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Determination of enantiomers of bupivacaine in serum using an on-line coupled three column liquid chromatographic system*

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Abstract: An on-line coupled HPLC system is described for the determination of the enantiomers of bupivacaine in serum. The method involves three steps: (i) pre-concentration and clean-up; (ii) the determination of the racemates on a reversed-phase column; and (iii) the separation of the racemates, heart-cut from the reversed-phase column, on an α-glycoprotein column. The method is suitable to determine the enantiomers in serum down to 0.1 $\mu g\ ml^{-1}$ and can be fully automated. The bupivacaine time course of patients will be shown.

Keywords: *Bupivacaine; enantiomers; liquid chromatography; on-line coupled; serum; α-glycoprotein column.*

Introduction

Bupivacaine is a local anaesthetic and nowadays frequently used for lumbar epidural anaesthesia. The compound has a chiral centre and is clinically administered as the racemate. The enantiomers are equally active as nerve blockers but the $R(+)$ form is more toxic than the $S(-)$ form [1]. It has been found that the enantiomers distribute unequally in the body after subcutaneous or intravenous administration with the $S(-)$ form having a longer duration of anaesthesia [2]. The origin of the difference in distribution of the enantiomers is not yet clear. In order to gain more insight into this phenomenon, the enantiomer ratio of bupivacaine in blood for a large number of patients should be determined and correlated with other variables in blood such as the concentration of plasma proteins. This means that it is useful to automate the analysis of the enantiomers of bupivacaine in serum samples.

Liquid chromatography has been shown to be suited for the separation and determination of the enantiomers of bupivacaine in biological samples [2, 3]. The most advanced method has been described by A. Walhagen and L.E. Edholm [3]. These authors applied a coupled-column system using an immobilized α-acid glycoprotein as the chiral column and a set of achiral reversed-phase columns. Although their system works satisfactorily, it is difficult to automate because bupivacaine is first isolated from plasma by an off-line extraction step.

In this paper we describe an on-line coupled three column liquid chromatographic system to determine the enantiomers of bupivacaine in serum. The method involves three on-line steps: (i) a clean-up and pre-concentration step; (ii) analytical separation of bupivacaine racemates from the matrix; and (iii) the separation of the racemates, heart-cut from the analytical column, on an α_1-acid glycoprotein column. Analysis of bupivacaine in serum of patients with the developed method will be shown and compared with an off-line method.

Experimental

Apparatus

Figure 1 shows a schematic diagram of the on-line column set-up. It consisted of:

(1) A medium pressure pump for the pre-column system (Eldex, Inacom, Veenendaal, The Netherlands).

(2) An injection valve (Rheodyne 7010, Berkeley, CA, USA) equipped with a 0.5 ml sample loop.

(3) A mixing coil 650 × 1 mm i.d.

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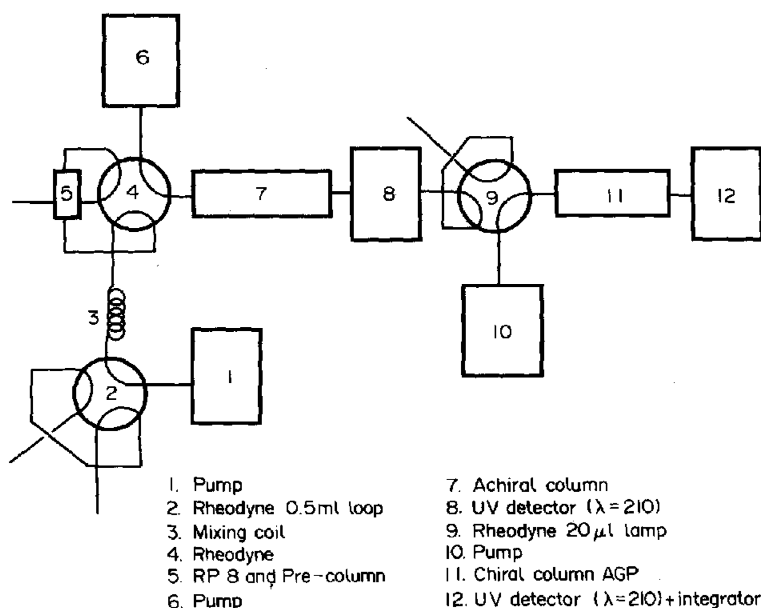

Figure 1
A schematic diagram of the on-line coupled three column system.

