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Determination of amide-type local anaesthetics by direct injection of plasma in a column-switching high-performance liquid chromatographic system using a pre-column with a semipermeable surface

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Abstract

A high-performance liquid chromatographic method using column switching was applied to the direct determination of two local anaesthetics, ropivacaine and bupivacaine, in human plasma. The method is intended to be used in a combined LC–GC system; here only the LC-part is described. After addition of internal standard, the samples were filtered and directly injected into a semipermeable surface (SPS) pre-column where the analytes were strongly retained and separated from many endogenous compounds by a short washing step. The retained analytes were transferred by a buffered methanol phase from the pre-column into a carbonaceous HPLC column and they were detected by UV detection at 254 nm. The SPS pre-column could withstand numerous (>200) direct injections of plasma samples (10 μl). The method has a detection limit of 8.2 ng and requires a total assay time of 15 min per plasma sample. Quantitative recoveries were obtained over the range 3.3–114 μg/ml with inter-day precisions of 1.6–5.2% (C.V.).

1. Introduction

Pretreatment of biological samples, such as solvent extraction, off-line solid-phase extraction and deproteinization, is necessary in conventional HPLC methods to prevent the accumulation of proteins, which results in clogging of the column and decrease in chromatographic performance, and to obtain an adequate selectivity. The procedures used for pretreatment are often tedious and give a low reproducibility.

Many efforts have been made to develop techniques that permit direct sample injection into liquid chromatographic systems [1]. These approaches include the use of column switching, micellar chromatographic systems and specialized HPLC packing materials. Of these, the packing materials, especially the so called restriced-access reversed-phase (RARP) types [2–4], have attracted most attention. The first paper applying these principles described the internal surface reversed-phase (ISRP) concept (Pinkerton *et al.* [5]. A number of columns packed with either the ISRP type, the shielded hydrophobic-phase (SHP) type [6], or the semipermeable surface (SPS) [7] type are now commercially

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available. Although these RARP materials are prepared by different methods, their properties are similar: large molecules (proteins) only reach the hydrophilic, non-adsorptive layers on the outer surface, access to the inner bonded phase is prevented. Small molecules, on the other hand, penetrate the outer surface and interact with the inner surface [2,4]. The ISRP materials [8–12] and the SHP materials [6,13,14] have been studied most extensively, but the retention properties and the character of the SPS materials, which utilize polyoxyethylene glycol as a hydrophilic layer bound to the conventional alkyl bonded phases (C_8, C_{18}, CN and phenyl), have also been reported [7,15]. The requirement to both retain the hydrophobic analytes on the RARP columns and remove the plasma proteins without causing precipitation in the columns often leads to contradictory variations in the composition of the organic modifier. This can be solved either by gradient elution or by using a column-switching technique. The column-switching technique enables the use of a wide range of stationary phases for specific applications. In addition, the use of RARP as pre-column enables the enrichment of the analytes on the column without extensive sample manipulation. Applications of the column-switching technique in combination with RARP as pre-column, particular with the ISRP material [9,10,12,16], have been published.

Bupivacaine, an amide-type local anaesthetic, is widely used in modern anaesthesiology. However, the side-effects of this drug, especially those with respect to the central nervous system and cardiac toxicity, may cause severe problems [17]. Ropivacaine is a recently developed local anaesthetic which possesses an anaesthetic profile similar to that of bupivacaine but with less toxicity [18–20]. High-performance liquid chromatographic methods to determine the concentration of bupivacaine in human plasma have been published [21–27]. A capillary gas chromatographic method for the analysis of ropivacaine has been published [28]. All these methods involved traditional extraction and evaporation procedures.

In this paper, we describe an HPLC system utilizing a pre-column based on SPS and an analytical column packed with a carbonaceous solid-phase combined by a column-switching technique. The SPS support used in this work contains hydrophilic polyoxyethylene "tails" and C_{18} alkyl groups; it was claimed to have similar retention characteristics as ordinary C_{18} phases and thereby should be applicable to the principles of reversed-phase systems [2,4]. In this study the SPS-phase was packed in a 10-mm cartridge and connected to the analytical column through a 10-port switching valve. Suitable conditions for the switching procedure have been determined, and the plasma volume and mass capacities of the pre-column have been evaluated. Eventually our purpose is to combine this system on-line with a gas chromatographic step thereby reaching lower detection limits; this work is on-going and will be described in a forthcoming paper.

