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Determination of terbutaline enantiomers in human urine by coupled achiral–chiral high-performance liquid chromatography with fluorescence detection

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Abstract

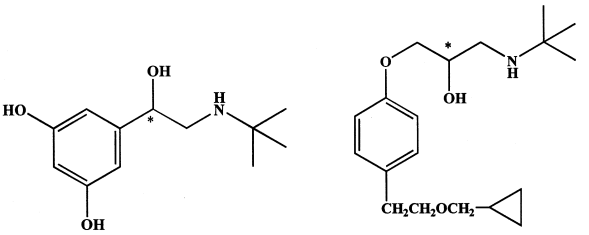
A coupled achiral–chiral high-performance liquid chromatographic system with fluorescence detection at excitation/emission wavelengths of 276/306 nm has been developed for the determination of the enantiomers of terbutaline, (*S*)-(+)-terbutaline and (*R*)-(−)-terbutaline in urine. Urine samples were prepared by solid-phase extraction with Sep-pak silica, followed by HPLC. The terbutaline was preseparated from the interfering components in urine on Phenomenex silica column and the terbutaline enantiomers and betaxolol were resolved and determined on a Sumichiral OA-4900 chiral stationary phase. The two columns were connected by a switching valve equipped with silica precolumn. The precolumn was used to concentrate the terbutaline in the eluent from the achiral column before back flushing onto the chiral phase. For each enantiomer the assay was linear between 1 and 250 ng/ml (R^2=0.9999) and the detection limit was 0.3 ng/ml. The intra-day variation was between 4.6 and 11.6% in relation to the measured concentration and the inter-day variation was 4.3–11.0%. It has been applied to the determination of (*S*)-(+)-terbutaline and (*R*)-(−)-terbutaline in urine from a healthy volunteer dosed with racemic terbutaline sulfate. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Terbutaline

1. Introduction

Enantiomers of racemic drugs often differ in their pharmacokinetic behaviour and/or pharmacololgical action [1].

Terbutaline, a sympaticomimetic drug-selective β_2-receptor agonist, is used in the treatment of asthma and lung disease (Fig. 1). The drug is usually



Terbutaline Betaxolol (internal standard)

Fig. 1. Structures of terbutaline and betaxolol (internal standard). Chiral centers in these molecules are indicated by an asterisk.

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administered as a racemate, but studies have shown that the (−)-enantiomer only has the desired therapeutic pharmacological effect [2]. For that reason it is of great importance that the enantiomers of such molecules can be separated, especially for biomedical analysis.

Among the different methods used for this purpose, high-performance liquid chromatography has often been applied in this field using a wide variety of chiral columns in which different resolution mechanisms have been applied [3]. However, its application to biological samples is not straightforward, because several complications like interferences from endogenous compounds and limits of the sensitivity can be expected. It has been shown that coupled column chromatography can be most useful in the determination of drugs in biological materials. Several configurations are possible and different modes of chromatography can be combined [4–7].

In biological analysis of terbutaline enantiomers, methods based on liquid chromatography–mass spectrometry [4] or coupled column chromatography with amperometric detection were reported [8,9]. However, these methods were presented only for separation and determination of the ratio of each enantiomer of terbutaline in plasma and intestinal juice. Determination of the total concentration of terbutaline (mixture of both enantiomers) in plasma samples demanded gas chromatography–mass spectrometry.

In recent years, capillary electrophoresis (CE), with its high resolving power, has attracted great interest for the analysis of different classes of compounds including enantiomers [10]. Boer et al. [11] and Sheppard et al. [12] reported chiral separation of terbutaline enantiomers by capillary electrophoresis using cyclodextrine as a chiral selector. While common levels of some β-agonists in human urine at therapeutic dosage are in the 1–100 ng/ml range, it was difficult to achieve this level of sensitivity with methods using CE. Most of these methods are not ideal for the measurement of terbutaline enantiomers in biological samples.

In this paper, we applied a column-switching method for the separation of (*S*)- and (*R*)-terbutaline in human urine samples. And for the separation of enantiomers, the Sumichiral OA-4900 column provided good separation. The assay was also applied to the stereoselective pharmacokinetic studies of terbutaline.

