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Direct injection of large volumes of plasma in a column-switching system for the analysis of local anaesthetics I. Optimization of semi-permeable surface precolumns in the system and characterization of some interference peaks ☆

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Abstract

Possibilities for accomplishing direct injection of large volumes (500 μl) of plasma samples into a column-switching HPLC system were investigated. A new format of precolumn containing a semi-permeable surface (SPS) support (1 cm × 1 cm) was used for the sample clean-up and trace enrichment and was combined with a Kromasil C_{18} column for the final separation. A stable chromatographic system with respect to the separation selectivity and separation time was constructed and evaluated. The main parameters were the hydrophobicity of the SPS column, pH of the eluents, concentration of the organic modifier in the eluents and the detection wavelength. Two main interference peaks that were eluted in front of the ropivacaine peak were systematically characterized by varying the loading conditions for the SPS precolumn. The SPS column could tolerate large volumes ($\leq$500 μl) of plasma injections with a total volume of more than 50 ml. The developed system is stable, which permits the detection of 30 ng/ml ropivacaine in human plasma.

Keywords: Column switching; Sample handling; Ropivacaine; Bupivacaine

1. Introduction

The invention of internal surface reversed-phase (ISRP) packing materials for HPLC [1] was a great breakthrough aiming at determination of drugs by direct injection of biological fluids into liquid chromatographic system. A family of such packing materials [2–6] has, since then, been presented to the market. They are generally termed restricted-access media (RAM) [5], since the packings are designed in such a way that a hydrophilic barrier allows passage of only small molecules to the hydrophobic part of the stationary phase, while sterically restricting macromolecules, such as proteins, to interact with this part of the stationary phase. Thus, developed RAM materials permit the isolation and quantification of the small molecules from biological matrices directly without extensive sample clean-up. The use of RAM columns to analyze drugs in biological fluid has been successfully applied in the pharmaceutical and clinical chemistry fields [7,8]. There are, however, two main drawbacks. One is the inflexibility in altering the organic modifier or pH in the mobile

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phase, which is usually required during reversed-phase chromatography, due to the necessity of eluting proteins in the void volume of columns under non-denaturing conditions. The other is the relatively poor detection limit caused by the limitation of the introduced sample size (50–100 μl) [8,9] and lack of reconcentrating capability since the sample pretreatment is omitted. These inherences, especially the limitation of detection, prevent the application of RAM from direct determination of drugs in biological matrices in certain circumstances. Most publications in this area demonstrate quantitative results at only therapeutic levels by using UV detection (μg/ml) [10], although a detection limit of 25 ng/ml for some sulfonamide antibiotics has been reported at 265 nm [9]. The pharmacokinetic and biopharmaceutical studies, however, require the analytical method to assay drugs present in the biological sample at lower concentration levels (ng/ml).

The limitations can be overcome by applying a column-switching approach. An advantage of the column-switching technique is the superior detection limit capabilities based on the allowance for injection of large sample volumes into the precolumn. In addition, the option of using different favourable mobile phases for the exclusion of proteinaceous compounds and for the separation of analytes from the system, respectively, can readily be achieved. Column-switching systems, employing conventional reversed-phase materials as precolumns, have greatly contributed to the direct determination of drugs in biological fluids [8,11,12]. However, the lifetime of precolumns, in most applications, was limited to 20–30 ml of plasma or serum injections when a large sample volume (≥500 μl) was applied. In order to avoid the rapid development of back pressure, some special procedures, such as protein precipitations, sample dilutions prior to injection, extra purge steps between injections, were still necessary [13–16], which led to deviation from the principle of direct injection. The addressed problem is due to the fact that the column packing materials are not designed to tolerate direct injection of protein-containing matrices. Coupling a precolumn of RAM packing to a conventional reversed-phase analytical column would be a promising combination, since the RAM should deal with protein-containing samples more effectively. Applications on this subject have been published [6,17–26]. So far, only one report concerning the injection of 500 μl of plasma showed the longevity of a precolumn packed with a RAM material [23], where a fluorescence detector was used.

Previously, we developed a column-switching method using a SPS C_{18} guard column as the precolumn to directly analyze local anaesthetics (ropivacaine and bupivacaine) in human plasma. The obtained sensitivity (0.2 μg/ml) was appropriate for therapeutic drug monitoring but not for pharmacokinetic studies, the reason being that the capacity of the SPS cartridge limited plasma injections to 50 μl [22]. In response to the need for the higher sensitivity enhanced by introducing larger volume of biological sample into the column-switching system, the Regis company recently produced a 10 mm × 10 mm I.D. format of the SPS column suitable for the injection 1–2 ml plasma or serum [27].

