differ pharmacologically. For example, *R*-(+)-bupivacaine is more toxic to the

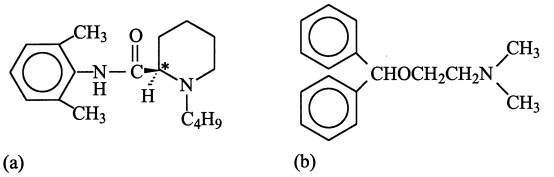

Fig. 1. Two-dimensional structures of bupivacaine (a) and diphenhydramine (b). The * denotes the chiral centre.

*Corresponding author.

0378-4347/98/$ – see front matter
PII: S0378-4347(98)00380-6

central nervous and the cardiovascular systems than *S*-(−)-bupivacaine [1,2]. Bupivacaine enantiomers also differ pharmacokinetically. For example, it has been found in sheep [1,3,4] and humans [5–7] that the clearance of *R*-(+)-bupivacaine is 20–40% greater than *S*-(−)-bupivacaine and it has been found that the tissue–blood distribution coefficient for many tissues is greater for *R*-(+)-bupivacaine than for *S*-(−)-bupivacaine [8]. Hence it is clear that an in depth understanding of the pharmacology of bupivacaine is not possible without a thorough study of its component enantiomers.

A number of high-performance liquid chromatography (HPLC) methods have been developed to measure the enantiomers of bupivacaine in biological fluids after administration of *rac*-bupivacaine [1,6,9–12]. These methods have differing levels of sensitivity and usefulness in describing the enantioselective pharmacokinetics and disposition of bupivacaine. We required a method for measurement of the bupivacaine enantiomers for use in an extensive pharmacokinetic study [13,14]. We now report an improved semi-automated technique for the enantioselective assay of bupivacaine, optimised for both high sensitivity and high throughput of samples. A one-step extraction technique with diphenhydramine as the internal standard (Fig. 1b) gave comparable sensitivity to previous methods but has the significant advantage of simplicity of sample preparation.

2. Experimental

2.1. Materials and reagents

Reference samples of bupivacaine hydrochloride and diphenhydramine hydrochloride were obtained from Sigma–Aldrich (Sydney, Australia). ChromAR HPLC grade *n*-hexane was supplied by BioLab Scientific (Melbourne, Australia); HiPerSolv grade propan-2-ol, AnalR grade sodium hydroxide and AnalR grade orthophosphoric acid were obtained from BDH Chemicals (Melbourne, Australia). Univar grade sodium dihydrogenorthophosphate and disodium hydrogenorthophosphate were obtained from Crown Scientific (Sydney, Australia). Water was purified by a Milli-Q system obtained from Millipore (Sydney, Australia).

A Medos JAK evaporator (Dynavac Engineering, Sydney, Australia) was used for concentration after the extraction procedure. The vacuum was achieved using a water aspirator and the temperature was held at 40°C.

2.2. Instrumentation

Analyses were performed on a Hewlett-Packard 1100 Series HPLC system equipped with an autosampler and diode array detector. The mobile phase consisted of 0.1 *M* (pH 6.6) sodium phosphate buffer with 4.3% propan-2-ol; the flow-rate was 0.9 ml/min. The column was a chiral α_1-acid glycoprotein (AGP) column (100 mm×4 mm I.D.) (ChromTech, Sweden). The wavelength of detection was 220 nm.

2.3. Sample preparation and chromatography

Heparinised blood samples, obtained from sheep or humans (as appropriate) before and after receiving *rac*-bupivacaine, were placed in Eppendorf tubes (1.5 ml) and stored until analysis. (In this series samples were stored at −80°C, however bupivacaine does not undergo significant degradation even if stored at room temperature).

A stock solution of 1 mg/ml *rac*-bupivacaine hydrochloride (as the monohydrate) in Milli-Q water was prepared. Standards in blood were then prepared by adding volumes of stock solution to drug-free blood to give final concentrations 0.1–50 μg/ml. The working internal standard solution (0.01 mg/ml diphenhydramine hydrochloride) was prepared in Milli-Q water.

