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Direct injection of large volumes of plasma in a column-switching system for the analysis of local anaesthetics II. Determination of bupivacaine in human plasma with an alkyl-diol silica precolumn☆

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Abstract

A column-switching high-performance liquid chromatographic system was applied for the determination of bupivacaine in plasma. A 500-μl plasma sample was directly introduced onto a C_{18}-alkyl-diol silica (ADS) precolumn separating analytes from proteins and polar endogenous compounds. The fraction containing bupivacaine and ropivacaine (internal standard) was back-flushed and transferred to a conventional reversed-phase column (Kromasil C_{18}) for final separation. A single ADS precolumn could withstand more than 50 ml of plasma injections without changing analytical performance. Quantitative studies showed a broad range of linearity (0.033–3.31 μg/ml) and high recovery (95–99.9%) with coefficients of variation less than 3.1%. The advantages of the ADS material are its high capability of sample clean-up, due to rapid elution of plasma proteins and endogenous compounds to waste, and its ability to elicit a stable baseline. As a result, UV detection could be performed at 210 nm and clean chromatograms with baseline separation for desired peaks were obtained within 15 min. The detection limit of this system was 10 ng/ml defined by a signal-to-noise ratio of 3:1. The concentration of bupivacaine in patients determined by this method agreed well with the values obtained from an alternative method, making the technique applicable for pharmacokinetic studies in humans.

Keywords: Column switching; Bupivacaine; Ropivacaine

1. Introduction

Bupivacaine, an amide-type local anaesthetic, is widely used in clinical practice. Previous studies have demonstrated that the systemic toxicity mainly involves the central nervous system and the cardiovascular system and these adverse effects vary with the concentration of bupivacaine in plasma [1,2]. It is, therefore, essential to determine pharmacokinetic profiles, which require highly sensitive methods with a detection limit at the ng/ml level.

Recently, high-performance liquid chromatographic determinations of bupivacaine, including its enantiomers and metabolites, in biological samples have been reported [3–11]. Most of these methods, however, employed conventional sample preparation procedures, such as liquid–liquid or solid phase extraction to remove proteins prior to injection. Problems such as complicated manipulations, low

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extraction yields and/or long analysis time are generally associated with these procedures.

Direct injection of plasma samples into liquid chromatographic systems based on column-switching techniques have been developed to simplify sample preparation procedures, to reduce the time required and to increase the precision of the assay. Butter *et al.* [9] have described an advanced coupled three-column system to analyze bupivacaine enantiomers in serum. The precolumn packed with conventional silica material was, however, unstable with a relatively short life span (<20 ml of serum), although a sophisticated mixing coil functioning to dilute the serum sample was inserted in the system. Based on our developed column-switching system for analyzing local anaesthetics in human plasma using a SPS C_{18} guard cartridge as the precolumn [6], we investigated the possibility of directly introducing large volumes (i.e., 500 μl) into a modified SPS C_8 precolumn (10 mm×10 mm I.D.) to enhance the detection sensitivity, as has been discussed previously [12]. This paper will focus on the application of a related system for determination of bupivacaine in human plasma, and ropivacaine, a recently introduced local anaesthetic, was chosen as the internal standard.

The precolumn packing used in this work was based on alkyl-diol silica (ADS) [13–15], a family of restricted-access materials (RAM), which consist of glycerylpropyl-(diol-) groups as the hydrophilic electroneutral phase and *n*-alkyl esters (C_4, C_8 and C_{18}) as the hydrophobic phase. Attempts were made to apply 210 nm UV detection using SPS C_8 as the precolumn in the former system to further increase the sensitivity, but this turned out to be unsuccessful because of noise and fluctuating baseline [12]. Use of the ADS C_{18} column, however, results in a clean chromatogram with a stable baseline, making this detection wavelength possible. The ADS C_{18} precolumn was coupled to an ODS bonded reversed-phase column via a 10-port switching valve in the back-flush mode. The different clean-up effects obtained from the SPS C_8 and ADS C_{18} columns will be discussed with respect to the physical properties of these packing materials. Data from quantitative studies and the application for analyzing human plasma samples using this system will be presented in this paper.