The work described in this paper involves a column-switching HPLC system, where such a SPS precolumn, to retain analytes and exclude plasma proteins, is connected to a conventional reversed-phase C_{18} column for analyte separation. Our attempts were to improve the sensitivity for determination of ropivacaine and bupivacaine by increasing the plasma volume up to 500 μl and to explore the possibility of chromatographic performance on this new format of precolumn from the practical point of view, particularly when the low wavelength of ultraviolet detection was incorporated in the system. To our knowledge, there is no publication on this topic.

The presence of interferences which perturb the assay of the target compounds is a general problem in the bioanalytical field when direct injection of biological samples onto a column-switching system is applied. Such interferences can arise either from the column-switching system [28,29], or from the plasma endogenous compounds or from co-administered drugs to patients [30,31]. Two interference peaks, eluting just before the ropivacaine peak, were noticed during the method development. By changing the mobile phase conditions for the loading step, we were able to recognize the characters of these two interfering peaks.

2. Experimental

2.1. Reagents and materials

Ropivacaine and bupivacaine hydrochloride monohydrates were received as gifts from Astra Pain Control AB (Södertälje, Sweden). Methanol of both analytical and gradient grade, 2-propanol of gradient grade and 1 *M* 9956 Titrisol sodium hydroxide solution were supplied by Merck (Darmstadt, Germany). 1-Octanesulfonic acid, sodium salt 98%, was purchased from Janssen Chimica (Geel, Belgium). Water used in all experiments was deionized water purified with a Milli-Q purification system (Millipore, Bedford, MO, USA). Phosphoric acid solution (1 *M*) was prepared from ortho-phosphoric acid (99% crystal) (Merck, Darmstadt, Germany) and filtered through a 0.45-μm Millipore membrane filter. The phosphate buffer, pH 7.7, with ionic strength 0.05, was obtained by mixing 36.68 ml of 1 *M* phosphoric acid with 68.34 ml of 1 *M* sodium hydroxide solution followed by diluting to 2 l with water. Millex-AP/GS syringe filter units (0.22 μm) were purchased from Millipore (Millipore S.A., Molsheim, France) and used for plasma filtration.

2.2. Chromatographic instrumentation and conditions

The chromatographic system included two pumps, pump 1 (LKB HPLC 2150, Bromma, Sweden) and pump 2 (Beckman 114 M, Berkeley, CA, USA), both of them equipped with SSI pulse dampeners, a ten-port valve (Valco, Houston TX, USA) with a 500-μl loop (Valco Europe, Switzerland), a LDC 3100 variable wavelength UV monitor (Riviera Beach, FL, USA) registered at 240 nm and a recorder (Kipp and Zonen BD 112, Holland). The semi-permeable surface (SPS), C_8 material, 5 μm, 10 mm × 10 mm I.D. (Regis, Morton Grove, IL, USA) was used as the precolumn. The analytical column (100 mm × 4.6 mm I.D.) and the guard column (10 mm × 4.6 mm I.D.), all from Hichrom (Hichrom Limited, Berkshire, UK), were packed with 5 μm Kromasil C_{18} and C_8, respectively (Eka Nobel AB, Bohus, Sweden). An inlet filter, with a 0.22-μm stainless-steel frit (Valco, Houston TX, USA) was inserted in front of the Kromasil guard column to protect the separation column further.

Mobile phase 1, the weak mobile phase, consisted of 5% 2-propanol in phosphate buffer, pH 7.7 (I= 0.05). A flow rate of 1.5 ml/min through the SPS precolumn was used. Mobile phase 2, the strong mobile phase, contained 63% methanol in phosphate buffer, pH 7.7 (I=0.05); the flow-rate was maintained at 1.0 ml/min. For conditioning both columns were provided with a flow-rate of 0.1 ml/min with respective mobile phase overnight. Both mobile phases were mixed and degassed by an ultrasonic vibrator (Bransonic 220, Bo Philip, Stockholm, Sweden) prior to use. A 500-μl plasma sample was introduced into the SPS precolumn unless otherwise indicated.

2.3. Plasma sample preparation

Blank human plasma was pooled from five to ten different healthy volunteers collected at the Blood Centre, Academic Hospital, Uppsala, Sweden. The spiked plasma was made by vortex mixing the ropivacaine and bupivacaine standard solution (solved in phosphate buffer) to the pooled plasma with less than 10% dilution factors. All injected plasma samples were prepared daily from freshly thawed human plasma and drawn through 0.22-μm filter units prior to injection.

