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DETERMINATION OF DRUG ENANTIOMERS IN BIOLOGICAL SAMPLES BY COUPLED COLUMN LIQUID CHROMATOGRAPHY AND LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Liquid chromatography–mass spectrometry (LC–MS) and coupled column chromatography can be used to overcome problems likely to occur in direct separation and determination of drug enantiomers in biological samples. This is exemplified here with the direct separation and determination of terbutaline in human plasma at the nmol/l level. A β-cyclodextrin column with an aqueous mobile phase was used for chiral separation. For coupled column chromatography, the concentration of each enantiomer was calculated from the enantiomeric area ratio and the racemate concentration. A deuterium-labelled internal standard was used in the LC–MS experiments.

INTRODUCTION

Racemates of chiral drugs are extensively used in therapy. An estimate made from the 1980 edition of the U.S. Pharmacopeial Dictionary of Drug Names showed that of the 486 synthetic chiral pharmaceuticals listed, 82% were used as racemates [1]. Although the desired pharmacological activity often resides in only one of the enantiomers [2], the pharmacokinetics (i.e., absorption, distribution, metabolism and excretion) have been predominantly evaluated from concentration measurements on the racemate. Such data might not reflect those of the active enantiomer, because enantiomers often show different pharmacokinetic behaviour [3]. Owing to the increasing awareness of the pharmacological and pharmacokinetic differences between enantiomers [2–7], the need to separate and determine them accurately in biological materials is likely to increase.

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Even if a drug is given as a pure enantiomer, methods that can discriminate between enantiomers will be needed, because racemization can occur both in vitro [8] and in vivo [9].

Separation of enantiomers can be performed either in the direct mode or indirectly after derivatization to diastereomers. If possible, the direct mode is usually preferred [10] but it necessitates a step of chiral recognition in the analytical procedure. In recent years, column liquid chromatography (LC) has become increasingly popular for chiral separations [10] and is at present the most important technique in this field. For direct separation, either a chemically bonded chiral stationary phase or a conventional stationary phase with a chiral mobile phase can be used. Applications of both types of chromatography have recently been reviewed [10,11]. A number of chemically bonded chiral stationary phases are commercially available, but most of them can only be used in the normal-phase mode. A review covering most of these phases available in late 1985 was given by Däppen et al. [12]. In spite of the number of chromatographic systems developed for direct separation, it is sometimes difficult to find one for a specific compound. Basically, there are two approaches which can be used, either the empirical (trial and error) or the more rational approach as exemplified by Pirkle et al. [13]. Some guidance on chromatographic systems useful for different classes of compounds can be obtained from the literature [10].

Hitherto, most of the work on direct chiral separations has been focused on analytes in a simple sample matrix and very little has been published on the direct separation of drug enantiomers in biological materials. Hermansson et al. [14] used a column packed with immobilized α_1-acid glycoprotein (Enantiopac®) for the direct determination of (*R*)- and (*S*)-diisopyramide in human plasma. The same phase was used by Ofori-Adjej et al. [15] for the determination of the enantiomers of chloroquine and desethylchloroquine in plasma and urine after oral administration of racemic chloroquine. Wainer et al. [16] used a Pirkle-type column for the direct separation and determination of propranolol enantiomers in serum after derivatization with a non-chiral reagent. Hay et al. [17] used a chiral mobile phase and a C_{18} chemically bonded packing material for the separation and determination of D- and L-thyroxine in serum.

Although LC is an increasingly popular and useful technique for the direct separation of enantiomers, its application to biological samples is not straightforward. Several complications can be expected, but so far they have received little attention. Some of the more severe complications are as follows.

(1) The chromatographic conditions are restricted [10]: there are fewer possibilities than with conventional LC of avoiding interferences from, e.g., endogenous compounds. Consequently, in most instances, a selective sample work-up must be performed prior to chiral separation.

(2) Most of the chromatographic systems developed for the direct separation of enantiomers are based on normal-phase chromatography, which might preclude the use of some selective detectors, e.g., the amperometric type. It also means that the analyte must be transferred from an aqueous to an organic phase before injection can be made. Traces of water might be difficult to remove and can destroy the chiral selectivity [18].