2. Experimental

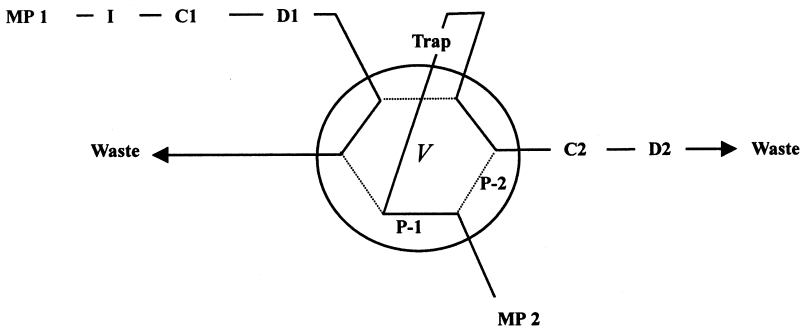
2.1. Chemicals

Terbutaline sulfate and the internal standard, betaxolol HCl, were obtained from Aldrich (Milwaukee, WI, USA). (*S*)-(+)-terbutaline and (*R*)-(−)-terbutaline were prepared by semi-preparative HPLC using a Sumichiral OA-4700 chiral column (250×10 mm I.D., 5 μm, SCAS, Osaka, Japan) and *n*-hexane–1,2-dichloroethane–methanol–trifluoroacetic acid (240:140:15:1, v/v/v/v) as a mobile phase at the Department of Pharmacy, Kangwon National University (Kangwon, Korea). Trifluoroacetic acid (analytical grade) was purchased from Aldrich (Milwaukee, WI, USA). Methanol, *n*-hexane, 1,2-dichloroethane, ethyl acetate and acetonitrile (HPLC grade) were obtained from Duksan Pure Chemicals Co. (Ansan, Kyeonggi, Korea).

2.2. Equipment

The HPLC equipment (Fig. 2) consisted of the following components: two LC-9A pumps, two RF-10AXL fluorescence detectors with excitation/emission wavelengths of 276/306 nm, an FCV-2AH six-port switching valve driven and time-controlled by a SCL-6B controller (Shimadzu, Kyoto, Japan). The samples were applied by a Rheodyne model 7725*i* sample loop injector with an effective volume of 200 μl.

For the LC columns, a Phenomenex silica (250×4.6 mm I.D., 5 μm, Torrance, CA, USA) and a Sumichiral OA-4900 (250×4.6 mm I.D., 5 μm, SCAS, Osaka, Japan) were used as an achiral column and a chiral column, respectively. A Phenomenex silica guard column (30×4.6 mm I.D., 5 μm, Torrance, CA, USA) was chosen as the trap column. The acquisition of chromatograms and integration were obtained using a C-R4A integrator (Shimadzu, Kyoto, Japan).



Valve Position	Time(minute)	Function
P-1	0.00 – 13.50	Waste & Data acquisition from achiral column
P-2	13.51 – 15.50	Load terbutaline and betaxolol on silica trap column
P-1	15.51– 40.00	Elute terbutaline and betaxolol from chiral column

Fig. 2. Schematic diagram of the column-switching apparatus in the HPLC system. P-1: valve position 1 (bold line), P-2: valve position 2 (dotted line), MP 1: achiral mobile phase, MP 2: Chiral mobile phase, I: injector, C1: Phenomenex silica column, C2: Sumichiral OA-4900 column, D1 and D2: fluorescence detector, Trap: Phenomenex silica guard column, V: switching valve.

2.3. Sample preparation

A 10-μl volume of internal standard solution (betaxolol HCl, 5 μg/ml, in water) was spiked into 1 ml urine. The mixture was vortex-mixed for 10 s and applied to a Sep-pak silica cartridge pre-conditioned with acetonitrile (1 ml) and water (1 ml). The cartridge was washed with water (1 ml) and then acetonitrile (2 ml). The analytes were eluted with 6 ml of methanol. The eluate was concentrated to dryness and the residue was reconstituted in the achiral mobile phase (400 μl) for an assay sample.

2.4. Chromatographic procedures

This column-switching system was able to introduce the objective compounds into the chiral column and remove the large biological materials from the HPLC system. A schematic diagram of the column-switching system is shown in Fig. 2.