Blood samples including standards (0.6 ml) were pipetted into Eppendorf tubes (1.5 ml) containing internal standard (diphenhydramine hydrochloride, 50 μl, 0.01 mg/ml) and made alkaline with 4 *M* sodium hydroxide (20 μl). *n*-Hexane (0.8 ml) was added and the tubes capped. The mixture was shaken for 1 min, frozen in dry ice for approximately 30 min, thawed (at 45°C in a water bath for 3–4 min) and centrifuged at 1500 *g* for 5 min. The samples were refrozen in dry ice for approximately 15 min, the organic layer was decanted into an Eppendorf tube and evaporated to dryness under vacuum at 40°C. The extract was reconstituted in phosphoric acid (pH 3, 70 μl 0.002 *M*), vortex mixed (10 s),

sonicated (1 min), centrifuged (1 min), then transferred into a 250-μl polypropylene insert. An aliquot (40 μl) was injected onto the HPLC system.

3. Results and discussion

The retention times for diphenhydramine, *R*-(+)-bupivacaine and *S*-(−)-bupivacaine were 7.7, 9.8, and 12.0 min, respectively. A typical chromatogram from an extracted blood sample is shown in Fig. 2 where the concentrations for *R*-(+)-bupivacaine, *S*-(−)-bupivacaine were both 0.89 μg/ml.

3.1. Sample preparation and chromatography

The impetus for development of this method was the need to perform comparative pharmacokinetic studies with a wide range of doses of *rac*-bupivacaine and levobupivacaine [*S*-(−)-bupivacaine] in sheep [13] and humans [14]. In the studies in sheep, numerous samples were taken with very low drug concentrations occurring towards the end of the sampling period. In order to meet the requirements of assay sensitivity and sample throughput, two parameters needed to be optimised.

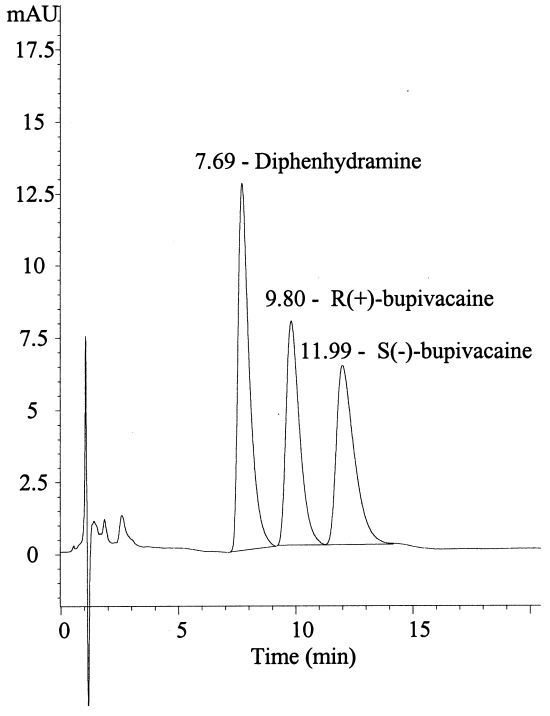

Fig. 2. Chromatogram showing the resolution of *R*-(+)-bupivacaine, *S*-(−)-bupivacaine and diphenhydramine in blood sampled from the coronary sinus 5 min after a 3 min coronary arterial infusion of 2.5 mg *rac*-bupivacaine hydrochloride.