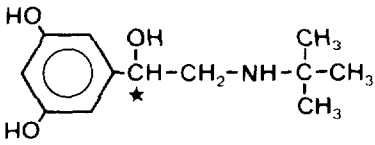

Fig. 1. Structure of terbutaline.

(3) The efficiency (plate number) of chiral columns is often low, which limits the sensitivity.

(4) Internal standardization can be more complicated in chiral work.

(5) The loadability can sometimes be limiting, as reported for protein columns [12].

The purpose of this work was to address some of these problems associated with direct separation and determination of drug enantiomers in biological samples. It has been shown that coupled column chromatography (CCC) can be most useful in the determination of drugs in biological materials [19]. Several configurations are possible and different modes of chromatography can be combined. Selectivity can be manipulated at will and sample work-up can be performed within the LC system. Although at present there are no applications of CCC to the bioanalysis of drug enantiomers, its potential use for such applications was recently pointed out [19].

Mass spectrometry (MS) in combination with LC provides an alternative to CCC for the determination of enantiomers in biological samples. Owing to the inherent selectivity of the mass spectrometer, sample clean-up can be kept to a minimum, but caution must be taken not to overload the chiral column with endogenous material. As both normal- and reversed-phase LC can be made compatible with MS through different inlet and ionization techniques, the use of various chiral chromatographic systems is possible. In recent publications the utility of LC–MS for the chiral separation and identification of drug enantiomers has been demonstrated. Crowther et al. [20] discussed the use of direct liquid introduction LC–MS with a Pirkle-type column for the identification of some non-steroidal anti-inflammatory agents. In the same paper the potential usefulness of LC–MS for the assay of drug enantiomers in biological samples was indicated by the determination of ibuprofen enantiomers in equine urine. A Pirkle-type chiral stationary phase and thermospray (TSP) with external ionization (filament-on) LC–MS was used by Lee et al. [21] to identify (*S*)-(+)-methamphetamine as a contaminant of the *R*-enantiomer. Parker et al. [22] used filament-off TSP LC–MS to detect enantiomers of stilbestrol analogues separated on a triphenylmethyl methacrylate column. Although MS also offers the unique possibility of using a stable isotope-labelled analogue as an internal standard, chiral phase LC–MS has not been used in any quantitative work.

The use of CCC and LC–MS to overcome some of the complications mentioned above will be exemplified by the application of the two techniques to the determination of terbutaline enantiomers in plasma. Terbutaline (Bricanyl®) (Fig. 1), a β_2-receptor agonist widely used in the treatment of asthma, contains one asymmetric carbon. The drug is given as a racemate and, in single-dose phar-

macokinetic studies, it is desirable to measure plasma concentrations of the unchanged drug in the range 0.4–40 nmol/l. Consequently, for routine determination of the racemate, sophisticated techniques such as CCC with amperometric detection [23] or gas chromatography (GC)–MS [24] are used. Effect studies have shown that only (−)-terbutaline is pharmacologically active [25]. The pharmacokinetics of the two enantiomers have been studied after administration of each isomer, but further studies are needed in order to evaluate the kinetics of the two enantiomers when the drug is administered as a racemate. For this reason, a method with the ability directly to separate and determine the two enantiomers of terbutaline was sought.

EXPERIMENTAL

Chemicals

Racemic terbutaline [1-(3,5-dihydroxyphenyl)-2-(*tert.*-butylamino)-ethanol], (+)- and (−)-terbutaline and deuterium-labelled terbutaline ([$^2H_6$]terbutaline, labelled in the *tert.*-butyl group) [24] were obtained from Draco (Lund, Sweden). Ammonium acetate, acetic acid, ammonium chloride, sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate dihydrate were of analytical-reagent grade and obtained from E. Merck (Darmstadt, F.R.G.). Glutathione was obtained from Sigma (St. Louis, MO, U.S.A.), methanol (HPLC grade) from Rathburn Chemicals (Walkerburn, U.K.) and ethanol (99.5%, spectroscopic grade) from Kemetylprodukter (Bromma, Sweden). Water was purified with a Milli Q system (Millipore, Bedford, MA, U.S.A.). Bond Elut C_{18} (200 mg packing material) was obtained from Analytichem International (Harbor City, CA, U.S.A.).

