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Simultaneous analysis of ketamine and bupivacaine in plasma by high-performance liquid chromatography

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Abstract

A reversed-phase HPLC technique for the simultaneous measurement of both bupivacaine and ketamine in plasma is described. Plasma samples (0.5 ml) were prepared using a rapid and simple back-extraction technique. Resolution of both analytes and the internal standard, desipramine, from medicines coadministered to surgical paediatric patients was obtained using a 5 μm cyano (CN) (250×4.6 mm) column and a mobile phase comprising methanol–acetonitrile–orthophosphoric acid–0.01 *M* sodium dihydrogenphosphate (200:80:2:718). Good sensitivity for both analytes was observed using UV detection at a wavelength of 215 nm. The method has been validated according to the criteria established by the *Journal of Chromatography B*. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ketamine; Bupivacaine

1. Introduction

New strategies to improve the treatment of both acute and chronic pain continue to be sought. Recently the coadministration of the *N*-methyl-D-aspartate (NMDA) receptor antagonist, ketamine, with the local anaesthetic bupivacaine has been shown to provide prolonged relief of acute pain following surgery [1–3] and to hold promise for the treatment of some chronic pain conditions refractory to present therapies [4]. In order to improve our understanding of the interaction between ketamine and bupivacaine and to optimise their possible synergy, a sound understanding of both the pharmacokinetics and pharmacodynamics of the drugs when coadministered to patients is required. Measurement of the plasma concentrations of both drugs is particularly interesting as it has been recently reported in animals that ketamine inhibits bupivacaine metabolism and prolongs its half-life [5]. A number of methods to measure either ketamine or bupivacaine in plasma using gas chromatography or high-performance liquid chromatography (HPLC) have been published and it would be feasible to measure each plasma sample twice using different analytical techniques. However laboratory resources (in terms of equipment, reagents and personnel) would be employed more efficiently, the cost of each analysis would be reduced substantially and the volume of plasma required for analysis would be reduced if bupivacaine and ketamine could be measured using a single analytical technique.

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To this end, a new method has been developed to measure simultaneously the plasma concentrations of bupivacaine and ketamine. The technique has good sensitivity and it is possible to measure both drugs following administration of low analgesic doses to paediatric patients, from whom only a limited volume of plasma can be obtained. For the measurement of both ketamine and bupivacaine in plasma, novel features of the technique developed include the use of a 5 μm cyano (CN) column, and the internal standard employed, desipramine. Plasma sample preparation, using diethyl ether extraction and acidic back-extraction, is relatively rapid and straightforward. Analytes are detected using ultraviolet (UV) absorption at 215 nm.

Ketamine and bupivacaine are used clinically as racemic mixtures of the *R*- and *S*-enantiomers. The technique developed does not resolve the enantiomers of either drug. Although enantioselective activity and disposition have been described for both drugs, measuring the concentration of the sum of the enantiomers can still provide information of interest. The method has been validated according to the criteria established by the Journal of Chromatography B [6].

2. Experimental

2.1. Chemicals and reagents

Ketamine hydrochloride (Mol Wt 274.2) and bupivacaine hydrochloride (Mol Wt 324.9) were purchased from Sigma (St. Louis, MO, USA). The internal standard desipramine hydrochloride was generously provided by Ciba Geigy (Rueil-Malmaison, France). Analytical grade sodium hydroxide, diethyl ether, sodium dihydrogenphosphate and orthophosphoric acid (85%) were obtained from Prolabo (Lyon, France). Analytical grade sulphuric acid (96%) and HPLC grade acetonitrile and methanol were supplied by Carlo Erba Reagenti (Val de Reuil, France). Deionised water (E-pure, Barnstead/Thermolyne, Dubuque, IO, USA) was used throughout.

2.2. Solutions

Aqueous stock solutions of ketamine (1 mg/ml), bupivacaine (1.25 mg/ml) and desipramine HCl (1 mg/ml) were prepared and stored in glass at 4°C. Aqueous working solutions of ketamine (1 μg/ml), bupivacaine (12.5 μg/ml) and desipramine HCl (10 μg/ml) were also stored at 4°C.

