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Note

Sensitive determination of bupivacaine in human plasma by high-performance liquid chromatography

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Bupivacaine hydrochloride is an amide-type local anaesthetic, which is used to provide intra- and postoperative epidural anaesthesia, e.g. in orthopaedics, abdominal surgery and paediatrics [1,2]. Systemic toxic reactions, especially cardiac arrhythmias and cerebral convulsions, may occur during bupivacaine epidural analgesia [3] because of fast absorption leading to high plasma peak levels [4]. For measuring bupivacaine plasma concentrations, especially in the later phase of elimination, a highly sensitive assay is required that is affected by neither plasma components nor other drugs given simultaneously.

High-performance liquid chromatographic (HPLC) methods for the determination of bupivacaine and other local anaesthetics with detection limits ranging between 10 and 175 ng/ml of plasma [5–10] as well as several gas chromatographic methods with detection limits ranging between 1.5 and 50 ng/ml of plasma [3,11–19] have been reported.

We have developed a rapid HPLC assay, which is linear down to a bupivacaine plasma concentration of 1 ng/ml. We used a re-extraction step to provide clean plasma extracts, which allow UV detection at 210 nm without interfering plasma peaks. The method (extraction and HPLC) was controlled by the addition of pentycaine as an internal standard.

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EXPERIMENTAL

Reagents and chemicals

Bupivacaine and pentycaine [naepaine, 2-(pentylamino)ethanol 4-aminobenzoate] were gifts from Astra Läkemedel (Södertälje, Sweden). Acetonitrile and water (both HPLC grade) were purchased from Baker (Gross-Gerau, F.R.G.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.) and were of the highest purity available.

Apparatus and HPLC conditions

The HPLC system consisted of a Merck/Hitachi L-6200 intelligent pump (Merck, Darmstadt, F.R.G.) coupled with a six-port injector (Rheodyne, Cotati, CA, U.S.A.), a Phenyl Hypersil NC-04 column, particle size 5 μm, 250 mm × 4 mm I.D. (Bischoff, Leonberg, F.R.G.) and a Merck/Hitachi 655A variable-wavelength UV monitor (Merck) fitted with a 2.2-μl flow-cell and operated at 210, 230 or 254 nm. A Merck/Hitachi D-2000 chromato-integrator was used for printing chromatograms and for data calculation.

The mobile phase was acetonitrile–10 m*M* potassium phosphate buffer (60:40, v/v) and was adjusted to pH 3.3 with phosphoric acid. The flow-rate was 1 ml/min and the column temperature was 22°C.

Plasma sample treatment

A 1-ml plasma sample spiked with 200, 500 or 1000 ng of pentycaine in 200 μl of doubly distilled water as internal standard was alkalinized with 200 μl of 2 *M* sodium hydroxide. After the addition of 200 μl of *n*-propanol, bupivacaine and pentycaine were extracted with 7 ml of hexane by careful shaking for 15 min. After centrifugation at 1000 *g* for 10 min, fractions of the hexane phase were transferred into several 1.5-ml test-tubes (Eppendorf, Hamburg, F.R.G.) and evaporated to dryness under nitrogen at ambient temperature. The residue was redissolved in 0.1 ml of hexane, and the re-extraction step was carried out after the addition of 0.1 ml of 20 m*M* phosphoric acid by vortex mixing for 20 s. After centrifugation, the hexane phase was discarded and a 50-μl aliquot of the phosphoric acid phase was injected into the chromatograph.

Calibration curves, precision and recovery

For calibration, plasma standards that had been spiked with fixed amounts of the internal standard and with bupivacaine concentrations ranging between 1 ng/ml and 3 μg/ml were prepared and assayed as described above. Calibration curves were plotted by correlating the peak-area ratio of bupivacaine to pentycaine against the corresponding bupivacaine concentrations. The accuracy of the method was evaluated in the concentration range from 5 ng/ml to 3 μg/ml in a blind study by assaying plasma samples spiked with known bu-

pivacaine concentrations. The intra- and inter-day precision of the assay was estimated by measuring plasma standards with 50 and 500 ng/ml bupivacaine at day 1 ($n=10$) and on the nine following days. The analytical recovery of the extraction and re-extraction was determined by comparing the bupivacaine peak heights in plasma extracts with peak heights of non-extracted aqueous bupivacaine solutions at concentrations of 50 and 500 ng/ml.