Sample work-up

Plasma samples were adsorbed on disposable solid-phase extraction columns (Bond Elut). A vacuum manifold was used to force the fluids through the extraction columns. The columns were conditioned twice with 3 ml of ethanol, twice with 3 ml of water and once with 2 ml of 0.1 *M* phosphate buffer (pH 7.5). In a test-tube, 2.0 ml of plasma were mixed with 1.0 ml of the phosphate buffer. When preparing samples for LC–MS analysis, the buffer containing the deuterated internal standard was used. The sample solution was forced through the column, followed by two 2-ml volumes of water. Collection tubes were placed under each column and the terbutaline-containing fraction was eluted with 1 ml of ethanol containing 5% of 50 m*M* ammonium chloride solution (pH 8.5). To protect terbutaline from degradation, 50 μl of a 50 m*M* glutathione solution were added to the tubes before evaporation to dryness under vacuum at 50°C.

Coupled column chromatography

A schematic diagram of the CCC system is shown in Fig. 2. The pre-treated samples were injected on to column 1, which was used for on-line sample clean-up. The terbutaline fraction from column 1 was collected in a loop and subsequently transferred to column 2 by switching of the six-port valve. The remainder

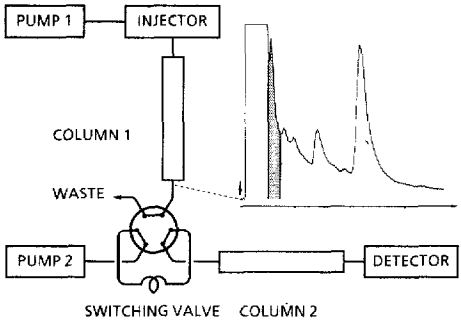

Fig. 2. Schematic representation of the coupled column system used for the determination of terbutaline enantiomers in plasma (cf. Table I).

of the eluate from column 1 was directed to waste. The retention time window for sampling from column 1 was determined beforehand with the amperometric detector coupled directly to the outlet of this column. The times for valve switching were microprocessor-controlled. The retention times of (−)- and (+)-terbutaline were used for identification of the enantiomers. For technical details, see Table I.

The concentration of each enantiomer was calculated from the peak area of each enantiomer and the total peak area. Peak areas were measured with an integrator. Racemate concentrations were determined according to previously described methods based on GC–MS [24] or LC with amperometric detection [23].

TABLE I

TECHNICAL DETAILS OF THE CCC SYSTEM USED FOR THE DETERMINATION OF TERBUTALINE ENANTIOMERS IN PLASMA (cf. FIG. 2)

Pumps: Waters Assoc. Model 45 (pump 1) and LKB 2150 (pump 2)
Flow-rates: 0.2 ml/min (pump 1) and 0.7 ml/min (pump 2)
Detector: LC-4B amperometric detector (Bioanalytical Systems) with a TL-5 glassy carbon electrode and an Ag/AgCl (NaCl, 3 *M*) reference electrode. Working potential, 0.90 V vs. Ag/AgCl
Valves: Valco Model CV-6-UHPa-N60 for injection of samples (manually, 100 μl) and for column switching (pneumatically, 300 μl)
Integrator: Waters 740 Data Module

Column	Dimensions (length × I.D., mm)	Packing material	Manufacturer	Mobile phase
1	100×2	Nucleosil-C_6H_5 (7 μm)	Macherey, Nagel (Düren, (F.R.G.)	0.01 *M* Ammonium acetate (pH 4.6)
2	200×4.5	β-Cyclodextrin (5 μm)	Prepared according to Fujimura et al. [32]	Methanol–0.05 *M* ammonium acetate (10:90, v/v) (pH 6.0)

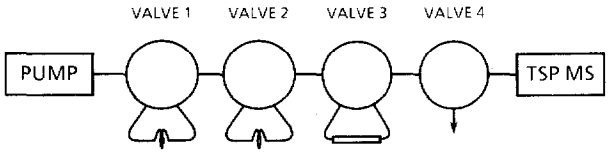

Fig. 3. Schematic representation of the equipment used for the LC–MS experiments (cf. Table II).