Firstly, the reconstitution volume of the sample extract needed to be minimal; 70 μl was used as this was the minimum volume for the autosampler. Secondly, a single liquid–liquid extraction was used to minimise losses during sample preparation.

n-Hexane was selected as the extraction solvent; its low-polarity precluded extraction of many potentially interfering organic substances from the matrix (blood); hence no back extraction was necessary. Extraction efficiency, determined by comparison of extracted blood samples with spiked water samples, was approximately 70%, resulting in a sufficiently robust assay. In the extraction procedure, the samples were frozen and thawed before centrifugation. This is an important step as emulsification can occur on mixing, and centrifugation does not break the emulsion. Freezing and thawing, however, breaks the emulsion and subsequent centrifugation gave two layers thus maximising extraction efficiency.

The high concentration of sodium hydroxide added to basify the blood samples assisted in minimising contaminants in the chromatograms. A ''blank'' blood sample taken from the same subject was run with each standard and sample set to verify possible interference; none of the blank samples were found to have peaks at the retention times of the enantiomers of bupivacaine or the internal standard.

The wavelength of detection of 220 nm was selected. This wavelength represented a compromise between baseline stability and maximum sensitivity. The variability of the baseline at 210 nm was unacceptable for lower concentration samples (Fig. 3). Although the areas of the peaks corresponding to *R*-(+)-bupivacaine and *S*-(−)-bupivacaine were lower at 220 nm than that at 210 nm, the greatly improved baseline stability gave better peak integration due to improved signal-to-noise (S/N) ratio, facilitating automation by eliminating the need to reprocess low concentration data.

The study for which this assay was developed generated thousands of samples, thus automation was

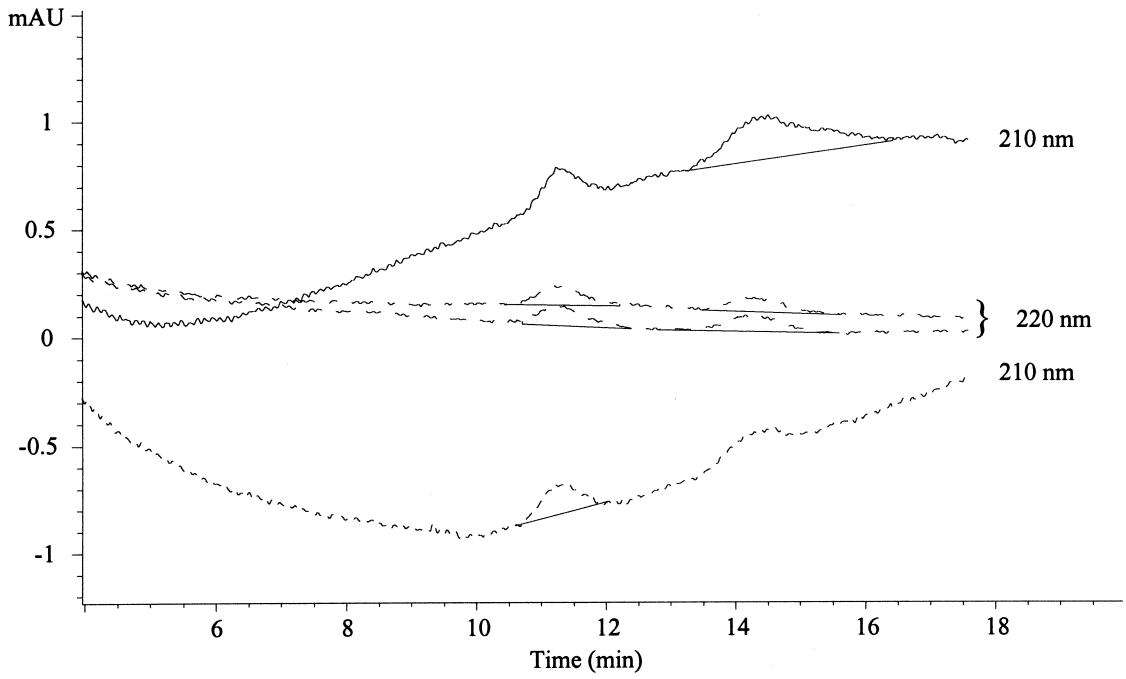

Fig. 3. Two samples each containing 0.2 μg/ml *rac*-bupivacaine, run with the detector set at 210 and 220 nm. The sensitivity of the assay may be improved using 210 nm but at the expense of poor integration and a poor baseline.

essential. The ease of sample preparation of this method allowed large numbers of samples to be processed. A typical batch contained seventy samples, including appropriate blank and standard curve samples. HPLC run time was the throughput limiting step.