A 200-μl urine sample was injected into an achiral column and racemic terbutaline and the internal standard were separated from urine interferences with a mobile phase consisting of *n*-hexane–ethyl acetate–1,2-dichloroethane–methanol–trifluoroacetic acid (240:200:120:25:1, v/v/v/v/v) at a flow-rate of 1.0 ml/min. After 13.5 min the fraction containing the compounds of interest, terbutaline and the internal standard, was sent into the trap column for 2 min. The compounds were then eluted from the trap column and transferred to the chiral column with the mobile phase consisting of *n*-hexane–ethyl acetate–1,2-dichloroethane–methanol–trifluoroacetic acid (240:220:160:35:1, v/v/v/v/v) at a flow-rate of 1.0 ml/min.

Ratios of terbutaline enantiomers to the internal standard vs. concentrations of terbutaline enantiomers were used for quantitative computations.

2.5. Validation studies

Spiked urine samples were prepared by adding known amounts of racemic terbutaline and the internal standard (betaxolol) to drug-free urine at six

concentration levels (2, 10, 50, 100, 250 and 500 ng/ml) and used for evaluation of linearity, accuracy and precision. The precision of the method was assessed by determining the intra-day assay coefficients of variation (C.V.) of the analysis ($n=6$) of spiked urine samples. The C.V. and accuracy for inter-day assay were evaluated by analysis of samples at three concentrations (10, 100 and 500 ng/ml), repeated for three different days.

2.6. Application of the method

This analytical method was applied to a pharmacokinetic study. Racemic terbutaline sulfate (5 mg) was administered once orally to a 24-year-old healthy male volunteer. Urine samples were obtained at 1, 3.5, 8.4, 12, 15.2, 24.7, 29.8, 33.2, 36, 38.8 and 45 h after intake of the drug and were stored at −20°C until analysis.

3. Results and discussion

3.1. Chromatography

The composition of the mobile phase was investigated to determine the optimal chromatographic conditions. In the column-switching technique, similar mobile phases have to be used for the two HPLC system because the solvent used for achiral HPLC could flow into the chiral HPLC system [13].

The enantiomers of terbutaline were separated by HPLC on Sumichiral OA-4900 chiral column using a mixture of *n*-hexane, 1,2-dichloroethane, methanol and trifluoroacetic acid (240:140:25:1, v/v/v/v) as a mobile phase [14]. The same mobile phase was used for the silica column. However, terbutaline was highly retained on silica stationary phase, i.e. the retention time was approximately 34 min. Although the concentration of 1,2-dichloroethane increased into 52%, the retention time of terbutaline did not changed.

Addition of ethyl acetate to the mobile phase decreased the retention of terbutaline on the silica column. The influence of the concentration of ethyl acetate on the capacity factors (k') of racemic terbutaline and the internal standard, betaxolol, are shown in Fig. 3. As the ratio of ethyl acetate increased, the capacity factor of the terbutaline decreased but capacity factor of internal standard did not change. We therefore selected *n*-hexane–ethyl acetate–1,2-dichloroethane–methanol–trifluoroacetic acid (240:200:120:25:1, v/v/v/v/v) as the optimum achiral mobile phase. Under these conditions, terbutaline and the internal standard were eluted as a single peak, with a peak width of approximately 1.2 min and the total fraction containing those two compounds was transferred to the trap column by a six-port switching valve.