2. Experimental

2.1. Reagents and materials

Ropivacaine and bupivacaine hydrochloride monohydrates and pentycaine hydrochloride (Fig. 1) were gifts from Astra Pain Control AB (Södertälje, Sweden). N-(2-Acetamido)-2-amino-ethanesulfonic acid (ACES) buffer, methanol, and isopropanol of gradient grade and acetic acid of analytical grade were purchased from Merck (Darmstadt, Germany). Triethylamine (TEA) was supplied by Fluka (Buchs, Switzerland). Deionized water was purified using a Milli-Q purification system (Millipore, Bed-

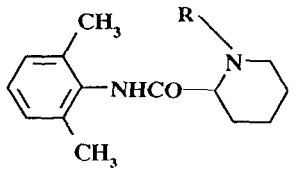

Fig. 1. Structures of (a) ropivacaine, R = C_3H_7; (b) bupivacaine, R = C_4H_9; and (c) pentycaine R = C_5H_{11}.

ford, MO, USA). Polypropylene syringes (2 ml) were used in conjunction with 0.22-μm, 13-mm diameter nylon syringe filter units (Millipore) for plasma filtration.

2.2. Preparation of spiked plasma and standard samples

In order to prepare calibration curves and carry out recovery and precision studies, blank human plasma was spiked with ropivacaine and bupivacaine, and with pentycaine as the internal standard. Standard solutions were prepared in 0.01 *M* ACES buffer (pH 7) by varying the amounts from the stock solution. Each standard solution contained the same amount of internal standard. A 50-μl aliquot of standard solution and 450 μl of heparinized plasma were vortex-mixed and then filtered through 0.22-μm nylon syringe filters prior to injection. All solutions were prepared daily from freshly thawed human plasma.

The non-plasma standards, used to determine the capacity of the SPS pre-column, were prepared in a similar way as the plasma standards except that 450 μl of water were added instead of plasma. The concentration of the internal standard and the final total volume were the same as for the plasma standards.

2.3. Chromatographic instrumentation and conditions

The HPLC system (Fig. 2) consisted of two pumps, pump 1 (LKB HPLC 2150, Bromma, Sweden) and pump 2 (Beckman 114M, Berkeley, CA, USA), equipped with SSI pulse dampeners. The column-switching device was a 10-port valve (Valco, Houston TX, USA). The pre-column was a semipermeable surface (SPS) guard cartridge, 5 μm particle size, 10 mm × 4.6 mm I.D. (Regis, Morton Grove, IL, USA). The analytical column 10 cm × 3.2 mm I.D. was obtained from Shandon Scientific (Chesire, UK) packed with 7 μm Hypercarb pH material. An inlet filter, with a 0.22-μm stainless-steel frit (Valco, Houston, TX, USA) was inserted before the Hypercarb column. The detector was a LDC 3100 variable wavelength UV monitor (Riviera Beach, FL, USA) registered at 254 nm.

A direct-injection HPLC system served to study the retention behaviour of the local anaesthetics on the analytical column and to evaluate the reliability of the column-switching system. The system consisted of the pump 2, the carbonaceous HPLC column and the LDC 3100 UV detector, which were the same as in the column-switching system, while a Rheodyne injector 7125 (Berkeley, CA, USA) equipped with a 10-μl loop substituted the 10-port valve.

