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Journal of Chromatography B, 658 (1994) 113–119

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Column liquid chromatographic determination of bupivacaine in human serum using solid-phase extraction

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First received 21 February 1994; revised manuscript received 13 April 1994

Abstract

A convenient and economical procedure for the extraction of bupivacaine and its major metabolites, desbutylbupivacaine and 4'-hydroxybupivacaine, from serum is described. A 0.25-ml aliquot of 4 μg/ml solution of N-pentyl-2,6-pipecoloxylidide used as the internal standard and an equal volume of the serum sample are applied to a 1-ml BondElut C_{18} silica extraction column which has been conditioned by subsequent washing once with 1 *M* HCl, twice with methanol and once with water. After passing the sample at a slow rate, the column is washed twice with water and once with acetonitrile. The desired compounds are then eluted with a 0.25-ml aliquot of a mixture of 35% perchloric acid–methanol (1:40, v/v). A 5-μl aliquot of the eluate is injected onto a 150 × 4.6 mm I.D. column packed with 5-μm C_8 silica particles, which is eluted at ambient temperature with a mobile phase of acetonitrile–10 m*M* KH_2PO_4 (25:80, v/v) at a flow-rate of 1.5 ml/min. The peaks are detected at 205 nm. The extraction recovery of all the compounds is in the range of 90%. The chromatogram is clean and the desired peaks are well separated from each other and from the extraneous peaks.

1. Introduction

Bupivacaine, an amide type local anaesthetic, is being increasingly used by continuous infusion via epidural or via extrapleural intercostal routes to control post-operative pain [1,2]. However, continuous infusion can lead to the accumulation of bupivacaine in toxic concentrations. Determination of serum bupivacaine concentration is potentially useful to titrate bupivacaine dose to avoid toxicity resulting from high concentrations of the drug. A number of gas chromatographic procedures have been described for the determination of bupivacaine in biological samples [3]. However, in recent years bupivacaine [4–13], bupivacaine and its metabolites [14,15] and chiral bupivacaine [16–20] have been determined by column liquid chromatographic (LC) procedures.

With rare exception, the biological samples have been extracted with water immiscible solvents and in most cases the extract evaporated prior to chromatography. Sullivan and Weiner [9] used simple protein precipitation with 1.5 volume of methanol and the supernatant was injected directly. This approach is not suitable for the determination of the metabolites of bupivacaine as the solvent peak is relatively large. Recently Butter *et al.* [20] have described an on-line coupled three-column LC system for

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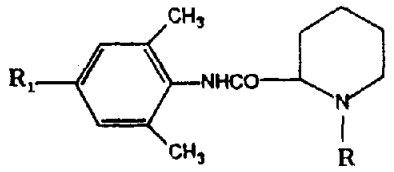
0378-4347/94/$07.00
SSDI 0378-4347(94)00214-P



1. R = C_4H_9,	R_1 = H	Bupivacaine
2. R = C_4H_9,	R_1 = OH	4′-Hydroxybupivacaine
3. R = H,	R_1 = H	Desbutylbupivacaine or 2′,6′-Pipecoloxylidide (PPX)
4. R = C_5H_{11},	R_1 = H	N-Pentyl-2′,6′-pipecoloxylidide (Pentyl-PPX)
5. R = CH_3,	R_1 = H	Mepivacaine

Fig. 1. Structures of bupivacaine, its metabolites and internal standards.

the determination of the enantiomers of bupivacaine in serum.

Off-line solid-phase extraction (SPE) is now being increasingly used to isolate analytes from biological fluids prior to their determination by LC [21] for speed, convenience and to avoid the use of solvents which are unfriendly for the environment. It seems that no off-line SPE has been described for the determination of bupivacaine so far. It was the objective of this investigation to optimize conditions for the SPE of bupivacaine and its major metabolites, desbutylbupivacaine (PPX) and hydroxybupivacaine (Fig. 1).

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Deionized water was distilled in an all-glass still.

Bupivacaine, 4′-hydroxybupivacaine, PPX and pentyl-PPX were obtained as gifts from Astra Pain Control AB (Södertälge, Sweden). Stock solution of bupivacaine at a concentration of 5 mg/ml and of other above mentioned compounds at a concentration of 1 mg/ml were prepared in methanol. The solutions were stored at −10°C.

