Evaluation of Solid-Phase Microextraction for the Study of Protein Binding in Human Plasma Samples

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Abstract

Solid-phase microextraction (SPME) in combination with capillary gas chromatography and a nitrogen–phosphorous detector is used to study protein binding in human plasma samples. Local anesthetics of the amide-type (ropivacaine, bupivacaine, mepivacaine, prilocaine, and lidocaine) are used as model compounds in this evaluation. Carbowax/divinylbenzene (CW/DVB), polyacrylate, and polydimethylsiloxane fibers are tested. Sampling on CW/DVB fibers give the highest recovery in plasma samples compared with other fibers. Ultrafiltrate spiked with each of the substances is used for the construction of calibration curves.

The protein binding is investigated at four different total concentrations from 0.5 to 15.0µM. The degree of protein binding increases when the solute concentration decreases.

Protein binding of the five solutes is investigated at four pH levels (6.4, 7.4, 8.4, and 9.4). It is found that protein binding increased with increasing pH. The influence of temperature variation (from 32°C to 40°C) on protein binding is also investigated. The protein binding decreases when the temperature increases. The methodology is validated and good correlation and precision are obtained. Back-calculated quality control samples give accuracy within 20% of theoretical values for all five substances. This study shows that SPME as a sample-preparation method gives the same protein binding for the studied local anesthetics as that achieved using earlier presented methods.

Introduction

Solid-phase microextraction (SPME) is a simple solvent-free sample-preparation method for gas chromatography (GC) and liquid chromatography (LC). This extraction technique is rapid and easy to handle. It is also easily automated and shows good linearity for many analytes. SPME has been introduced as an alternative to current sample-preparation technology. Today, the technique has been applied to extract a wide range of analytes in many areas (1,2). The extraction is based on the partitioning of the analyte between the organic phase on the fused-silica fiber and the matrix. Many factors (such as pH, temperature, salt concentration, and stirring) affect the equilibrium constant and the equilibration time (1). A number of publications on SPME involving drugs and their metabolites in human urine or plasma have been reported (2–19). However, only a few publications deal with the optimization of SPME for plasma analysis (10–12,15–19).

The protein binding of amide-type local anesthetics was first reported 30 years ago (21,22). Minor changes in the binding capacity can cause severe reactions (e.g., cardiovascular and central-nervous side effects). It is mainly the free nonprotein-bound fraction of local anesthetics that is responsible for these side effects (23,24). It has been shown that most of the binding is because of association with α_1-acid glycoprotein (AGP), one of the so-called acute-phase or stress proteins. It has also been demonstrated that the extent of binding varies significantly in different types of patients, depending largely on the plasma level of their AGP (25–30). The binding of drugs to protein influences drug pharmacokinetics and causes pharmacological effects. A majority of acidic drugs bind to albumin in plasma, and basic drugs mainly bind to AGP.

The aim of this study is to evaluate SPME as a sample-preparation tool in order to determine the protein binding of local anesthetics of the amide type in human plasma. The influence of pH, temperature, and analyte concentration on the protein binding was studied for five solutes: ropivacaine, bupivacaine, mepivacaine, prilocaine, and lidocaine. SPME followed by GC with a nitrogen-phosphorous detector (NPD) was compared with methods described in the literature. The merits of SPME as a sample-preparation method to determine total concentrations of local anesthetics include reproducibility, easy handling, and automation. Furthermore, a minimum number of

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steps are required as compared with other methods (18).

Ropivacaine, bupivacaine, and mepivacaine are chemical homologues. Ropivacaine is a new amide-type local anesthetic drug that is mainly used for surgery and post-operative pain relief. Also, it has a lower central nervous and cardiotoxic potential than bupivacaine (19). Lidocaine is widely used in anesthesiology. It also has antiarrhythmic effects and is used as a therapeutic agent in the treatment of cardiac disorders (30).