(4) An injection valve (Rheodyne).

(5) A 10 × 2.1 mm i.d. pre-column (Chrompack, Middelburg, The Netherlands). The column was mounted in the loop of valve (4).

(6) A high pressure pump for the achiral column (Millipore, Model 510, Bedford, MA, USA).

(7) A 150 × 2.1 mm i.d. achiral column.

(8) A UV detector (Applied Biosystems, model 757, Foster City, CA, USA) equipped with a 2.3 μl cell + an integrator (I) (Shimadzu, C-R3A, Kyoto, Japan). The heat exchanger of the detector was removed to minimize extra peak broadening. The wavelength was set at 210 nm.

(9) An injection valve (Rheodyne) equipped with a 20 μl loop.

(10) A high pressure pump for the chiral column.

(11) A 100 × 4.0 mm i.d. chiral column.

(12) A UV detector (Applied Biosystems) + an integrator (II) (Shimadzu); the wavelength was set at 210 nm.

The three columns were operated with different mobile phases. For the pre-column a mixture of THF–0.1 M phosphate buffer (pH 8.0) (2.5:97.5, v/v) was used; for the achiral column, the mobile phase consisted of 2-propanol–0.05 M phosphate buffer (pH 3.0) (15:85, v/v) and for the AGP column a mixture of 2-propanol–0.025 M phosphate buffer (pH 7.0) (7:93, v/v) was used. The flow rates for the pre-column, the achiral column and the AGP column were set at 1.0, 0.2 and 0.9 ml min^{-1}, respectively.

Materials

The organic solvents (Merck, Darmstadt, Germany) were of analytical quality and used without further pretreatment. Bupivacaine (Astra, Södertalje, Sweden) was kindly donated by Dr Kerstin Gröningson. Haloperidol was obtained from Sigma (St Louis, MO, USA). The packing for the pre-column was RP 8, 25–40 μm (Machery & Nagel, Düren, Germany) and the pre-column was dry packed; for the achiral column Inertsil ODS 5 μm (GL Sciences Inc., Tokyo, Japan) was used and the chiral column was an α_1-acid glycoprotein (AGP) column obtained from Pharmacia (Uppsala, Sweden). The LC column used for the off-line measurements was RP18 DB (Supelco, Bellefonte, PA, USA).

Procedures

On-line method. Put valves (2), (4) and (9) in the load position. Fill the loop of valve (2) with the serum sample and transport the sample to the pre-column by switching valve (2). Flush the pre-column for 5 min (5 ml) and then connect the pre-column to the achiral column by switching valve (4) to the inject position and start the integrator I. After 2 min valves (2)

and (4) are switched back to the load position and the pre-column is reconditioned. The elution of the bupivacaine racemates from the achiral column is monitored with detector (8) and by proper timing, a fraction of the peak is trapped in the 20 μl loop of valve (9) and injected on the chiral column by switching valve (9) in the inject position and the integrator II is started. After the elution of the enantiomers, valve (9) is switched back to the load position and the system is ready for the next cycle.

The concentration of the bupivacaine enantiomers can be calculated from the total racemate concentration as found on the achiral column and from the ratio of the peak area of the enantiomers determined on the chiral column.

