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Short Communication

Improved clean-up procedure for the high-performance liquid chromatographic assay of bupivacaine enantiomers in human plasma and ultrafiltrate in the nanogram per milliliter range

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Abstract

A clean-up procedure to obtain a minimal detectable concentration of 5–10 ng bupivacaine enantiomer per milliliter human plasma is described. The procedure consists of precipitation of plasma proteins using acetonitrile, followed by solid-phase extraction using a cyano column. The eluate is then made alkaline, and bupivacaine is extracted using *n*-hexane. After evaporation of *n*-hexane, the residue is redissolved in the eluent used for HPLC analysis. The HPLC method has been described previously. The minimal detectable concentrations using this method are *ca.* 8 and 10 ng/ml for *R*-(+)- and *S*-(−)-bupivacaine, respectively. For both enantiomers, r^2 is >0.995 over the range of 9.5–760 ng/ml enantiomer.

1. Introduction

Bupivacaine is a member of the family of aminoacylaniline local anaesthetics which possess a chiral carbon atom. From a clinical point of view, the pharmacokinetics of the individual bupivacaine enantiomers is important. During the last decennia a number of studies have been published dealing with the stereoselective effects of the bupivacaine enantiomers [1–4].

In clinical practice, only the racemate of bupivacaine is used. This is important, because the individual enantiomers may modulate each other's vasomotor activity. Recently, some pharmacokinetic studies have been performed after extrapleural administration of the racemate to man [5], and intra-atrial administration of the individual enantiomers to sheep [2]. These studies indicated that there are differences in the pharmacokinetics of the enantiomers, although the nomenclature used is not always consistent.

A detailed study of the pharmacokinetics of the individual bupivacaine enantiomers after intravenous administration of the racemate in human volunteers was performed to obtain reliable estimates of the basic pharmacokinetic parameters, such as clearance, volume of distribution, and elimination half-life. Besides these parameters, binding of the enantiomers to plasma proteins had to be determined.

A prerequisite to obtain approval for this type of study from the Ethics Committee of Leiden

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University Hospital was that the possibility of achieving toxic levels of bupivacaine was negligible. Therefore, plasma levels of the enantiomers should not exceed 1 μg ml^{-1}. Consequently, in the analytical method to be used, a limit of detection of *ca.* 5–10 ng ml^{-1} was thought to be necessary, in order to delineate the pharmacokinetic variables with an acceptable accuracy.

Although several HPLC methods for the assay of bupivacaine enantiomers in plasma from humans [6,7] and sheep [2] have been reported, these were not applicable to the intended study either because the limit of detection was too high or because even a limited number of assays already caused a considerable decrease in column performance. Therefore, an improved clean-up procedure had to be developed to meet the requirements.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Gilson Model 305 pump, a Gilson Model 231 automatic sample injector (Gilson, Middleton, WI, USA), and an LKB 2141 variable wavelength detector (LKB, Bromma, Sweden). The column was a Bakerbond chiral α_1-acid glycoprotein column (100 × 4.0 mm I.D.) and was used together with a guard column of the same material (10 × 4.0 mm I.D.). Both were obtained from Baker (Deventer, Netherlands). Bakerbond cyano (CN) SPE columns (Baker) were used for the solid-phase extraction.

2.2. Chemicals

Marcaine (solution for injection, containing racemic bupivacaine hydrochloride) was obtained from Astra Pharmaceutica (Rijswijk, Netherlands), and racemic bupivacaine hydrochloride was obtained from Sigma (St. Louis, MO, USA). Isopropanol, acetonitrile, and *n*-hexane were all HPLC-grade solvents, obtained from Westburg (Leusden, Netherlands). Phosphoric acid 85%, $Na_2PO_4 \cdot H_2O$, and NaH_2PO_4 were of analytical grade from Merck (Darmstadt, Germany). Fresh blank plasma was obtained from the University Hospital Utrecht (Utrecht, Netherlands).

2.3. Sample preparation

Acetonitrile (2 ml) was slowly added to a 1-ml aliquot of plasma while whirlmixing and left for 5 min at room temperature. After centrifugation for 10 min at 3000 *g*, the supernatant was transferred to a clean test tube and diluted with 15 ml of bidistilled water. This solution was then applied to a Bakerbond SPE cyano column, which had previously been activated with two 1-ml aliquots of the eluent consisting of 50 m*M* NaH_2PO_4 (adjusted to pH 3.0 with 1 *M* phosphoric acid)–methanol (1:1, v/v). Flow was maintained by suction using a vacuum of 20 kPa.

Subsequently, the diluted supernatant was applied to the SPE column under vacuum (20 kPa). Then, the column was washed with three 1-ml volumes of bidistilled water. Bupivacaine was eluted from the column using the above mentioned eluent. The volume of the eluate was reduced to *ca.* 0.5 ml under a stream of nitrogen in order to evaporate the methanol from the eluate. Then, 1 ml of bidistilled water and 50 μl 1 *M* NaOH were added, followed by whirlmixing for 30 s. A 6-ml volume of *n*-hexane was used to extract bupivacaine from the alkaline mixture. After mixing for 10 min on a rotator and centrifugation for 5 min at 3000 *g*, 5 ml of the hexane fraction was transferred to a clean test tube and evaporated under nitrogen. The residue was then redissolved in 80 μl of the eluent used for HPLC analysis, and left for *ca.* 2 h. This eluent consisted of 0.01 *M* NaH_2PO_4 (pH 6.8)–isopropanol (94:6, v/v). Finally, 50 μl was injected onto the HPLC system.

The eluent flow-rate was 1.0 ml/min, and the detection wavelength was 210 nm.