Experimental

Reagents and materials

Ropivacaine, bupivacaine, mepivacaine, prilocaine, and lidocaine (Figure 1) in hydrochloride form were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Methanol LiChrosolv grade, NaOH, acetic acid, and NaCl were obtained from Merck (Darmstadt, Germany). Nitrobenzene was used as the internal standard and obtained from Merck (Darmstadt, Germany). AGP was purchased from Sigma-Aldrich (Steinheim, Switzerland).

For each compound a stock solution was prepared in methanol and stored at 4°C (a stability of 8 months). Working solutions were prepared by appropriate dilution in phosphate buffer (pH 7.4, μ = 0.2), which was stable for more than one month at room temperature.

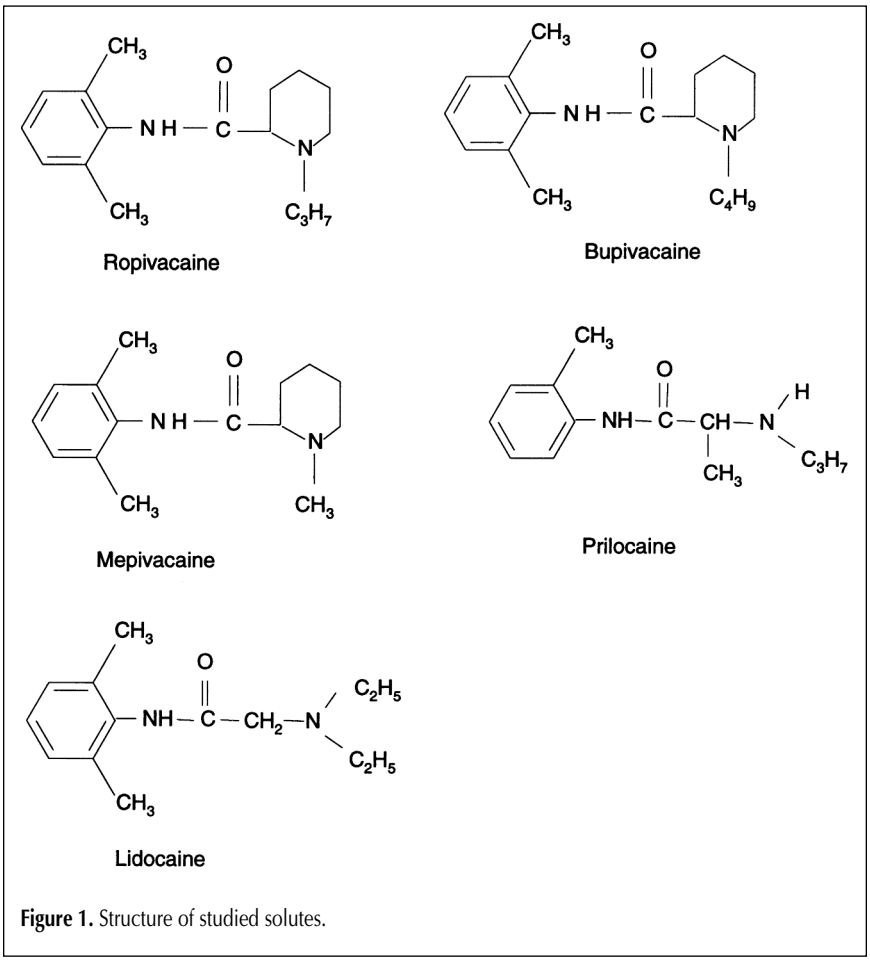

Figure 1. Structure of studied solutes.