Off-line method. With the off-line method the bupivacaine racemate is extracted from serum and subsequently determined by HPLC on an achiral RP 18 column, using a mixture of acetonitrile–methanol–0.05 M ammonium phosphate buffer (pH 6.8) (35:35:30, v/v/v) as mobile phase. For the extraction, 500 μl serum was spiked with 1 μg of haloperidol (IS) and alkalinized with 0.1 ml of 0.1 M Na_2CO_3 solution. Then the sample is extracted with 7 ml of a mixture of dichloromethane–*n*-heptane (1:4, v/v). The organic phase is evaporated to dryness and the residue is redissolved in 100 μl mobile phase. An aliquot of 20 μl is injected onto the HPLC column.

Results and Discussion

When designing an on-line coupled LC column system one is faced with problems concerning the compatibility of the mobile phases and dispersion of the sample. Usually the single separation steps can be well optimized by tuning the mobile phase composition. However, the on-line transfer of one mobile phase into another mobile phase might destroy the separation on the second column. Therefore in practice, usually a compromise in the mobile and/or stationary phase composition must be found. In addition to this, the column dimensions must be adapted to avoid problems with the detectability of the sample due to dilution of the sample.

In order to optimize our on-line three column system, the effects of the mobile phase composition, type of packing and column dimensions on the performance have been investigated.

The chiral AGP column

The retention behaviour of enantiomers on an AGP column as function of the composition of the mobile phase and temperature is well documented [4]. The organic modifier concentration and pH of the mobile phase appear to be the important parameters to adjust the selectivity and retention. In most cases *n*-propanol or 2-propanol is used as modifier. For bupivacaine the effects of pH and the concentration of 2-propanol on the selectivity factor, α, and the capacity factor, k', were investigated and the results are given in Figs 2 and 3. From these figures it can be seen that pH has a large effect on the capacity factor as well as on the selectivity factor, while the organic modifier concentration significantly influences

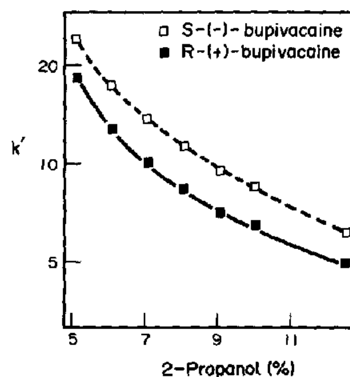

Figure 2
Effect of 2-propanol on k' of the enantiomers of bupivacaine on the AGP column. Mobile phase: 0.025 M phosphate buffer (pH 7.0)–2-propanol.

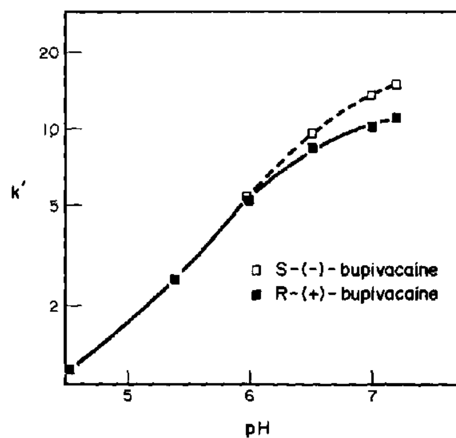

Figure 3
Effect of the pH of the mobile phase on k' of the enantiomers of bupivacaine on the AGP column. Mobile phase: 0.025 M phosphate buffer–7% 2-propanol.

the capacity factor but hardly influences the selectivity factor. From these observations it can be concluded that good separation conditions are obtained for the separation of the enantiomers of bupivacaine when using a mobile phase with 7% of 2-propanol and a buffer of pH 7.0. Since the composition of the racemates heart-cut from the achiral column will be different it is important to have sufficient buffer capacity to avoid changes in pH during the separation. This last requirement was investigated by changing the buffer concentration from 0.01 M to 0.025 M phosphate pH 7 at constant organic modifier composition. It appears that the larger the buffer concentration the smaller the capacity factors (drop of about 30%) but the larger the selectivity factors (1.27–1.35). On the basis of these findings the final mobile phase composition for the chiral separation was adjusted to 2-propanol–0.025 M phosphate buffer pH 7.0 (7:93, v/v). Figure 4 shows a typical chromatogram of the enantiomers of bupivacaine under these conditions.