2.3. Chromatographic technique

A HPLC system manufactured by Gynkotek HPLC (Softron, Germering, Germany), which comprised a P580 HPLC pump, a GINA 50 autoinjector, a UVD170S UV–Vis detector measuring UV absorption at 215 nm, and the integration software Chromeleon Chromatography Data Systems Ver 3.14, was used. The mobile phase, comprising methanol–acetonitrile–orthophosphoric acid–0.01 *M* sodium dihydrogenphosphate (200:80:2:718), was used at the final pH of 2.34. A reversed-phase Ultremex 5 μm CN column (250×4.6 mm) (Phenomenex, Torrance, CA, USA) was selected. The flow-rate was 1 ml/min which resulted in a back pressure of 155 bar. All chromatography was performed at ambient temperature.

2.4. Sample preparation

The extraction technique was based on that reported by Adams et al. [7] and is shown in Fig. 1. Briefly, following basification of plasma (0.5 ml), the analytes were extracted into diethyl ether and back extracted into 0.025 *M* sulphuric acid. The aqueous phase was dried and reconstituted in 100 μl of 0.01 *M* sodium dihydrogenphosphate. Tapered screw top polypropylene tubes (Falcon Blue Max Jr., Becton-Dickinson Labware, Franklin Lakes, NJ, USA) were used throughout, facilitating drying of the aqueous back extract. Mixing steps described in Fig. 1 were performed at a rate of 30 inversions per minute for 10 min (Agitelec, J. Toulemonde & Cie, Paris, France).

2.5. Assay validation

2.5.1. Assay selectivity

Aliquots of plasma from four healthy drug-free subjects were extracted and chromatographed using the technique described in Sections 2.3 and 2.4. In addition three plasma samples from blood bank donors were examined. Solutions of the comedications administered to patients during surgery, includ-

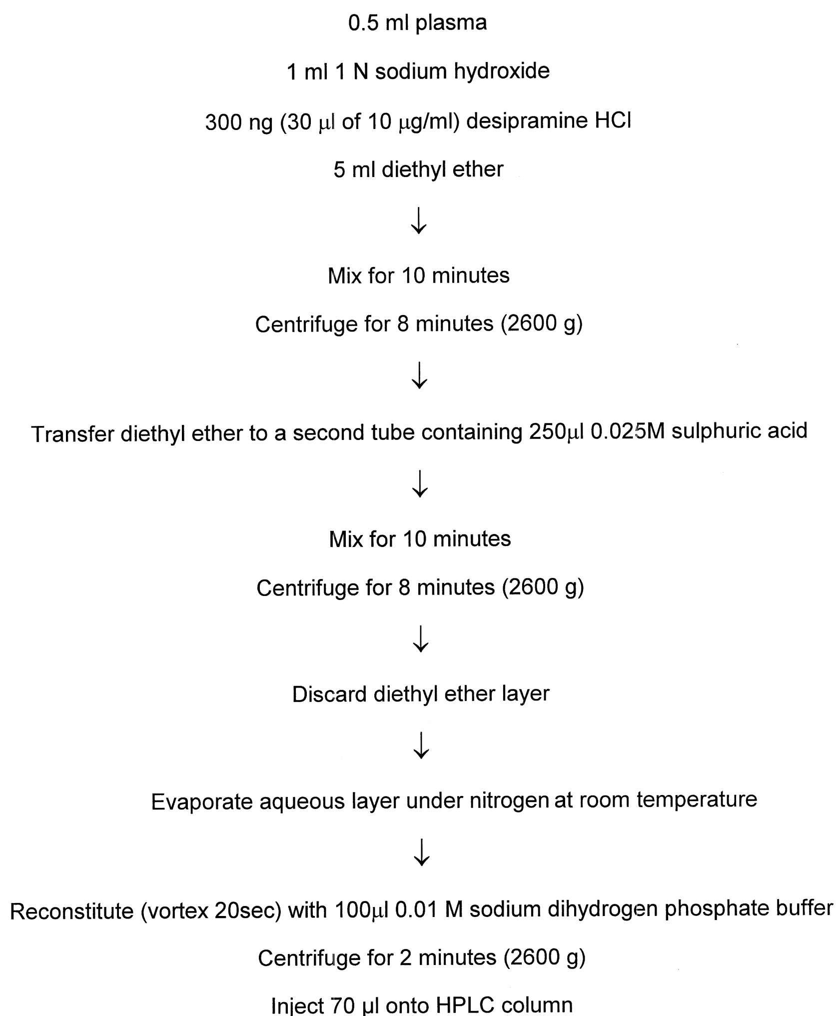

Fig. 1. Flow chart of the plasma extraction technique.

