Chromatographic Behavior of Bupivacaine and Five of its Major Metabolites in Human Plasma, Utilizing Solid-Phase Extraction and Capillary Gas Chromatography

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Abstract

Bupivacaine and its five known metabolites were determined in underivatized form using capillary gas chromatography. Gas chromatography with mass spectrometry (GC–MS) and nitrogen-phosphorus detection was used for the determination of bupivacaine and its metabolites. A comparison between seven commercially available columns of differing polarity and from different manufacturers was made. Peak symmetries and retention factors varied significantly between the columns. The nonpolar columns (dimethylpolylsiloxane), Ultra1 and CP-Sil, gave good peak symmetries and an analysis time of 9 min with complete baseline separation for all of the analytes studied. The stability of the substances at different injector temperatures, 200–350°C, was studied. The limit of detection was 0.13–0.16 pmol using GC–MS in positive chemical ionization mode. Different conditions for the extraction of analytes from plasma samples using solid-phase extraction (SPE) were investigated. Different sorbents (C_2, C_8, C_{18}, and ENV+, divinyl-polystyrene) were tested. The ENV+ phase gave recoveries of between 82–120% for all the analytes studied. Several attempts at varying the washing and elution steps in order to obtain a high recovery and clean extract were investigated.

Introduction

Bupivacaine is a local anaesthetic that gives infiltration, nerve block and epidural and intrathecal anaesthesia. It is a long-acting aniline local anesthetic often administered by epidural injection and is commonly injected into surgical wound sites to reduce pain for up to 20 h after surgery. Although bupivacaine is markedly cardiotoxic and more toxic than ropivacaine, adverse drug reactions (ADRs) are rare when it is administered correctly. The determination of bupivacaine was performed by liquid chromatography (LC) and gas chromatography (GC) (1–13). The major metabolites of bupivacaine are 3-hydroxy-1-butyl-2,6-pipecoloxylidide (3-bupivacaine), 4-hydroxy-1-butyl-2,6-pipecoloxylidide (4-bupivacaine), 2,6-pipecoloxylidide hydrochloride (PPX), 3-hydroxy-2,6-pipecoloxylidide hydrochloride (3-OH-PPX), and 4-hydroxy-2,6-pipecoloxylidide hydrochloride (4-OH-PPX). There are a number of methods using liquid chromatography–mass spectrometry (LC–MS) (1) or LC–UV (2), capillary electrophoresis (3), and GC (2,5,7,9,11) that have been developed to quantify only bupivacaine levels in biological samples or with some of its metabolites. These methods differ mainly by their extraction procedure, limits of quantitation, volume of sample or total run-time. The published GC methods aimed to separate and determine only bupivacaine (2,5,7,9,11). In this work, we present a method for the separation and determination of bupivacaine and five of its known metabolites (Figure 1) in plasma samples utilizing gas chromatography–nitrogen-phosphorus detection (GC–NPD) and GC–MS. Only one publication described the determination of bupivacaine and its five metabolites using LC–UV (4). Compared to this method, our method reduced the retention time by one-fourth and improved the limit of detection

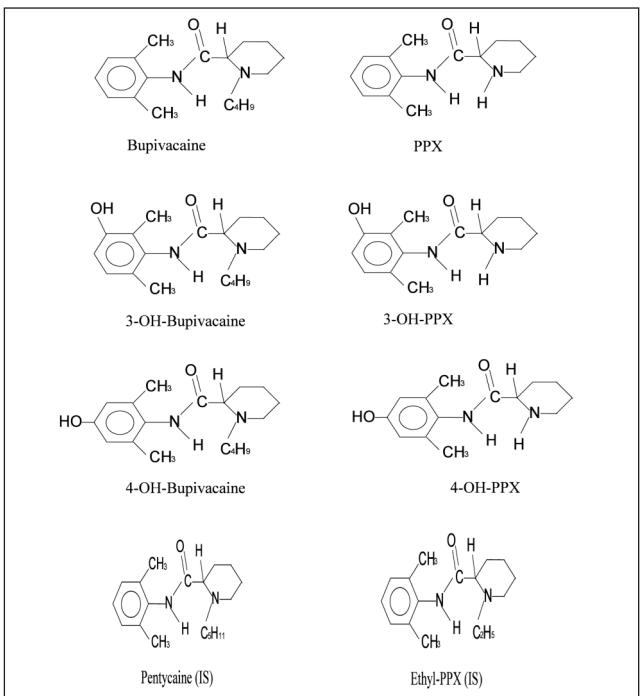

Figure 1. The structure of bupivacaine and its metabolites.