3.2. Assay characteristics

Accuracy, determined by six repeat analyses of blood containing 0.5, 5 and 50 μg/ml *rac*-bupivacaine gave coefficients of variation (C.V.s) of 3.3, 1.4 and 1.6% for *R*-(+)-bupivacaine, respectively and 3.7, 2.0 and 1.5% for *S*-(−)-bupivacaine, respectively. Precision, determined by five repeat injections of the same aqueous sample containing 1, 10 and 100 μg/ml gave C.V.s of 2.2, 0.7 and 0.8%, respectively for *R*-(+)-bupivacaine and 3.2, 1.2 and 0.9%, respectively for *S*-(−)-bupivacaine. Using 0.6-ml samples, the estimated limits of detection for *R*-(+)-bupivacaine and *S*-(−)-bupivacaine were both 15 ng/ml in blood (*S*/*N* 3:1). The limit of quantitation was 50 ng/ml for *R*-(+)-bupivacaine and *S*-(−)-bupivacaine (*S*/*N* 10:1).

Samples and standards were quantified by the measurement of peak areas. Calibration curves from 0.1 to 50 μg/ml were linear, with all correlation coefficients being >0.99. Typical calibration curves [for *R*-(+)-bupivacaine and *S*-(−)-bupivacaine] had slopes of 1.169 and 1.156 with coefficients of determination (R^2) of 0.9999 and 0.9997, respectively.

An example of application of this analytical method to studying bupivacaine regional pharmacokinetics is shown in Fig. 4. In this study coronary sinus blood concentrations of *R*-(+)-bupivacaine and *S*-(−)-bupivacaine were measured to 30 min after a 3-min infusion of 2.5–12.5 mg *rac*-bupivacaine·HCl into the left coronary artery of a sheep. In other studies the method has been used to determine the whole body pharmacokinetics of the bupivacaine enantiomers in sheep and humans after intravenous [13] and epidural [14] administration after doses of up to 350 mg of *rac*-bupivacaine.