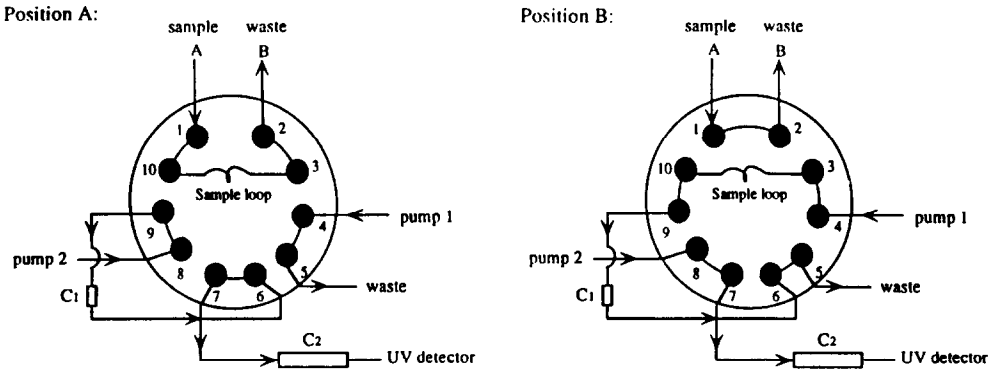

Fig. 2. Schematic diagram of the column-switching HPLC system. Position A: injection and full flush. Position B: loading. Pump 1: mobile phase A = 3% isopropanol in 0.01 *M* ACES buffer (pH 7). Pump 2: mobile phase B = 10 m*M* acetic acid and 4 m*M* triethylamine in methanol. C1: 10 × 4.6 mm I.D. SPS C_{18} column. C2: 100 × 3.2 mm I.D. Hypercarb column. Flow direction is indicated by arrows.

The strong mobile phase, mobile phase B, was 10 m*M* acetic acid and 4 m*M* triethylamine in methanol, and the weak mobile phase, mobile phase A, consisted of 3% isopropanol in 0.01 *M* ACES buffer adjusted to pH 7.0 by adding 5 *M* ammonia solution. Mobile phase A was filtered through a 0.45-μm Millipore membrane and degassed by a suction pump prior to use. Flow-rates were 0.5 ml/min for mobile phase B, and 0.8 ml/min for mobile phase A. Samples were introduced into the SPS pre-column by using an injection volume of 10 μl unless otherwise noted.

3. Results and discussion

3.1. Regulation of retention on Hypercarb

The mobile phase for the analytical column should have such properties that an adequate selectivity towards endogenous compounds is obtained; further, the peak performance should be acceptable and the time for analysis reasonably short. In addition, since the intention is to combine this LC-procedure with a final GC-step, the mobile phase must be compatible with the conditions used in GC and preferably consist of pure organic solvents.

The solid phase, Hypercarb, which consists of spherical particles of porous graphitic carbon, is more hydrophobic than other commercially available reversed-phase materials. Initial experiments using pure methanol as mobile phase gave tailing peaks with relatively long retention times. With 4 m*M* TEA in the mobile phase, the peak shape of the analytes was symmetrical. The retentions could then be regulated by adding acetic acid to the mobile phase (Fig. 3). The decrease in retention is probably due to ionization of the analytes, which gave decreasing binding of the amine compounds to the solid phase with increasing acetic acid concentration. The most suitable composition of the mobile phase was found to be 10 m*M* acetic acid in 4 m*M* triethylamine in methanol. This composition gives the best compromise between selectivity and assay time.

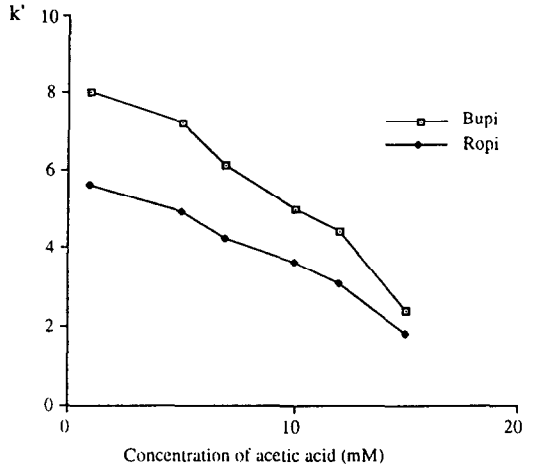

Fig. 3. Regulation of retention by varying the concentrations of acetic acid in methanol with 4 m*M* triethylamine.

3.2. Optimization of column-switching conditions

To apply a column-switching method, the following parameters need to be investigated: (1) conditions for suitable retention of the drugs on the pre-column; (2) an appropriate time period before switching the flow to the analytical column, giving a balance in flushing retained drugs from the SPS column to the carbonaceous column and effectively directing most of the proteins and hydrophilic endogenous compounds to waste; (3) matching the characters of the mobile and solid phases in the two columns in order to get efficient analyte enrichment on the analytical column after the transfer of eluate.