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of all studied analytes. The thermostability of bupivacaine and its metabolites was not investigated before. The present work showed that the bupivacaine and its metabolites are not stable over 280°C. Our method can be an alternative method for LC–MS to avoid the ion suppression effects. Additionally, this paper shows the possibility of using GC for metabolic profiles by studying different types of capillary columns as well as to examine the possibility of separating bupivacaine and its metabolites without derivatization and study the parameters affecting the peak performance. Avoiding derivatization would be highly advantageous in analysis in connection with, for example, structure elucidation, metabolic profiling, drug purity, and quantitative determination in bioanalysis. In the present study, the analysis was carried out using different fused-silica columns from different manufacturers covering a wide range of polarity to achieve good separation. It is well-known that the nature of the capillary column as well as the deactivation method for the analytical column is of fundamental importance for the separation (14,15).

Experimental

Chemicals

Bupivacaine, metabolites, and internal standards (Figure 1) were supplied by the Department of Chemistry, AstraZeneca (Södertälje, Sweden). Ethyl-PPX was used as internal standard only for PPX, while pentycaine was used as internal standard for the other analyses. Acetonitrile, methanol, formic acid, and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were analytical-grade.

Table I. GC Columns Specifications

Column	Manufacturer	Description	Length (m)	i.d. (mm)	Film thickness (µm)
HP1	Agilent	Dimethylpolylsiloxane	15	0.32	0.25
HP5	Agilent	5%-Diphenyl-95%-dimethylpolysiloxane	30	0.32	0.25
Ultra1	Agilent	Dimethylpolysiloxane	25	0.32	0.17
Ultra2	Agilent	5%-Diphenyl-95%-dimethylsiloxane	25	0.32	0.17
CP-Sil CB5	Chrom-pack	Dimethylpolysiloxane	25	0.32	0.25
HP35	Agilent	35%-Diphenyl-65%-dimethylsiloxane	30	0.32	0.25
BPX35	SGE	35% Phenyl (equiv) polysilphenylene-siloxane	25	0.32	0.25

Table II. ASPEC Procedures for the Extraction of Plasma Samples Using ENV+ Sorbent

Liquid dispensed	Dispensing flow rate (µL/s)	Pressuring air volume (µL)	Flow rate (µL)
Conditioning			
Methanol	50	600	100
Buffer pH 8.0 (I = 0.01)	50	600	100
Loading			
1.0 mL	15	800	100
Washing			
Buffer (pH 8.0)–methanol (1:1, v/v), 1.0 mL	50	1200	100
Elution			
Methanol, 1.0 mL	50	800	100

GC columns

CP-Sil CB5 (dimethylpolysiloxane) was obtained from Chrompack Nederland BV (Raritan, NJ). BPX35 (35% phenyl-equivalent polysilphenylene-siloxane) was purchased from SGE (Austin, Texas), while HP1 and Ultra1 (dimethypolylsiloxane), HP5 and Ultra2 (5% diphenylmethylpolysiloxane), and HP35 (35% phenyl dimethylpolysiloxane) were obtained from Agilent (Palo Alto, CA). All the columns used were of fused silica and were cross-linked (Table I).

Apparatus

GC–NPD

An HP model 5890 series II GC (Agilent) equipped with a Hewlett-Packard model 7673A automatic liquid sampler, a split-splitless inlet system, and an NPD was used. Helium was used as the carrier gas with a constant velocity of 27 cm/s. The column temperature was set at 120°C for 5 min, and the column was then programmed to heat up from 120°C to 280°C at 40°C/min. The injector and detector temperatures were 280°Cand 300°C, respectively. The injector temperature was 280°C. The software used for data processing was Chemstation version A.06.02 (Dayton, OH).