Application of the method

The assay was used to determine bupivacaine plasma concentrations in patients with balanced anaesthesia combined with epidural bupivacaine analgesia for abdominal surgery. Pulmonal-arterial blood samples were withdrawn over a period of 75 min; after centrifugation the plasma samples were assayed as described.

RESULTS AND DISCUSSION

The determination of plasma bupivacaine concentrations for the evaluation of pharmacokinetic parameters in patients under epidural anaesthesia requires a very sensitive assay, especially in the later phase of elimination. In

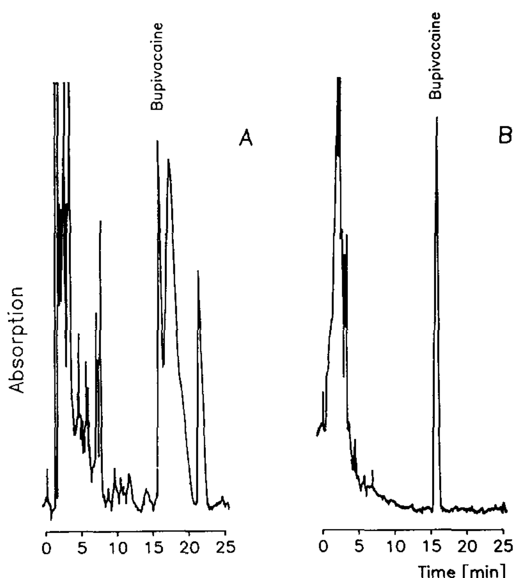

Fig. 1. HPLC separation of a plasma sample spiked with 1 μg/ml bupivacaine. The chromatograms were obtained at 254 nm without (A) and with (B) re-extraction of bupivacaine from the hexane phase into 20 mM phosphoric acid. The plasma sample was from a patient who had received flunitrazepam, pancuronium, midazolam, dihydroergocristine methanesulphonate, clopamide and reserpine.

addition, high specificity is necessary owing to the variety of supplementary drugs given to these patients. The reported HPLC method using UV detection at 254 nm does not allow the determination of bupivacaine plasma concentrations below 50 ng/ml [5]. In our experience, the lower wavelengths used by other authors [7–10] promising a better sensitivity produced manifold increase of interfering plasma and drug peaks, especially in low concentration ranges, if only a single step extraction was performed. Therefore, we used a re-extraction step to transfer bupivacaine from the hexane phase into an acidic aqueous phase and to remove interfering plasma components. The best results were obtained when HPLC was carried out with a phenyl column instead of the commonly used C_{18} columns.

Fig. 1 shows chromatograms (obtained at 254 nm) of a blank plasma spiked with 1 μg/ml bupivacaine withdrawn from a patient who received flunitrazepam, pancuronium, midazolam, dihydroergocristine methanesulphonate, clopamide and reserpine. Without re-extraction, the chromatogram showed peaks interfering with the bupivacaine peak (A), whereas no interferences with the bupivacaine peak could be observed (B) if the re-extraction step was carried

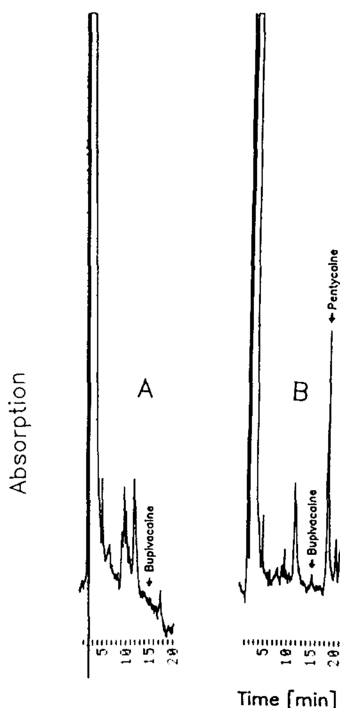

Fig. 2. Limit of detection for bupivacaine at 210 nm. (A) Plasma sample without bupivacaine and pentycaine; (B) plasma sample spiked with 1 ng/ml bupivacaine and 25 ng/ml pentycaine as internal standard.

out. After re-extraction, lower wavelengths (230 and 210 nm) could be used without interference from plasma components and with an evident increase in sensitivity. At a signal-to-noise ratio of 3:1 the lower limit of detection at 210 nm for bupivacaine from plasma samples (1 ml) was ca. 1 ng/ml (Fig. 2), and the extraction recovery at a bupivacaine concentration of 50 ($n=3$) and 500 ($n=3$) ng/ml was $93 \pm 0\%$.