ing lignocaine (Astra France, Rueil-Malmaison, France), clonidine (Boehringer Ingelheim France, Reims, France) and midazolam (Produits Roche, Neuilly-sur-Seine, France) were injected directly on column to check for chromatographic interference. Furthermore plasma samples obtained from paediatric patients receiving all medications used during surgery including bupivacaine but not ketamine were extracted and the chromatograms examined for possible interference at the retention times of interest. These samples enabled the specificity of the technique at the retention times of ketamine and the internal standard to be verified. Plasma samples from surgical patients free of bupivacaine could not be obtained to verify the specificity of the bupivacaine retention time when surgical comedications have actually been administered to patients.

2.5.2. Linearity of calibration

Calibration curves (six standards in duplicate) were prepared over the concentration ranges 10 to 400 ng/ml ketamine and 125 to 4000 ng/ml bupivacaine by pipetting appropriate volumes of ketamine and bupivacaine working solutions into drug-free plasma and assaying 0.5 ml aliquots using the technique described in Section 2.4. The calibration curves were calculated by least-squares linear regression analysis of the concentration of the ana-

lyte in plasma versus the ratio of the peak height of ketamine or bupivacaine to that of the internal standard, desipramine. The slopes, intercepts and coefficients of determination of the calibration curves were tabulated for each analytical run.

2.5.3. Repeatability

The within-day repeatability of the measurement of both ketamine and bupivacaine was assessed by assaying at least five plasma samples of the same concentration on the same day. The between-day repeatability was determined by measuring the concentration of ketamine and bupivacaine in aliquots of the same quality control samples assayed on different days. Low and high quality control samples (QC) were prepared by adding appropriate volumes of stock solutions of ketamine and bupivacaine to 20 ml of drug-free plasma. Aliquots (0.6 ml) were stored at −20°C and thawed just prior to use. The concentration was calculated from the calibration curve produced that day.

2.5.4. Accuracy

The accuracy of the technique was determined during a single analytical run by assaying 10 samples of drug-free plasma to which different amounts of ketamine or bupivacaine were added. The relationship between the analyte concentration added and the analyte concentration measured was investigated using least-squares linear regression. If the concentration measured and added concurred, the slope of the regression line should be 1.0.

2.5.5. Stability

The stability of the plasma extracts prior to injection on column was evaluated by preparing a calibration curve in duplicate and injecting one set of samples immediately after reconstitution and one set of samples stored in the injection vials at room temperature for 24 h after reconstitution. The latter samples were injected in series with a freshly prepared calibration curve. Stability of the analytes during storage of plasma samples was studied using aliquots of QC samples stored frozen at −20°C for up to three months.

2.5.6. Limits of detection and quantification

The limits of quantification of the ketamine and bupivacaine assays were the concentrations of the lowest standards included in the calibration curves. The limit of detection was determined from the detector response after direct injection on column of decreasing amounts of standard solutions of each analyte. A signal-to-noise ratio of 4 was taken as the limit of detection.

2.5.7. Absolute recoveries

The efficiency of the extraction technique was assessed by comparing the peak heights of ketamine and bupivacaine injected directly on column (differing volumes of the 1 μg/ml and 12.5 μg/ml solutions, respectively) with the peak heights observed following injection of plasma extracts containing the same amount of each analyte. The calculation accounted for the injection on column of only 70 μl of the 100 μl reconstitution volume.

2.5.8. Application of technique to patient samples

The technique described has been used to measure bupivacaine and ketamine concentrations in plasma samples from paediatric patients administered bupivacaine (1 mg/kg) and ketamine (0.5 mg/kg) centrally to provide prolonged analgesia following surgery.

3. Results

Using the mobile phase and column described, the retention times of ketamine, bupivacaine and the internal standard, desipramine, were 6.7 min, 8.0 min and 11.5 min, respectively. The chromatographic run time selected was 15 min. Representative chromatograms are shown in Fig. 2. In all samples of drug-free plasma studied, endogenous peaks were not observed at the retention times of ketamine, bupivacaine and the internal standard (Fig. 2A). Medications coadministered during the surgical procedure, including lignocaine (retention time 5.6 min), clonidine (retention time 5.5 min) and midazolam (retention time 14.8 min), were well resolved from the retention times of interest. Plasma samples from paediatric patients administered all medications except ketamine were free from interference at the