Serum standard of bupivacaine (10 μg/ml) + PPX (2 μg/ml) + 4′-hydroxybupivacaine (0.8 μg/ml) was prepared by mixing 100 μl of stock bupivacaine, 100 μl of stock PPX and 40 μl of stock hydroxybupivacaine solution in a 50-ml volumetric flask and making up the volume with drug free serum. This standard was serially diluted with drug free serum to prepare six serum standards.

2.2. Extraction

The required number of 1-ml BondElut C_{18} extraction columns (Varian, Harbor City, CA, USA) was placed on a VacElut system. The columns were washed once with 1 *M* HCl, twice with methanol and once with water, each time aspirating the liquid completely with suction. A 0.25-ml aliquot of the working internal standard solution, prepared by mixing 40 μl of stock pentyl-PPX solution with 10 ml of 0.1 *M* NaH_2PO_4, was placed on each column and then a 0.25-ml aliquot of the sample was applied. Mild suction was applied so that the liquid passed through the column at a slow rate of approximately 1 ml/min. The columns were washed twice with water and once with acetonitrile making sure that each column was drained completely after every wash. The tips of the columns were wiped with tissue and placed on 16 × 100 mm tubes containing correspondingly labelled 1.5-ml plastic sample cups. An aliquot of 0.25 ml of methanol containing 2.5 ml/100 ml of 35% perchloric acid was applied to each column. The liquid was allowed to pass through the column bed by gravity and finally drained

completely by centrifugation for 20 s. The cups were covered with aluminium foil and loaded in the autosampler. A 5-μl aliquot of the eluate was injected onto the chromatographic system.

2.3. Chromatography

A modular chromatographic system consisting of a Model LC-6A pump, a Model SPD-10A absorbance detector, a Model Sil-9A autosampler and a Model CR501 integrator plotter (all from Shimadzu Scientific Instrument Co., Columbia, MD, USA) was used. A 150 × 4.6 mm I.D. Ultrasphere Octyl reversed-phase column packed with 5-μm bonded silica particles (Beckman Instruments, San Ramon, CA, USA) protected by a 15 × 3.2 mm I.D. RP-8 guard cartridge packed with 7-μm silica particles (Applied Biosystems, SanJose, CA, USA) was used as the analytical column. Mobile phase A consisting of 10 m*M* KH_2PO_4–acetonitrile (80:25, v/v), pH 5.2, was pumped at a flow-rate of 1.5 ml/min with an operating pressure of 9.8 MPa. Mobile phase B consisting of 10 m*M* KH_2PO_4–acetonitrile (80:40, v/v), pH 5.2, was pumped at a flow-rate of 1 ml/min. Chromatography was performed at ambient temperature.

3. Results and discussion

Extraction of basic drugs with the use of reversed-phase silica extraction columns is a complex process and requires optimization of different steps for every compound [21,22]. To optimize the pH of the matrix for optimal recovery, we prepared an aqueous sample containing 5 μg/ml of each compound (bupivacaine + PPX + hydroxybupivacaine and pentyl-PPX). Aliquots of this sample were mixed with an equal volume of 0.1 *M* phosphate buffers of pH 4.5, 5.5, 6.5, 7, 7.5 and 8 and 0.1 *M* carbonate buffers of pH 9, 9.5 and 10.5 and passed through the washed extraction columns. The eluates from each column were collected. Chromatographic analysis of these eluates did not show any peak at any pH. With the limit of detection at 25 ng/ml, it can be concluded that all four compounds have been adsorbed on the extraction column up to 99% of the amount present in the original sample in the pH range tested, *i.e.* 4.5–10.5.

Further optimization of extraction was carried out only at the extreme pHs of 4.5 and 10.5. After adsorption of the samples at these two pHs, the columns were washed twice with water. No compounds were detected in the water washings in either case. The columns were then washed with 1 ml of acetonitrile. The acetonitrile washing obtained from the pH 4.5 column did not show any compound. However, the acetonitrile wash of the pH 10.5 column showed the presence of *ca.* 60% hydroxybupivacaine, *ca.* 20% PPX and less than 10% of bupivacaine and the internal standard. It appears that at pH 4.5, bupivacaine, its metabolites and the internal standard are adsorbed on the C_{18} silica column primarily as cations and at higher pH, hydrophobic interactions become important for the retention of the analytes [22]. Acetonitrile can elute compounds bound to C_{18} silica by hydrophobic forces but can not elute compounds bound to silanol groups of the sorbent by cation-exchange forces.