GC–MS

The GC–MS used is an HP-5890 series II GC with a split-splitless inlet system and is connected to a Finningan-MAT SSQ 710 B instrument (Finningan-MAT, San Jose, CA). GC separation was performed using a 30 m × 0.25 mm with film thickness 0.25 µm CP-Sil capillary column. The flow rate was 1 mL/min, and the temperature was programmed to rise from 75 to 280°C at 40°C/min. Chemical ionization positive ion mode using ammonia as reagent gas was used. The software used for data processing was ICIS executive version 8.2.1 beta 5 (Thermo Scientific, Waltham, WA).

Sample preparation and extraction procedures

An ASPEC system from Gilson (Villiers-LeBel, France) was used for solid-phase extraction and a Turbovap LV Nitrogen Degasser from Zymark (Hopkinton, MA) was used for evaporation.

Sample preparation was done as follows: To a 250 µL plasma sample, 50 µL internal standard and 1700 µL of phosphate buffer pH 8.0 (I = 0.1) were added, mixed, and then centrifuged (3000 rpm for 3 min).

Solid-phase extraction

The total volume obtained from the sample preparation was used for solid-phase extraction. Bond Elute C_2, C_8, C_{18}, and ENV+ (hydroxylated polystyrene-divinylbenzene copolymer) (100 mg) were tested. The procedure was automated using an ASPEC system. The sequences used are described in Table II. After extraction, the eluate is evaporated to dryness with nitrogen at 40°C. The dry sample is reconstituted in 100 µL of ethanol and heptane (1:9, v/v).

Results and Discussion

GC behavior of bupivacaine and its metabolites

Polar compounds such as amines, carboxylic acids, alcohols, and phenols are in most cases derivatized prior to their analysis by GC to shorten the analysis time and avoid peak tailing. Strong interaction between the solutes and column surface can in some cases lead to complete adsorption of these compounds. The surface inertness is an important parameter influencing column efficiency (14).

Peak symmetry

In capillary columns, there are different sites of adsorption. The first of these is attributed to metallic impurities (Na, Al, Ca, Fe, Mg, Cu) found in the glass matrix (15). The metallic impurities can act as Lewis acids sites. The synthetic fused-silica materials are essentially free of metallic impurities. The second and most important adsorption site is attributed to the silica surface structure (hydroxy groups). The hydroxy groups (silanol groups) are available as proton donors for hydrogen bonding. Highly basic or acidic compounds are thus difficult to determine by GC. A completely deactivated surface is required, or the compounds must be derivatized to nonpolar products. Several types of deactivation method have been used to overcome the adsorption problems. The different deactivating methods used for different capillary columns can affect the separation performance in different ways. The manufacturers often keep these methods secret. Fused-silica capillary tubing from different manufacturers may differ in its inertness and characteristics (14).

When the underivatized analytes were applied to different capillary columns, different effects on the peak symmetry were observed (Table III). The HP1, HP5, Ultra1, and Ultra2 columns (from the same manufacturer but having different deactivating methods) showed significant differences in peak performance. The HP5 column showed poor peak symmetry, especially for compounds containing hydroxy or amine groups. The peak symmetry improved significantly using the Ultra1 column. CP-Sil CB5 showed even better peak symmetry than HP5 (Table III). For the intermediate polar columns PBX35 and HP35, BPX35 showed excellent performance with peak symmetry for all of the compounds studied (Table III). BPX35 was used for method validation with GC–NPD.

Table III. Asymmetry Factors of the Analytes on Different Columns*

Analyte/Column	HP1	Ultra1	Ultra2	HP5	CP-Sil	HP35	BPX35
Bupivacaine	1.05	1.01	1.05	1.28	1.02	1.02	1.02
PPX	1.47	1.11	1.10	1.56	1.20	1.16	1.06
3-OH-PPX	1.15	1.10	1.14	1.43	1.23	1.45	1.20
4-OH-PPX	1.61	1.28	1.49	2.08	1.35	1.52	1.14
3-OH-Bupi	1.08	1.02	1.04	1.35	1.09	1.16	1.10
4-OH-Bupi	1.18	1.12	1.23	1.49	1.10	1.20	1.02

* Assymetry factor is calculated by dividing the base of second half peak to the base of first half peak (b/a) at 10% of peak height.