A loading condition test focusing on determination of the conditions for retention on the SPS pre-column was undertaken with a 0.1 m*M* mixture of the two drugs at different mobile phase compositions and different loading times. The peak areas and peak heights of ropivacaine and bupivacaine were calculated with the mobile phase 0.01 *M* ACES buffer pH 7 in 3 min loading as reference. Table 1 shows the results measured as relative deviations at the respective experimental conditions. The deviations of the peak areas of both analytes were $\leq 2\%$ either in different mobile phases or at different loading

Table 1
Loading condition test on SPS C_{18} column[a]

Mobile phase condition[b]	Relative deviations 3 min loading				5 min loading			
	AR[c]	AB[c]	HR[c]	HB[c]	AR	AB	HR	HB
(A) 3% Isopropanol	+0.02	−0.01	+0.07	+0.06	+0.02	−0.01	+0.07	+0.06
(B) 8% Isopropanol	+0.02	0	+0.08	+0.1	−0.1	−0.05	−0.03	−0.01
(C) 3% Methanol	+0.02	−0.01	+0.05	+0.03	−0.02	−0.01	+0.02	+0.02
(D) 8% Methanol	−0.01	+0.01	+0.05	+0.03	0	+0.02	+0.03	+0.02

[a] Fractional difference with calculations based on relative deviation in peak areas and heights compared with analytes in 0.01 *M* ACES buffer pH 7 in 3 min loading.
[b] Organic modifier in 0.01 *M* ACES buffer pH 7.
[c] AR = peak area of ropivacaine, AB = peak area of bupivacaine, HR = peak height of ropivacaine, HB = peak height of bupivacaine.

times; only when 8% isopropanol in 0.01 *M* ACES was used for 5 min loading, larger deviations were obtained. This meant that part of the analytes was lost to waste at such strong eluent strength. The peak heights were generally relatively higher than those of the reference runs which indicated that the addition of organic modifier could accelerate the elution rate of drugs from the SPS support and improve the peak performance.

The data indicated that the most suitable switching conditions were 3 min loading under the weak mobile phase, 3% isopropanol in 0.01 *M* ACES buffer pH 7, with a flow-rate of 0.8 ml/min before the valve switched to the strong mobile phase B. Ropivacaine, bupivacaine and the internal standard pentycaine were eluted from the small SPS column in half a minute using the strong mobile phase B, suggesting that only a short flushing time was required. Between 20–30 consecutive injections with 10 μl plasma could be made daily after which a washing process with the weak mobile phase at the flow-rate of 0.1 ml/min was undertaken overnight. No noticeable increase in back-pressure was observed for the analytical column, indicating that loading for 3 min corresponding to 2.4 ml of mobile phase A was sufficient to remove most of the plasma proteins. In comparison, it has been reported [29] that proteins eluted from a 5-cm long ISRP column in 2.5–3 ml volume of mobile phase.

3.3. Comparison of direct and switching injections

The volume of the fraction containing the analytes of interest was *ca.* 0.25 ml flushing with strong mobile phase. If such a relatively large fraction is transferred to the analytical column, the risk to introduce extra band broadening by the transfer process must be considered. The strategy to decrease this extra band broadening is to use different retention principles in the chromatographic systems. The mobile phase B used for transfer of the fraction from the SPS pre-column must have a stronger eluting power on the SPS column than on the analytical column so that an enrichment effect can be obtained in the injection zone. In the transfer process, mobile phase B will be mixed with mobile phase A decreasing its eluting strength. The effective injected volume (V_e) at the top of the analytical column can be estimated by the following formula

$$V_e = V_i/(1 + k')$$

where V_i is the sample volume transferred to the analytical column, k' is the capacity ratio of the analytes on the analytical column using a mixture of weak and strong mobile phase obtained during the switching process ($k' > 25$, based on the experimental results; see Table 2).