Equipment

The GC analysis was performed using an Agilent Technologies (Palo Alto, CA) model HP 5890 Series II equipped with a split–splitless injector and NPD. A ChemStation data system (HP 3365 Series II version A.05.02) was used for data processing. The column used was an HP50 (50% phenyl dimethylpolysiloxane) fused-silica capillary column (25-m × 0.20-mm i.d., 0.31-μm film thickness), which was also obtained from Agilent Technologies. Helium was used as the carrier and make-up gas. Helium, air, and hydrogen were of high-purity grade and obtained from AGA (Lidingö, Sweden). The gas flow rates were measured using a digital flowmeter (Fisons, J&W Scientific, Folsom, CA). The oven temperature was programmed for an initial hold of 3 min at 80°C in order to focus the solutes in the beginning of the column, and after that the temperature was increased at 20°C/min to 280°C. The detector temperature was 280°C, and the injector temperature was kept at 250°C. The injector was operated in splitless mode with the purge activation time adjusted to 3 min.

Plasma samples were ultrafiltered using Centrisart I (cutoff 20,000 u) filter tubes (Sartorius AG, Goettingen, Germany), and a Centrifug Hettich Rotanta/AP (Hettich, Tuttlingen, Germany) was also used in this study. The pH was measured using a pH-meter Beckman 110 ISFET (Beckman Instruments, Fullerton, CA).

SPME

Autosampler Varian 8200 CX with an SPME III sample agitation (Varian Chromatography Systems, Walnut Creek, CA) was used. The autosampler was equipped with an auto-therm controller with a temperature range of 5–70°C. A Carbowax/Divinylbenzene (CW/DVB) coated fused-silica fiber (Supelco, Bellefonte, PA) with a 65-μm film thickness was used. The sample volume selected was 1.00 mL. The type of sampling was immersion, not headspace. Prior to the first extraction, the fibers were conditioned at 250°C for 1 h according to the manufacturer's recommendation.

Procedures

Ultrafiltrate from human plasma was prepared by thawing the human plasma that was stored in a freezer at –20°C. The plasma was added (maximum 2.5 mL) to centrifugal tubes (Centrisart I, cutoff 20,000 u) and was centrifuged at 3000 rpm for 20 min. Then, the ultrafiltrate was removed and stored at 4°C, but only for a limited time (less than one week).

The runs were divided into two steps: (*a*) first the immersion step of the fibers in the test tubes for 25 min (17,18) and (*b*) then the immersion step into the GC

(5 min) with subsequent analysis. During the absorbing step, agitation shortened the time needed to reach equilibrium (17). The total time for one run was 40 min. Every 30 min, a new sample was started in the SPME step. Sample pH was adjusted to 7.4 by adding a few microliters (4–8 µL to 1.0 mL plasma) of 5% acetic acid in water (v/v).

A calibration curve was constructed to define a relationship between the concentration of the analytes and the response obtained in the runs. Seven calibration levels with a concentration range of 20–19000nM in ultrafiltrated plasma were used. The five drugs investigated in this work were tested against both AGP and albumin. It was clearly shown that for these basic drugs, AGP is the protein that gives protein binding. In the test tubes, the concentration of the proteins were the same as the average in human blood (0.9 mg/mL). To determine analyte binding to protein, each solute was separately added to the ultrafiltrate at a concentration range of 0.5–15.0µM. At this stage, all tests were carried out in ultrafiltrated plasma and phosphate buffer.

It was difficult to find an internal standard that did not bind to the protein and give a well-defined chromatographic peak signal from the detector. A number of substances such as pentycaine, azobenzene, and nitrophenol were examined. The signal of the internal standard (as peak area) was compared with and without AGP in ultrafiltrated plasma. Unfortunately, after several experiments, it was obvious that all substances bound to protein (> 30%). After additional tests, nitrobenzene was found suitable, having minimal protein binding (< 2%).

Results and Discussion

Method development

It is well-known that the protein binding of local anesthetics in plasma must be taken into consideration in connection with the interpretation of blood-level data. Minor changes in the binding capacity can cause severe reactions (e.g., cardiovascular and central-nervous side effects). Earlier studies have shown that pH and temperature influence the protein binding of local anesthetics in plasma. Therefore, the methods used for the determination of the free concentrations in blood plasma have been quite complex, and they involve a number of steps. First, pH is adjusted by adding CO_2 to plasma samples. Sample-preparation methods such as dialysis or ultrafiltration is also used. Second, after a pretreatment, the ultrafiltrate is injected on HPLC. When using a coupled-column system, the pretreatment is performed on a precolumn (26–32).