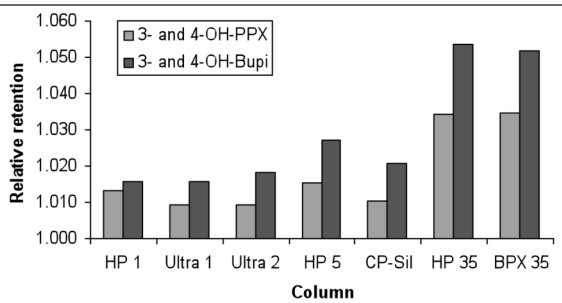

Figure 2. The relative retention of 3- and 4-OH-ropivacine; 3- and 4-OH-PPX on the studied columns.

Retention time

Table IV shows the retention factors of the bupivacaine and its metabolites on the columns studied. The retention factors of the analytes for the different columns are calculated as follows:

$k = (t_R - t_M)/t_M$, t_M = L/u min, relative retention (α) = k_2/k_1

where k = retention factor, t_R= retention time of sample compound (min), t_M = void time for mobile phase (min), L = length of column (cm), u = mobile phase linear velocity (cm/s).

The precision of the retention factor values in Table IV was determined by analysis of six repeat samples in one analytical run. Precision (CV%) was defined as the percentage of standard deviation of the observed values divided by their mean values: [(standard deviation) / mean value] × 100. The precision ranged from 4.0 to 10%.

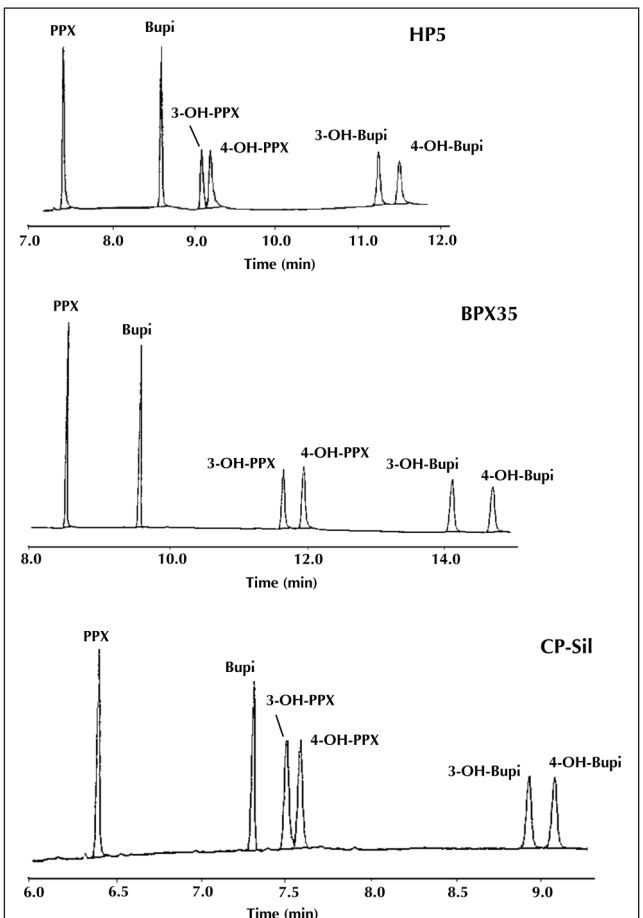

Figure 3. GC chromatogram of the extracted plasma sample of bupivacaine and its metabolites on HP5, BPX35, and CP-Sil columns.

Figure 2 shows relative retention (α) of 3-, 4-OH-PPX, and 3-, 4-OH-bupivacaine. BPX35 and HP35 showed higher α values compared to other columns that gave better separation of 3- and 4-OH-PPX /bupivacaine. Baseline separation of 3- and 4-OH-PPX was obtained with PPX35 and HP 35 (Figure 3).

Figure 3 shows a chromatogram of extracted plasma sample bupivacaine and its metabolite on a HP5, CP-Sil, and BPX35 columns. Bupivacaine and its metabolites were well-separated on BPX35. Under the optimized chromatographic conditions described, they are eluted within 10 min (nonpolar column) and 15 min (intermediate polar columns).

