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COUPLED-COLUMN CHROMATOGRAPHY ON IMMOBILIZED PROTEIN PHASES FOR DIRECT SEPARATION AND DETERMINATION OF DRUG ENANTIOMERS IN PLASMA

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SUMMARY

Columns packed with immobilized α_1-acid glycoprotein and albumin were used in coupled-column chromatography to increase their utility for determining low concentrations of enantiomers in biological samples. The two enantiomers eluted from the protein columns were trapped and compressed on two separate columns, packed with hydrophobic stationary phase, and subsequently transferred to a fourth column for final separation. The overall effect was an increase in efficiency and selectivity. Examples are given of separations of the enantiomers of terbutaline, metoprolol, oxazepam and bupivacaine in plasma. For quantitative determination a single calibration can be used for both enantiomers.

INTRODUCTION

Liquid chromatography (LC) on columns is currently the most useful technique for the separation and determination of enantiomers in biological samples. LC allows great latitude in the chromatographic conditions for special applications. Separation can be achieved either indirectly after derivatization to diastereomers or directly[1]. In the latter instance, a chiral stationary or chiral mobile phase can be used.

Progress in direct separation by LC has been summarized in several books and papers[1–4]. Some guidance on how to choose suitable chromatographic systems for special applications can be obtained from the current literature[5]. However, most of the applications described are for samples in an uncomplicated matrix. As was pointed out in a recent paper[6], application of direct separation to biochemical analysis is not straightforward, especially when very low concentrations of drugs and their metabolites are to be determined. Some of the more severe problems were addressed, *e.g.*, restricted chromatographic conditions, low efficiency, internal standardization and loadability. It was shown that LC with coupled columns and the combination of LC with mass spectrometry can be used to overcome some of these problems. Recently,

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Chu and Wainer[7] utilized LC with coupled columns for the measurement of warfarin enantiomers in serum.

In this work, coupled-column chromatography (CCC) has been further explored with columns packed with immobilized proteins for use in biochemical analysis. Two stationary phases of this type are commercially available, α_1-acid glycoprotein and albumin. Although it has been shown that this type of phase can be used for the separation of a number of compounds, their applications to biochemical analysis are limited. Most important, broad asymmetric peaks are often obtained, which will hamper their use in quantitative work at low concentrations. A means of circumventing this problem is to concentrate the samples eluted as broad peaks by utilizing the effect of peak compression. There are several ways to achieve this, but in principle, peak compression can best be obtained if an analyte is introduced into a column in a "non-eluting" solvent. In reversed-phase chromatography, water is a "non-eluting" solvent, and large volumes can be introduced into columns packed with a hydrophobic chemically bonded phase, *e.g.*, C_{18}. In biochemical analysis, peak compression was used to allow the injection of large sample volumes[8,9], and in CCC to avoid band broadening when analytes were transferred from one column to another[10]. In most applications of protein columns high concentrations of water are used in the mobile phase[11,12].

Thus, in principle, peaks eluted from a protein column can be trapped on a column with a hydrophobic stationary phase for compression and subsequently transferred to another hydrophobic stationary phase for final separation. In this way, the efficiency and symmetry of peaks can be improved, and this will also increase the overall sensitivity and selectivity. This principle has been used here and is exemplified for some selected chiral drugs in plasma samples. The possibility of determining plasma concentrations by CCC is also discussed.

EXPERIMENTAL

Materials

The structures of the chiral drugs used in this study are shown in Fig. 1. Racemic bupivacaine hydrochloride, (*R*)-bupivacaine and (*S*)-bupivacaine, used as the hydrochlorides, were obtained from Astra (Södertälje, Sweden). Racemic metoprolol was used as the succinate and *S*-(−)-metoprolol as hydrochloride. Both were obtained from Hässle (Mölndal, Sweden). The racemate of oxazepam was obtained from KabiVitrum (Stockholm, Sweden). Racemic terbutaline was used as the sulphate and (−)-terbutaline and (+)-terbutaline were used as the hydrobromides. All three were obtained from Draco (Lund, Sweden). Albumin (A7030) and glutathione were obtained from Sigma (St. Louis, MO, U.S.A.), ethanol (99.5%, spectroscopic grade) from Kemetylprodukter (Bromma, Sweden) and methanol and acetonitrile from Rathburn Chemicals (Walkerburn, U.K.). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Bond-Elut C_{18} cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.). All other chemicals were of analytical-reagent grade and were obtained from E. Merck (Darmstadt, F.R.G.).