The first part of this study was to determine protein binding of ropivacaine, bupivacaine, mepivacaine, prilocaine, and lidocaine as model substances in human plasma using SPME as the sample preparation in combination with capillary gas chromatography and compare the results with literature data. The second aim was to validate the method.

In our earlier studies, CW/DVB, polyacrylate, and polydimethylsiloxane fibers were evaluated for the extraction of lidocaine and its metabolites from human plasma (17). The CW/DVB fiber gave the highest recovery (2–4 times) in plasma samples as compared with the other fibers. In this study, a CW/DVB fiber was used to determine the protein binding of the studied solutes. Acetic acid in water (5%, v/v) was used to adjust pH to 7.4. Ultrafiltrated plasma was spiked with the analytes and the internal standard (2µM). Every analyte was injected with and without AGP. This was done at four different concentrations (0.5–15.0µM). The free concentration (%) was calculated by dividing the free concentration with AGP added by the total concentration without AGP. The protein binding (%) was then calculated by the following equation:

$$(1 - \text{free concentration}) \times 100 \qquad \text{Eq. 1}$$

The effect of compound concentrations, pH, and temperature variation on protein binding was investigated.

Protein binding

Determination of analyte bound to protein at different analyte concentrations

Figure 2 shows the total (350nM) and free concentrations of bupivacaine. The results from this study were compared with the results from literature. The pH had to be adjusted before each run. Acetic acid (5%, v/v) was used for that purpose. The protein binding was studied at four different concentrations (0.5µM, 2µM, 3µM, and 15µM) (Table I). The results showed increased protein binding with decreased concentration. This was expected because the equilibrium shifted as the concentration varied. In Table II, the results from the literature are compared with the results from this study. The results from

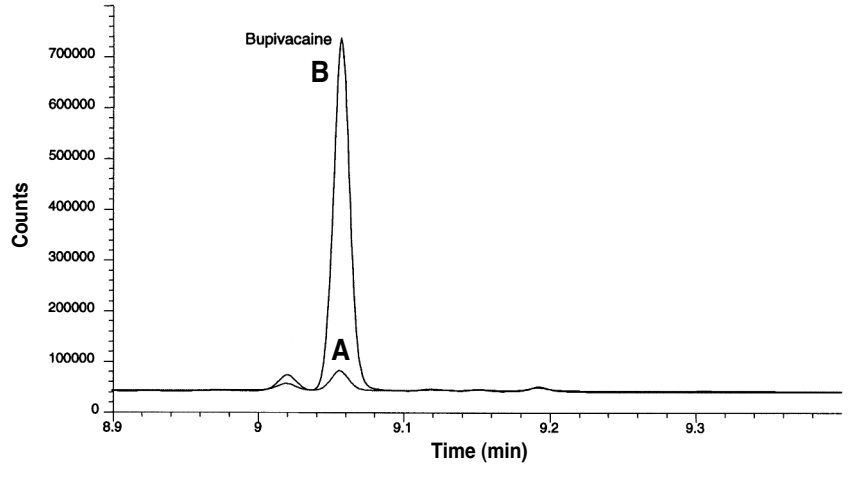

Figure 2. Gas chromatogram of 350nM bupivacaine (A) with and (B) without the presence of AGP in the sample matrix.

this study showed favorable agreement with earlier published data (26–32,34).

Effect of pH

The binding of analyte to protein was studied at four different pH levels from 6.4 to 9.4 (Table III). The pH was adjusted by adding a few microliters (4–8 µL to 1.0 mL plasma) of acetic acid (5%) for lowering pH and NaOH (5%) for raising pH. The result showed increased analyte binding with increased pH in the evaluated interval.