2. Experimental

2.1. Reagents and materials

These were described previously [12].

2.2. Chromatographic instrumentation and column-switching procedure

These were as described earlier [12] except that the UV wavelength was set at 210 nm and that the analytical column (Kromasil C_{18}, 5 μm, 100 mm× 4.6 mm I.D.) was maintained at 30°C using a thermostated water bath. A dry packed C_{18}-alkyl-diol silica, 25 μm, 25 mm×4 mm I.D., sealed with 1 μm sieves (Dr. Boos, University of Munich, Germany), was used as the precolumn.

The plasma loading time was 7 min instead of the 10 min used in the former paper [12]. The injection volume was 500 μl unless otherwise indicated.

2.3. Mobile phase conditions

Mobile phase 1, the weak mobile phase used for loading plasma samples onto the ADS precolumn was 5% isopropanol in phosphate buffer, pH 7.4 (I=0.05), with a flow-rate of 1.5 ml/min. Mobile phase 2, the strong mobile phase consisted of 63% methanol in phosphate buffer, pH 7.4 (I=0.05). The flow-rate was kept at 1.0 ml/min.

2.4. Preparation of spiked plasma and standard samples

Ropivacaine and bupivacaine were dissolved in phosphate buffer pH 7.4 (I=0.05) to give a concentration of 2 mg/ml as stock solutions. Standard solutions of bupivacaine were obtained by diluting the stock solution to the desired concentration range in the buffer and containing 5.90 μg/ml of ropivacaine as the internal standard. Spiked plasma and standard samples for preparing calibration curves were made by vortex mixing 10% (in volume) of each standard solution with either pooled blank plasma or the weak mobile phase. All plasma samples were prepared daily from freshly thawed human plasma and filtered through 0.22-μm Millex-AP/GS syringe filter units prior to injection.

2.5. Sample preparation of patient/volunteer plasma samples

Frozen human plasma samples containing bupivacaine (Astra Pain Control AB, Södertälje, Sweden) were received and stored at −20°C until analysis. Plasma samples were thawed, pipetted into conical vials and vortex-mixed with the internal standard and using the same dilution factors as used for the calibration curve. It was necessary to centrifuge samples for 5 min at 7000 rpm (3000 *g*) for separating coagulated proteins from the plasma matrix, since the samples had been subjected to many freeze-thaw cycles and stored for a long time. The samples were then passed through the 0.22-μm Millex-AP/GS syringe filter units before being injected into the HPLC system (Note: it is probably unnecessary to include this step if a high speed centrifuge is available).

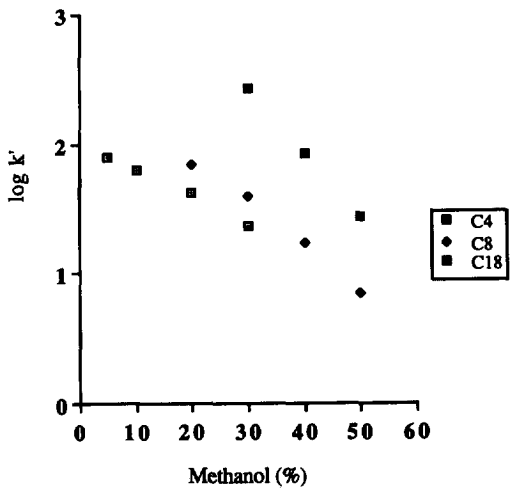

Fig. 1. Effect of methanol content on the retention of bupivacaine on ADS columns with different *n*-alkyl esters on the internal surfaces. Simple LC system: column size: 25 mm×4 mm I.D. 25 μm; mobile phase: methanol in phosphate buffer, pH 7.4 (I= 0.05); injection volume: 10 μl; detection: UV 210 nm.