The achiral column

The achiral column has the function of separating the bupivacaine racemates from the

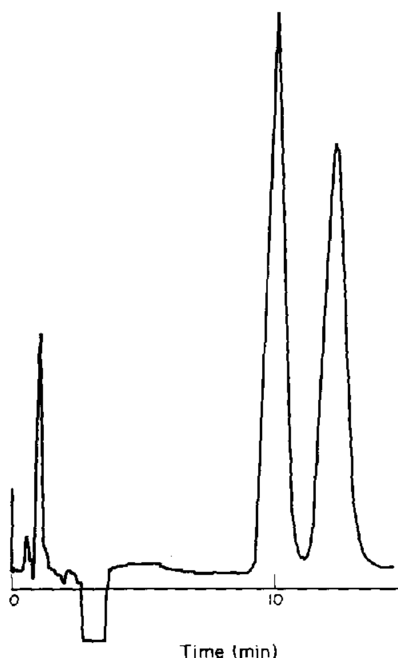

Figure 4
Separation of the enantiomers of bupivacaine on the AGP column. Conditions: 0.025 M phosphate buffer (pH 7.0)–2-propanol (93:7, v/v). Sample: 2 μg ml^{-1} of bupivacaine; detector setting: 0.01 AU full scale.

matrix compounds. Then a part of the racemate peak is trapped in the sample loop of an injection valve (heart cut) and subsequently injected onto the chiral AGP column. From the point of view of detectability and quantitative reliability of the separated enantiomers, the whole peak should be trapped. However, injection of too large a volume onto the chiral column will destroy the separation efficiency. From additional experiments on the effect of the injection volume on the AGP column it was found that the injection volume cannot be larger than 20 μl in order to preserve the separation efficiency. This means that the dilution of bupivacaine must be kept small in order to be able to trap a sufficiently high racemate concentration in the 20 μl sample loop. In our experience it proved impossible to collect the entire analyte peak and transfer it to the chiral column. It was decided to collect only a fraction of the analyte peak. This necessitates the use of a second detector for quantitative precision. Even with this arrangement, the fraction collected must be as large as possible in order to get enough signal in the detection of the chiral separation. This points to the reduction of the RP-column diameter and to a mobile phase system in which bupivacaine elutes in a reasonable retention time and which is compatible with the mobile phase used with the AGP column.

From previous reports on the analysis of bupivacaine by reversed chromatography [3, 5, 6] good retention and symmetrical peaks are obtained when using mixtures of 40–60% (v/v) acetonitrile in phosphate buffer pH 3.0 as mobile phase. However, this mobile phase was found to be incompatible with the AGP column and injection of the trapped fraction destroys the separation of the enantiomers. Therefore it was decided to replace acetonitrile by 2-propanol. The compatibility of the mobile phase was investigated by injecting 20 μl of bupivacaine dissolved in different 2-propanol–phosphate buffer (pH 3.0) mixtures on the AGP column. It appeared that mobile phases up to 15% (v/v) 2-propanol could be injected without influencing the separation of the enantiomers. Therefore the mobile phase composition for the achiral column was fixed on 2-propanol–phosphate buffer (pH 3.0) (15:85, v/v) and the optimization of the achiral column could be reduced to finding a commercial reversed-phase small bore column on which bupivacaine elutes with a reasonable k'.

Several end-capped RP ODS columns (Supelco, Machery & Nagel, Inertsil) were investigated. The most suitable column was Inertsil and Fig. 5 shows a typical chromatogram of bupivacaine racemates obtained on the achiral column and of separation of the enantiomers on-line transferred from the achiral column onto the AGP column.