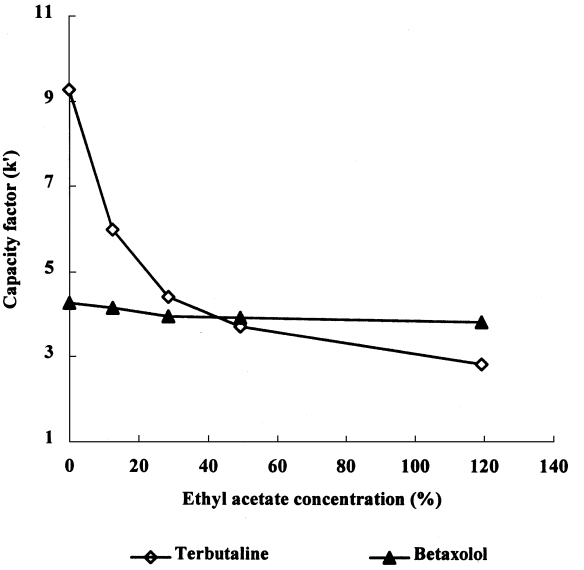


Fig. 3. The effects of the ethyl acetate concentration in the achiral mobile phase on the capacity factors of terbutaline and betaxolol (internal standard) in the achiral HPLC system. Column: Phenomenex silica, 250×4.6 mm I.D.; mobile phase: the indicated volume percent of ethyl acetate was added to a mobile phase consisting of *n*-hexane–1,2-dichloroethane–methanol–trifluoroacetic acid (240:120:25:1, v/v/v/v); flow-rate: 1.0 ml/min; fluorescence detector: excitation wavelength 276 nm, emission wavelength 306 nm.

The trapping capacity of a silica precolumn was examined by altering the transfer time. Fig. 4 shows the relationship between peak areas of the analytes and trapping windows. The peak areas of terbutaline and betaxolol reached the highest levels at the transfer window of 1.5 min and the peak areas of those and the peak area ratio of terbutaline to internal standard also did not changed when the transfer time of 2.0 min was used. The peak areas of those

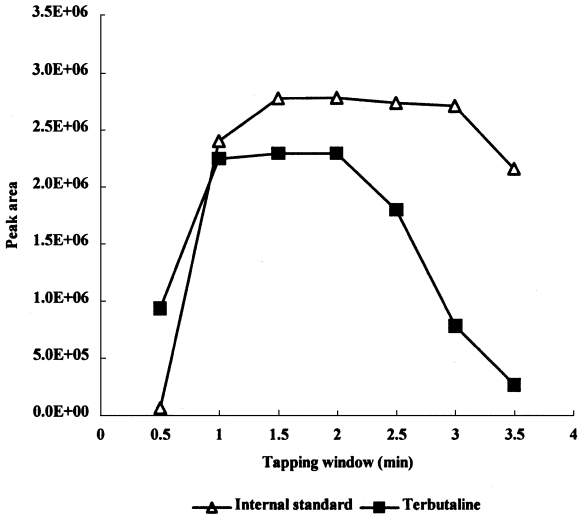


Fig. 4. Relationship between peak areas of the analytes and trapping windows.

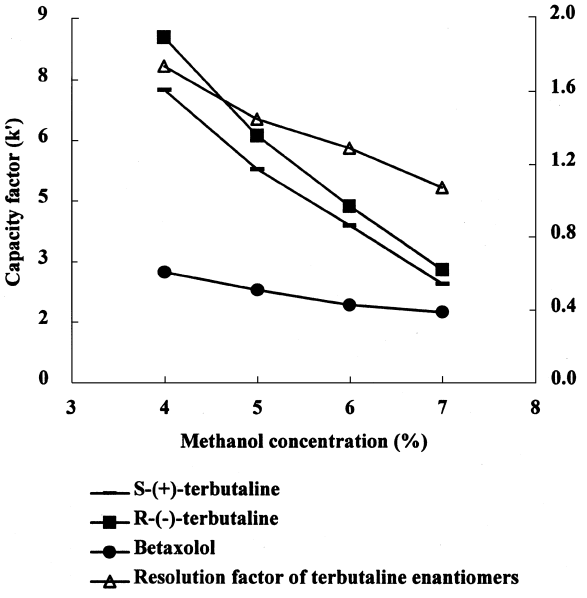


Fig. 5. The effects of methanol concentration in the chiral mobile phase on the capacity factor (k') of terbutaline and betaxolol and resolution factor (R_s) of terbutaline enantiomers. Column: Sumichiral OA-4900, 250×4.6 mm I.D.; mobile phase: the indicated volume percent of methanol was added to a mobile phase consisting of *n*-hexane–ethyl acetate–1,2-dichloroethane–trifluoroacetic acid (240:200:120:1, v/v/v/v); flow-rate: 1.0 ml/min; fluorescence detector: excitation wavelength 276 nm, emission wavelength 306 nm.

decreased with the transfer time ≥2.5 min. These results indicate that the transfer of a peak containing terbutaline and internal standard is complete within 1.5 min and the trapping capacity is not less than 2.0 min. Thus, a 2-min window for the switch was selected and satisfactory results were obtained in validation studies.