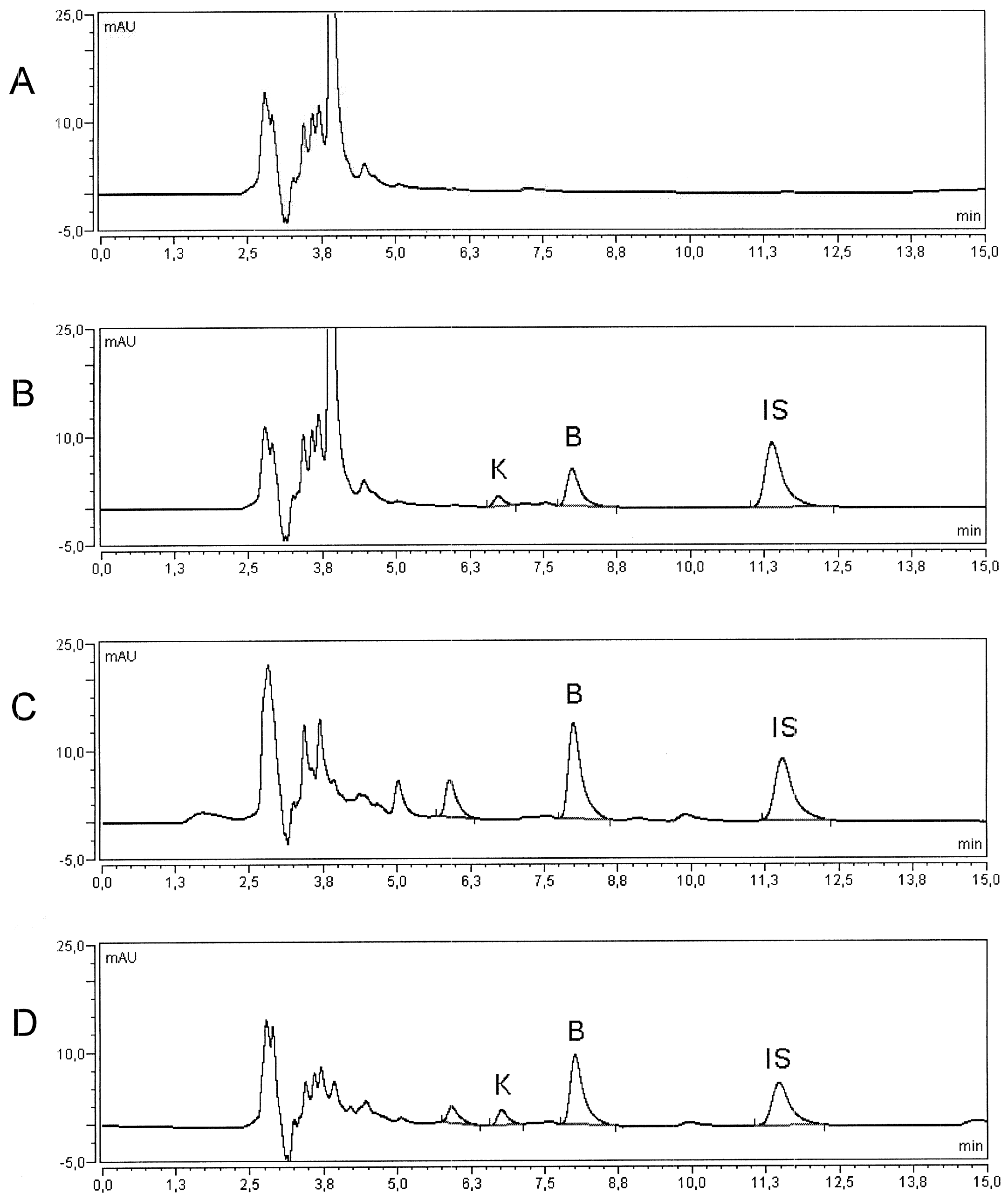

Fig. 2. Representative chromatograms of ketamine (K) and bupivacaine (B) detected by ultraviolet absorption at 215 nm. (A) Drug-free plasma. (B) Drug-free plasma with ketamine (K: 50 ng/ml), bupivacaine (B: 250 ng/ml) and desipramine (I.S. 300 ng/ml) added. Chromatograms (C) and (D) are plasma samples from paediatric patients 10 min after administration of surgical anaesthesia including (C) bupivacaine (B: 550 ng/ml) and (D) ketamine (K: 96 ng/ml) and bupivacaine (B: 563 ng/ml).

retention times of ketamine (Fig. 2C) and the internal standard (not shown), and the bupivacaine peaks did not display shoulders or tailing, suggesting that additional peaks were not eluting close to the retention time of bupivacaine. In chromatograms of extracts of plasma from paediatric surgical patients, peaks attributable to drugs and/or their metabolites were present at other retention times. The technique developed therefore has appropriate selectivity to measure the concentrations of bupivacaine and ketamine in patient plasma samples.