The pre-concentration column

The pre-concentration column is used to remove the serum proteins and to preconcentrate bupivacaine prior to the analysis on the achiral column. The pre-concentration step is necessary to improve the detectability of bupivacaine but also to minimize the injection volume on the small bore achiral column. To meet this last requirement a 10 × 2.1 mm i.d. pre-column was used. The pre-column was dry packed and had a void volume of about 25 µl. Preferably a system is selected from which the serum proteins elute unretarded where bupivacaine is strongly adsorbed and can be desorbed in a small volume without disturbing separation on the achiral column. Bupivacaine is a lipophilic weak base (pK_a = 8.1) and can be well adsorbed on reversed-phase packings at basic pH or under acidic conditions as a cation on an ion-exchanger or as an ion-pair on reversed-phase materials. Explorative investigations of these systems, including the effect of particle size on the life-time of the column, revealed that the sorption of bupivacaine at basic pH on reversed-phase packings with a relatively large particle size yielded the most promising results. Therefore, several silica-based and polymeric reversed-phase packings, including a shielded hydrophobic packing, were tested with respect to their efficacy in the removal of serum proteins, and in the pre-concentration and desorption of bupivacaine. Some of these packings are commercially available, others were collected from disposable cartridges. The performance of the various packings with respect to clean-up, pre-concentration and desorption was tested by on-line coupling of the pre-column to the achiral column and comparing the chromatograms

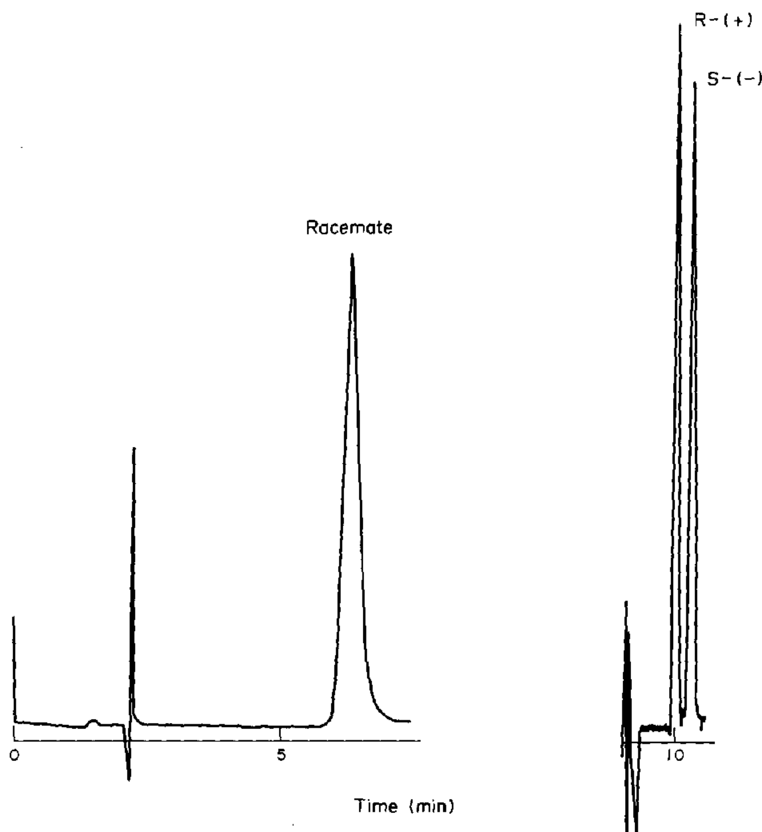

Figure 5
Chromatogram of bupivacaine racemate and the enantiomers with the on-line coupled reversed-phase and chiral column. Conditions: RP column: 0.05 M phosphate buffer (pH 3.0)–2-propanol (85:15, v/v); detector setting: 0.125 AU full scale. AGP column: same conditions as in Fig. 4. Detector setting: 0.01 AU full scale.