3. Results and discussion

3.1. Precolumn selection

Ropivacaine and bupivacaine standard solutions were injected into C_4, C_8 and C_{18} ADS precolumns with different percentages of methanol in the phosphate buffer as the mobile phases, in a simple LC system to test the hydrophobicities of these internal surfaces. Fig. 1 shows the dependence of the capacity ratios of bupivacaine on the methanol content with different solid phases. The difference in k' between the C_8 and C_4 surfaces is about 2-fold, while the C_{18} phase gives 4–8 times higher retention than the C_8 phase; the difference decreasing with increasing methanol content. However, the capacity ratios for the analytes obtained from the ADS column were about half those obtained from the SPS column, comparing surfaces with the same alkyl chain length using 10% methanol in the buffer as the mobile phase. Since the ADS columns had smaller void volume (<0.2 ml), due to larger particle size, the break-through volume of ropivacaine on the ADS C_8 was about 14 ml calculated from 10% methanol in the buffer, which could cause premature elution of ropivacaine during the loading procedure. Hence, ADS C_{18}, which gave 6–7 times higher capacity ratios of analytes compared with SPS C_8 applied in [12], seemed to be the most suitable surface to sufficiently adsorb analytes. The loading mobile phase was 5% isopropanol in phosphate buffer, pH 7.4, and the mobile phase used to transfer analytes to the second column for the separation was 63% methanol in the same buffer.

3.2. Comparison of ADS and SPS precolumn for sample clean-up

To evaluate the effect of the ADS precolumn on sample clean-up, blank plasma was injected into the column-switching system using ADS as the precolumn. The system with ADS C_{18} column (Fig. 2), provided a cleaner and less disturbed chromatogram with a more stable baseline, compared with a SPS C_8 column (Fig. 7 in [12]). Further, after introducing 500 μl of plasma into the ADS C_{18} column, only 10 ml of weak mobile phase was needed for the washing procedure compared to 15 ml for the SPS C_8 column. These observations revealed that, in contrast with its higher retentivity of the analytes on the inner phase, the ADS had less retentivity and better retention selectivity towards the endogenous compounds and other unwanted substances in the

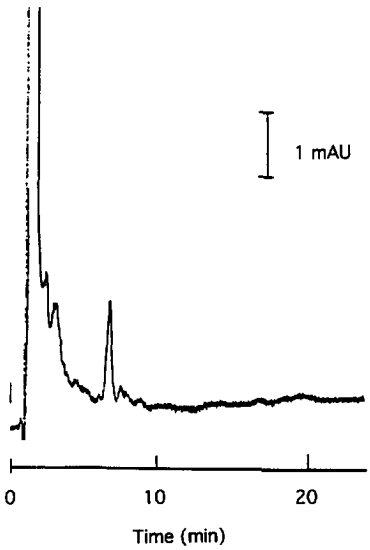

Fig. 2. Blank chromatogram obtained by using ADS C_{18} as the precolumn using a column-switching system. Sample: pooled plasma from the same batch as reference [12]; detection: UV 240 nm. Other conditions as described in Section 2.

plasma matrix. The physical properties of the two column materials [13,16,17] are listed in Table 1. The ADS phase is generally expected to give less retention, because of the lower surface area. Packing materials synthesized from different silicas and prepared by different methods can have significant differences in retention character and retention selectivity. Furthermore, the hydrophilic surface of ADS packing is the small glycerylpropyl-(diol-) group, which seems to retain interferences present in plasma to a lesser extent than the shielding polyoxyethylene polymer on the SPS phase. In addition, the more compatible plug size transferring from the ADS to the separation column may have the additional benefit of stabilizing the baseline. This is probably another reason for successful use of the low wavelength of 210 nm for direct determination of bupivacaine in plasma.