We chose to use pH 4.5 for the extraction of bupivacaine as it would allow to wash the non-basic impurities to waste with acetonitrile without the loss of desired analytes. The compounds adsorbed on the extraction column at pH 4.5 were best eluted with 0.25 ml of methanol containing 2.5 ml/100 ml of 35% perchloric acid as compared to 0.25 ml of methanol or of acetonitrile containing 2.5 ml/100 ml of 35% perchloric acid. The overall recovery of all the analytes from serum samples is in the range of 85–95% and there is no change in the ratio of drug or metabolite peak area to that of the internal standard after extraction. Fig. 2A shows a chromatogram of an unextracted mixture of the analytes dissolved in acidified methanol and Fig. 2B shows a chromatogram of an extract of an aqueous sample. The final concentration expected for 100% recovery in the extract is the same as in the control sample of Fig. 2A. These Figures show a stable baseline even at 205 nm, good extraction recovery and absence of extra-

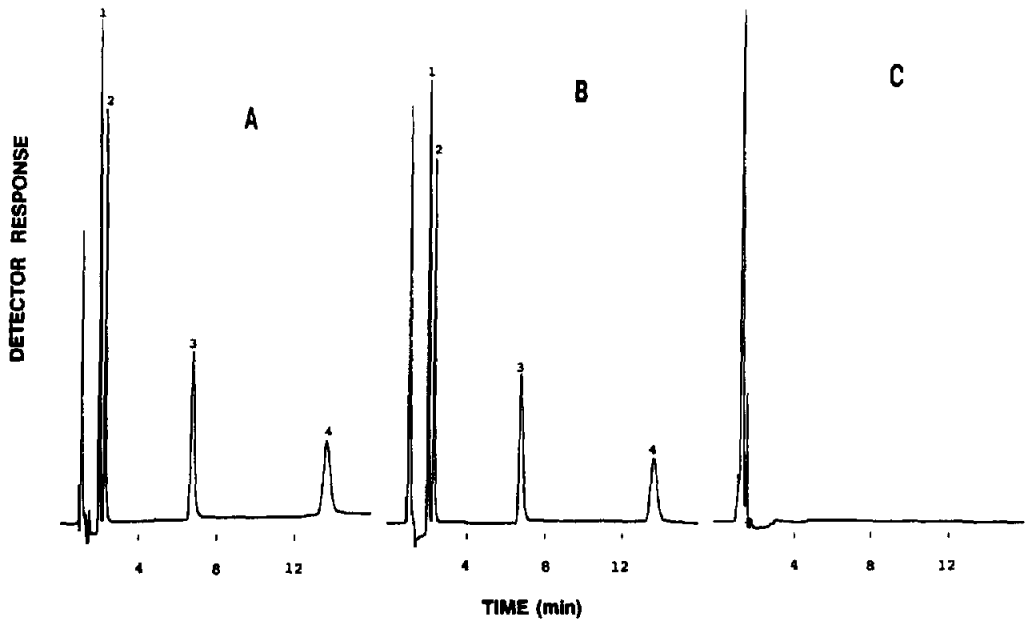


Fig. 2. Chromatograms of (A) 5-μl injection of an unextracted mixture of 2 μg/ml of each of desbutylbupivacaine (peak 1), 4'-hydroxybupivacaine (peak 2), bupivacaine (peak 3) and N-pentyl-2',6'-pipecoloxylidide (peak 4), (B) 5-μl injection of an extract of a 2 μg/ml aqueous mixture of each compound, and (C) 5-μl injection of an extract of drug free serum. Mobile phase A was used in all cases.

neous peaks originating from the extraction column or reagents. A chromatogram of an extract of drug free serum (Fig. 2C) shows the absence of extraneous peaks from the serum matrix after the solvent front.