Liquid chromatography–mass spectrometry

The chromatographic system used in the LC–MS experiments is shown schematically in Fig. 3. To optimize the TSP conditions, the 5-ml loop at valve 1 was filled with the terbutaline solution and, with valve 3 in the column bypass mode, switched into the flow. This afforded approximately 3 min of terbutaline signal from the mass spectrometer, during which time the TSP conditions could be studied. The interface was operated at a vaporizer temperature of 154 °C and a jet (source block) temperature of 234 °C, giving an aerosol temperature of 210 °C. The repeller potential was kept at 130 V. To achieve maximum sensitivity, the mass spectrometer was operated in the selected-ion monitoring mode. The signal was repetitively monitored from the MH^+ ions of terbutaline (m/z 226) and [$^2H_6$]terbutaline (m/z 232) during 0.2 s per interval, giving a total scan rate of 0.45 s/a.m.u. For technical details, see Table II.

Standard samples for calibration were prepared by adding known amounts of terbutaline and the deuterium-labelled internal standard to blank plasma. The samples were then taken through the complete analytical procedure. The amount of (+)-terbutaline was determined from a calibration graph constructed by plotting the peak-height ratio of (+)-terbutaline to (+)-[$^2H_6$]terbutaline against the amount of (+)-enantiomer. A straight line was fitted to the data by least-squares regression analysis. A corresponding calibration graph was constructed for (−)-terbutaline.

TABLE II

TECHNICAL DETAILS OF THE LC–MS SYSTEM USED FOR THE DETERMINATION OF TERBUTALINE ENANTIOMERS IN PLASMA (cf. FIG. 3)

Pump:	Spectra-Physics SP8700
Flow-rate:	1.2 ml/min
Detector:	Finnigan 4500 mass spectrometer and Finnigan thermospray interface
Valves:	Rheodyne Models 7125 (valve 1, injection of MS optimization solution, 5 ml), 7000 (valve 3, column bypass valve) and 7001 (valve 4, MS isolation valve). Valco Model C6W (valve 2, sample injection, 100 μl) with a Model A60 air actuator
Column:	250×4.6 mm I.D. Cyclobond I, β-cyclodextrin (Astec, Whippany, NJ, U.S.A.)
Mobile phase:	0.1 *M* ammonium acetate (pH 5)

RESULTS AND DISCUSSION

Coupled column chromatography

With the CCC system shown in Fig. 2, a selected part (heart-cut) of the eluate from column 1 can be transferred to the chiral column for further separation. As heart cutting allows for very clean samples to be injected on to the chiral column it should be useful for application to the direct chiral separation of drugs in biological samples. The configuration should be useful in both reversed- and normal-phase chromatography.

For proper use of CCC, some practical details must be considered [19]. Band broadening must be kept to a minimum, which can be accomplished if peak compression can be obtained or if the injected volume is small and extra-column effects are minimized. The fraction transferred to the chiral column must not disturb the chiral separation or interfere with the detection. In cases where incompatability with mobile phases exists, the remedy might be to inject a small volume [26].

Both β-cyclodextrin and α_1-acid glycoprotein can be used for the separation of the enantiomers of terbutaline with an aqueous mobile phase, which was necessary for amperometric detection [27]. However, only partial separation was obtained with the α_1-acid glycoprotein phase. As commonly observed with protein phases, the plate number was very low. β-Cyclodextrin gave a much better resolution and plate number, and was chosen for further work.

The separation and retention of the terbutaline enantiomers on the β-cyclodextrin column was affected by several parameters, e.g., pH, type and amount of organic modifier, injection volume and amount of substance injected. Fig. 4 shows the increased selectivity that can be obtained for terbutaline enantiomers in plasma using CCC compared with a single-column system. For sensitivity reasons, the whole terbutaline peak had to be collected and transferred to the β-cyclodextrin column. Peak compression and hence reduction of band broadening were obtained because mobile phase 2 was a stronger eluent than mobile phase 1 on column 2. This is illustrated in Fig. 5.