The pK_a values of the local anesthetics evaluated in this study were between 7 and 8. At pH 6.4, the substances were in charged form, resulting in poorer protein binding. When pH increases to 9.4, the substances became uncharged. The uncharged substances underwent hydrophobic interaction with the proteins, resulting in higher analyte binding to protein.

Effect of temperature

The analyte binding to protein was studied at three different temperatures from 32°C to 40°C (Table IV). The results showed decreased binding with increased temperature. When the temperature was raised, the binding between the local anesthetic and the protein broke up, resulting in lower binding.

Table I. Influence of Solute Concentration on Protein Binding*

	Protein Binding (%)			
Compound	0.5µM	2.0µM	3.0µM	15.0µM
Ropivacaine	99	92	94	90
Bupivacaine	95	96	95	93
Mepivacaine	81	81	76	72
Prilocaine	39	35	35	35
Lidocaine	88	80	75	49

* Conditions in the vial: temperature at 37°C, pH 7.4.

Table II. Comparison of Protein Binding Between This Study and Earlier Studies*

	Ropivacaine		Bupivacaine		Mepivacaine		Prilocaine		Lidocaine	
Concentration (µM)	Present Study (%)	Literature (%)	Present Study (%)	Literature (%)	Present Study (%)	Literature (%)	Present Study (%)	Literature (%)	Present Study (%)	Literature (%)
0.5	98	–	95	96	81	82	39	30	88	80
2.0	92	94	96	96	81	81	35	30	80	75
3.0	94	93	95	95	76	78	35	30	75	70
15.0	90	–	93	94	72	70	35	30	49	55
References	(30)		(26,30)		(26)		(26,31,32)		(26,29,34)	

* Conditions in the vial: temperature at 37°C, pH 7.4.

Method validation

Selectivity of the method

No significant interfering peaks were detected at the chromatographic retention times of the analytes when comparing chromatograms of spiked human plasma with blank plasma from different persons. Figure 3 shows that the SPME technique is selective as a sample-preparation tool for the studied analytes.

Calibration

For the construction of the calibration curve, seven levels were used for all five analytes. The calibration standards were prepared in ultrafiltrated plasma and phosphate buffer to examine media effect on the SPME accuracy. The spiked ultrafiltrate samples (QC) were compared with these different calibration curves. When using ultrafiltrate, pH had to be adjusted to 7.4 prior to each run. This was done by the addition of 4–8 µL of acetic acid (5%) to 1.0 mL plasma. The pH was allowed to vary between 7.35 and 7.45.

Calibration curves in phosphate buffer (pH 7.4, µ = 0.2) gave good correlation for all five substances, but when spiked QC samples were compared with the curves obtained in phosphate buffer, the back-calculated concentration values differed up to 40% from the theoretical values, which was much more than the allowed (15–20%). Ultrafiltrate contains a large number of different substances, some of these could be competing with the studied solutes resulting in lower recovery.

Table III. Influence of pH on Protein Binding*

	Protein Binding (%)			
Compound	pH 6.4	pH 7.4	pH 8.4	pH 9.4
Ropivacaine	72	94	96	95
Bupivacaine	78	95	98	97
Mepivacaine	28	76	87	88
Prilocaine	30	35	46	48
Lidocaine	53	75	86	88

* Conditions in the vial: temperature at 37°C, concentration approximately 3µM.

Table IV. Influence of Temperature on Protein Binding*

	Protein binding (%)		
Compound	32°C	37°C	40°C
Ropivacaine	94	94	91
Bupivacaine	96	95	87
Mepivacaine	79	76	74
Prilocaine	45	35	26
Lidocaine	83	75	77

* Conditions in the vial: pH 7.4, concentration approximately 3 µM.