2.4. Column-switching procedure

Fig. 1 shows a schematic diagram of the chromatographic system set-up. The complete procedure was as follows.

Loading and injection of sample

The sample loop was filled with a plasma sample when the switching valve was at position A. The valve was then actuated to position B, the weak mobile phase carrying the sample through the loop to the precolumn, where the analytes were captured by the SPS column; the proteins and hydrophilic endogenous compounds, on the other hand, were excluded from the column to waste. During this loading step, the strong mobile phase passed the inlet filter, the

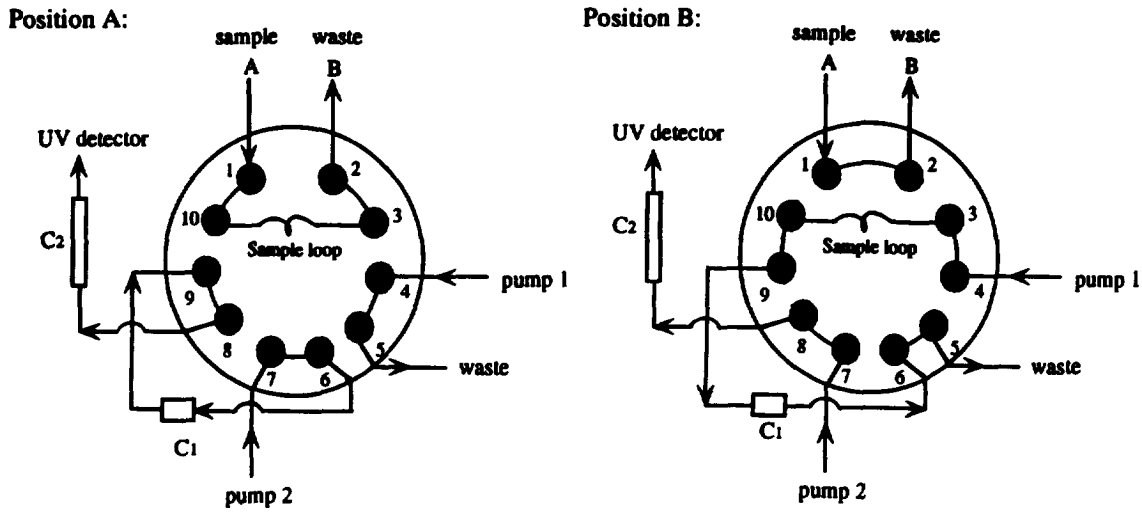

Fig. 1. Schematic diagram of the column-switching HPLC system. Position A: injection and back flush. Position B: loading. Pump 1: mobile phase 1, 5% 2-propanol in phosphate buffer, pH 7.7 (I=0.05); flow rate: 1.5 ml/min. Pump 2: mobile phase 2, 63% methanol in phosphate buffer, pH 7.7 (I=0.05); flow rate: 1.0 ml/min; C1: SPS C_8 column, 10 mm × 10 mm I.D., 5 μm; C2: Kromasil C_{18} column, 100 mm × 4.6 mm I.D., 5 μm; detection: 240 nm. Flow direction is indicated by arrows.

guard column, the analytical column and the UV detector.

Separation of analytes

After 10 min, the valve was switched back to position A, the retained analytes were swept by the strong mobile phase from the SPS column through the inlet filter and the guard column to the top of the analytical column in a back-flush mode. Two minutes later, the switching valve was changed back to position B to recondition the SPS column with the weak mobile phase to be ready for the next injection. In the meantime, the separation was performed by the strong mobile phase and detected by UV at 240 nm.

3. Results and discussion

In drug analysis of biological materials, the analytes of interest are often present at very low concentrations (μg/ml–ng/ml) whereas proteins and other potentially interfering compounds exhibit hundred- to thousand-fold higher levels. Therefore, to be able to directly inject plasma samples into a column-switching system, one is faced with the problem of how to select different mobile phases to meet hard requirements on selectivity. The weak mobile phase, which is used for loading plasma samples onto the SPS precolumn, should possess such properties that it can direct most of the proteins and hydrophilic endogenous compounds effectively to waste and simultaneously retain the analytes completely. The choice of the strong mobile phase applied for the final separation should depend on the possibility of regulating the retention on the analytical column and adjusting the separation selectivity within a reasonable assay time. Furthermore, compatibility of these two mobile phases involves the achievement of an enrichment effect on the top of the analytical column after switching and the minimization of the disturbance of system peaks on the separation performance. The composition of the weak mobile phase and the loading time are of vital importance to exclude proteins and endogenous compounds to waste, especially in the case where large volumes of plasma are introduced into the HPLC system directly. This will be discussed in a separate paper [32]. The selected condition for loading 500-μl plasma samples onto the SPS column was 5% 2-propanol in phosphate buffer, pH 7.7 (I=0.05), for 10 min at the flow-rate of 1.5 ml/min.

