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High-performance liquid chromatographic method for the quantitation of bupivacaine, 2,6-pipecoloxylidide and 4′-hydroxybupivacaine in plasma and urine

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ABSTRACT

A high-performance liquid chromatographic method with ultraviolet detection at 210 nm for quantitation of bupivacaine and two of its metabolites from plasma and urine is described. The compounds are extracted into *n*-hexane–isopropanol (5:1), evaporated and the reconstituted residue injected onto a reversed phase C_{18} column. Standard curves for all compounds were linear ($r^2 > 0.999$) in the range 20–2000 ng/ml, with a limit of detection of 10 ng/ml. The inter-day coefficients of variation ranged between 2.7 and 12.2%. The method was applied to analyse bupivacaine and metabolite concentrations in patients on long-term epidural bupivacaine–fentanyl infusions.

INTRODUCTION

Bupivacaine, (±)-1-butylpipecolo-2′,6′-xylidide, is an amide-type local anaesthetic agent, which is used extensively in anaesthesia for nerve blocks and for intra- and post-operative pain management.

Although the biotransformation products of bupivacaine in man have not been entirely identified, desbutylbupivacaine or 2,6-pipecoloxylidide (PPX) and 4′-hydroxybupivacaine (Fig. 1) are two of the known metabolites of bupivacaine, which are formed by N-dealkylation and ring hydroxylation of the parent drug, respectively [1]. Studies in mice indicate that PPX is approximately one eighth as toxic as bupivacaine [2]. Measurement of the concentrations of bupivacaine and these metabolites in biological fluids may be useful in understanding the pharmacokinetics and toxicity following bupivacaine administration in man.

Several gas chromatographic methods and one high-performance liquid chromatographic (HPLC) technique have been described for the determination of racemic bupivacaine and PPX concentrations in plasma and urine [3–6]. However, only one method has been reported for the simultaneous determination of bupivacaine, PPX and 4′-hydroxybupivacaine in plasma and urine [7]. This HPLC method, described by Lindberg and *et al.* [7], while being sensitive and specific, requires a lengthy sample work-up procedure.

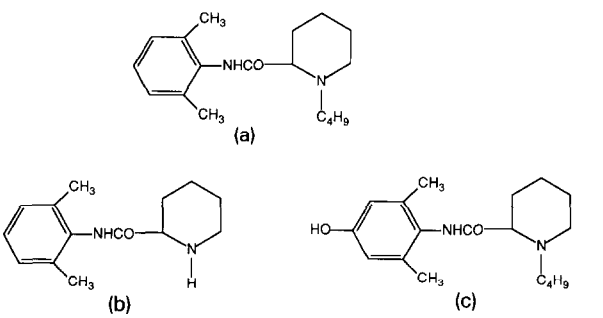

Fig. 1. Structures of (a) bupivacaine, (b) 2,6-pipecoloxylidide (PPX or desbutylbupivacaine) and (c) 4′-hydroxybupivacaine.

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The HPLC method with UV detection described here permits the determination of concentrations of these compounds at the same level of sensitivity as those previously described with the added advantage of a rapid one-step extraction procedure.

EXPERIMENTAL

Reagents and materials

Bupivacaine hydrochloride, PPX, 4′-hydroxybupivacaine and 1-pentyl-2-(2′,6′-xylylcarbamoyl)piperidine hydrochloride (internal standard) were kindly supplied by Astra Pharmaceuticals (Sydney, Australia). *n*-Hexane, acetonitrile and methanol (Mallinkrodt, Clayton, Australia) and isopropanol (Prolabo, Paris, France) were of HPLC grade. All other chemicals were analytical grade. Pyrex tubes were washed sequentially in nitric acid, Extran and distilled water and silanized with a 1% ethereal solution of hexamethyldisilazane before use.

