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The application of capillary gas chromatography-selective ion mass spectrometry for the separation, identification and quantification of phenolic bupivacaine metabolites from human urine

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Abstract

A capillary gas chromatographic method has been developed and validated for the separation of phenolic metabolites from human urine following the intravenous infusion of racemic bupivacaine, with subsequent formal identification and quantification by on-line mass spectrometry. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Bupivacaine; 3-Hydroxybupivacaine; 4-Hydroxybupivacaine; Human metabolism; Gas chromatography-mass spectrometry; Selective ion monitoring

1. Introduction

(±)-1-Butyl-2′,6′-pipecoloxylidide (bupivacaine; Fig. 1) is a long-acting local anaesthetic drug used clinically as the racemate and employed mainly for spinal anaesthesia [1]. It is known to be metabolised by cytochromes P450, but its full metabolic profile has not been established. Amongst the urinary metabolites identified, aromatic hydroxylation on the xylidide ring to give both 3-hydroxybupivacaine and 4-hydroxybupivacaine with subsequent glucuronic acid conjugate

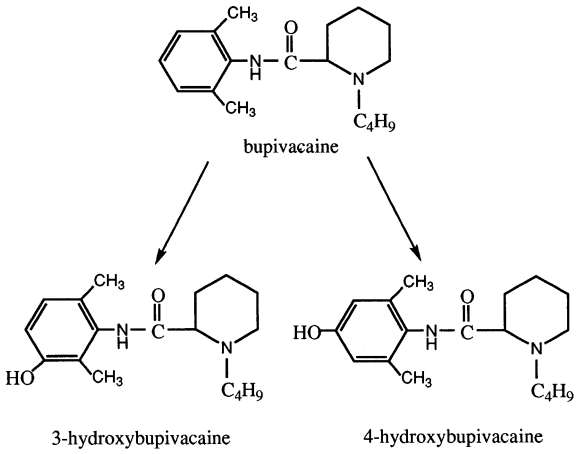

Fig. 1. The structures of bupivacaine and its two phenolic metabolites, 3-hydroxybupivacaine and 4-hydroxybupivacaine.

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formation appears to be a major route (Fig. 1) [2–6]. In this short paper a capillary gas chromatographic separation is described for the two hydroxy-xylidide metabolites excreted in human urine together with identity confirmation by mass spectrometry and subsequent quantification by selective ion monitoring.

2. Experimental

2.1. Chemicals and reagents

Bupivacaine, 3-hydroxybupivacaine and 4-hydroxybupivacaine were gifts from Chiroscience, Cambridge, England. Lignocaine (2-diethylamino-2′,6′-acetoxylidide) was supplied by the local pharmacy. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) was obtained from Aldrich, Gillingham, England. β-Glucuronidase and phenolphthalein glucuronide were purchased from Sigma, Poole, England. All other reagents were of analytical grade and were freely available within the laboratory.

2.2. Sample collection

Urine samples were collected at timed intervals (0–6, 6–12, 12–24 h) from three male volunteers who had received 40 mg racemic ($\pm$)-bupivacaine as an intravenous infusion over a period of 8 min (5 mg free base ml^{-1} min^{-1}). The total urine volumes were recorded and mutiple aliquots frozen (-70°C) until analysis. Samples were only thawed once and then discarded. Multiple analyses required the initial concurrent storage of multiple aliquots.

2.3. Sample preparation

Aliquots of urine (1 ml) were adjusted to pH 5 with glacial acetic acid (2 μl) and incubated with β-glucuronidase (5000 units, Type H-1) in acetate buffer (1 ml, pH 5) for 18 h at 37°C. Positive controls containing phenolphthalein glucuronide and negative controls containing boiled enzyme plus phenolphthalein glucuronide were taken through the same procedures.

Internal standard (5 μg lignocaine) was added to each of the hydrolysed urine samples (2 ml) which were then made alkaline by the addition of sodium carbonate buffer (0.2 ml, 1 M, pH 11). After thorough mixing, the samples were extracted with diethyl ether (2×5 ml) by rotating on a tumble mixer (28 rpm, 10 min). Following centrifugation, the organic layer was transferred by aspiration to a clean tube and evaporated to dryness at 40°C in a gentle nitrogen stream.