Table 1
Physical properties of the ADS and SPS packing materials

	ADS	SPS
Basic material	LiChrospher Silica	Rexchrom Silica
Inner phase	C_{18}	C_8
Outer phase	$-CH(OH)CH_2OH$	$[-O-CH_2-CH_2-O-]_n$
Surface area* (m^2/g)	174	210
Pore volume (ml/g)	>0.80	0.52
Pore diameter (nm)	5–6	10
Particle size (μm)	25	5
Column size (mm)	25×4	10×10

* Modified material

3.3. Temperature effect

As a wavelength of 210 nm was used for detection, it was necessary to control the separation performance at constant temperature to increase the stability of the system. It was found that temperature influenced the resolution between the ropivacaine peak and an interference peak. Increasing temperature gave rise to preferential separation of ropivacaine from the interference as shown in Fig. 3. However, a column temperature of 30°C was used in the present work as a higher temperature (32°C) brought about more detection noise.

3.4. Analysis of ropivacaine and bupivacaine in plasma

Fig. 4 shows representative chromatograms obtained from analysis of bupivacaine in different media, with ropivacaine as the internal standard. Comparing the chromatogram, where 590 ng/ml of ropivacaine and 33.1 ng/ml of bupivacaine were dissolved in the eluent (Top), with the chromatogram obtained from the same amount of spiked analytes in

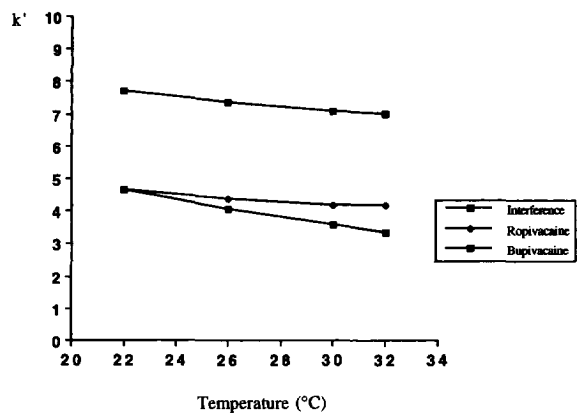

Fig. 3. The separation selectivity between ropivacaine and the interfering peak as a function of temperature. Column-switching system: conditions as described in Section 2.

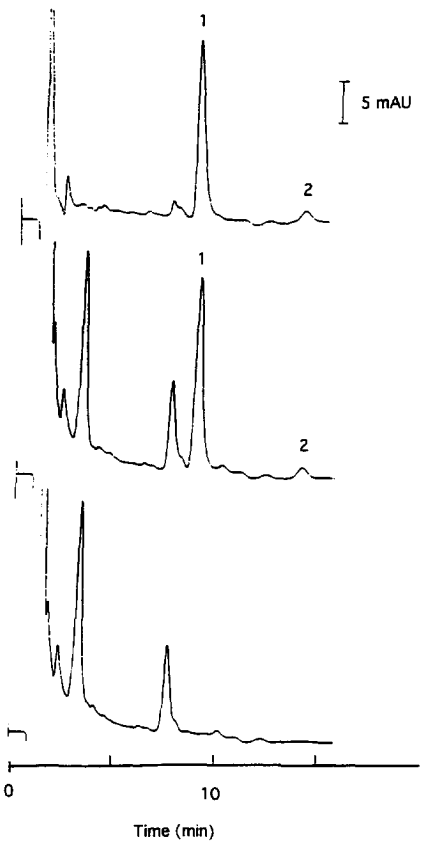

Fig. 4. Determination of bupivacaine in human plasma. (Top) Chromatogram obtained from (1) 590 ng/ml of ropivacaine and (2) 33.1 ng/ml of bupivacaine dissolved in the eluent. (Middle) chromatogram obtained from spiking (1) 590 ng/ml of ropivacaine and (2) 33.1 ng/ml of bupivacaine in pooled plasma. (Bottom) blank chromatogram obtained from pooled plasma.

plasma (Middle), the clean-up effect from plasma by the ADS column and the enrichment effect in the system are obvious. The detection limit of bupivacaine was 10 ng/ml defined by a signal-to-noise ratio of 3:1. The developed method can also be applied to determine ropivacaine with bupivacaine as the internal standard. The system performed well throughout the whole study including a total of 45 ml of plasma injections. The ADS precolumn was found to have a long life span, i.e. the pressure was raised only 15 extra bars after 90 injections. Flushing with ~30 ml of water and ~30 ml of methanol was usually conducted after daily work. The performance of the analytical column was checked regularly as previously described [12]. The results illustrated that there was no influence of large volumes of plasma injections on the chromatographic performance of the analytical column. However, similar to the previous system [12], the on-line inlet filter was replaced every 20–30 injections.