When k' is high, the extra band broadening

Table 2
Comparison of peak areas and heights in direct and switching injections

	AR[a]		AB[a]		HR[a]		HB[a]	
	direct	switch	direct	switch	direct	switch	direct	switch
Mean	1.40	1.44	1.34	1.30	4.68	4.26	3.57	3.24
C.V.	4.8	3.2	5.8	2.3	4.7	2.2	4.8	2.3
Deviation in peak height (%)						9.0		9.2

[a] AR = peak area of ropivacaine, AB = peak area of bupivacaine, HR = peak height of ropivacaine, HB = peak height of bupivacaine.

can be minimized. For this reason, it is essential to have a more hydrophobic solid phase (here porous graphitic carbon) in the analytical column than in the pre-column which results in a trace enrichment effect in the transfer process.

The maximum efficiency that could be obtained with the whole column-switching system was evaluated from 10 injections onto the column-switching system (Fig. 2) compared with 10 injections with a small volume (10 μl) directly onto the analytical column with a 0.1 m*M* mixture of ropivacaine and bupivacaine. The mobile phases of both systems and the switching conditions were described earlier. There was no significant difference in peak areas between the two injection methods (Table 2), only a slight extra band broadening was induced by the switching technique giving 9% lower peak heights. The results show that an effective enrichment of the analytes was obtained on the top of the analytical column when the analytes were flushed from the SPS pre-column to the Hypercarb column.

3.4. Plasma analysis with the column-switching system

The arrangement of the column-switching system is shown in Fig. 2. When a plasma sample was injected into the 10-μl sample loop, the switching valve was in position A. Then the switching valve was actuated to position B, the weak mobile phase carrying the sample through the SPS column, where the analytes were strongly retained; the proteins and hydrophilic endogenous compounds, however, were excluded and directed to waste. With the valve at this position, pump 2 introduced the strong mobile phase through the inlet filter and the analytical column, then through the UV detector to waste. After 3 min, the switching valve was actuated to position A, the strong mobile phase swept the retained analytes from the SPS support in a foreflush mode to the analytical column where separation was performed, and the compounds were detected by UV (at 254 nm). The valve switched back to position B after 2 min to allow the SPS column to re-equilibrate with weak mobile phase before the next injection.

Fig. 4 shows a typical chromatogram obtained from human plasma spiked with 6.6 μg/ml of ropivacaine and 7.6 μg/ml of bupivacaine, respectively. During the analysis of numerous plasma samples using this column-switching technique, no interference from endogenous plasma compounds was observed. The method performed well throughout the study including over 400 spiked human plasma sample injections.

3.5. Capacity test for SPS pre-column

In an effort to determine the influence of the concentration of spiked plasma samples on the SPS pre-column, a series of samples ranging more than fiftyfold in concentration was analyzed by using the column-switching system. Calculations were based on the calibration curves with pure eluent as the matrix (see Table

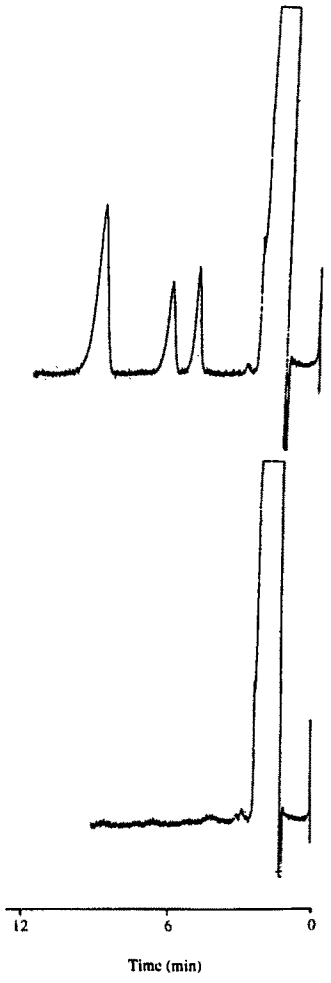

Fig. 4. (Top) Chromatogram obtained from analysis of (1) ropivacaine 6.6 μg/ml, (2) bupivacaine 7.6 μg/ml, and (3) internal standard pentycaine 21.4 μg/ml. (Bottom) Chromatogram obtained from blank plasma. The range of UV detection was 0.05 AUFS, other condition as in Experimental.