The configuration of the CCC system is shown in Fig. 2. The pretreated sample was injected into the protein column (column 1) for separation of enantiomers. For collection and quantitative transfer of each enantiomer eluted from column 1 into

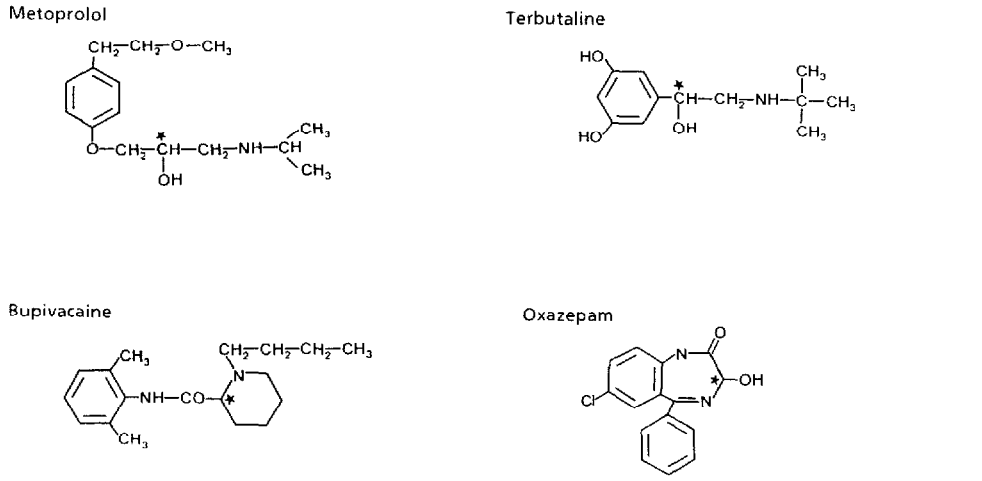

Fig. 1. Structure of chiral drugs used.

columns 2 and 3, a suitable retention time "window", defined by exact times for switching the six- and ten-port valves, was chosen. The "window" was determined by coupling column 1 directly to the detector, and injecting representative amounts (calculated from estimated plasma concentrations) of the racemates. When the first enantiomer started to emerge from column 1, the six-port valve was switched so that the eluate was entering column 2 for trapping of that enantiomer. When the second enantiomer started to emerge from column 1, the ten-port valve was switched so that it could be trapped on column 3. At the same time mobile phase 2 was directed to column 2 for elution of the trapped enantiomer into the achiral column (column 4). After elution of the first enantiomer from that column, the second enantiomer was eluted from column 3 into column 4. All of the eluate from column 1, except for the enantiomers, was directed to waste.

The CCC system was composed of two LKB 2150 pumps (LKB, Bromma, Sweden), a Valco Model CV-6-UHPa-N60 valve (Valco, Houston, TX, U.S.A.) for

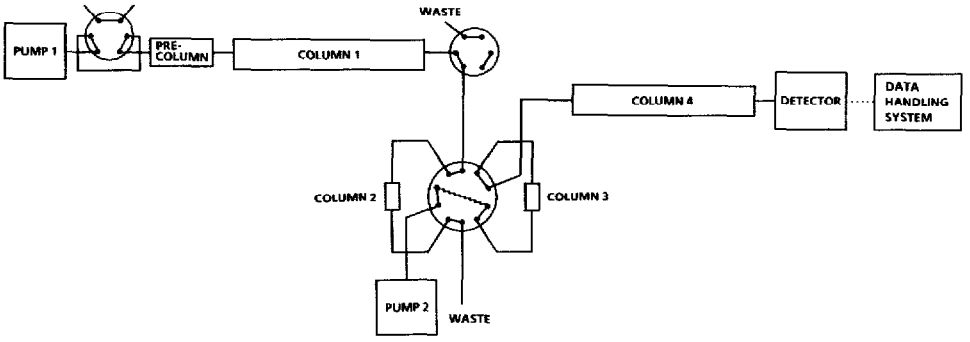

Fig. 2. Schematic diagram of the coupled-column chromatography system used for the separation of some selected chiraBl drugs in plasma: column 1 = chiral (protein); columns 2 and 3 = achiral for trapping; column 4 = achiral.