Chromatographic methods for the determination of drugs in biological samples often require an internal standard. In quantitative work on enantiomers, finding an internal standard might be difficult. In such instances, an alternative approach is first to determine the concentration of the racemate by a non-chiral method. Next, the chiral separation is performed and the peak areas for each enantiomer are used to calculate their respective concentrations. CCC, with the heart-cutting technique, offers an advantage in the determination of enantiomers because, irrespective of where the cut is taken and how wide it is, the enantiomeric ratio will be the same (Fig. 6). This should be helpful in instances where selectivity is a problem because very small cuts could be taken anywhere on a chromatographic peak. However, care must be taken to check that there is no chiral separation before the chiral column. For example, optically active endogenous compounds in the mobile phase could form diastereomeric pairs with the analyte.

Immobilization of, e.g., albumin could, in principle, produce a chiral chromatographic system [28]. This can be tested by injection of representative amounts

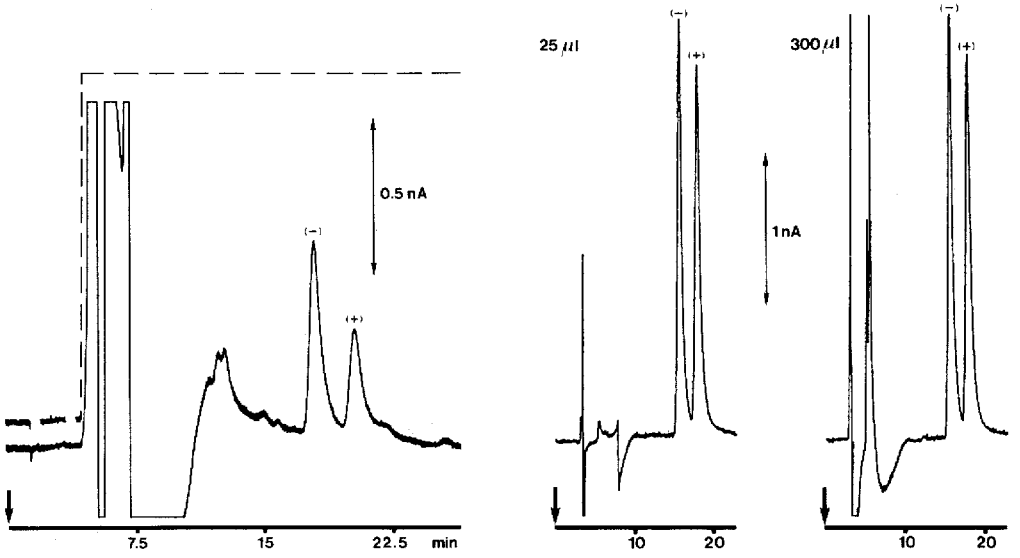

Fig. 4. Chromatograms of a plasma sample containing terbutaline enantiomers [(−)- 11.6 nmol/l and (+)- 6.03 nmol/l]. The lower chromatogram was obtained with the CCC system. The upper chromatogram (broken line) was obtained by direct injection of the crude plasma extract onto the β-cyclodextrin column.

Fig. 5. Chromatograms of the terbutaline enantiomer obtained using the CCC system, demonstrating the peak compression. The resolution was not affected by increasing the volume injected from 25 to 300 µl. Same amount of racemate injected.

of the racemate and calculation of the peak areas for the respective enantiomers. Fig. 4 shows a chromatogram of a plasma sample obtained from a patient given an oral dose of terbutaline.

Liquid chromatography–mass spectrometry

The sensitivity that can be achieved in TSP LC–MS depends strongly on the chemical structure of the analyte and the concentration of organic modifier in the mobile phase [29,30]. Terbutaline was effectively ionized in the TSP ion source and produced an intense MH^+ ion with minimal fragmentation (Fig. 7). In addition, the aqueous ammonium acetate buffer used with the β-cyclodextrin column is the ideal medium for direct ionization (filament-off) TSP, contributing to the favourable conditions. To achieve the ultimate sensitivity required in order to measure terbutaline at levels down to a few picomoles, it was necessary to optimize the temperature of the TSP interface [31]. The sensitivity remained stable over a working day.