The calibration curves for both ketamine and bupivacaine in plasma were linear over the concentration ranges studied. Successive ketamine calibration curves displayed good inter-day reproducibilities (mean±standard deviation, SD; relative standard deviation, RSD; $n=10$) of the slope (0.0044±0.0002; 5.7%), intercept (−0.05±0.02; 38%) and coefficient of determination (0.992±0.008; 0.8%). For the bupivacaine calibration curves the inter-day reproducibilities (mean±SD; RSD; $n=9$) of the slope (0.0033±0.0002; 6.5%), intercept (−0.20±0.09; 46%) and coefficient of determination (0.992±0.007; 0.7%) were also acceptable.

The within- and between-day repeatabilities of the ketamine and bupivacaine plasma assays are given in Table 1. The technique displays good within- and between-day repeatability for both ketamine and bupivacaine. As anticipated greater variability in the between-day repeatability is observed at low relative to high concentrations.

The ketamine concentrations added and measured in 10 plasma samples to assess the accuracy of the technique are given in Table 2. There was excellent agreement between the two concentrations. The relationship between the ketamine concentration added and measured is described by the least-squares linear regression line [ketamine measured]=0.995· [ketamine added]−1.214, $r^2=0.996$. The slope of this line is 0.995, which is very close to the ideal value of 1. The accuracy of the method over the concentration range studied is therefore very good. The relationship between the bupivacaine concen-

Table 1
Within- and between-day repeatabilities (mean±SD) of the ketamine and bupivacaine plasma assays

Concentration added (ng/ml)	n	Concentration measured (ng/ml)	RSD (%)
Within-day repeatability			
Ketamine			
10	6	8±1	10.8
25	6	25±3	11.0
100	6	102±10	9.4
200	6	200±10	5.1
Bupivacaine			
125	6	113±9	7.8
400	6	408±29	7.0
1000	6	1020±60	5.8
1600	5	1446±61	4.0
3000	6	3114±201	6.5
Between-day repeatability			
Ketamine			
25	8	26±4	13.3
50	10	48±4	7.3
200	10	211±13	6.1
400	8	399±18	4.4
Bupivacaine			
150	5	162±26	16.0
1000	7	1060±80	7.5
3000	6	3003±81	2.7
4000	7	3996±379	9.5

Table 2
Accuracy of the ketamine plasma assay[a]

Sample	Ketamine (ng/ml)	
	Added	Measured
1	12	12
2	24	24
3	48	47
4	18	18
5	112	117
6	0	ND
7	130	124
8	70	60
9	36	35
10	96	98

[a] Comparison of ketamine concentrations added to plasma and the concentrations measured (ND: not detected).

trations added to plasma and the concentrations measured in an additional 10 accuracy samples is shown in Fig. 3. For this analyte there is also agreement between the added and measured concentrations (slope 1.12), indicating that the bupivacaine assay also has acceptable accuracy.

The slopes and intercepts of ketamine and bupivacaine calibration curves injected on column immediately following preparation and after storage at room temperature for 24 h prior to injection were comparable. The reconstituted plasma extracts are therefore stable under these conditions for at least one day. Ketamine and bupivacaine concentrations were stable in frozen plasma quality control samples for at least three months.

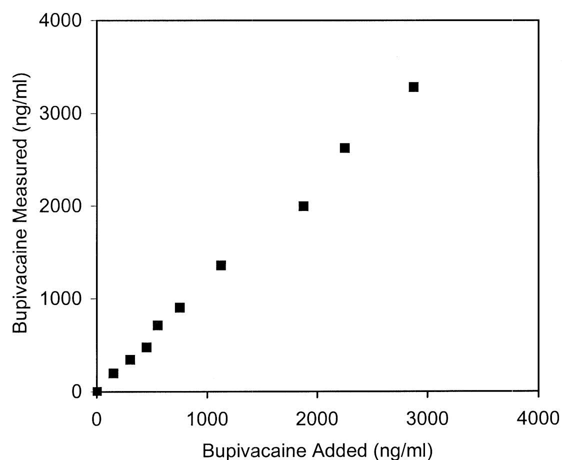

Fig. 3. Accuracy of the bupivacaine plasma assay showing the relationship between the bupivacaine concentration added and the bupivacaine concentration measured. The least-squares linear regression line is described by the relationship $y=1.12x+24$; $r^2=0.998$.