3). The recoveries of ropivacaine and bupivacaine are shown in Figs. 5 and 6, respectively. The figures clearly indicate that the SPS pre-column was almost unaffected by high concentrations of the solutes. The reasons for the relatively high recoveries, especially at low concentrations, have not been evaluated in detail. One reason might be the heterogeneity of the plasma matrix. The compounds are protein bound to >90%, which means that the drugs

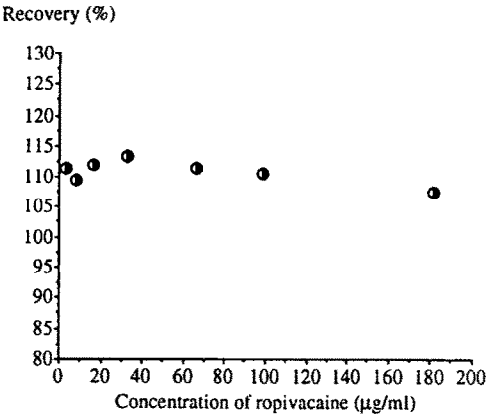

Fig. 5. Recovery of ropivacaine from spiked plasma as a function of sample concentration.

will be concentrated to the protein part of the samples. Another reason could be slight adsorption losses of the analytes from the standard solutions. Both effects should be more pronounced at low analyte concentrations; this is in accordance with the results.

Various volumes of spiked ropivacaine and bupivacaine plasma were also injected into the SPS pre-column. Percentage recoveries of ropivacaine and bupivacaine from plasma were quantified by comparing with calibration curves in eluent. It can be observed (Fig. 7) that the recoveries from the SPS pre-column were dependent on the plasma injection volume. This may be due to continuous plasma volume overload on

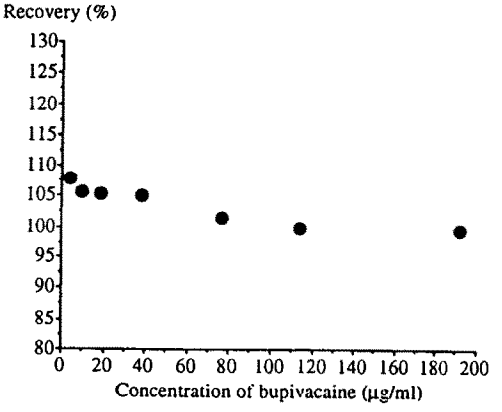

Fig. 6. Recovery of bupivacaine from spiked plasma as a function of sample concentration.

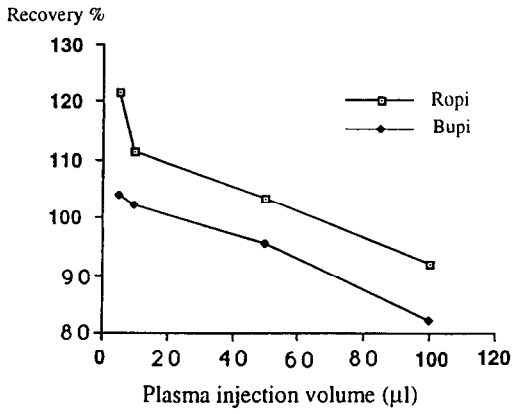

Fig. 7. Recoveries of ropivacaine (16.4 μg/ml) and bupivacain (19.0 μg/ml) as a function of sample volume.

the SPS support, resulting in the drug being eluted to waste bound to the proteins. It is suggested that a 10–50 μl injection volume loaded on the SPS pre-column can provide the desired sensitivity with acceptable recoveries.

The durability of the SPS guard cartridge was also studied. It was found that the pre-column could tolerate at least 200 direct injections of 10-μl plasma samples before replacement of the SPS guard cartridge was required. No influence on the performance of the analytical column was observed.

3.6. Linearity and sensitivity

Table 3 illustrates the calibration curves based on the peak-area ratios *versus* concentration over the range 1.6–120 μg/ml. Since the recoveries varied slightly with different types of media, calibration curves of ropivacaine and bupivacaine both in spiked plasma and in aqueous solution were prepared for different purposes. A linear response was obtained from 1.6–120 μg/ml and 1.9–200 μg/ml for ropivacaine and bupivacaine, respectively, both in plasma and in the eluent. Typical correlation coefficients ranged from 0.9990 to 0.9999.

The detection limit of ropivacaine was 8.2 ng at a signal-to-noise ratio of 3. When 50 μl is injected, this corresponds to *ca.* 0.2 μg/ml.