Regression parameters for all the calibration curves in ultrafiltrate are given in Table V. The results showed a linear response for all analytes in the calibration range studied. Correlation coefficient values between 0.993 and 0.9992 were obtained for all analytes. The calibration curves indicated that the method was suitable for quantitative analysis.

Blank injections were made after the injections of the highest concentration in order to investigate whether there were any carryover effects. The blank contained no interfering peaks. The result showed that no analytes could be detected on the fiber to interfere with the blank separation.

The calibration curves showed good correlation and the back-calculated concentration values of the quality controls in ultrafiltrate were within the accepted limit (variation less than 20% of theoretical values). The back-calculated concentrations of spiked plasma samples were also within the 20% allowance.

Calibration curves in the ultrafiltrate–phosphate buffer mixture (1:1) were also prepared. This was done for all analytes. As was previously the case, the correlation was good for all five solutes. Spiked plasma samples were made for every solute and tested against the calibration curves. The back-calculated values of the samples showed variation higher than 20% of the theoretical value.

Accuracy and precision

Intra-assay and interassay analyses of the quality control samples at two levels (112.8nM and 564nM) were made. The results are shown in Table VI.

The coefficient of variation percentage values were less than the 20% allowance for both interassay and intra-assay. This indicates that the SPME method has a sufficiently good precision.

Test of the fiber lifetime

The manufacturer claims that the fiber should last 60–100 runs. A fiber was tested for 80 runs. The test was divided into four parts: two different concentrations in phosphate buffer and the same concentrations in ultrafiltrate. The aim of the test was to check for how many injections the fiber could be used. The coating remained on the fiber after 80 runs, but the signal decreased by approx-