The relationship between the ratios of the peak areas of bupivacaine and its metabolites to those of the internal standard is linear and the curves pass through the origin ($y = 0.000 + 0.288x$, $r^2 = 1$ for bupivacaine; $y = 0.003 + 0.305x$, $r^2 = 1$ for hydroxybupivacaine and $y = 0.005 + 0.341x$, $r^2 = 1$ for PPX). For chromatography, only 5 μl of the 250 μl of the eluate are injected. Under these conditions, each compound can be quantitated down to 25 ng/ml. For improved sensitivity, up to 25 μl of the eluate can be injected without distortion of desired peaks. In the described extraction procedure, no concentration of the analytes occurs as the sample volume and the eluate volume are the same, *i.e.* 0.25 ml. However, the sample volume can be increased up to 0.9 ml keeping the volume of the internal standard and of the eluate at 0.25 ml. The extraction columns have been used 4 times without loss of recovery or appearance of additional extraneous peaks. Analysis of serum spiked with high and normal concentrations of bupivacaine and its metabolites using a mixture of new and used extraction columns, showed good within- and between-batch precision. The precision data is summarized in Table 1.

Fig. 3A shows a chromatogram of an extract of a serum standard spiked with 5 μg/ml of bupivacaine, 1 μg/ml of PPX and 0.4 μg/ml of hydroxybupivacaine and Fig. 3B shows a chromatogram of an extract of a serum sample collected from a patient after 48 h of continuous infusion of bupivacaine. Some of the patients receive additional analgesics to control post surgery pain. The retention times of the commonly prescribed analgesics are described in Table 2. These compounds are well separated from the analytes of interest and do not interfere with the quantitation of bupivacaine or its metabolites. Acidic and neutral compounds are not extracted efficiently by the described procedure.

Table 1
Precision of method ($n = 8$ in all cases)

	Desbutylbupivacaine		4'-OH-Bupivacaine		Bupivacaine	
	Mean (μg/ml)	C.V.%	Mean (μg/ml)	C.V.%	Mean (μg/ml)	C.V.%
Within-batch: A	0.50	2.9	0.20	3.3	2.4	1.6
Within-batch: B	1.9	5.1	0.77	4.1	9.8	1.6
Between-batch: A	0.46	4.3	0.20	7.3	2.4	5.1
Between-batch: B	1.9	0.8	0.77	1.5	9.9	5.1

However, all types of basic compounds including the weakly basic benzodiazepines are co-extracted with bupivacaine in the described extraction procedure.

As seen in Fig. 3A, the chromatographic run time for each sample is quite long which is not desirable for clinical laboratories. The chromatogram also shows that bupivacaine is widely separated from the metabolites and from the internal standard. The chromatographic run time could be reduced if an alternative internal standard could be used. For this purpose, we evaluated the use of mepivacaine, another analogue of bupivacaine, as the internal standard for the determination of bupivacaine. Mepivacaine behaves similarly to bupivacaine in the extraction procedure in that there is no change in the ratios of peak areas of bupivacaine/mepivacaine after extraction. However, mepivacaine could not be used as the internal standard as it elutes very close to hydroxybupivacaine. With the described procedure, mepivacaine can be determined in