The lower limits of quantification of the ketamine and bupivacaine assays were 10 ng/ml (or 5 ng/0.5 ml) and 125 ng/ml (62.5 ng/0.5 ml), respectively, the concentrations of the lowest calibration standards prepared. These concentrations are appropriate for the paediatric plasma samples analysed and are well above the limits of detection of both analytes. The limit of detection of ketamine was 1 ng on-column (signal-to-noise ratio of 4:1) and that of bupivacaine was 0.8 ng.

Using the diethyl ether back-extraction technique described, the absolute recovery of ketamine from plasma (mean±SD), assessed at concentrations between 10 and 100 ng/ml was 79.9±7.5% ($n=4$). The absolute recovery of bupivacaine (mean±SD; $n=4$) following plasma extraction was 58.3±4.1% (125–3000 ng/ml). The lower extraction efficiency of bupivacaine did not compromise the analytical technique as plasma bupivacaine concentrations are relatively high.

The technique described has been used to measure the plasma concentrations of ketamine and bupivacaine in more than 200 plasma samples from 76 paediatric patients. An example of the plasma concentrations observed in a paediatric patient following epidural administration of ketamine and bupivacaine is shown in Fig. 4.

4. Discussion

The aim of the technique developed was to measure simultaneously the plasma concentrations of both ketamine and bupivacaine administered at low analgesic doses. Good sensitivity and selectivity from coadministered drugs were therefore critical. By requiring only one assay to measure both drugs, efficiencies in terms of equipment, reagents and personnel are maximised. An additional important benefit for the measurement of drug concentrations in paediatric patients, is that only 0.5 ml plasma is needed for the measurement of both analytes.

Although gas chromatographic and gas chromatographic–mass spectroscopic methods for the mea-

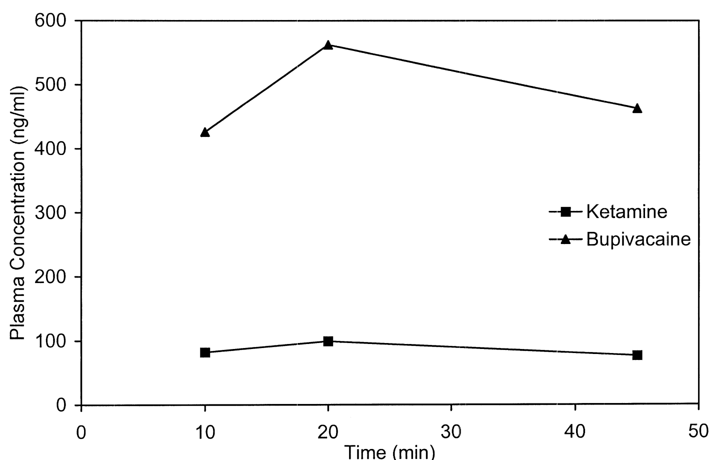

Fig. 4. Ketamine and bupivacaine plasma concentrations in a paediatric patient administered ketamine (0.5 mg/kg) and bupivacaine (1.0 mg/kg).

surement of ketamine or bupivacaine have been described, for the present analysis a high-performance liquid chromatographic technique was developed. Previous methods for ketamine and bupivacaine reported in the literature have involved the use of reversed-phase C_{18} [7–10], C_8 [11] or phenyl [12] columns and consequently these packings and the mobile phases suggested were initially studied. However, in our analyses, ketamine and bupivacaine peak shapes and relative retention times were optimal using a CN (5 μm) column and the methanol–acetonitrile–phosphoric acid–phosphate buffer employed by Seay et al. [12]. The choice of mobile phase and column were critical to ensure baseline resolution of ketamine and bupivacaine from coadministered drugs such as lignocaine and clonidine. Good peak shape and a realistic run time (15 min) were achieved without elevation of the column temperature.