3.7. Recovery and precision

Recovery and precision of this method were determined by preparing spiked plasma samples and using calibration curves in plasma. The values of inter-day and intra-day recovery and precision are shown in Tables 4 and 5 for ropivacaine and bupivacaine, respectively. These data revealed that the drugs were completely

Table 3
Calibration curves[a]

Matrix	Concentration (μg/ml)	Equation	Correlation coeffcient
Ropivacaine			
1. Eluent[b]	1.64–6.56	$y = 0.0181x + 0.0022$	0.9999
2. Eluent	7.81–187.56	$y = 0.0091x - 0.0001$	0.9997
3. Plasma[b]	1.64–6.56	$y = 0.0162x + 0.0090$	0.9990
4. Plasma	6.05–120.98	$y = 0.0102x + 0.0065$	0.9998
Bupivacaine			
1. Eluent[b]	1.90–7.61	$y = 0.0160x + 0.0017$	0.9990
2. Eluent	8.61–206.58	$y = 0.0092x + 0.0040$	0.9996
3. Plasma[b]	1.90–7.61	$y = 0.0153x + 0.0042$	0.9999
4. Plasma	6.42–128.44	$y = 0.0093x + 0.0176$	0.9999

[a] Four different concentrations for curves 1 and 3 and eight different concentrations for curves 2 and 4 were constructed. Each concentration injected at least 3 times.
[b] Another but the same brand of detector has been used.

Table 4
The intra-day inter-day precisions and recoveries for ropivacaine

Spiked conc. (μg/ml)	Found conc. (μg/ml)	Recovery (%)	C.V. (%)	n
Intra-day				
3.28	3.34	101.9	1.8	6
16.40	17.01	103.7	2.7	6
32.78	32.30	98.5	2.6	6
98.37	98.25	99.9	2.4	6
Inter-day[a]				
3.28	3.34	101.8	4.2	18
16.40	16.81	102.5	3.6	18
32.78	32.50	99.2	3.4	18
98.37	95.25	96.8	4.2	18

[a]Over a period of twelve days.

recovered (96–103%) with coefficient of variations ranging from 1.6 to 5.2%.

4. Conclusions

The described coupled-column HPLC system to determine ropivacaine and bupivacaine in plasma is simple, rapid and reliable. The sample work-up consists only of plasma filtration and addition of internal standard and the total assay time is *ca.* 15 min per plasma sample. The system is easily maintained and gives quantitative recoveries with high precision provided that the pre-column is washed after *ca.* 30 injections and replaced after *ca.* 200 injections at regular intervals.

The SPS pre-column has a large capacity for injecting plasma volumes, ≤ 50 μl is recommended for the 10-mm cartridges. An essential feature of the method is the use of a carbonaceous analytical column, which is strongly hydrophobic, making it possible to transfer large volumes from the pre-column and still giving an

Table 5
The intra-day inter-day precisions and recoveries for bupivacaine

Spiked conc. (μg/ml)	Found conc. (μg/ml)	Recovery (%)	C.V. (%)	n
Intra-day				
3.81	3.73	98.0	2.4	6
19.03	18.75	98.5	2.0	6
38.01	38.11	100.3	1.8	6
114.18	112.52	98.5	1.6	6
Inter-day[a]				
3.81	3.75	98.4	2.6	18
19.03	18.75	98.5	1.8	18
38.01	38.75	102.0	2.2	18
114.18	109.18	95.6	5.2	18

[a]Over a period of twelve days.

adequate enrichment on the column-top eliminating extra-column band broadening effects.

The sensitivity obtained in this method may be appropriate for therapeutic drug monitoring (TDM) purposes. However, pharmacokinetic studies require lower detection limits. This may be achieved, as already indicated in the paper, by combining the LC-system with a GC-separation taking advantage of the more sensitive detectors available in this technique, *i.e.* AFID. However, the development of alternative LC-systems may also be possible: firstly, by using a lower wavelength for detection (*i.e.* 210 nm), which would require refinement of the selectivity of the present system; secondly, to construct a system that permits the introduction of larger injection volumes (≥ 100 μl), which would require a modified pre-column procedure. Work in these directions is in progress.

5. Acknowledgements

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