Freshly prepared ethereal diazomethane (1 ml, c. 500 μg) (from KOH on *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, 'Diazald' [7]) was added to the residue and the tube capped. After 15 min, methanol (100 μl) was added to increase the rate of methylation of phenolic hydroxyl groups. The recapped tubes were then allowed to stand at room temperature for 24 h, after which the excess diazomethane was removed under a stream of dry nitrogen and the residue reconstituted in acetonitrile (50 μl) ready for analysis by GC-MS.

Calibration curves of peak area ratios of compound to internal standard versus compound concentration were constructed by adding authentic compounds to blank urine (1 ml) over the range 5.0 ng to 5.0 μg ml^{-1} for bupivacaine and 5.0 ng to 10 μg ml^{-1} for 3-hydroxybupivacaine and 4-hydroxybupivacaine.

2.4. Gas chromatography-mass spectrometry

Aliquots (1 μl) of the methylated urine extracts were injected (split mode 1:6) onto a GC-MS system comprising a HP5890 series II gas chromatograph connected to a HP5971 mass selective detector operated in the electron impact mode controlled by HPG1034C software from the MS Chemstation (Hewlett Packard, Cheshire, England). The fused-silica capillary column (30 m $\times$ 0.25 mm i.d.) was coated (film thickness 0.25 μm) with HP5MS (crosslinked 5% phenylmethyl silicone) with a helium gas flow rate of 1 ml min^{-1}. The oven temperature programme was held at 70°C for 2 min and then increased at 20°C min^{-1} to 270°C where it was held for a further 6 min (total 18 min run) before being returned to 70°C. The injection port was held at 250°C. The GC-MS interface temperature, the ionization energy

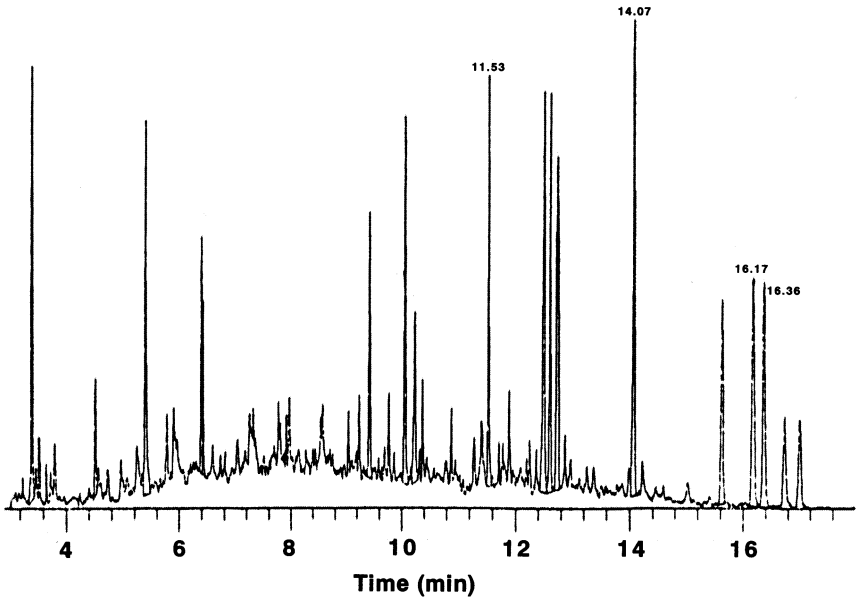

Fig. 2. Capillary gas chromatogram of human urine extract showing peaks for lignocaine (added internal standard) (Rt. 11.53 min), bupivacaine (Rt. 14.07 min), 3-hydroxybupivacaine (Rt. 16.17 min) and 4-hydroxybupivacaine (Rt. 16.36 min). The hydroxybupivacaines are present as their methoxy derivatives.

and the ion source temperature of the electron impact mass spectrometer were 280°C, 70 eV and 175°C respectively.