obtained with spiked serum and standard solutions. Although pH >9 favours the adsorption of bupivacaine on reversed packings, an aqueous 10 mM phosphate buffer (pH 8) was used to avoid deterioration of the silica-based packings. With this mobile phase composition, the k' of bupivacaine on most reversed packings was found to be >100 and a washing step up to 5 ml could be tolerated. Moreover, at pH 8 no precipitation of serum proteins occurs. The adsorbed bupivacaine was desorbed from the column by back flush using the same mobile phase as applied for the achiral column, i.e. 2-propanol–50 mM phosphate buffer (pH 3.0). The results of the investigations are presented in Table 1. From this table it can be seen that the pre-concentration and clean-up are satisfactory on most packings, except with the RP 4 and Hisep packing. However, except on the RP 8 the desorption of bupivacaine is poor on all investigated packings, with asymmetric elution profiles usually being obtained. On the basis of Table 1 the RP 8 silica packing (60 Å) for on-line coupling on the achiral column was selected. With this packing, additional experiments were carried out into the long-term stability of the material and the column-to-column reproducibility of the pre-column. Over a period of time, 60 injections of 500 µl serum were made, using 0.1 M phosphate buffer (pH 8.0) + 2.5% THF as mobile phase. The k' values changed gradually from 5.50 to 5.62. Column-to-column reproducibility for k' was found to be 5.52 ± 0.07 ($n = 5$) (Fig. 6).

It was noticed that the pressure drop of the pre-column slowly increases with the number of serum injections. This effect is probably caused by irreversible adsorption of serum constituents (e.g. proteins). The problem could be satisfactorily solved by washing the pre-column, after the back flush, with 5 ml of a

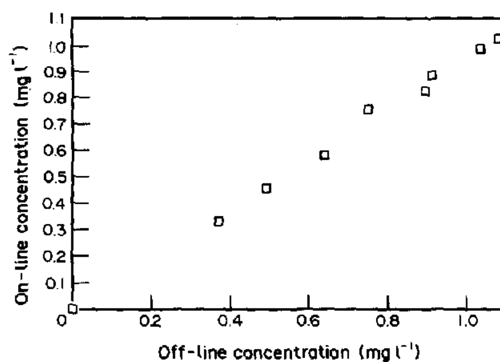

Figure 6
Correlation of the on-line and off-line method for the determination of the bupivacaine racemates.

solution of 2-propanol–10 M phosphate (pH 8.5) (2:8, v/v), followed by reconditioning of the pre-column with the washing liquid.

The recovery of bupivacaine from serum appeared to be significantly smaller (about 60–70%) to that obtained with aqueous standard solutions. It is known that bupivacaine strongly binds to serum proteins and this might be the cause of the low recovery from serum. If this is the case recovery may be improved by reducing drug–protein binding.

In order to reduce protein binding, the pH of the serum sample can be lowered to pH <5. However under these conditions, the k' of bupivacaine becomes small and this is unfavourable with respect to pre-concentration. Increasing the buffer concentration is another way to decrease the protein binding. Increasing the buffer concentration (used to adjust serum pH) from 10 to 100 mM resulted in a 10% increase in recovery. The effect of addition of an organic modifier to the serum sample on the recovery was investigated with three different organic modifiers; acetonitrile, THF and 2-propanol. The organic modifier concentration must be kept small to avoid a large decrease in

Table 1
Comparison of reversed-phase packings for the pre-column

Firm	Type	Material	Particle size (µm)	Pore size	Pre-concentration	Clean-up	Desorption	Stability
M & N	RP 4	silica	25–40	300 Å	good	bad	good	good
M & N	RP 8	silica	25–40	60 Å	good	good	good	good
M & N	RP 18	silica	25–40	100 Å	good	good	poor	good
Baker	RP 2	silica	40–50	80 Å	good	good	bad	good
R & H	XAD-2	organic	50–100	—	good	good	bad	good
Merck	C 18	organic	30	—	good	good	bad	good
Polysorb	MP 1	organic	30	—	good	good	bad	good
Hisep	RP 18	silica	5	—	bad	poor	poor	good

