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Capillary gas chromatographic method for the determination of *n*-butyl-*p*-aminobenzoate and lidocaine in plasma samples

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Abstract

A fast capillary gas chromatographic method with nitrogen-selective detection is described that allows selective and reproducible quantification of *n*-butyl-*p*-aminobenzoate (BAB) and lidocaine in plasma. The sampling and sample storage conditions are critical for the quantification of BAB. Diisopropyl fluorophosphate, an organophosphorus pesticide, has to be added during sampling to prevent the rapid decomposition of BAB by cholinesterases.

1. Introduction

n-Butyl-*p*-aminobenzoate (BAB), an ester local anesthetic, is known since 1928. Because of its limited water solubility, approximately 170 mg l^{-1}, the documented pharmaceutical use of BAB is limited to dermal preparations [1] e.g. dusting powders, ointments etc.

Recently, epidural administration in humans of an aqueous suspension of BAB has been shown to produce a long-lasting (up to months) differential nerve block [2,3]. In dogs, a significant increase in pain threshold without motor blockade was observed after repeated epidural administration of the BAB suspension [4].

The pharmacokinetics of BAB after epidural administration have never been studied for the obvious reason that BAB, because of its low solubility in water, was deemed unsuitable for the preparation of the conventional formulation for epidural administration, e.g. a solution. In a study of Nakazone et al. [5], aimed at determining the brain uptake in rats of local anesthetics (including BAB), a reversed-phase high-performance liquid chromatographic (HPLC) method was used. Statistical validation parameters of the method are lacking and no data are presented on the stability of BAB in plasma during sampling and the subsequent storage. This may

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be of crucial importance as ester local anesthetics are very susceptible to hydrolysis by the enzyme cholinesterase. Procaine and chloroprocaine for instance, ester local anesthetics like BAB, decompose by hydrolysis with half-lives of less than 1 min [6,7]. The concentrations measured by Nakazone et al. [5] may therefore grossly underestimate the actual concentrations in vivo.

To elucidate the mechanism(s) responsible for the observed long-lasting effects of BAB after epidural administration as a suspension, a pharmacokinetic study is clearly indicated. The sensitivity of the HPLC method of Nakazone et al. [5] (limit of quantification approximately 1–2 μg/ml, deduced from a published plasma curve) has to be improved. The method should, preferably, also allow the quantification of lidocaine, as lidocaine is clinically administered as a test-dose before the BAB suspension to assure proper catheter placement.

The present paper describes a validated [8] sensitive and selective gas chromatographic (GC) method for the quantification of BAB and lidocaine in plasma with special emphasis on the sampling procedure, necessary to secure the stability of BAB in plasma.

2. Experimental

2.1. Reagents and materials

Analytical-grade chemicals were used, unless indicated otherwise. BAB was a gift of Abbott (Chicago, IL, USA). Lidocaine and mepivacaine hydrochloride were supplied by BUFA (Uitgeest, Netherlands), methanol, acetone, chloroform and dihydrogenphosphate dihydrate by Merck (Darmstadt, Germany). Diisopropyl fluorophosphate (DFP) was purchased from Sigma (Axel, Netherlands). Blank pooled serum was obtained from the Department of Clinical Chemistry (Catharina Ziekenhuis, Eindhoven, Netherlands).

For the determination of cholinesterase in serum a photometric test (Granutest 3) of Merck was employed.

A stock solution of 100 m*M* DFP in acetone was prepared and stored at −20°C.

For every experiment, aqueous standard solutions of BAB and lidocaine were freshly prepared from aqueous stock solutions stored at −20°C.

2.2. Instrumentation

The GC system consisted of a Carlo Erba VEGA 6300 gas chromatograph equipped with a flameless nitrogen–phosphorus detector (NPD-80-FL) operated in the N mode controlled by the NPD800 module (heating current 2.67 A, polarization voltage 4.0 V), a Carlo Erba A200S autosampler (Interscience, Breda, Netherlands) and an IBM PS2 55SX computer with System Gold chromatography software version 7.11 (Beckman, Mijdrecht, Netherlands).