The quantification of compounds was achieved by monitoring selected ions (base peak) at m/z 86 for lignocaine and 140 for bupivacaine, 3-hydroxybupivacaine and 4-hydroxybupivacaine [8]. The concentrations were determined by peak area ratio of the compound to internal standard and the previously established calibration curves. Any urine sample where the concentration was beyond (above) the standard curve range was diluted with water and then re-examined.

3. Results and discussion

The four compounds examined were clearly resolved and the retention times obtained with this system were, 11.53 min for lignocaine (internal standard), 14.07 min for bupivacaine, 16.17 min for 3-hydroxybupivacaine and 16.36 min for 4-hydroxybupivacaine (Fig. 2). The latter two compounds are, of course, as their methoxy derivatives.

The electron impact mass spectra of lignocaine, bupivacaine and the two methylated phenolic metabolites were unimpressive and showed complimentary fragmentation patterns, with the spectra for all bupivacaine compounds being virtually identical. Molecular ions (M^+) were discernable (lignocaine m/z 234, bupivacaine m/z 288, 3-methoxybupivacaine m/z 318 and 4-methoxybupivacaine m/z 318) but only at a fraction of a percent abundance. The base peaks (100% abundance) in all cases resulted from the fragment ions remaining following cleavage of the carbon–carbon linkage on the carbonyl side of the anilide bond furthest away from the aromatic moiety, leaving the diethylaminomethylene fragment ($C_5H_{12}N$) for lignocaine (m/z 86) and the butylpiperidine fragment ($C_9H_{18}N$) for bupivacaine, 3-methoxybupivacaine and 4-methoxybupivacaine (m/z 140). Other fragment ions were insignificant.

The correlation of peak-area ratios (compound/internal standard) resulting from the injection of bupivacaine ($y = 0.166x + 0.004$), 3-hydroxybupivacaine ($y = 0.155x - 0.003$) and 4-hydroxybupi-

Table 1
Quantification of bupivacaine, 3-hydroxybupivacaine and 4-hydroxybupivacaine in urine following the intravenous infusion of bupivacaine (40 mg base) to three human volunteers

Collection period (h)	Urine volume (ml)	Percentage administered dose		
		Bupivacaine	3-Hydroxybupivacaine	4-Hydroxybupivacaine
Subject 1				
0–6	455	0.62	4.65	2.07
6–12	525	0.01	0.42	0.22
12–24	1240	NQ	0.22	0.12
0–24	2220	0.63	5.29	2.41
Subject 2				
0–6	1150	0.09	2.07	0.95
6–12	655	NQ	0.39	0.23
12–24	1515	NQ	0.20	0.12
0–24	3320	0.09	2.66	1.30
Subject 3				
0–6	515	0.09	2.36	1.02
6–12	335	0.07	0.46	0.27
12–24	625	NQ	0.20	0.14
0–24	1475	0.16	3.02	1.43

NQ, below the level of detection (5 ng ml^{-1}).

vacaine ($y = 0.153x - 0.002$) were statistically significant for all compounds ($r^2 > 0.999$; $P << 1\%$) with instrument response being linear over the full calibation range employed which spanned 5.0 ng to 5.0 μg for bupivacaine and 5.0 ng to 10.0 μg for its two hydroxylated metabolites. Results obtained from several determinations undertaken on multiple aliquots of the same urine sample showed coefficients of variation of $5.0 \pm 0.8\%$ for all three compounds ($n = 5$). The variability observed over a period of one month between aliquots of the same urine sample analysed at weekly intervals resulted in coefficients of variation from 5.0 to 7.0% ($n = 4$)

The sensitivity and specificity of the assay permitted the measurement of bupivacaine and its hydroxyxylidide metabolites in the urine samples provided by the three volunteers. Only very small amounts of bupivacaine ($< 1\%$ dose) were found in the urine following administration indicating removal by metabolism rather than renal elimination. In all urine samples there was approximately twice as much 3-hydroxybupivacaine as 4-hydroxybupivacaine; nevertheless, the total amounts excreted as these two metabolites accounted for $< 10\%$ (8.33, 4.05 and 4.61%) of the administered dose suggesting other metabolic pathways and/or elimination routes (Table 1).

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