3.1. Retention properties of analytes and the enrichment effect in the system

Retention properties of analytes

Ropivacaine and bupivacaine are weak bases with pK_a 8.1, and the pH is a suitable parameter to regulate k', as demonstrated in Fig. 2. A variation of pH gave, as expected, no selectivity difference for the closely related compounds. The pH for analysis of these substances was chosen at the neutral region (here 7.7) for two reasons: firstly, the analytes gave an appropriate retention on the bonded silica C_{18} surface; secondly, such a pH was preferable to keep eventually retained plasma proteins on the precolumn in a native state. In addition, increasing concentrations of methanol were found to decrease the retention of analytes on the Kromasil column as anticipated (Fig. 3). Similarly, the logarithm of the capacity ratio of the analytes decreased linearly on the SPS C_8 column with increasing percentage of 2-propanol in the buffer (Fig. 4). By extrapolation, the capacity ratios of ropivacaine and bupivacaine using 5% 2-propanol in the phosphate buffer on this column were estimated as 86 and 316, corresponding to retention volumes of 43 and 158 ml, respectively, which clearly showed that there was no risk of losing analytes during the loading step (when a volume of 15 ml was used). Similar retention testing on the SPS C_8 column was also made by varying the methanol content in the buffer. The results demonstrated that either methanol or 2-propanol can be used as modifier to regulate retention on SPS C_8. The solvent strength of 2-propanol was, however, 3.6 times stronger than that of methanol. The selection of 5%

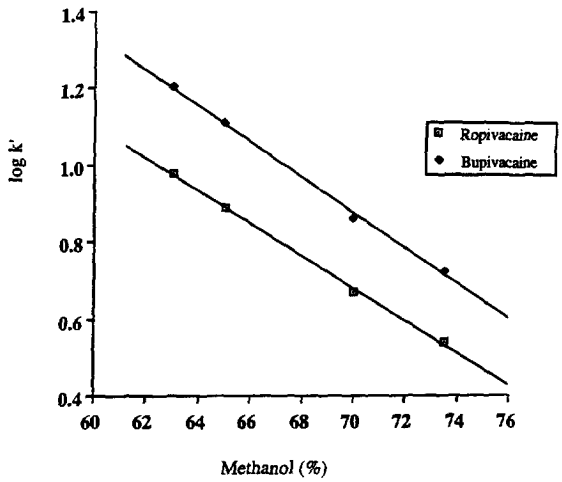

Fig. 3. Regulation of retention by varying methanol concentrations in phosphate buffer, pH 7.7 (I=0.05), on the Kromasil C_{18} column. Mobile phase: methanol in phosphate buffer, pH 7.7 (I=0.05); injection volume: 10 μl; detection: 210 nm.

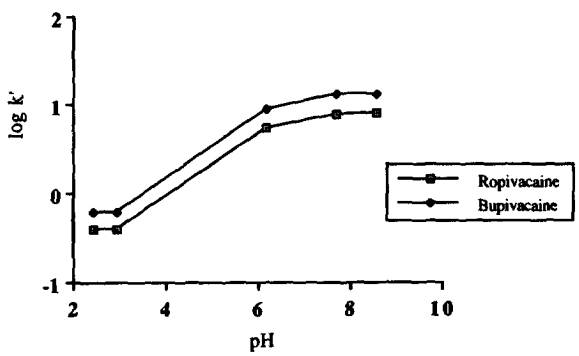

Fig. 2. Dependence of retention on pH. Simple LC system: Column: Kromasil C_{18}, 100 mm × 4.6 mm I.D., 5 μm; mobile phase: 65% methanol in phosphate buffer (I=0.05); injection volume: 10 μl; detection: 210 nm.

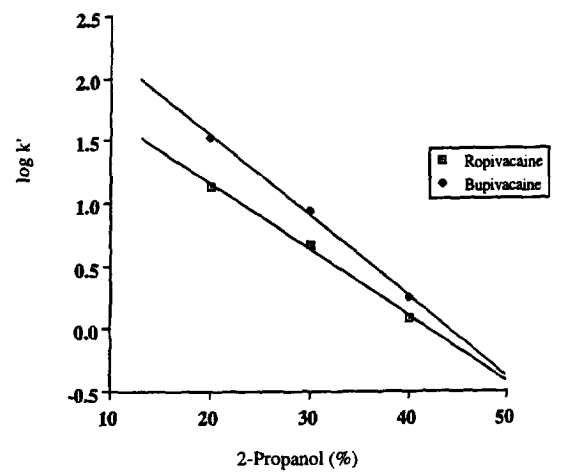

Fig. 4. Relationship between capacity ratios of the analytes and the content of 2-propanol in phosphate buffer, pH 7.7 (I=0.05), on the SPS C_8 column. Injection volume: 10 μl; detection: 210 nm.