After sample work-up (see Experimental), the plasma extract could be injected directly into the β-cyclodextrin column. Fig. 8 shows the mass chromatograms of the blank plasma and an authentic plasma sample containing terbutaline. The crude plasma extracts gave rise to a sloping and noisy baseline, which limited the sensitivity of the method. However, linear calibration graphs were obtained down to 4 pmol. The sum of the amounts of (+)- and (−)-terbutaline measured

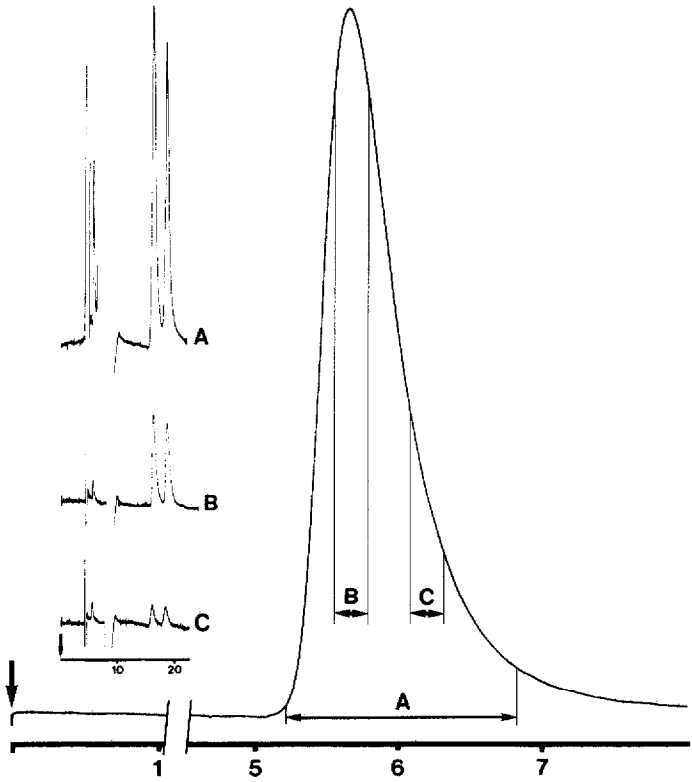

Fig. 6. Chromatographic peak of the racemate of terbutaline obtained after separation on column 1 (cf. Fig. 2). Inset chromatograms (A-C), obtained by transferring different fractions to the chiral column (column 2), show the same enantiomeric peak-area ratio irrespective of where the cut was taken.

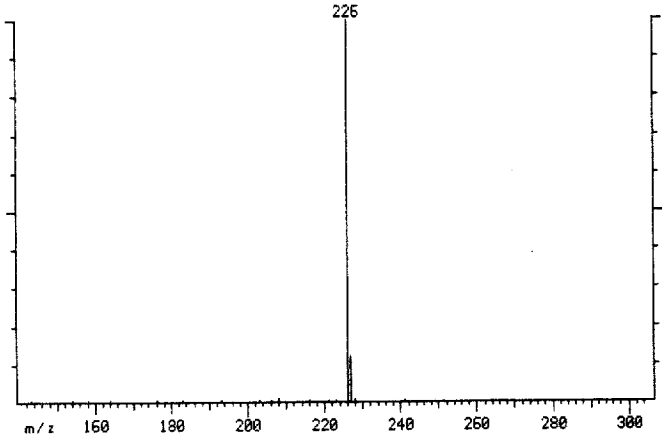

Fig. 7. Thermospray mass spectrum of terbutaline (m/z 226, MH^+).

for the sample shown in Fig. 8 correlated very well with the total amount of racemic terbutaline in an identical sample, as measured by a non-chiral method [23].