As plasma bupivacaine concentrations are higher than ketamine plasma concentrations, ketamine sensitivity was most critical and detection was optimised for this analyte. When dissolved in the mobile phase selected, the ketamine UV absorption spectra produced a peak at 215 nm. This wavelength, which has also been used by other authors [12,13] was therefore selected. Ketamine has also been monitored at 210 [7,8] or 220 nm [14]. Low wavelength UV absorption has been used to measure bupivacaine concentrations in plasma [9–11,15] and the wavelength selected also provides good sensitivity for this analyte.

The diethyl ether/acidic back-extraction from plasma is relatively rapid and straightforward. The method is based on that reported for ketamine by Adams et al. [7], and similar extraction techniques have been published for bupivacaine [10,11,15]. The major modification is evaporation of the aqueous back extract under nitrogen and reconstitution of the residue in a small volume of mobile phase buffer. This additional step concentrates the extract, elevates the pH of the solution injected and ensures that residual ether dissolved in the aqueous phase is not injected on column. The absolute recoveries of both analytes from plasma are acceptable and the technique developed has good sensitivity. The low plasma concentrations resulting from analgesic doses of ketamine and bupivacaine can therefore be readily measured using just 0.5 ml of plasma (Fig. 4).

A number of basic compounds were assessed as possible internal standards. As anticipated they were extracted from plasma using the technique described. However baseline resolution from ketamine or bupivacaine or the coadministered medications lignocaine and midazolam was not always observed. Interference in the analysis of ketamine or bupivacaine may potentially be observed with high concentrations of the drugs cisapride (retention time 8.4 min), metoclopramide (retention time 7.1 min) and propranolol (retention time 6.4 min). Desipramine eluted at a

retention time free from endogenous interference or coeluting drugs and, as confirmed by the good accuracies and between- and within-day repeatabilities of the method developed, was a suitable internal standard for both analytes.

In summary a technique to simultaneously measure plasma concentrations of ketamine and bupivacaine has been developed and validated. Both analytes are extracted well using a simple back-extraction technique and the method has good sensitivity for both ketamine and bupivacaine. Simultaneous extraction and chromatography means that only 0.5 ml of plasma is required to measure both analytes and the technique is therefore suitable for studies in paediatric patients. Calibration curves are linear, within- and between-day repeatabilities are within 16% and accuracies are good. The method developed has been used to measure simultaneously the plasma concentrations of bupivacaine and ketamine in paediatric patients and such studies will further our understanding of the clinically important interaction between these two drugs.

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References

[1] B. Cook, D.J. Grubb, L.A. Aldridge, E. Doyle, Br. J. Anaesth. 75 (1995) 698.
[2] D. Semple, D. Findlow, L.M. Aldridge, E. Doyle, Anaesthesia 51 (1996) 1170.
[3] D. Findlow, L.M. Aldridge, E. Doyle, Anaesthesia 52 (1997) 1110.
[4] T.C. Lin, C.S. Wong, F.C. Chen, S.Y. Lin, S.T. Ho, Can. J. Anaesth. 45 (1998) 175.
[5] M. Gantenbein, C. Abat, L. Attolini, P. Pisano, N. Emperaire, B. Bruguerolle, Life Sci. 61 (1997) 2027.
[6] W. Lindner, I.W. Wainer, J. Chromatogr. B 707 (1998) 1.
[7] H.A. Adams, B. Weber, M.B. Bachmann, M. Guerin, G. Hempelmann, Anaesthesist 41 (1992) 619.
[8] S. Bolze, R. Boulieu, Clin. Chem. 44 (1998) 560.
[9] H. Kastrissios, M.F. Hung, E.J. Triggs, J. Chromatogr. 577 (1992) 103.
[10] P. Le Guévello, P. Le Corre, F. Chevanne, R. Le Verge, J. Chromatogr. 622 (1993) 284.
[11] R.L.P. Lindberg, J.H. Kanto, K.K. Pihlajamäki, J. Chromatogr. 383 (1986) 357.
[12] S.S. Seay, D.P. Aucoin, K.L. Tyczkowska, J. Chromatogr. 620 (1993) 281.
[13] G. Geisslinger, S. Menzel-Soglowek, H.D. Kamp, K. Brune, J. Chromatogr. 568 (1991) 165.
[14] J.O. Svensson, L.L. Gustafsson, J. Chromatogr. B 678 (1996) 373.
[15] P. St. Louis, I. Das, J. Rybczynski, B. Bissonnette, E.J. Hartley, Clin. Biochem. 24 (1991) 463.