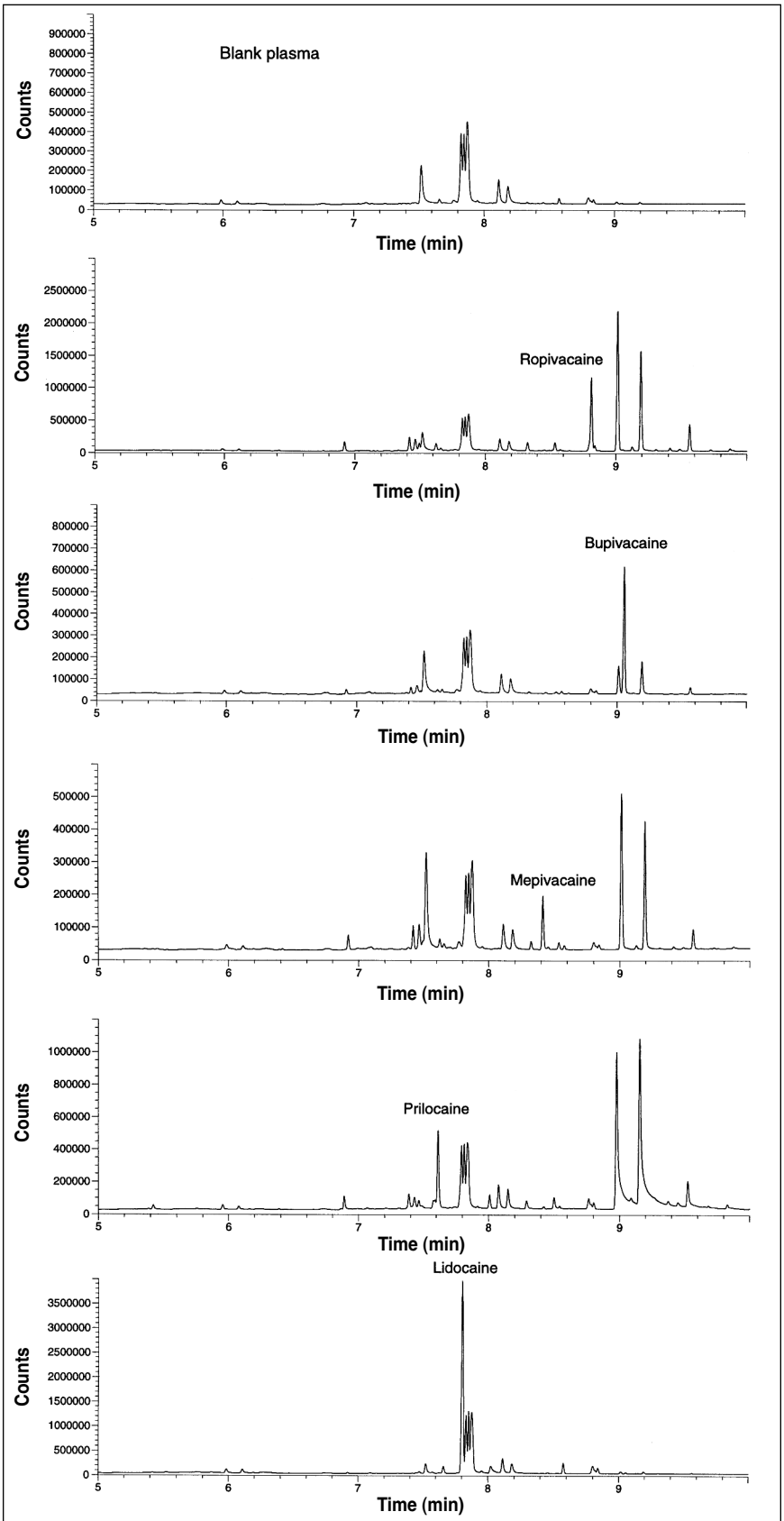

Figure 3. Gas chromatograms showing the total concentration of the studied solutes in plasma samples (same plasma, 200–300nM concentrations). Conditions in the vial: temperature 37°C and pH 7.4. GC conditions: splitless injection at 80°C; purge activation time, 3 min; isothermal for 3 min; then temperature programmed at 20°C/min to 280°C; injector temperature, 250°C; and detector temperature, 280°C.

imately 30% for the last run compared with the first run. It should be mentioned here that some fibers did not last for more than 30–40 runs.

Conclusion

A simple and robust method for the determination of the free concentrations in plasma of five local anesthetics involving SPME–GC–NPD has been demonstrated. The results from this study showed a good agreement with earlier published data. The results demonstrate that the SPME method can be used to study the free concentrations of local anesthetics. The limitation of SPME as a sample-preparation method is that it has low analytical recovery, thus low sensitivity. Also, the validation of the method showed that SPME gave a higher coefficient of variation compared with earlier methods described in the literature, but the acceptance criteria for the study validation were well in line with the international criteria (31).

Table V. Linear Calibration Range and Correlation Coefficient in Ultrafiltrated Plasma

Analyte	Calibration range (nM)	Slope	Intercept	Correlation coefficient (n = 3)
Ropivacaine	20–19000	0.0011	0.017	0.9992
Bupivacaine	20–18000	0.0013	0.008	0.9990
Mepivacaine	20–13000	0.0007	0.022	0.9964
Prilocaine	29–19000	0.0030	–0.041	0.9930
Lidocaine	29–18000	0.0012	0.027	0.9990

Table VI. Intra- and Interassay Precision and Accuracy

Analyte	Concentration (nM)	Mean accuracy percentage (n = 6)	Precision (RSD%)* Intra-assay (n = 4)	Precision (RSD%)* Interassay (n = 3)
Ropivacaine	112	98.0	–1.3	–15
	564	103	2.6	3.3
Bupivacaine	87.5	102	12	11
	700	87.0	5.2	14
Mepivacaine	106	111	12.1	18.7
	586	108	13.5	11.3
Prilocaine	112	80	3.5	11.5
	750	106	4.7	4.4
Lidocaine	117	108	11.8	14
	784	111	2.1	1.9

* RSD%, relative standard deviation percentage.

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