M & N, Machery & Nagel (Düren, Germany); Baker, J.T. Baker (Deventer, The Netherlands); R & H; Polysorb, Interaction Chemicals Inc. (Mountain View, CA, USA); Hisep, Supelco (Bellefonte, PA, USA).

k' on the pre-column. With all modifiers, a significant increase in recovery was found when mixing the serum with small concentrations of organic modifiers (up to 5%, v/v) before the injection on the pre-column. The most effective was THF and with 2.5% (v/v) and 100 mM phosphate buffer, a recovery of 100% was obtained. This finding indicates the use of 2.5% THF–100 mM phosphate buffer also in the washing liquid, providing the clean-up and pre-concentration is not negatively influenced. Fortunately this was found not to be the case.

The mixing of the serum sample with THF and buffer is an additional action and should be integrated in the on-line system. In order to mix the organic modifier and buffer on-line with the sample before it is transported to the pre-column, use can be made of the dispersion of the sample as occurring in the coil placed between valves (2) and (4). The serum plug is pushed through the coil, and due to the flow

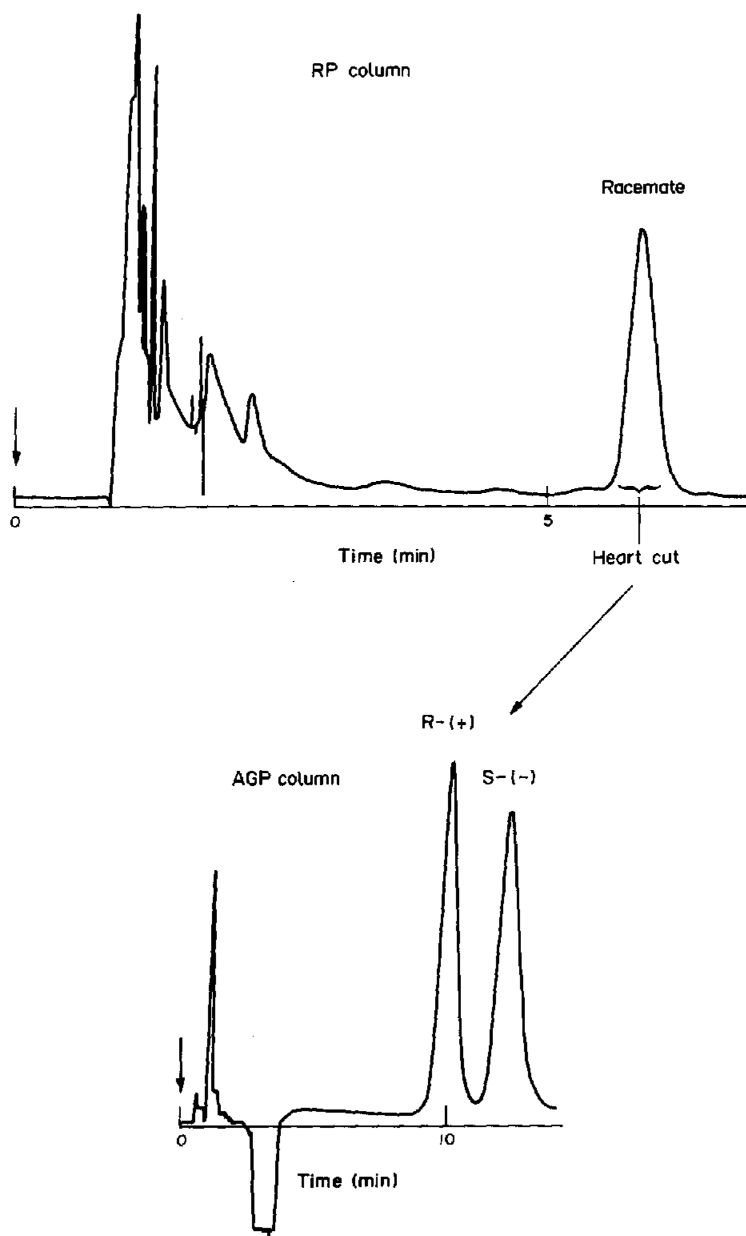

Figure 7
Chromatogram of bupivacaine racemates and enantiomers in serum of a patient after lumbar epidural anaesthesis obtained with the on-line coupled three column system.

profile, the sample is mixed with the washing liquid before it reaches the pre-column.