2-propanol in the buffer was based on the fact that 2-propanol is known to bring about less protein denaturation and a better clean-up effect than methanol [32,33].

Enrichment effect

Since there is a considerable difference in capacity ratios of the analytes between the weak and strong mobile phases employed in the coupled column system, a significant enrichment effect would be expected. This effect might simply be calculated by the following formula [34]:

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

where V_e is the effective injected volume on the top of the analytical column, V_i is the sample volume transferred from the precolumn to the analytical column, and k' is the capacity ratio of the analyte in the analytical column by using the weak mobile phase.

Taking the observed values obtained from the retention profiles in Fig. 3 and Fig. 4 into account, with $V_i \leq 1.5$ ml (transferred fraction containing both analytes) and $k' = 1900$, resulted in $V_e \sim 1$ μl. The estimation was consistent with our observation by comparison of peak performance in the column-switching system with the simple LC system.

3.2. Optimization of the column-switching system

As mentioned in the introduction, the main goal of the study was to develop a quantitative method by injecting 500-μl plasma samples into the coupled column system. Initially, 65% methanol in the eluent was used with a detection wavelength of 210 nm. The detection of low concentrations (ng/ml level) could easily be reached by this system; the noisy baseline, however, made reliable quantification of analytes very difficult. Further optimization studies to create a stable system was needed.

Hydrophobicity of SPS surfaces

The trace enrichment effect in the column-switching system is, in general, non-selective. In other words, this effect also concentrates some interfering compounds. The use of a less retentive precolumn is advantageous from the standpoint of reducing the risk of interferences, as long as the analytes are sufficiently retained on the precolumn. The precolumn SPS C_8 gave less disturbance than SPS C_{18} in the blank chromatogram as illustrated in Fig. 5. Moreover, such a relatively hydrophilic surface is favourable to gaining an enrichment effect on the top of the analytical column by the transfer process, since the analytes are eluted in a small volume.

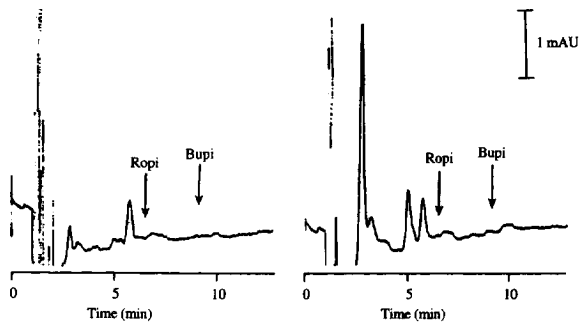

Fig. 5. Influence the plasma clean-up process with respect to the hydrophobicity of SPS columns. (Left) SPS C_8; (Right) SPS C_{18}. Mobile phase for the analytical column: 70% methanol in phosphate buffer, pH 7.7 (I=0.05); detection: 254 nm. Other conditions as in Experimental.

Detection wavelength

The choice of wavelength depends upon the UV absorption spectra of the analytes, the presence of interfering peaks and the noise level generated by the separation system. Table 1 summarizes the dependence of the detection limits for ropivacaine and bupivacaine on the applied wavelengths: the estimations were based on the peak height of ropivacaine with a signal-to-noise ratio of 3:1. With decreasing

Table 1
Dependence of detection wavelength on the determination of concentration of local anaesthetics. Column-switching system: Column 1: SPS C_8, 5 μm, 10 mm × 10 mm I.D.; eluent 1: 5% 2-propanol in phosphate buffer (I=0.05); loading volume: 15 ml. Column 2: Kromasil C_{18}, 5 μm, 100 mm × 4.6 mm I.D.; eluent: 65% methanol in phosphate buffer pH 7.7 (I=0.05). Other conditions as in Experimental

Wavelength (nm)	Limit of detection (ng/ml)
210	5–10
230	≈15
240	30
254	100

wavelength, the UV absorbance of the analytes increased; however, the baseline fluctuation was concomitantly increased. A compromise on the choice of wavelength was necessary; herein 240 nm was used.