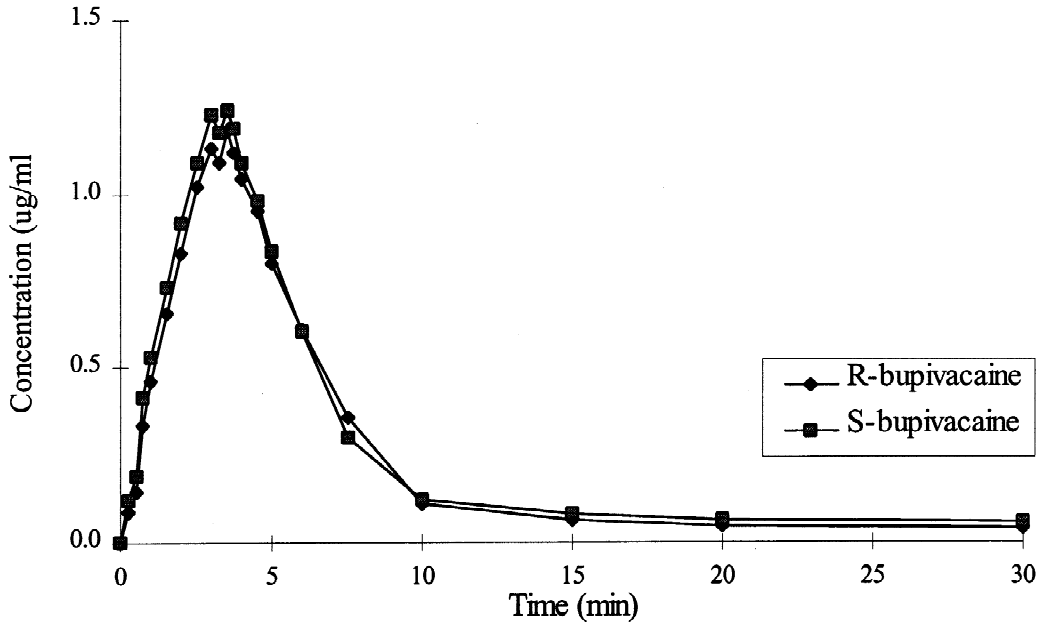

Fig. 4. Application of the method in determining regional concentration–time profiles of *R*-(+)-bupivacaine and *S*-(−)-bupivacaine in blood. In this example blood was sampled from the coronary sinus after a 3-min infusion of 2.5 mg *rac*-bupivacaine·HCl into the left coronary artery of a sheep in order to determine the amount of drug recirculated into the whole body. Examples of the whole body pharmacokinetics of bupivacaine enantiomers have been published elsewhere [13,14].

3.3. Significance

Although administered in equimolar concentrations, enantiomers of many racemic drugs may behave pharmacokinetically as independent drugs. Recent studies have described significant differences in *R*-(+)-and *S*-(−)-bupivacaine enantiomer concentrations in the body after administration of *rac*-bupivacaine – a result of enantiomeric differences in total body and hepatic clearance, tissue distribution and blood–plasma distribution coefficients [1,4,6–8]. Thus, measurement of (total) bupivacaine concentration is no longer optimal for pharmacokinetic and pharmacodynamic studies because of differences between the bupivacaine enantiomers. Fortunately advances in chiral separation chromatography have made possible routine measurements of bupivacaine enantiomers. Previously reported methods have been successful for routine measurements to different degrees.

In 1987 Lee et al. [9] reported the enantiomeric separation of bupivacaine with a limit of detection of 500 ng/ml using 1 ml of serum. Butter et al. [10] devised an on-line analysis of enantiomeric separation of bupivacaine with a detection limit of 100 ng/ml using 500 μl serum samples. Neither of these methods was suitable for our pharmacokinetic studies, as they lacked the required degree of sensitivity. Clark et al. [11] reported a simple sample preparation which used large extraction volumes, however it proved cumbersome when large number of samples were to be analysed.

More recently, Groen et al. [12] reported limits of detection of 8 and 10 ng/ml for *R*-(+)-bupivacaine and *S*-(−)-bupivacaine, respectively. Although this suggests greater sensitivity than the method reported here, similar sensitivity could be achieved with the present method by changing the wavelength of detection to 210 nm as used by Groen et al. [12]. At 210 nm, sensitivity increases by a factor of at least 2 but the baseline is much less stable so that automated integration becomes unreliable at very low concentrations (Fig. 3). However at 220 nm, the baseline is more stable and does not compromise the integrity of

the integration which was needed for the low dose–low concentrations of our studies. Moreover the method of Groen et al. [12] did not report the use of an internal standard, a significant deficiency considering that the method included the following steps: protein precipitation with acetonitrile, solid-phase extraction; concentration; basification; extraction; concentration and reconstitution [12]. In contrast the method reported here involves a single extraction technique (incorporating an internal standard) followed by concentration and reconstitution.

4. Conclusions

The semi-automated HPLC method reported here has been used to process more than 12 000 blood samples. Although others have reported methods in which the accuracy, precision and limit of detection are similar to the present method, their sample preparation has been more complex, the volume of sample needed to achieve the low limit of detection has been larger or instability of the baseline precluded automated data processing.

Acknowledgements

The authors are pleased to acknowledge the technical advice of Dr. J.L. Huang and Mr. S. Edwards as well as the financial support of Chiroscience Ltd., Cambridge, UK.

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