The analytes were eluted from the trap column with the chiral mobile phase in back-flush mode. The difference between the chiral mobile phase and achiral mobile phase usually causes large baseline disturbances after column switching [15]. This disturbance should be avoided, especially for sensitive analysis, which requires fast elution of the analyte. The difference in composition of the true mobile phase should be minimal.

Because peak-sharpening effects generally can be obtained by enhancing the elution power (solvent strength) of the eluents, the effects of the concentration of methanol in the chiral mobile phase were investigated (Fig. 5) and a methanol concentration of 6% was selected to obtain a reasonable separation in a reasonable time. However, when the assays of blank samples spiked with terbutaline and the internal standard from human urine were performed, the (*S*)-terbutaline peak was not completely separated from that of the interfering substance. To improve the separation of the analytes, the effect of varying the ratio of ethyl acetate to 1,2-dichloroethane in the mobile phase was examined. Fig. 6 shows the effects of the ratio of ethyl acetate to 1,2-dichloroethane in the chiral mobile phase on the retention time of terbutaline, the internal standard and the interference peak. The use of *n*-hexane–ethyl acetate–1,2-dichloroethane–methanol–trifluoroacetic acid (240:220:160:35:1, v/v/v/v) as a chiral mobile phase resulted in baseline separation of terbutaline enantiomers, the internal standard and the interference peak. Under the chiral conditions, the retention times of (*S*)-(+)-terbutaline, (*R*)-(−)-terbutaline and internal standard were 23.47, 24.86 and 20.15 min, respectively. For the terbutaline enantiomers, the stereoselectivity (α) was 1.22 and the resolution factor (R_s) was 1.65. Fig. 7 shows chromatograms obtained from analysis of drug-free human urine and urine samples. No interferences with either terbutaline enantiomers or the internal standard were detected from blank urine.

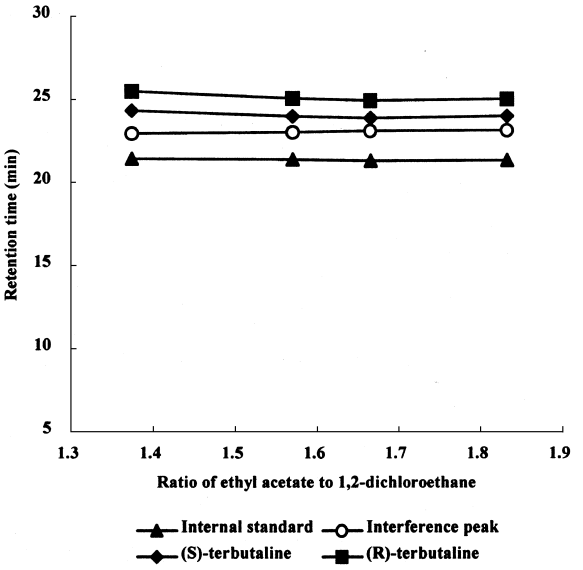


Fig. 6. The effects of the ratio of ethyl acetate to 1,2-dichloroethane in the chiral mobile phase on the retention time of terbutaline, the internal standard and the interference peak. Column: Sumichiral OA-4900, 250×4.6 mm I.D.; mobile phase: the indicated ratio of ethyl acetate to 1,2-dichloroethane was added to a mobile phase consisting of *n*-hexane–methanol–trifluoroacetic acid (240:35:1, v/v/v); flow-rate: 1.0 ml/min; fluorescence detector: excitation wavelength 276 nm, emission wavelength 306 nm.

The enantiomeric elution order was determined by chromatographing the individual enantiomers of terbutaline separately under the same chromatographic conditions. The peak that eluted first was identified as (*S*)-(+)-terbutaline and the second peak was identified as (*R*)-(−)-terbutaline.