Interference peaks

Two interference peaks eluting closely before the ropivacaine peak were observed in the course of sample analysis. It was initially suspected that the interferences were introduced by the filter units used for filtering plasma samples prior to injection. Different types of filter units purchased from Millipore or Waterman were studied. However, none of them offered any significant contribution to these two peaks. By decreasing the pH of the buffer from 7.7 to 6.7 for both mobile phases, no improvement on the selectivities between ropivacaine and interferences was observed. However, the retention behaviour of analytes and interference peaks could be regulated by the methanol modifier in phosphate buffer, pH 7.7, as shown in Fig. 6. Although all three peaks could be separated from each other with increasing methanol content in the buffer, the interesting ropivacaine peak was disturbed by the front due to short retention time. The optimal mobile phase for the analysis of ropivacaine and bupivacaine was 63% of methanol in phosphate buffer, pH 7.7.

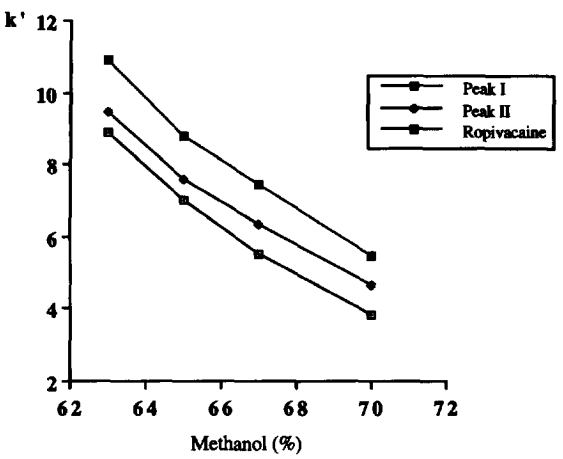

Fig. 6. Effect of methanol concentration on the separation selectivity of ropivacaine with interfering peaks. Plasma sample spiked with 68 ng/ml of ropivacaine. Other conditions as in Experimental.

3.3. Stability of the column-switching system

Stability of the analytical column towards plasma injections

The effect of multiple direct injections of large plasma volumes on the performance of the applied C_{18} column was investigated. The analytical column was checked regularly via a simple LC system with 10-μl injections of the standard analyte solutions. Table 2 shows the results of this procedure. After 45 ml of plasma injections there was only a slight change in capacity factors, while other chromatographic properties such as column efficiency, selectivity and flow resistance (not shown) remained constant.

Lifetime of the SPS column

The behaviour of the SPS packing material together with another type of RAM material, alkyl-diol silica, with regard to plasma loadings will be discussed in detail in a forthcoming paper [35]. In summary, the studies showed that over 50 ml of plasma (refer to the injection volume of 500 μl) could be loaded on the SPS column. The only precaution taken was to keep the column in the weak eluent at a low flow-rate (0.1 ml/min) when the daily work was finished.

Table 2
Influence of multiple plasma injections on chromatographic performance of the Kromasil column. Simple LC system: Column: Kromasil C_{18}, 5 μm, 100 mm × 4.6 mm I.D.; eluent: 65% methanol in phosphate buffer, pH 7.7 (I=0.05); flow-rate: 1.0 ml/min; detection: 210 nm, 0.01 AUFS; sample: 1.22 μg/ml of ropivacaine and 3.92 μg/ml of bupivacaine solved in the eluent; injection: 10 μl

Plasma volume	Ropivacaine		Bupivacaine		α
(ml)	k'	H (μm)	k'	H (μm)	
0	7.85	22.7	12.90	21.5	1.64
8.5	7.81	23.1	12.88	21.2	1.65
15.0	7.75	23.0	12.75	21.1	1.64
21.0	7.70	22.8	12.67	21.8	1.64
31.5	7.68	23.0	12.63	21.4	1.64
40.0	7.61	23.4	12.44	21.4	1.63
45.0	7.57	23.1	12.40	21.3	1.64

Repeatability

The stability of the column-switching system was tested by evaluating the repeatability of plasma samples spiked with 105 ng/ml of ropivacaine and 1.33 μg/ml of bupivacaine, respectively. Absolute recoveries (from plasma compared with standard solutions) were close to 100% as described in more detail elsewhere [36]. The coefficients of variation ranged from 2.8 to 4.3%, based on the calculation of peak areas of ropivacaine and bupivacaine with ten sequential injections in the system. Fig. 7 shows a representative chromatogram. Owing to the use of different wavelengths, the appearance of interfering peaks in Fig. 7 was different from the left chromatogram of Fig. 5.