The GC column was a Restek RTX-200 capillary column (cross-linked trifluoropropylmethyl polysiloxane; 30 m × 0.53 mm I.D., film thickness 0.25 μm) (LC-Service, Emmen, Netherlands). The split–splitless injector was used in the splitless mode with a splitless time of 10 s. Helium was used as the carrier gas (70 kPa). Hydrogen, air and helium (make-up gas) pressures to the detector were 27, 70 and 50 kPa, respectively.

The temperatures of the injector block and the detector were 250 and 300°C, respectively. The samples were injected at an oven temperature of 65°C. This temperature was constant during the splitless period and then raised at a programmed rate of 8°C/min until a temperature of 225°C was reached.

Bakerbond octadecyl LD (C_{18}) SPE columns (Baker) of 3 ml volume were used for the solid-phase extraction in combination with the Baker spe-12G vacuum column processor.

2.3. Sample preparation

DFP-containing Vacutainer test-tubes were prepared by adding 20 μl of a DFP stock solution (100 m*M* in acetone) to a 5-ml Vacutainer test-tube (no additive).

To prepare study samples a 2-ml volume of serum (blank or spiked with BAB and lidocaine)

was added to either a plain or a DFP-containing Vacutainer test-tube. A 1-ml volume of the internal standard (10 μg/ml mepivacaine hydrochloride in 0.2 *M* dihydrogenphosphate dihydrate) was added and vortex-mixed briefly.

2.4. Extraction procedure

The required amount of Bakerbond C_{18} SPE columns was placed on the vacuum column processor. The columns were washed once with 1 ml of the eluent (chloroform–methanol, 9:1), twice with 1 ml of methanol and twice with 1 ml of water, each time aspirating the liquid completely with suction using a vacuum of −20 kPa. Hereafter the 3-ml study sample was transferred to the column. Mild suction was applied so that the liquid passed through the column at a flow-rate of approximately 0.5 ml/min. The columns were washed twice with 1 ml of water and dried by air suction during 1 min (air-flow approximately 10 ml/min). The tips of the columns were wiped with tissues. BAB was eluted from the columns using three aliquots of 300 μl of the eluent. The eluate was evaporated to dryness under nitrogen. The residue was dissolved in 50 μl of methanol. A 1-μl aliquot was injected into the chromatographic system.

3. Results

Typical chromatograms of extracted blank plasma and plasma spiked with BAB and lidocaine are shown in Fig. 1. The retention times of BAB, lidocaine and internal standard (mepivacaine) are 10.16, 12.07 and 13.21 min, respectively. Chromatogram B shows complete baseline separation between peaks.

3.1. Sample stability

DFP, an organophosphate, is a potent irreversible inhibitor of acetylcholinesterase (ACh) [9,10]. The protective effect of DFP during and after sampling against the ACh-mediated hydrolysis of BAB was tested.

Plasma samples of twelve patients were analyzed for ACh activity [11]. Hereafter a 2-ml fraction was transferred to a DFP-containing Vacutainer test-tube, gently mixed and again analyzed for ACh activity. ACh values before addition of DFP were in the range 2.26–5.57 kU/l. After transferring to DFP-containing test-tubes the ACh activity was reduced to less than 1% of the original value in all twelve samples.

Sample stability was tested by spiking plasma to concentrations of 10, 100 and 1000 ng/ml BAB and lidocaine. Spiked samples were added to DFP-containing or blank Vacutainer test-tubes and stored at either −20°C, 5°C or room temperature. The samples were repeatedly analyzed at intervals during a period of 27 days.

DFP clearly protects BAB from hydrolysis by ACh (Fig. 2). Hydrolysis at room temperature proceeds very rapidly with and without DFP. Lidocaine, an amide local anesthetic, is stable at all test conditions. The optimal conditions are: sampling in DFP-containing Vacutainer test-tubes (final DFP concentration in the sample approximately 1.0 m*M*) and storage at −20°C. Under these conditions, samples are stable for at least 27 days.

3.2. Linearity and sensitivity

The limit of detection, defined as the concentration corresponding to a signal-to-noise ratio of 3, was determined in quintuplicate and found to be 3 ng/ml for BAB and 2 ng/ml for lidocaine.