injection of samples (loop volumes 20–100 μl), and a Valco A-C6U and AC10W (ten-port) valve for column switching. A Model 490 UV detector (Waters Assoc., Milford, MA, U.S.A.) set at 210 nm was used for bupivacaine and a Spectromonitor 111 UV detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) set at 230 nm for oxazepam. A Model Rf 535 fluorescence detector (Shimadzu, Tokyo, Japan) set at λ_{ex} = 272 nm and λ_{em} = 306 nm was used for detection of metoprolol. For detection of terbutaline an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a TL-5 glassy carbon electrode and an Ag/AgCl (NaCl, 3 *M*) reference electrode was used. The working potential was 0.9 V. A Chromguard precolumn (Chrompack, Middelburg, The Netherlands) (10 × 3 mm I.D.) packed with a 20-μm pellicular, chemically bonded CN phase was used to protect the protein column.

For separation of the enantiomers (column 1) of bupivacaine, metoprolol and terbutaline, columns (100 × 4 mm I.D.) packed with immobilized α_1-acid glycoprotein of a newly manufactured type from ChromTech (Stockholm, Sweden) were used. Enantiopac columns (100 × 4 mm I.D.) obtained from LKB were used in some additional experiments. Columns packed with bovine serum albumin are commercially available, but in this study bovine serum albumin was immobilized *in situ* on to Nucleosil 300-5 silica (Macherey, Nagel & Co., Düren, F.R.G.) by a procedure described elsewhere[13]. This column was used for separation of the enantiomers of oxazepam.

The mobile phases used for separation of the enantiomers (mobile phase 1) consisted of 20 m*M* phosphate buffer (pH 7.5) for terbutaline, 10 m*M* phosphate buffer (pH 7)–2-propanol (91:9, v/v) for bupivacaine, 20 m*M* phosphate buffer (pH 7)–2-propanol (99.75:0.25, v/v) for metoprolol and 50 m*M* phosphate buffer (pH 6.8)–1-propanol (98:2, v/v) for oxazepam. The flow-rates were 0.8 ml/min and 1.0 ml/min (oxazepam).

Waters Guard-Pak columns (4 × 5 mm I.D.) packed with μBondapak C_{18} was used as trapping columns (columns 2 and 3). For achiral chromatography (column 4), the chemically bonded phases 5-μm Nucleosil C_{18} and Nucleosil C_8 (bupivacaine) were used packed in 100 × 4 mm I.D. (bupivacaine), 100 × 4.6 mm I.D. (metoprolol, terbutaline) and 200 × 4.6 mm I.D. (oxazepam) stainless-steel columns. The mobile phases (mobile phase 2) consisted of 50 m*M* ammonium acetate buffer pH 6.0–methanol (90:10, v/v) for terbutaline, 50 m*M* phosphate buffer (pH 3.0)–acetonitrile (72:28, v/v) for bupivacaine, 500 m*M* phosphate buffer (pH 3.0)–water–acetonitrile (10:70:20. v/v/v) for metoprolol and 2 m*M* potassium dihydrogenphosphate–acetonitrile–methanol (42:1:57, v/v/v) for oxazepam. The flow-rates were 1.0 ml/min and 1.5 ml/min (oxazepam).

Methods

Bond-Elut C_{18} solid-phase extraction columns were used for sample work-up by published methods[6,14,15] for terbutaline, oxazepam and metoprolol. Bupivacaine was extracted from plasma with hexane from alkaline medium by a previously described method[16]. The sample work-up procedures were followed in detail, *e.g.*, the same volumes of plasma as given therein were used.

Standards were prepared by adding racemate to plasma at relevant concentrations. For metoprolol and oxazepam calibration studies were also undertaken and the

concentration ranges were 7–3500 nmol/l and 0.8–17 μmol/l, respectively. For calibration the area under the chromatographic peak was measured and correlated with the actual concentrations.

The maximum overall efficiency (number of theoretical plates) that could be obtained was determined by injection of standard solutions of the respective drugs directly into the achiral column (column 4) with a loop of negligible volume (20 μl). These values were compared with those obtained by using the whole CCC system.

Known injected amounts of the respective racemates were used to calculate the recovery in the transfer from column 1 to column 4. Peak areas after passage through the whole CCC system were determined at relevant concentrations and compared with those obtained after direct injection into column 4. For calculation of peak areas the Nelson Analytical (Cupertino, CA, U.S.A.) software program Access Chrom was used. The total absolute recovery, including sample work-up, was only determined for metoprolol by adding known amounts of the racemate to blank plasma and taking it through the whole analytical procedure. For bupivacaine, metoprolol and terbutaline pure enantiomers were used to determine their order of elution on the protein columns.