2.4. Intravenous bupivacaine

Venous blood samples, from a volunteer who received 30 mg racemic bupivacaine (Marcaine) intravenously during a 10-min infusion in a forearm vein, were taken at several time points over an 8-h period. After centrifugation for 15 min at

3000 *g* the obtained plasma was stored at −18°C until analysis.

3. Results

Typical chromatograms are shown in Fig. 1. Since the optically pure bupivacaine enantiomers were not available, we had to assume that the order of elution from the system was the same as that described previously [8].

3.1. Absolute detection limit

The absolute limit of detection in eluent, defined as the concentration corresponding to a signal-to-noise ratio of 2, was determined in six-fold. At a concentration of 3.0 ng enantiomer per ml, the signal-to-noise ratios were 4 and 3 for *R*-(+)- and *S*-(−)-bupivacaine, respectively, with C.V.s of 10.8% and 7.5%, respectively. Consequently, the absolute limits of detection were approximately 1.5 and 2 ng per enantiomer for *R*-(+)- and *S*-(−)-bupivacaine, respectively.

3.2. Minimal detectable concentration

For the determination of the minimal detectable concentration in plasma, spiked plasma samples with a final concentration of 4.76 and 9.52 ng of each enantiomer per ml were prepared and analyzed in five-fold. The minimal detectable concentration was defined as the concentration in plasma corresponding to a signal-to-noise ratio ≥ 5. It is concluded that the minimal detectable concentrations are *ca.* 8 and 10 ng/ml for *R*-(+)- and *S*-(−)-bupivacaine, respectively.

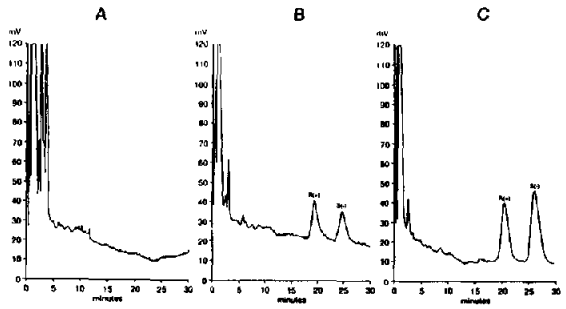

Fig. 1. Typical chromatograms of (A) blank plasma, (B) plasma spiked to a concentration of 36 ng enantiomer per ml, and (C) a plasma sample taken 180 min after intravenous administration of 30 mg racemic bupivacaine.

3.3. Linearity in plasma

Calibration curves of racemic bupivacaine were made using eleven spiked plasma samples at concentrations of 9.5–760 ng enantiomer per ml. Linearity was determined for all concentrations and for the lowest five concentrations (9.5–47.7 ng enantiomer per ml). The calibration curves were linear for both enantiomers. For *R*-(+)-bupivacaine r^2 was 0.998 for all concentrations and 0.983 for the five lowest concentrations, respectively. For *S*-(−)-bupivacaine r^2 was 0.999 for all concentrations and 0.986 for the five lowest concentrations, respectively.

3.4. Intra-day variability

The intra-day variability was 6.0%, 5.0%, and 2.9% at concentrations of 50, 250 and 500 ng *R*-(+)-bupivacaine per ml, respectively. For *S*-(−)-bupivacaine the intra-day variability was 10.4%, 4.9%, and 2.9% at the respective concentration levels.

3.5. Plasma levels after intravenous administration

Typical plasma concentration–time profiles of *R*-(+)- and *S*-(−)-bupivacaine are given in Fig. 2. The plasma concentration of *R*-(+)-bupivacaine ranged between 294 and 14.5 ng per ml, and that of *S*-(−)-bupivacaine between 401 and 14.8 ng per ml.

4. Discussion and conclusions

It was already known that the enantiomers of bupivacaine could be resolved using α_1-acid glycoprotein columns [2,6,7]. However, the sample preparation methods described in those

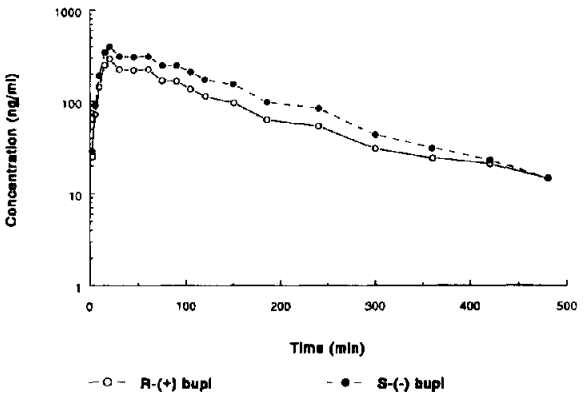

Fig. 2. Plasma concentration *vs.* time curves of *R*-(+)- and *S*-(−)-bupivacaine in a volunteer who received 30 mg racemic bupivacaine intravenously.

papers had a too high limit of detection to study the pharmacokinetic parameters after intravenous administration of bupivacaine.

In the present paper an adjusted clean-up procedure is described, which enables the determination of plasma concentrations of bupivacaine at the nanogram per milliliter level. The observation that the plasma concentration of *S*-(−)-bupivacaine is higher than that of *R*-(+)-bupivacaine after a single intravenous dose of racemic bupivacaine is in agreement with the data obtained after intrapleural administration [6].

From the data obtained after intravenous administration of bupivacaine to one volunteer, it is concluded that the method described here offers the opportunity to derive pharmacokinetic parameters with an acceptable accuracy after low doses. Furthermore, the method can also be applied to the determination of the plasma protein binding of bupivacaine enantiomers, since very low concentrations can be detected (data not shown in this paper).

This method will be used to derive all basic pharmacokinetic parameters of the bupivacaine enantiomers, including protein binding in healthy volunteers as well as in patients.

5. References

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