The limit of quantification (LOQ), defined as the plasma concentration corresponding to a signal-to-noise ratio ≥ 5, was determined by analyzing in quintuplicate plasma samples spiked to a final concentration of 5 and 10 ng/ml BAB. The LOQs of BAB and lidocaine were 6 and 3 ng/ml, respectively.

Calibration curves of BAB were established in spiked plasma samples and methanol. Calibration curves of working standards of BAB and lidocaine in plasma, run on each analysis day, were constructed from the least-squares linear regression of the peak-area ratios versus concentrations over the range 5–1000 ng/ml. Typical, linear response functions for BAB and

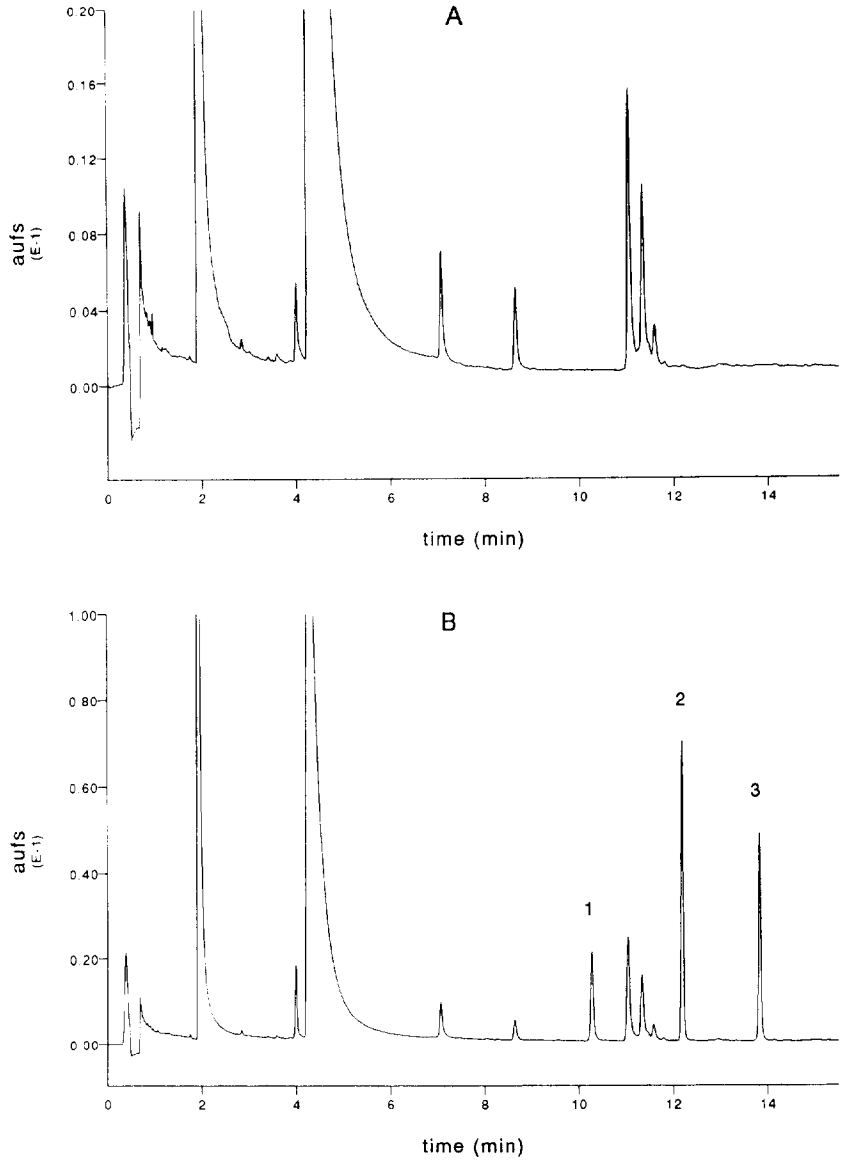

Fig. 1. Chromatograms of (A) a blank plasma extract, (B) a blank plasma spiked with BAB (peak 1, 100 ng/ml), lidocaine (peak 2, 100 ng/ml) and internal standard mepivacaine (peak 3).

lidocaine in plasma are $y = 13831.36x + 4.985948$ ($r = 0.999$) and $y = 5105.737x - 1.82088$ ($r = 0.999$).