With 100 injections of plasma samples using this column-switching technique, no replacement of the analytical column and the guard column were needed. The on-line inlet filter, on the other hand, was renewed every 20–30 injections when the pressure typically elevated by 2 bars.

3.4. Characterization of interference peaks

In order to get a better comprehension of the developed system, it was our purpose to recognize and characterize interference peaks I and II which possessed retentions close to ropivacaine as demonstrated in Fig. 7. However, the selectivities between the ropivacaine and these peaks could be regulated by the methanol content in the eluent as discussed above.

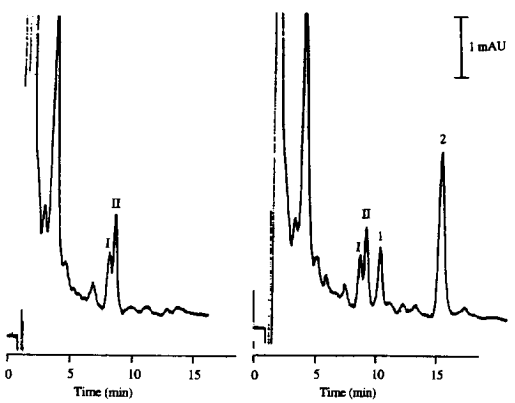

Fig. 7. Analysis of ropivacaine and bupivacaine in plasma. (Left) blank chromatogram obtained from pooled plasma; (Right) chromatogram obtained from spiked plasma of (1) ropivacaine 105 ng/ml, (2) bupivacaine 1.33 μg/ml. For conditions see Experimental.

Identifying the origin of interference peaks

It was quite straightforward to find the sources of peaks I and II. Peak II originated from the plasma matrix. Different batches of plasma from pooled or individual donors have been checked and all gave rise to this peak. Peak I arose from the column-switching process which occurred even by loading the weak mobile phase. The nature of this peak was studied by varying the conditions for the loading procedure on the column-switching system.

Different loading compositions

Similar chromatograms were obtained when 5% 2-propanol either in water or in the buffer were used for the loading step. This illustrated that peak I was not caused by impurities from the buffer.

Different loading volumes

Fig. 8 illustrates the chromatograms obtained from different loading volumes. The peak height increased when extending the loading volumes up to 22 ml; after that the peak remained unchanged. This implied that peak I could be induced by either methanol or 2-propanol.

Different amounts of methanol in the loading mobile phase

If the content of the organic modifier was adjusted more closely to the strong mobile phase used for the analytical column, this interference peak was diminished and finally eliminated as shown in Fig. 9. No doubt, peak I derived from the deviation of the methanol content in the two eluents.

So far, it can be concluded that peak I was a system peak generated by a detectable impurity in methanol. When the valve was located at position B, two different equilibria were established along the precolumn and the analytical column with different mobile phases. When the plug containing the weak mobile phase was transferred to the analytical column, the equilibrium on the top of the analytical column was disturbed and the system peak then appeared. A system peak is defined as a migration

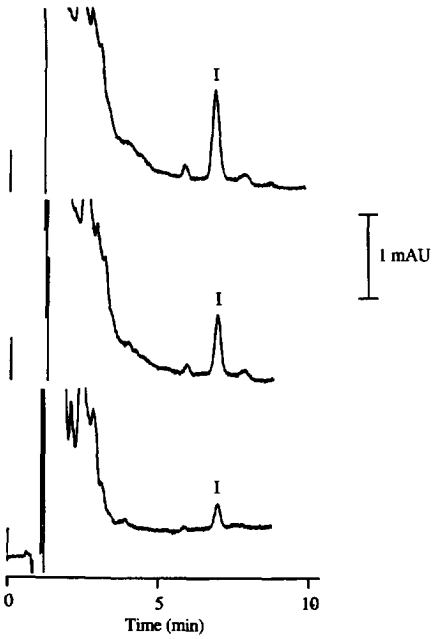

Fig. 8. Effect of loading volumes on the peak performance of the interference peak I. (Top) loading with over 22 ml; (Middle) loading with 18 ml; (Bottom) loading with 6 ml. Mobile phase for the analytical column: 65% methanol in phosphate buffer, pH 7.7 (I=0.05). No sample injection was made. Other conditions as described in Experimental.