The on-line sample pre-treatment system was compared with the off-line method by spiking pooled serum samples and determining the racemate concentration. The results of these measurements are given in Fig. 6. As can be seen, a good correlation between the two methods is found (intercept = 0.033, slope = 0.998, r = 0.9965).

The performance of the on-line coupled three column system

The performance of the on-line coupled system was investigated with standard solutions of bupivacaine racemate and spiked pooled serum samples. The calibration with standard solutions and spiked serum in the range of 0.2–2 μg ml^{-1} (seven points) appeared to be linear for standard solutions, intercept = 0.011, slope = 0.9959, r = 0.9999 and for spiked serum, intercept = 0.046, slope = 0.9847, r = 0.994). The enantiomer ratio with standard solution was found to be 1 ± 0.006 (n = 30) and for serum 1.03 ± 0.013 (n = 29). The limit of determination (6 SD) with standard solutions is approximately 0.1 μg ml^{-1} and for serum 0.17 μg ml^{-1}. The recovery of bupivacaine from spiked serum over the same concentration range was found to be 97–100% with a RSD of 4.7% at 0.2 μg ml^{-1} and 1.3% at 2 μg ml^{-1} (n = 6). By proper timing four analyses per hour can be performed.

Determination of bupivacaine in patients

Figure 7 shows a typical chromatogram of a serum of a patient after lumbar epidural anaesthesia with bupivacaine. The time course of the enantiomers of bupivacaine for several patients was determined by this method, and a typical curve is given in Figure 8. This curve clearly demonstrates the difference in concentration of the enantiomers just after the treatment and the change of the enantiomer ratio in time. The same curves were found with other patients. This behaviour agrees well with that reported in literature [2].

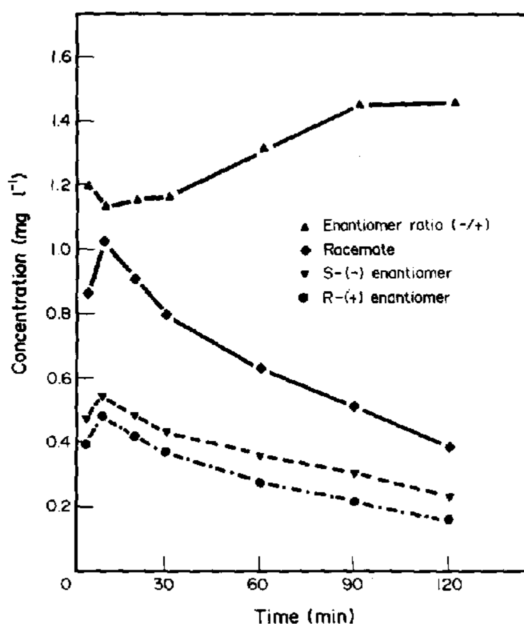

Figure 8
The time course of the enantiomers of bupivacaine in a patient lumbar epidural anaesthesis. First sample taken after 5 min.

Conclusions

This on-line coupled three column system appears to be very well suited for the determination of the enantiomers of bupivacaine in serum of patients down to 0.1 μg ml^{-1}. The system can be fully automated and four analyses per hour can be realized. So far the weakest point in the system is the stability of the pre-column in that it is advisable to repack the pre-column after 30–40 serum injections. Efforts are now being made to find a more stable packing for the precolumn.

This approach to the determination of the enantiomer concentration via the racemate concentration on an achiral column and the enantiomer ratio on a chiral column looks attractive for other chiral drugs in biological samples.

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