3.3. Extraction recovery, accuracy and precision

Recoveries from plasma samples spiked to a concentration of 100 ng/ml ($n = 6$ for BAB, lidocaine and mepivacaine) were calculated by comparing peak-area ratios of extracted samples with those obtained from injection of pure substances dissolved in methanol. The recoveries of BAB, lidocaine and mepivacaine were 84%, 91% and 92%, respectively

The intra-assay precision was determined from the analysis of six samples of each of three

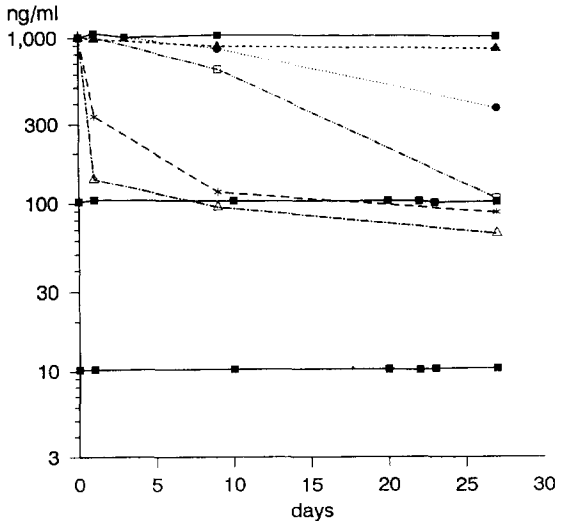

Fig. 2. Recovery of BAB in time under various sampling and storage conditions. Initial BAB concentrations (day 0) are 10, 100 and 1000 ng/ml, respectively; (■) +DFP and $T = -20°C$ ($n = 6$); (▲) $T = -20°C$ ($n = 3$); (●) +DFP and $T = 5°C$ ($n = 3$); (*) $T = 5°C$ ($n = 3$); (□) +DFP and $T = 20°C$ ($n = 3$); (△) $T = 20°C$ ($n = 3$).

concentrations of BAB and lidocaine. The results are summarized in Table 1.

Inter-assay precision was evaluated by analyzing aliquots of spiked plasma at 10, 100 and 1000 ng/ml BAB and lidocaine on five separate days over a period of one month. Inter-assay coefficients of variation are shown in Table 2.

3.4. Selectivity and specificity

Plasma blanks of five patients and pooled plasma (130 patients) were analyzed. The concentrations of BAB and lidocaine, obtained by extrapolation from a calibration graph, were 0.52 ng/ml (range −0.16–1.6) and 1.3 ng/ml (range 0.3–1.8). The selectivity of the assay was illustrated by the absence of interfering endogenous substances in the chromatogram of a plasma blank (Fig. 1A). An extensive interference study was conducted with drugs often used in pain treatment. Pure substances, including opioids, NSAIDs, antipsychotic and antidepressant drugs (and their metabolites) were dissolved in metha-

Table 1
Intra-assay precision ($n = 6$)

Spiked concentration (ng/ml)	*n*-Butyl-*p*-aminobenzoate		Lidocaine	
	Mean concentration found (ng/ml)	C.V. (%)	Mean concentration found (ng/ml)	C.V. (%)
10	10.2	6.1	10.5	5.6
100	102.3	4.1	105.0	3.0
1000	992.4	1.5	1025.3	1.7

Table 2
Inter-assay variability ($n = 5$)

Spiked concentration (ng/ml)	*n*-Butyl-*p*-aminobenzoate		Lidocaine	
	Mean concentration found (ng/ml)	C.V. (%)	Mean concentration found (ng/ml)	C.V. (%)
10	9.7	8.5	10.2	6.7
100	101.6	4.4	103.5	3.7
1000	987.3	2.2	1030.8	2.8

nol and injected (Table 3). The potential for interference was judged by the presence of an interfering peak in the chromatogram (i.e. t_R interfering peak = t_R BAB, lidocaine or internal standard ± 0.1 min.). Of the 52 drugs tested only desmethyltrimipramine, doxepine and desipramine showed a peak that could potentially interfere with the t_R of mepivacaine, the internal standard.