RESULTS AND DISCUSSION

The strategy for the separation of the enantiomers on protein columns (column 1) was taken from the literature[11,12]. In each instance, complete separation could be achieved by using high concentrations of water, and thus the heart-cut sample could be compressed by using a hydrophobic packing material for trapping the enantiomers eluted from the chiral column (column 1). The eluate from column 1 could either be directly transferred to column 4 or trapped in a loop or on a small column. Sampling via a loop or direct transfer of the relatively large volumes used here (1.4–4.2 ml) was tried, but gave rise to pressure surges and a high background, making quantitative work at low concentrations more difficult or impossible. Instead, small columns packed with a hydrophobic packing material and relatively large particles (> 20 μm) were chosen. Small columns reduced the trapped volumes to less than the void volume of the column (< 100 μl). Pressure surges during valve switching were also smoothed out.

The amount of retardation and thus peak compression in general are determined by the capacity factor for the analyte and can be judged from the following equation[10]:

$$V_i = V_0 \cdot \frac{1}{1 + k_i^{ci}}$$

where V_i = the contracted volume containing compound i, V_0 = trapped sample volume transferred to column i and k_i^{ci} = capacity ratio of compound i when using the injection medium of trapped volume as the mobile phase on column i.

In the two step transfer (Fig. 2) of the enantiomer from the protein column (column 1) via the trapping column (column 2 or 3) to the final achiral column (column 4), it is important not to introduce extra band broadening by the transfer process. For that reason, mobile phase 1 must be a stronger eluting solvent on column 1 than on the trapping column (column 2 or 3). Further, mobile phase 2 must be a stronger eluting solvent on the trapping column than on column 4.

TABLE I

EFFICIENCY OBTAINED FOR SOME SELECTED DRUG ENANTIOMERS ON PROTEIN PHASES ALONE AND AFTER COUPLED-COLUMN CHROMATOGRAPHY

Protein phase	*Drug*	*Number of theoretical plates*				*Amount injected (nmol)*
		Chiral column (column 1)		*CCC (column 4)*		
		First-eluted enantiomer	*Second-eluted enantiomer*	*First-eluted enantiomer*	*Second-eluted enantiomer*	
α_1-Acid glycoprotein	Metoprolol	800	920	3800	3800	0.04
	Terbutaline	1300	1400	3500	3500	0.05
	Bupivacaine	2200	1600	3500	3500	8
Albumin	Oxazepam	700	430	6400	6400	13

The maximum efficiency that could be obtained for the whole CCC system was estimated by injection of standards in small (negligible) volumes directly into column 4. In all instances, the chromatographic parameters and the packing material used in column 4 were based on earlier published methods[6,15,17,18]. It was shown that maximum efficiency of the CCC system was obtained in all instances, and this indicates an optimal design of the CCC system.

In Table I and Figs. 3 and 4, examples are given to show how the efficiency was improved when CCC was used. It should be noted that no attempt was made to choose the most efficient achiral column. Thus, an even greater increase in efficiency could possibly have been obtained.