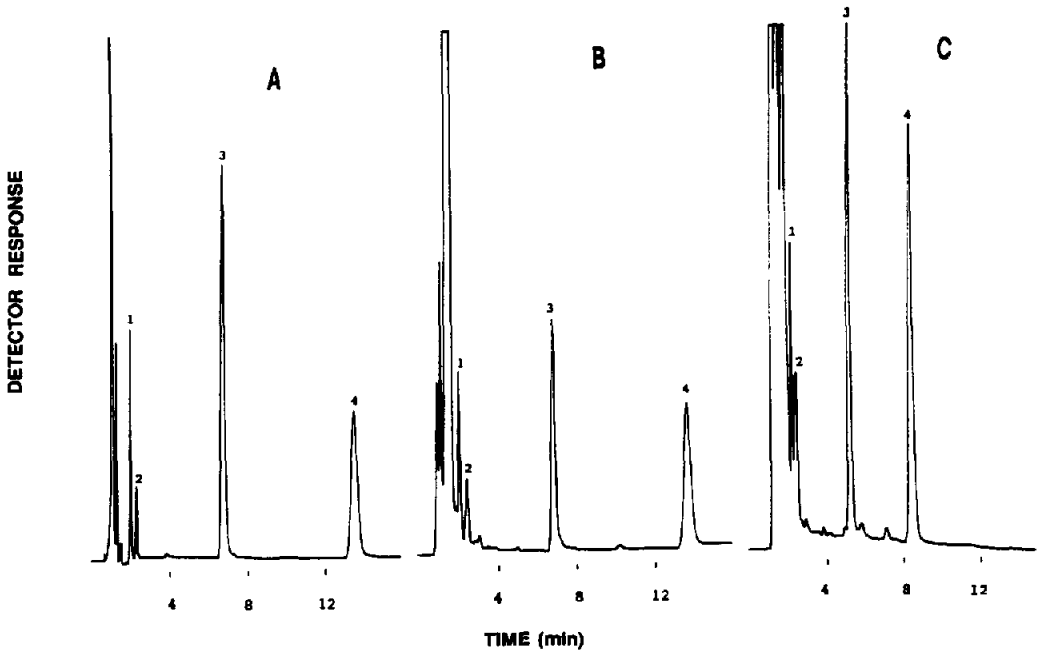


Fig. 3. Chromatograms of (A) a 5-μl aliquot of an extract of serum standard of desbutylbupivacaine (1 μg/ml) + 4'-hydroxybupivacaine (0.4 μg/ml) + bupivacaine (5 μg/ml), (B), and (C) 5-μl aliquots of an extract of serum of a patient collected 48 h after continuous infusion of bupivacaine. Mobile phase A was used for (A) and (B) and mobile phase B was used for (C). Peak identification as in Fig. 1.

Table 2
Retention time of compounds

Compounds	Retention time (min)	
	Mobile phase A (Flow-rate = 1.5 ml/min)	Mobile phase B (Flow-rate = 1.0 ml/min)
Acetaminophen[a]	a	a
Codeine	a	a
Epinephrine	a	a
Morphine	a	a
Desbutylbupivacaine	2.1	2.3
4'-Hydroxybupivacaine	2.5	2.5
Mepivacaine	2.6	2.5
Meperidine	4.3	3.6
Bupivacaine	7.2	5.2
Fentanyl	11.4	6.8
N-Pentyl-2',6'-pipecoloxylidide	14.5	8.5
Diazepam	b	21

[a] Not extracted by the described procedure.
a = Less than 1.5 min; b = no peak up to 25 min.

serum using bupivacaine as the internal standard.

At present it seems that the concentration of racemic bupivacaine alone is adequate to decide if an excessive amount of bupivacaine has been accumulated in a patient receiving continuous infusion of bupivacaine. When the quantitation of bupivacaine metabolites is not required, the run time can be considerably reduced by increasing the acetonitrile content of the mobile phase. Fig. 3C shows a chromatogram of the same extract as Fig. 3B. With this mobile phase, the limit of quantitation of bupivacaine has been lowered to 10 ng/ml. All the other commonly prescribed analgesics are still adequately separated from bupivacaine and the internal standard. However, the metabolites of bupivacaine are not adequately separated from the solvent peaks in the extracts of patients samples. Linearity and precision of this fast procedure are similar to those obtained using slower mobile phase. Drug free serum spiked with nominal concentrations of bupivacaine of 5 μg/ml and 78 ng/ml when analyzed in duplicate gave average values of 4.7 μg/ml and 76 ng/ml respectively. Negative biases of *ca.* 6% and 2.6% between the spiked and observed values indicate that the procedure is fairly accurate.

Table 3
Serum bupivacaine concentrations

Patient No.	Bupivacaine (μg/ml)	
	24 h	48 h
1	3.6	4
2	1.6	3.8
3	1.6	2.4
4	2.2	6.1
5	1.4	3
6	3.6	4.2
7	1.8	2.9
8	3.3	4.1
9	3	5
10	1.4	2.2

This procedure was applied to the determination of bupivacaine concentrations in serum samples obtained from patients who had undergone thoracotomy 24 h and 48 h after the administration of a bolus of 0.3 ml/kg followed by a continuous infusion via extrapleural intercostal route of 0.1 ml/kg/h of a 5 mg/ml solution of bupivacaine containing 5 μg/ml of epinephrine. The results are summarized in Table 3. The results indicate that bupivacaine is

accumulating with time. None of these patients showed any clinical symptoms of toxicity. Bupivacaine infusion could have been decreased in some cases if the test results had been available during the treatment. The toxic threshold for bupivacaine is considered to be 4 μg/ml [24].