Aqueous stock solutions (40 μg/ml) of each of bupivacaine, PPX and 4′-hydroxybupivacaine were prepared. These were further diluted with distilled water to give standard solutions with final concentrations of 0.1, 0.2, 0.5, 1, 2, 4, 5, 10 and 20 μg/ml. Daily working standards were prepared by spiking 0.7-ml aliquots of drug-free plasma or urine with 0.1 ml of aqueous standard of each of the three compounds. The internal standard solution was prepared by dissolving the compound in distilled water to a concentration of 5 μg/ml.

Sample preparation

To 1 ml of human plasma or urine in a silanized 10-ml screw-cap Pyrex tube was added 0.1 ml of internal standard solution (500 ng) and this was then alkalinized with 0.1 ml of sodium carbonate buffer (1 *M*, pH 10). The sample was mixed by shaking by hand for 5 s and extracted with 6 ml of *n*-hexane–isopropanol (5:1) by rotating on a tumble mixer at 28 rpm for 10 min. After centrifugation at 2000 *g* for 10 min, the organic layer was transferred by aspiration to a clean tube and evaporated to dryness with a gentle nitrogen stream at 40°C. The residue was reconstituted with 120 μl of mobile phase and 100 μl were injected onto the column.

Chromatographic conditions

The analyses were performed on a μBondapak C_{18} (300 mm × 3.9 mm I.D., 10 μm silica particle size) column (Waters Assoc., Newstead, Australia) attached to a Waters Model 501 HPLC pump, coupled with a Rheodyne Model 7125 injector (Analytical Instruments, Scarborough, Australia) with 200-μl capacity loop, Waters Model 481AZ variable-wavelength UV detector and Rikadenki (Tokyo, Japan) chart recorder set at 15 cm/min. The mobile phase, methanol–acetonitrile–sodium phosphate buffer (0.02 *M*, pH 6) (15:40:45, v/v) was pumped at 1.2 ml/min. The column effluent was monitored at a wavelength of 210 nm at a detector sensitivity of between 0.05 and 0.2 a.u.f.s.

Application of the method

Plasma samples from five patients who received long-term epidural bupivacaine–fentanyl infusions were assayed for bupivacaine and its two known metabolites. Patients' venous blood samples were withdrawn at twelve hourly intervals for at least 60 h during the infusion and then every 2 h post-infusion for a further 12 h. Plasma was separated by centrifugation and deep frozen at −20°C prior to assay.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram obtained from a sample of blank plasma spiked with 500 ng of internal standard and a typical chromatogram obtained from a patient plasma sample. The elution order of the compounds was PPX, 4′-hydroxybupivacaine, bupivacaine and internal standard at retention times of 3.6, 4.8, 9.2 and 13.2 min, respectively, with a total run time of 14.4 min. The drug and metabolite peaks were all sharp, symmetrical and well resolved and there was no interference from endogenous compounds. Interference from a number of other clinically used drugs was assessed by injecting

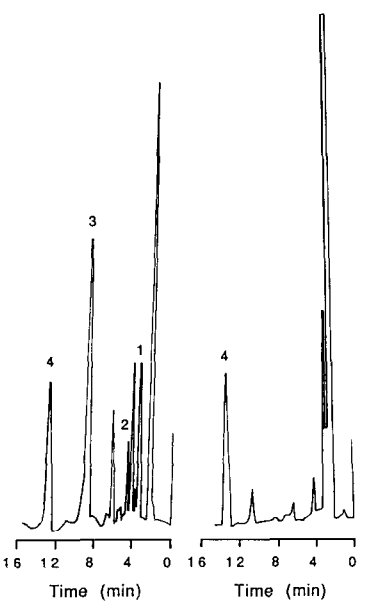

Fig. 2. (Left) Chromatogram of an extract of human plasma following epidural bupivacaine–fentanyl infusion showing PPX (1; 325 ng/ml), 4′-hydroxybupivacaine (2; 180 ng/ml), bupivacaine (3; 530 ng/ml) and internal standard (4). (Right) Chromatogram of a plasma blank spiked with 500 ng of internal standard (4).

aqueous samples of these agents onto the column and comparing their retention times with those of the peaks of interest. Fentanyl, morphine and oxycodone were found not to interfere with the assay.