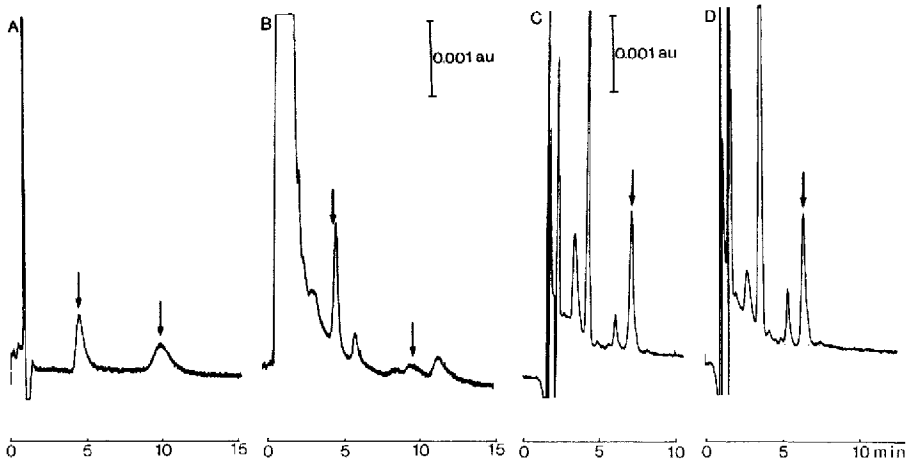

Fig. 3. Chromatograms obtained after separation of racemic oxazepam. (A) 70 pmol (racemate) of standard solution injected directly into the albumin column (column 1). (B) Blank plasma after separation on the albumin column alone. Arrows indicate positions of the enantiomers of oxazepam. (Only the racemate was used in the experiment and therefore no identification of the respective enantiomers is given.) (C) Spiked plasma, containing 3.3 μmol/l of racemic oxazepam after separation by CCC. Amount injected: 33 pmol (racemate). Arrow indicates oxazepam enantiomer (first-eluted enantiomer). (D) As in (C) (second-eluted enantiomer).

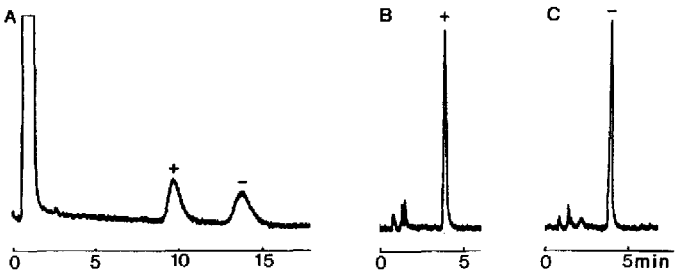

Fig. 4. Chromatogram obtained after separation of racemic metoprolol. (A) 35 pmol (racemate) of standard solution injected directly into the α_1-acid glycoprotein column (column 1). (B) Spiked plasma, containing 70 nmol/l of racemic metoprolol after separation by CCC. Amount injected: 58 pmol (racemate). (+)-Metoprolol, first-eluted enantiomer. (C) As in (B). (−)-Metoprolol, second-eluted enantiomer.

In Fig. 3 the selectivity achieved by using CCC is obvious. A more pronounced effect is exemplified in Fig. 5. In this instance, the gain in efficiency is only two- to three-fold (see Table I), but the selectivity is increased dramatically by CCC.

The recovery in the transfer from column 1 to column 4 was 100% in all instances. The absolute recovery, including sample work-up, was estimated to be 85% for metoprolol at 0.18–18 μmol/l.

In general on the achiral column, enantiomers at equal concentration will give the same area under the chromatographic peak. This is useful in practice because a single calibration graph can be used for their determination. For example, when peak area *versus* concentration was plotted for each enantiomer of metoprolol and

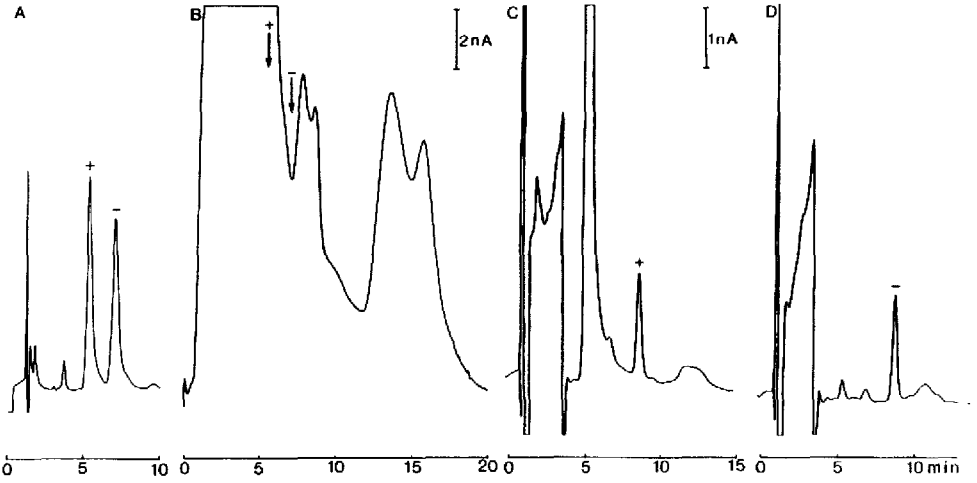

Fig. 5. Chromatograms obtained after separation of racemic terbutaline. (A) 50 pmol (racemate) of standard solution injected directly into the α_1-acid glycoprotein column (column 1). (B) Blank plasma after separation on the α_1-acid glycoprotein column alone. Arrows indicate positions of the enantiomers of terbutaline. (C) Spiked plasma, containing 50 nmol/l of racemic terbutaline after separation by CCC. Amount injected: 21 pmol (racemate). (+)-Terbutaline, first-eluted enantiomer. (D) As in (C). (−)-Terbutaline, second-eluted enantiomer.

oxazepam, overlapping calibration graphs were obtained for each pair of enantiomers ($r = 0.999$).