In conclusion the described procedure is convenient, economical and environmentally friendly for the rapid determination of bupivacaine.

Acknowledgements

We thank Astra Pharma, Mississauga, Canada for providing financial assistance and for getting the pure compounds from Astra Sweden. Miss Asha Gupta helped in the preparation of the manuscript.

References

[1] S. Sabanathan, A.J. Mearns, P.J. Bickford Smith, J. Eng, R.G. Berrisford, S.R. Bibby and M.R. Majid, *Br. J. Surg.*, 77 (1990) 221.
[2] V.W.S. Chan, F. Chung, D.C.H. Cheng, C. Seyone, A. Chung and T.J. Kirby, *Can. J. Anaesth.*, 38 (1991) 733.
[3] R.N. Gupta, in G. Zweig and J. Sherma (Editors), *Handbook of Chromatography: Drugs*, Vol. III, CRC Press, Boca Raton, FL, 1989, p. 219.
[4] R.L.P. Lindberg and K.K. Pihlajamäki, *J. Chromatogr.*, 309 (1984) 369.
[5] H.-R. Ha, B. Funk, H.R. Gerber and F. Follath, *Anesth. Analg.*, 63 (1984) 448.
[6] U.-W. Wiegand, R.C. Chow, E. Lanz and E. Jähnchen, *J. Chromatogr.*, 311 (1984) 218.
[7] R.L. Eyres, C. Hastings, T.C.K. Brown and R.C. Oppenheim, *Anesth. Intens. care*, 14 (1986) 131.
[8] R.J. Campbell, K.F. Ilett, And L. Dusci, *Anesth. Intens. Care*, 14 (1986) 343.
[9] N.W. Sullivan and L. Weiner, *J. Chromatogr.*, 417 (1987) 236.
[10] A. Ruzafa, M.C. Pastor, J.L. Aguilar and R. Galimany, *J. Liq. Chromatogr.*, 14 (1991) 2937.
[11] H.C. Michaelis, W. Geng, G.F. Kahl and H. Foth, *J. Chromatogr.*, 527 (1990) 201.
[12] I. Murillo, J. Costa and P. Salvà, *J. Liq. Chromatogr.*, 16 (1993) 3509.
[13] P. Le Guévello, P. Le Corre, F. Chevanne and R. Le Verge, *J. Chromatogr.*, 622 (1993) 284.
[14] R.L.P. Lindberg, J.H. Kanto and K.K. Philajamäki, *J. Chromatogr.*, 383 (1986) 357.
[15] H. Kastrissios, M.-F. Hung and E.J. Triggs, *J. Chromatogr.*, 577 (1992) 103.
[16] E.J.D. Lee, S.B. Ang and T.L. Lee, *J. Chromatogr.*, 420 (1987) 203.
[17] A. Walhagen and L.-E. Edholm, *J. Chromatogr.*, 473 (1989) 371.
[18] B.J. Clark, A. Hamdi, R.G. Berrisford, S. Sabanathan and A.J. Mcarns, *J. Chromatogr.*, 553 (1991) 383.
[19] H. Soini, M.-L. Riekkola and M.V. Novotny, *J. Chromatogr.*, 608 (1992) 265.
[20] J.J. Butter, J.C. Kraak and H. Poppe, *J. Pharm. Biomed. Anal.*, 11 (1993) 225.
[21] R.J. Ruane and I.D. Wilson, *J. Pharm. Biomed. Anal.*, 5 (1987) 723.
[22] B. Law, S. Weir and N.A. Ward, *J. Pharm. Biomed. Anal.*, 10 (1992) 167.
[23] V. Marko and K. Radovà, *J. Liq. Chromatogr.*, 14 (1991) 1645.
[24] M.J. Cousins and P.O. Bridenbaugh, *Neural Blockade in Clinical Anesthesia and Management of Pain*, Lippincott, Philadelphia, PA, 1988, p. 122.