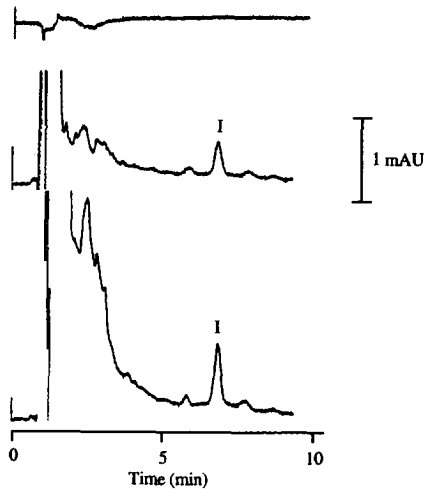

Fig. 9. Effect of loading composition on the peak performance of the interference peak I. (Top) loading with 65% methanol in the buffer; (Middle) loading with 20% methanol in the buffer; (Bottom) loading with 5% 2-propanol in the buffer. Mobile phase for the analytical column: 65% methanol in phosphate buffer, pH 7.7 (I=0.05); loading volume: 18 ml. No sample injection was made. Other conditions as described in Experimental.

zone with a deviating concentration from that of the mobile phase. Similar findings have been reported by Arvidsson [28]. However, no pronounced improvement on the system peak was obtained by changing the quality of methanol from analytical grade to the gradient one, which was suggested as a remedy in that paper.

Identifying the character of interference peaks

Alternating pH in the loading mobile phase

In order to investigate the characters of the two interfering compounds, pH of the aqueous plugs was varied from acidic to basic range by using phosphate buffers. The retention data (Table 3) indicated that the system peak, peak I, has weak acidic property since the retention increased with decreasing pH in the eluent. Peak II seemed to have a neutral character.

Addition of anion to the loading eluent

Studies on the retentions of peaks I and II were also carried out by varying the concentrations of octanesulfonic acid at different pH values in the weak mobile phase. The results are given in Table 4.

Table 3
Regulation of retention of interference peaks by varying pH in the loading eluent. Column-switching system: Column 1: SPS C_8, 5 μm, 10 mm × 10 mm I.D.; eluent 1: 5% 2-propanol in phosphate buffer (I=0.05); loading volume: 18 ml. Column 2: Kromasil C_{18}, 5 μm, 100 mm × 4.6 mm I.D.; eluent: 65% methanol in phosphate buffer pH 7.7 (I=0.05)

Plug pH	Peak I[a]		Peak II[b]
	t_R(min)	Δt_R^c	t_R (min)
2.4	6.8	+0.3	6.8
7.7	6.5	0	6.8
8.6	6.3	−0.3	6.8

[a]Peak I was created by loading the SPS column with the eluent.
[b]Peak II was obtained from injecting 500 μl pooled plasma.
[c]The retention of 5% 2-propanol in phosphate buffer, pH 7.7, was used as the reference.

Table 4
Effect on addition of anion in the loading eluent. All conditions are as given in Table 3

Plug composition	Peak I[a] t_R (min)	Peak II[b] t_R (min)
pH 7.7	6.5	6.8
0.5 m*M* OS[c] in pH 7.7	6.3	6.8
pH 2.4	6.8	6.8
0.5 m*M* OS[c] in pH 7.7	6.8	6.8
2 m*M* OS[c] in pH 7.7	6.8	6.8

[a] and [b] are the same as in Table 3.
[c] OS denotes 1-octanesulfonic acid.

With octanesulfonic acid at pH 7.7, the retention of peak I decreased slightly, while it was unaffected at acidic pH. This confirmed that peak I was acidic and its anion competed with octanesulfonic acid at pH 7.7, leading to a decrease in retention, whereas at lower pH, peak I was completely uncharged and therefore no effect on its retention was expected. The results also supported the hypothesis that peak II was generated from a neutral compound, since its retention was unaffected by octanesulfonate.

4. Conclusions

In the present study, the utility of the new format of the SPS precolumn in the column-switching system was evaluated by direct injection of 500-μl plasma samples for analysis of local anaesthetics. The system was stable with an estimated detection limit of 30 ng/ml. Suitable eluents chosen for the system permitted the analytes to elute within 15 min when 500 μl plasma were injected directly.

The enhancement of the detection sensitivity by increasing the volume of plasma introduced onto this format of SPS column was technically feasible; the choice of UV wavelength, however, needed to be compromised because of baseline noise in the system; 240 nm was applied. The SPS precolumn could tolerate the injection of $\geq$50 ml total plasma volumes without the need of special protection.

The characters of two main potentially interfering peaks were determined by varying the conditions for the loading procedure. A neutral compound originated from plasma, while the other with acidic character seemed to be an impurity from the mobile phase component, methanol.

In view of the results obtained, the method is of importance for bioanalysis with direct injection of plasma in the column-switching system. Further efforts in improving the detection limit by using a wavelength of 210 nm will be described in a subsequent paper [36].

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