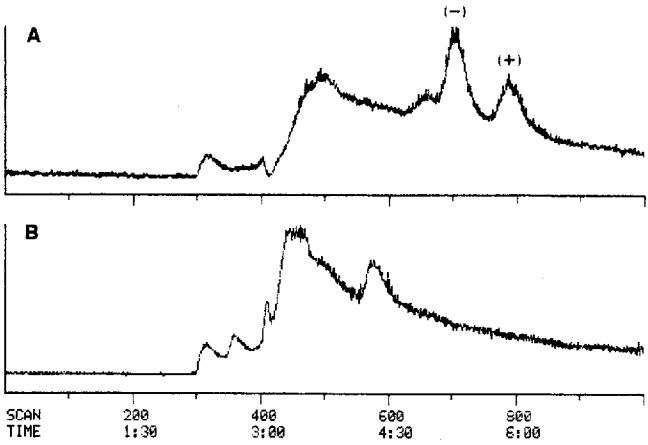

Fig. 8. Mass chromatograms (m/z 226) obtained by direct injection of Bond Elut C_{18}-treated extracts into the TSP LC–MS system. (A) Authentic plasma sample containing terbutaline; (B) blank plasma. Concentrations determined: (−)- 25 nmol/l and (+)- 15 nmol/l. Total racemate concentration: 40.3 nmol/l.

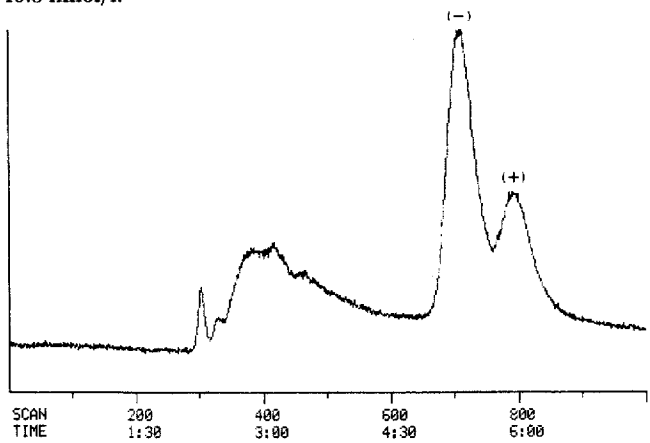

Fig. 9. Mass chromatogram (m/z 226) of an authentic terbutaline-containing plasma sample after purification on column 1 (cf. Fig. 2) and injection into the TSP LC–MS system. Concentrations determined: (−)- 24 nmol/l and (+)- 10 nmol/l. Total racemate concentration: 37.1 nmol/l.

In principle, the above-described CCC system could be used in combination with MS if further purification of the sample is needed. In this work, the pre-separation step on column 1 (cf., Fig. 2) was used off-line to remove the disturbing chromatographic front caused by the crude plasma extract. A mass chromatogram of an authentic plasma sample, analysed by this technique, is shown in Fig. 9. Again, there was good agreement between the sum of (+)- and (−)-terbutaline and the amount of racemate measured previously. If LC–MS is to be used on a routine basis for the determination of drug enantiomers in plasma, it would probably not be advisable to inject crude samples. Instead, they could be injected into a CCC system, which would allow for on-line sample work-up with automated procedures [19].

CONCLUSION

CCC and LC–MS can be used to overcome some of the problems in the direct separation and determination of drug enantiomers in biological samples. With CCC, selective on-line sample work-up can be carried out and, if combined with a heart-cutting technique, excellent selectivity can be obtained. An alternative approach for the determination of enantiomers based on relative peak-area measurements of the respective enantiomers is also suggested. This procedure requires the racemate concentration to be known, but makes the use of an internal standard unnecessary.

When TSP LC–MS is used, the sample work-up can be kept to a minimum because of the selectivity provided by the mass spectrometer. However, care must be taken not to overload the chiral column or contaminate the mass spectrometer with endogenous material. Internal standardization is less complicated in LC–MS because isotopically labelled standards can be used. Both CCC and LC–MS should be applicable to the direct separation and determination of drug enantiomers in biological samples using either reversed- or normal-phase chromatography. The techniques can be used either separately or in combination.

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