Thermostability

Thermostability of bupivacaine and its metabolites was investigated. Figure 4 shows the effect of different injector temperatures (200–350°C) on the peak area for all studied analytes. The optimum temperature was 260–280°C. The peak areas decreased when the injector temperature was less than 240°C. This may be due to the fact that the compounds did not completely gasify and then adsorb on the injector liner. At high temperatures (300–350°C) the peak areas decreased, which is most probably due to the fact that the compounds break down in the injector. The optimum injector temperature was 280°C.

Extraction method development

Different sorbents such as C_2, C_8, C_{18}, and ENV+ were investigated. The effect of elution solutions on the analytes recoveries was investigated. Methanol–methanol–acetone (1:1), 0.5% TFA in methanol–acetone–ethyl acetate (2:1:1), and 0.5% TFA in methanol–acetone (1:1) were tested. In addition, the pH of the plasma sample on the extraction recovery was investigated at 7.0, 7.5, 8.0, and 8.5. The recoveries increased for all of the analytes when the pH was increased. The optimum pH was 8.0. The pKa values of bupivacaine and its metabolites ranged from 6 to 8 at pH 8.0. The analytes are uncharged except bupivacaine (pKa ≈ 8). In addition, using ENV phase (polystyrene polymer) may have mixed retention. Mechanism was involved, and good recovery for bupivacaine can also be obtained.

To determine extraction recoveries, plasma samples spiked with bupivacaine and its metabolites at a concentration of 1 µM were prepared and analyzed. Extraction recoveries were calculated by comparison of the peak areas of extracted solution with the peak areas of the direct injection of the same concentration in methanol. The best recoveries obtained using different sorbents were in order as follows: ENV+ > C_{18} > C_8 > C_2 at pH 8.0, and methanol gave the best recovery as elution solution. Thus ENV+ as sorbent and methanol as elution solution were used to get optimum recovery (Table II). Extraction degree was calculated as follow:

$$\text{Recovery \%} = \frac{Peak_{\,extract-analytes} / Peak_{\,extract-IS}}{Peak_{\,analytes-reference\ solution} / Peak_{\,IS-reference\ solution}} \times 100$$

The recovery at the optimum conditions were 95% for bupivacaine, 83% for PPX, 82% for 3-OH-PPX, and 85% for 4-OH-PPX. The recovery for 3- and 4-OH-bupivacaine was 120%. This high recovery may be due to matrix effect or more a suitable IS was needed). The extraction degree is shown in Table V.

Calibration curve

To determine calibration curves, plasma samples spiked with bupivacaine and its metabolites were prepared and extracted by ENV+ (Table II) and analyzed using GC–NPD and GC–MS using a PBX35 column. Ethyl-PPX (2 µM) was used as IS for PPX, and

Table IV. Retention Factors of the Analytes on Different GC Columns

Analytes/Column	HP 1	Ultra 1	Ultra 2	HP 5	CP-Sil	HP 35	BPX 35
Bupivacaine	5.83	3.13	3.11	3.64	3.70	4.00	4.85
PPX	5.14	2.69	2.69	2.98	3.14	3.38	4.11
3-OH-PPX	6.00	3.20	3.26	3.92	3.80	5.26	6.32
4-OH-PPX	6.08	3.23	3.29	3.98	3.90	5.44	6.54
3-OH-Bupi	7.05	3.80	3.88	5.13	4.79	6.73	8.09
4-OH-Bupi	7.16	3.86	3.95	5.27	4.89	7.09	8.51

Table V. Between-batch Accuracy and Precision of Bupivacaine and its Metabolites Using ENV+ Sorbent and Methanol as Elution Solution

Analyte	Extraction degree % (*n* = 6)	Inter-day precision* (RSD%, *n* = 12)
Bupivacaine	95	2.6
PPX	83	3.1
3-OH-PPX	82	4.2
4-OH-PPX	85	5.9
3-OH-Bupivacaine	120	4.8
4-OH-Bupivacaine	120	4.1

* GC–MS.