Daily calibration curves of working standards for each of bupivacaine, PPX and 4′-hydroxybupivacaine were constructed from the least-squares linear regression of peak-height ratios of the respective compound to internal standard *versus* concentration of the compound. Standard curves for each compound were linear ($r^2 > 0.999$) in the range 20–2000 ng/ml. Typical calibration curves (correlation coefficients in parentheses) were $y = 0.003x - 0.005$ ($r^2 = 1.0000$) for bupivacaine, $y = 0.004x - 0.035$ ($r^2 = 0.9996$) for PPX and $y = 0.004x - 0.011$ ($r^2 = 0.9999$) for 4′-hydroxybupivacaine. The limit of detection, defined as the concentration that could be detected at a baseline to noise ratio of 3:1, was 10 ng/ml for all three compounds.

The precision and accuracy of the technique (calculated according to ref. 8) were determined from the analysis of eight samples of each of five concentrations of bupivacaine, PPX and 4′-hydroxybupivacaine over the concentration range 20–2000 ng/ml in both plasma and urine. The concentrations found experimentally were determined by inverse prediction from daily working calibration curves. Mean found concentrations agreed well with the spiked concentrations for each of the compounds (Table I).

Inter-day precision was determined from aliquots of spiked, pooled plasma at low (50 ng/ml), medium (400 ng/ml) and high (2000 ng/ml) concentrations on six separate days over a period of one month. Coefficients of variation for inter-day precision are shown in Table II.

Recoveries from plasma and urine of each of the compounds over the range 50–2000 ng/ml were calculated. Plasma extracts and aqueous samples of the same known concentrations were prepared and internal standard was added prior to injection onto the column. Recoveries were calculated by comparing peak-height ratios of extracted samples with those obtained from aqueous injections at five concentrations. Recoveries of bupivacaine, PPX and 4′-hydroxybupivacaine were 95, 84 and 80%, respectively, from plasma, and 97, 71 and 86%, respectively, from urine. The lower recovery of PPX from urine was unexpected and may perhaps be attributed to fluctuations in urinary pH, which was not controlled in our experiments.

The recovery of the 4′-hydroxybupivacaine metabolite was found to be influenced by heat. While the application of heat accelerates the evaporation of solvent from extracted samples, a temperature rise from 40 to 50°C was found to decrease recovery of this metabolite by approximately 30%. Thus, the use of excessive heat may have accounted for the poor recovery of this metabolite with a similar extraction solvent [7]. Silanization of Pyrex extraction tubes and maintenance of a low heat of evaporation (40°C) was necessary to avoid this problem.

Fig. 3 is a representative plasma concentration–time profile for a patient who received a continuous epidural infusion of bupivacaine–fentanyl (2 mg bupivacaine–20 μg fentanyl per ml) at

TABLE I

ASSAY PRECISION AND ACCURACY (n = 8)

Spiked concentration (ng/ml)	Plasma			Urine		
	Mean concentration found (ng/ml)	C.V. (%)	Accuracy (%)	Mean concentration found (ng/ml)	C.V. (%)	Accuracy (%)
Bupivacaine						
25	26.7	9.8	7.0	28.4	6.0	13.7
50	53.8	3.7	7.6	48.8	4.8	2.4
100	97.1	6.9	2.9	99.0	4.6	1.0
400	377.2	1.7	5.7	392.6	4.3	1.8
2000	2101.5	3.3	5.1	1942.0	2.4	2.9
PPX						
20	18.0	16.2	10.2	23.0	7.7	14.8
50	46.7	7.6	6.6	45.2	9.3	9.7
100	92.9	2.7	7.1	102.1	4.8	2.1
400	410.7	2.6	2.7	375.5	5.0	6.1
2000	1961.0	2.8	2.0	2008.1	3.4	0.4
4'-Hydroxybupivacaine						
20	21.4	9.0	7.1	22.8	5.2	13.8
50	46.0	4.3	8.1	53.4	6.8	6.9
100	91.2	2.6	8.8	103.1	8.1	3.1
400	420.1	3.3	5.0	377.3	1.7	5.7
2000	1917.9	2.9	4.1	2073.6	1.7	3.7