3.5. Calibration curves

The calibration curve of bupivacaine with spiked plasma in the range of 0.033–3.310 μg/ml gave good linearity based on the calculations of either the peak-area ratios or the peak-height ratios versus concentrations (Table 2). In order to calculate the absolute recovery from plasma, a calibration curve of bupivacaine in the standard solution was also made. A plot of peak-area ratios in plasma versus peak area ratios in eluent gives a straight line (linear regression $y=3.5710^{-2}+0.987x$, $r^2=0.998$), which clearly demonstrates that the method determines the total concentration of the drug under the designed conditions irrespective of high protein-binding (>90%) of the analyte in plasma and an injection volume of 500 μl.

3.6. Reproducibility

Recovery and precision of this method were measured by spiking plasma at the low and middle concentration levels. The peak area ratios were used for the calculations. Table 3 summarizes these data. Recoveries ranging from 95% to 99.9% for intra- and inter-day assays were obtained with coefficients of variation of less than 3.1%.

3.7. Determination of bupivacaine in human plasma samples

The accuracy of this technique was examined by determining bupivacaine concentrations in human

Table 2
Calibration curves[a]

Concentration (μg/ml)	Calculation	Linear regression	Correlation coefficient
0.033–3.31	A_S/A_I^b	$y=1.01710^{-2}+2.514x$	0.996
	H_S/H_I^c	$y=1.35110^{-2}+1.917x$	0.999

[a] Six different concentrations were analyzed with 3 injections at each concentration.[b] Peak-area ratios.[c] Peak-height ratios.

Table 3
Reproducibility

Spiked conc. (μg/ml)	Determined conc. (μg/ml)	Recovery (%)	C.V. (%)	Number
Intra-day				
0.116	0.110	95.0	2.5	6
1.327	1.315	99.0	0.6	6
Inter-day[a]				
0.116	0.111	95.9	3.1	12
1.327	1.337	99.9	1.6	12

[a] Over a period of six days.

plasma samples that had been assayed earlier by an alternative method at the laboratory of Astra Pain Control AB. Though the samples had been stored for more than four years and had gone through many freeze-thaw cycles, it was still possible to quantify the concentrations of bupivacaine based on the chromatograms. Table 4 lists the results which agree well with those values reported earlier.

4. Conclusions

The applicability of enhancing detection sensitivity by increasing injected plasma volumes, together with UV detection at 210 nm, was demonstrated by determination of bupivacaine in human plasma using the novel restricted-access precolumn packing, ADS, in a column-switching system. The sample preparation was minimized and required only the addition of the internal standard and the filtering (or centrifugation at high speed) of the plasma. The chromatographic system was stable, reliable and allowed the detection of 10 ng/ml of bupivacaine in plasma. Quantitative studies showed high recovery and precision. Bupivacaine in human plasma was successfully determined by this method. The developed system can be applied not only to determine bupivacaine in human plasma using ropivacaine as the internal standard or *vice versa*, but probably also to assay other drugs with similar chemical characteristics in biological fluids.

Like the SPS column, the ADS column exhibits a long life span, that is, more than 50 ml of undiluted plasma could be readily loaded on one precolumn, which shows that RAM type of materials can be very useful in the bioanalysis of drugs.

Table 4
Determination of bupivacaine in patients

Patient No.	Reported conc. (μM)[a]	Determined conc. (μM)[b]
1	0.27	0.28
1	0.30	0.33
2	0.26	0.25
3	0.10	0.08
3	0.35	0.40

[a] Astra Pain Control AB, Södertälje, Sweden.[b] Calculations based on peak area ratios.

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