The calibration curve was linear for samples over the concentration range studied, 1 ng/ml to 3 μg/ml ($r=0.99995$). In the low concentration range (1–100 ng/ml) the least-squares linear regression line, which represents the best fit of the bupivacaine data, has a slope of 0.05792 and a y-intercept of 0.0184 (where y is the peak-area ratio of bupivacaine to pentycaine and x is the bupivacaine concentration, ng/ml) ($r=0.9998$).

The accuracy of the method is shown in Table I; the theoretical concentrations (spiked concentration) agreed well with the assayed concentrations (found concentration) with an mean accuracy of 3.0%.

The mean concentration, standard deviation and coefficient of variation for intra and inter-day precision are shown in Table II.

The selectivity of the assay was ascertained by chromatographing, without extraction, aqueous solutions of several drugs commonly used in anaesthesia. None of the drug peaks, except the peak of fentanyl, interfered with the bupivacaine or the pentycaine peak. Because of the identical retention times of fentanyl and bupivacaine it was ascertained that fentanyl could not be extracted by this method. The drugs and their corresponding retention times are listed in Table III.

This method was used to determine bupivacaine concentrations in plasma

TABLE I

ACCURACY OF THE ASSAY

Concentration of bupivacaine (ng/ml)		Accuracy[a] (%)
Spiked	Found	
5	4.9	2.0
20	21.6	8.0
70	68.2	2.6
160	162.2	1.4
600	602.0	0.3
1100	1120.3	1.8
2000	1974.0	1.3
3000	2811.0	6.3
Mean		3.0

[a]Calculated according to ref. 20.

TABLE II

INTRA- AND INTER-DAY PRECISION FOR BUPIVACAINE

	Spiked concentration (ng/ml)	Found concentration (mean ± S.D., $n=10$) (ng/ml)	C.V. (%)
Intra-day	50	50.5 ± 1.86	3.69
	500	505.3 ± 5.41	1.07
Inter-day	50	49.7 ± 2.41	4.84
	500	511.5 ± 7.58	1.48

TABLE III

SELECTIVITY OF THE ASSAY

Drug	Retention time (min)
Bupivacaine	15.50
Pentycaine	17.00
Methohexital	2.50
Flunitrazepam	3.00
Pancuronium bromide	3.00
Suxamethonium chloride	3.00
Diazepam	3.50
Propofol	5.50
Etomidate	5.50
Droperidol	11.50
Fentanyl	15.50[a]
Midazolam	16.25
Promethazine	22.50
Piritramide	29.60

[a]Fentanyl is not extractable under these conditions.

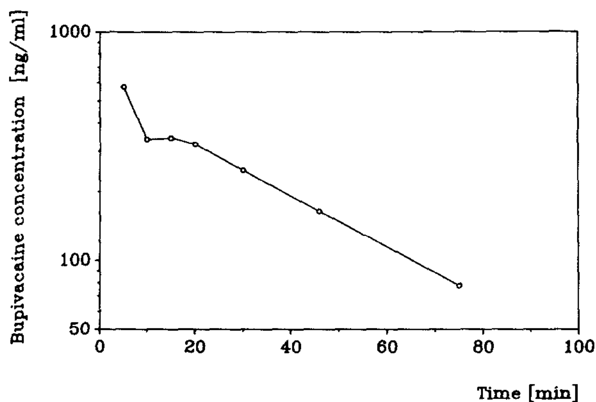

Fig. 3. Bupivacaine concentration–time curve in a patient who received bupivacaine epidural anaesthesia for abdominal surgery. The plasma samples were withdrawn from the pulmonal artery.

samples drawn from the arteria pulmonalis in patients receiving epidural anaesthesia for abdominal surgery. Fig. 3, for example, shows a bupivacaine concentration–time curve. According to the protocol of the study to which this patient belonged the sample withdrawal period was limited. Therefore, no samples were available with concentration values expected near the limit of detection.

Our method offers the advantage of an increased sensitivity compared with other HPLC methods, and its application will be useful to obtain pharmacokinetic data of bupivacaine, especially at low plasma concentrations, in patients receiving various supplementary drugs.

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