3.2. Linearity and limit of detection

The calibration curves were obtained by analyzing spiked urine samples. Calibration curves for each enantiomer showed good linearity in the concentration range 1–250 ng/ml in urine. The Eq. of the calibration line obtained for (*S*)-(+)-terbutaline is: $Y=0.0090X+0.0055$ and for (*R*)-(−)-terbutaline is: $Y=0.0091X+0.0064$. The correlation coefficients of (*S*)-(+)-terbutaline and (*R*)-(−)-terbutaline were 0.9999.

The limit of detection, estimated under the described conditions at a signal-to-noise ratio of 3, was 0.3 ng/ml.

3.3. Accuracy and precision

The accuracy and precision of the method were determined by replicated analysis of blank human urine spiked with six concentrations of terbutaline enantiomers within the range 1–250 ng/ml. Six replicates of each concentration were analyzed on each of three separate days. The results obtained are shown in Tables 1 and 2. The intra-day precision for each concentration was 4.9–11.6% for (*S*)-(+)-terbutaline and was 4.6–10.9% for (*R*)-(−)-terbutaline. The inter-day precision was 4.5–11.0% for (*S*)-(+)-terbutaline and was 4.3–7.0% for (*R*)-(−)-terbutaline. The accuracy, determined for each concentration, ranged from 99.4% to 107.5%.

3.4. Application

The method described was applied to a pharmacokinetic study of terbutaline enantiomers in human urine samples obtained after the oral administration of tablet containing 5 mg terbutaline sulfate. Typical urinary excretion data of terbutaline enantiomers from a healthy male volunteer is shown in Fig. 8. From the 0–45-h urine, the amounts of terbutaline enantiomers were determined. Unchanged terbutaline accounted for 5.07% of the dose and the (*S*)-(+)-terbutaline)/(*R*)-(−)-terbutaline ratio was 0.76. (*R*)-(−)-terbutaline had a higher excretion rate than (*S*)-(+)-terbutaline.

4. Conclusion

When CSPs are used in bioanalysis, the presence of co-extracted contaminants may cause problems in analyte retention, resolution and column stability. Some of these problems associated with CSPs can be overcome by using coupled column chromatography. Combination of a silica column as an achiral phase with a Sumichiral OA-4900 column as a chiral column provided a satisfactory separation of terbutaline enantiomers with an R_s value of 1.65. Coupled column chromatography with fluorescence detection was sufficient to selectively detect ter-