Table VI. The LOD For Analytes Using GC–NPD and GC–MS

Analyte	GC–NPD (pmol) (*n* = 3)	GC–MS (pmol) (*n* = 3)
Bupivacaine	0.17	0.13
PPX	0.78	0.16
3-OH-PPX	2.2	0.15
4-OH-PPX	2.3	0.15
3-OH-Bupivacaine	2.2	0.14
4-OH-Bupivacaine	2.3	0.14

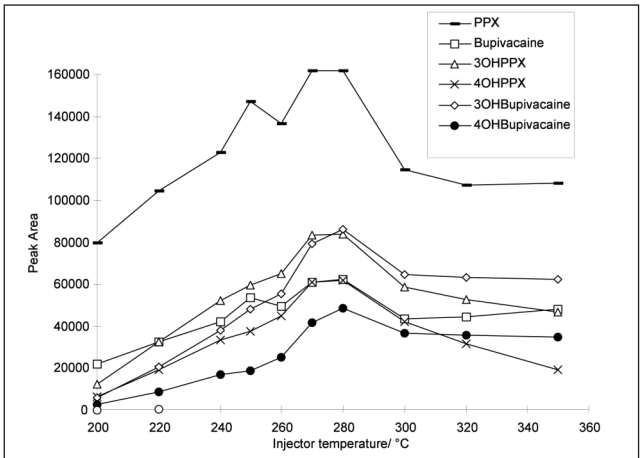

Figure 4. The effect of different injector temp. (200–350°C) on the peak area.

pentycaine (2 µM) was used as IS for the other analytes. The samples were quantified using internal standardization. Standard curves were prepared using five to seven different concentrations for each analyte. The concentration range of each individual standard curve was chosen according to the actual concentration in the plasma samples. Non-weight linear regression was used for quantification. The calibration curves were obtained by plotting the peak area ratio between analytes and IS. Ethyl-PPX was used as IS for PPX. Ethyl-PPX and was eluted between PPX and bupivacaine and a base separation was obtained on the all studied columns [Rs (Ethyl-PPX/bupivacaine) > 1.2]. Pentycaine was used as internal standard for the other analytes and was eluted between 4-OH-PPX and 3-OH-bupivacaine. Pentacine was separated from the both metabolites [Rs (pentycaine/3-OH-bupivacaine) > 1.2]. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

where y is peak area ratio, x is the concentration, a is the curvature, b is the slope, and c is the intercept. The calibration curves were quadric and the weight was $1/x$. The method showed good relationship between the analytes response and the analytes concentration in the concentration range of 0.1 to 20 µM ($r^2 > 0.99$, $n = 6$). In addition, the backcalculated values of the calibration points showed good agreement with the theoretical concentrations (≤ ±15%) of the nominal concentrations using quadratic calibration equation comparing to linear calibration equation. A typical linear calibration equations are $y = 0.0006x + 0.029$ and $y = 0.0004x + 0.009$ for bupivacaine and PPX, respectively. The quadratic calibration equation for bupivacaine and PPX are $y = -3E\text{-}8x^2 + 0.0004x + 008$ and $y = -2E\text{-}8x^2 + 0.0002x + 005$, respectively.

Reproducibility

Reproducibility was evaluated by analyzing plasma samples containing two different concentrations of bupivacaine and its metabolites on the same day (intra-day reproducibility) and over five consecutive days (inter-day reproducibility). The accuracy and inter-day reproducibility of bupivacaine and its metabolites are reported in Table V.

The limit of detection of the studied analytes (LOD) was about 0.15 pmol using GC–MS and between 0.17 and 2.3 pmol using GC–NPD (Table VI).

Conclusions

This paper clearly shows that it is possible to chromatograph underivatized metabolites of bupivacaine with high peak performance using GC. Seven different columns were tested, and on some columns peak tailing was observed, but other columns showed excellent performance with both narrow and symmetrical peaks. The optimal injection temperature was 250°C. The limit of quantitation for bupivacaine and its metabolites were between 130–160 fmol using GC–MS. The results from this paper demonstrates that GC of underivatized metabolites may be a useful analytical tool in e.g., structure elucidation (in combination with mass spectrometry), impurity testing, and for quantitative determination of bupivacaine and its metabolites.

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