an initial rate of 3 ml/h. While the rate of infusion overall was not constant (as is usually the case in clinical practice), concentrations of bupivacaine approximated a steady state after about 40 h (mean concentration 380 ± 61 ng/ml). The concentrations of each of the metabolites remained fairly constant for the duration of the infusion, with approximate steady-state plasma concentrations for PPX and 4'-hydroxybupivacaine of 159 ± 22 and 28 ± 6 ng/ml, respectively. Metabolite concentrations increased transiently after the infusion was ceased to approximately 200 ng/ml for PPX and 100 ng/ml for 4'-hydroxybupivacaine, and had begun to decline by the 12th h post-infusion (172 and 69 ng/ml, respectively). No accumulation of metabolites was evident in the patients studied. In examining such data, it would also be appropriate to consider stereoisomerism since bupivacaine is administered as a racemic mixture [9,10]. However, while an assay capable of detecting bupivacaine metabolites and incorporating enantiomeric separations would be very useful, a recently published HPLC assay for the detection of bupivacaine and its enantiomers suggests that, in order to do so, a lengthy analysis and an elaborate and costly apparatus would be required [11].

TABLE II

COEFFICIENTS OF VARIATION FOR INTER-DAY RESULTS

Concentration (ng/ml)	Coefficient of variation (n = 6) (%)		
	Bupivacaine	PPX	4'-Hydroxy-bupivacaine
Plasma			
50	4.4	9.0	4.3
400	2.7	2.8	6.9
2000	3.4	8.9	5.3
Urine			
50	6.7	10.4	5.9
400	3.5	12.2	4.9
2000	4.9	4.6	2.9

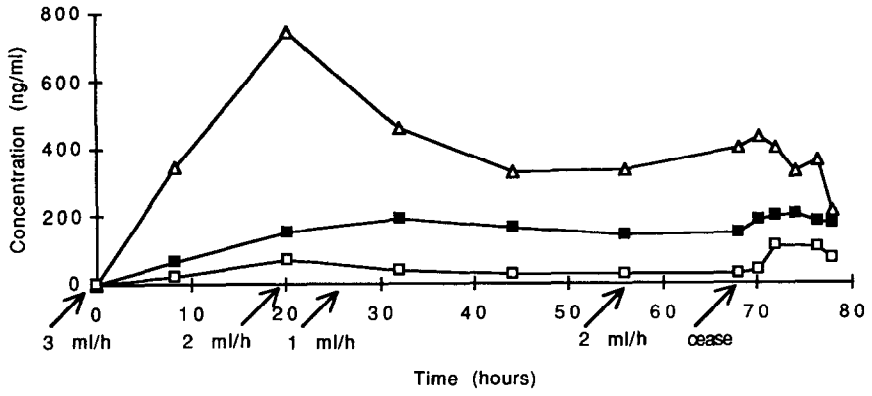

Fig. 3. Plasma concentration–time profiles of bupivacaine (△), PPX (■) and 4′-hydroxybupivacaine (□) during a 68-h continuous infusion of bupivacaine–fentanyl (2 mg bupivacaine–20 μg fentanyl per ml; initial rate 3 ml/h) and for 12 h post-infusion. Arrows indicate changes in rate of infusion.

While plasma metabolite concentrations following long-term continuous infusion of bupivacaine have not been previously reported, our measured concentrations are of the same order of magnitude at 24 h post-infusion as those reported by Rosenberg *et al.* [12] following a 24-h continuous interscalene infusion. Our measured PPX concentrations were also similar to those noted following multiple epidural bolus injections in pregnant women at term [13].

In conclusion, the HPLC assay described for the simultaneous determination of racemic bupivacaine, PPX and 4′-hydroxybupivacaine in plasma and urine employs a rapid, one-step extraction procedure and is linear, sensitive and specific for the compounds of interest. Application of the method may be useful in determining the relationship, if any, between known metabolite concentrations and bupivacaine toxicity and in further elucidating the pharmacokinetic profile of bupivacaine.

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