In the examples given here, simple mobile phases (phosphate buffers and propanol) were used on the protein columns, because chiral separation can often be achieved under such conditions. However, it has been shown that the addition of different kinds of modifiers to the mobile phase can be used to increase the chiral selectivity[11] and extends the applicability of protein columns to more compounds. It is believed that the proposed approach for application of CCC to chiral separation on immobilized protein columns can also be used in such instances.

The load capacity of protein columns is often low, and this might sometimes preclude the use of a protein column as the first column in the CCC system. In our experience, protein columns are not rugged and can lose their usefulness rapidly if handled incorrectly. Therefore, crude samples should not be injected directly into these columns. Instead, use of sample work-up and the use of a precolumn, as used in this work, is recommended.

For the proposed technique to work, resolution on the chiral column must be complete at all relevant concentrations. In Fig. 6, an example is given of the separation of bupivacaine on a first-generation α_1-acid glycoprotein column. Although the resolution could be judged as good (Fig. 6A), severe peak tailing can be seen. It was impossible to recover the respective enantiomers completely without contamination with the other enantiomer and it is not easy to observe the fronting on the second peak. Injection of equal amounts of the pure enantiomers revealed that severe fronting on the second enantiomer was hidden under the first-eluted enantiomer (Fig. 6B). In Fig. 6C, a separation is shown of the same amount of enantiomers of bupivacaine on a recently available α_1-acid glycoprotein column from ChromTech. In this instance, the resolution was nearly complete. As was mentioned above, the efficiency and symmetry of peaks on protein columns are often poor. Although the resolution seems to be complete, caution is recommended when utilizing the approach described in this work.

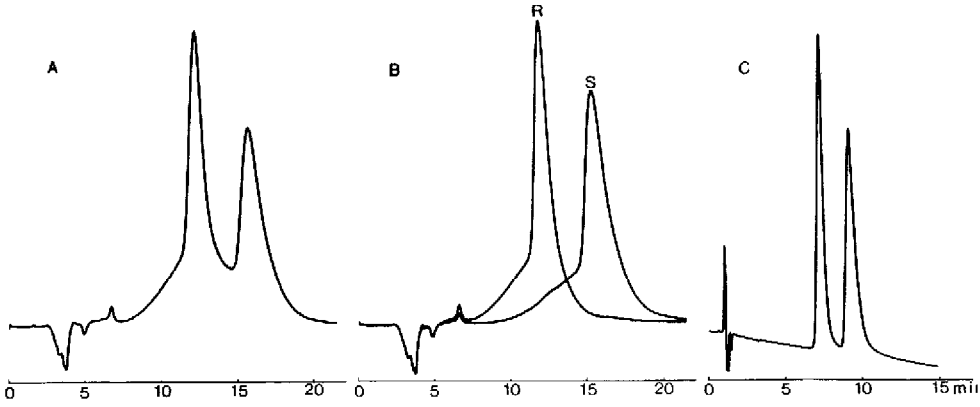

Fig. 6. Chromatograms of bupivacaine. (A) Racemic mixture separated on a first-generation α_1-acid glycoprotein column (Enantiopac). Amount injected: 3 nmol (racemate). (B) Enantiomers injected separately. Amount injected: 1.5 nmol (each enantiomer). (C) Racemic mixture separated on a newly manufactured α_1-acid glycoprotein column (ChromTech). Amount injected: 1.3 nmol (racemate).

CONCLUSION

The use of columns packed with immobilized proteins for the separation and determination of enantiomers in biological samples can be further extended if they are combined with achiral columns in a CCC system. It is possible to obtain an increase in efficiency (sensitivity), peak symmetry and overall selectivity. To achieve this it is necessary to run the whole CCC system in the reversed-phase mode, and to place the protein column first. For many of the enantiomers which have been separated on protein columns methods based on achiral reversed-phase LC have been described in the literature for the determination of the sum of the enantiomers in biological samples. In principle, it is therefore possible to combine such an achiral LC system directly with the chiral LC system to obtain a complete method for the determination of the enantiomers. This is exemplified here for four compounds of pharmaceutical interest.

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