3.5. Plasma levels after epidural administration

A typical plasma concentration–time profile of BAB after epidural administration in a dog of a single dose (4 ml) of the 9.1% BAB suspension is given in Fig. 3.

4. Conclusion

The present method, using solid-phase extraction and automated capillary GC with nitrogen-selective detection, allows selective and reproducible quantification of BAB and lidocaine in plasma. Our study underlines the importance of validating the sampling and sample storage conditions in the development of an analytical method. The addition of the irreversible ACh inhibitor DFP to the sample proved to be essential to prevent hydrolysis of the analyte. The sensitivity is approximately 1000 times improved compared to the HPLC method of Nakazone et al. [5] and seems sufficient for the determination of pharmacokinetic parameters after epidural administration of the BAB suspension. Approxi-

Table 3
Drugs and metabolites tested for interference with the assay of BAB and lidocaine

Drug	t_R	Drug	t_R
Zuclopentixol	0.54	Maprotiline	14.21
Methylaminobenzoate	6.85	Pentazocine	14.54
Ethylaminobenzoate	7.73	Dosulipine	14.62
Pethidine	8.00	Clomipramine	14.92
Fluoxetine	9.47	Nordosulipine	15.00
BAB	10.16	Tetracaine	15.19
Fluvoxamine	10.48	Desmethylclomipramine	15.36
Prilocaine	11.20	Zopiclone	15.60
Lidocaine	12.07	Chlorpromazine	15.68
Amitryptiline	12.36	Codeine	15.82
Mianserine	12.41	Bupivacaine	15.89
Methadon	12.54	Levomepromazine	16.23
Trimipramine	12.58	Oxubuprocaine	16.75
Nortryptiline	12.74	Paroxetine	17.26
Imipramine	12.78	Clozapine	19.89
Dextropropoxyphene	12.92	Thioridazine	21.07
Procaine	13.01	Opipramol	21.08
Desmethyltrimipramine	13.12	Morphine	21.26
Doxepine	13.12	Pimozide	21.53
Desipramine	13.19	Desmethylmianserine	21.54
Mepivacaine	13.21	Desmethylmaprotiline	21.56
Acetaminophen	13.35	Fluspirilene	21.56
Protryptiline	13.37	Haloperidol	21.60
Etidocaine	13.72	Pipamperon	22.44
Buprenorphine	13.74	Trazodon	23.87
Bezitramide	13.75	Indomethacine	>25
Diclofenac	13.77	Perfenazine	>25
Promazine	13.92		

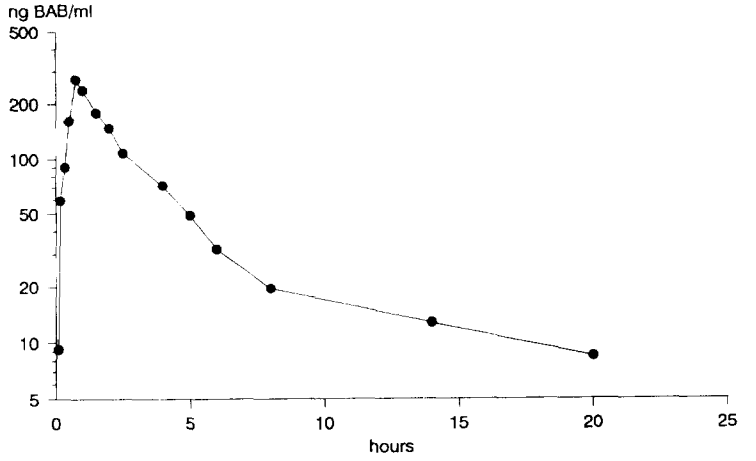

Fig. 3. Plasma concentration–time profile in the dog after epidural administration of 3.5 ml of the 9.1% BAB suspension (single dose).

mately 25 determinations can be performed per day by one person.

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