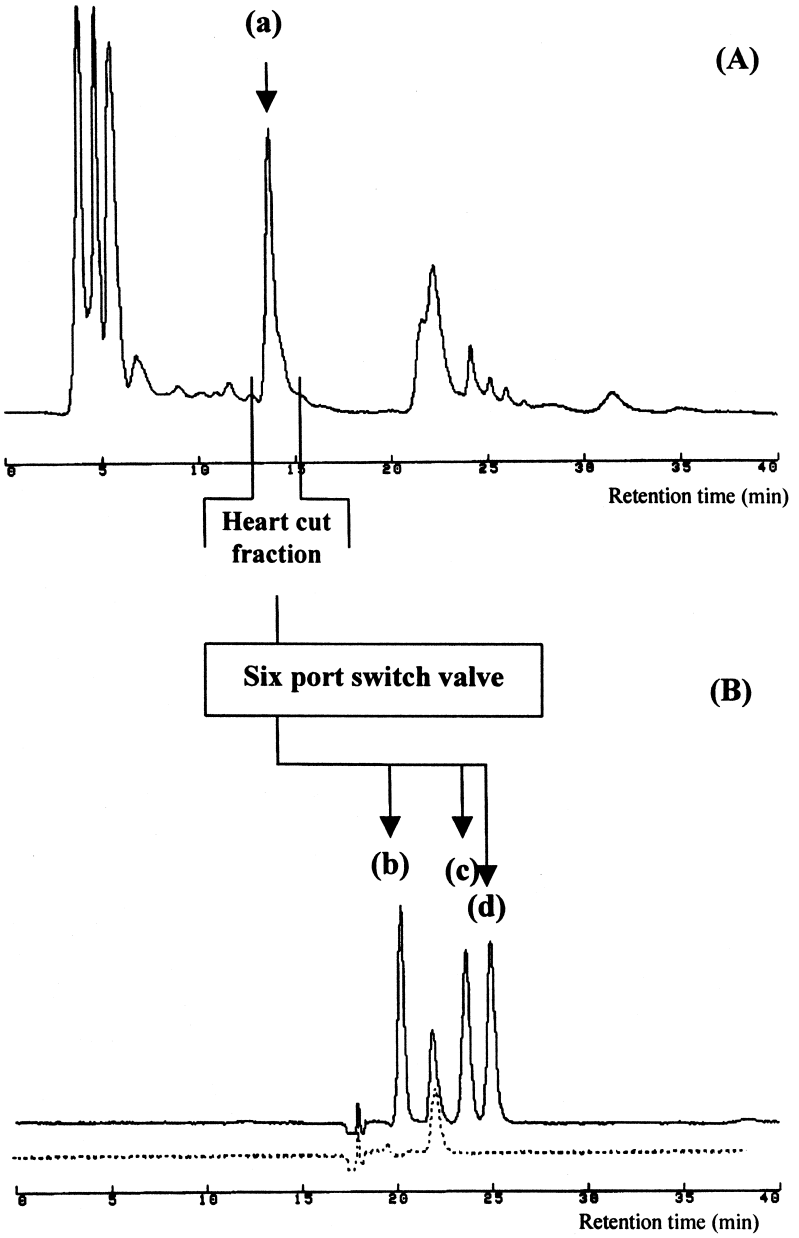


Fig. 7. Representative chromatograms of a 3.5-h urine sample after oral administration of 5 mg terbutaline sulfate on the coupled achiral–chiral chromatographic system. (A) achiral chromatogram (B) chiral chromatogram after column switching, Peak a; racemic terbutaline and internal standard (betaxolol), Peak b; internal standard (betaxolol), Peak c; (*S*)-(+)-terbutaline (111 ng/ml), Peak d; (*R*)-(−)-terbutaline (113 ng/ml), dotted line; chiral chromatogram of blank urine sample after column switching. Achiral column: Phenomenex silica, 250×4.6 mm I.D.; chiral column: Sumichiral OA-4900, 250×4.6 mm I.D.; achiral mobile phase: *n*-hexane–ethyl acetate–1,2-dichloroethane–methanol–trifluoroacetic acid (240:200:120:25:1, v/v/v/v); chiral mobile phase: *n*-hexane–ethyl acetate–1,2-dichloroethane–methanol–trifluoroacetic acid (240:220:160:35:1, v/v/v/v); flow-rate: 1.0 ml/min; fluorescence detector: excitation wavelength 276 nm, emission wavelength 306 nm.

Table 1
Intra-day precision for the (*S*)-(+)-terbutaline and (*R*)-(−)-terbutaline in human urine (n=6)

Added conc. (ng/ml)	(*S*)-(+)-terbutaline			(*R*)-(−)-terbutaline		
	Measured conc. (ng/ml)	Accuracy (%)	C.V. (%)	Measured conc. (ng/ml)	Accuracy (%)	C.V. (%)
1	1.1	107.5	11.6	1.0	99.9	10.9
5	5.2	103.2	5.9	5.2	103.2	5.3
25	25.4	101.5	8.6	25.3	101.3	8.5
50	49.7	99.4	8.6	49.9	99.7	8.6
125	124.4	99.5	4.9	124.3	99.5	4.6
250	250.4	100.1	6.5	250.3	100.1	6.4

Table 2
Inter-day precision for the (*S*)-(+)-terbutaline and (*R*)-(−)-terbutaline in human urine (n=3)

Added conc. (ng/ml)	(*S*)-(+)-terbutaline			(*R*)-(−)-terbutaline		
	Measured conc. (ng/ml)	Accuracy (%)	C.V. (%)	Measured conc. (ng/ml)	Accuracy (%)	C.V. (%)
5	5.1	101.4	11.0	5.1	101.8	7.0
50	49.5	99.0	6.0	49.6	99.1	6.4
250	249.5	99.8	4.5	250.2	100.1	4.3

butaline in human urine. The present method is convenient and simple and has been fully validated and proved to be suitable for the stereoselective pharmacokinetic studies of terbutaline.

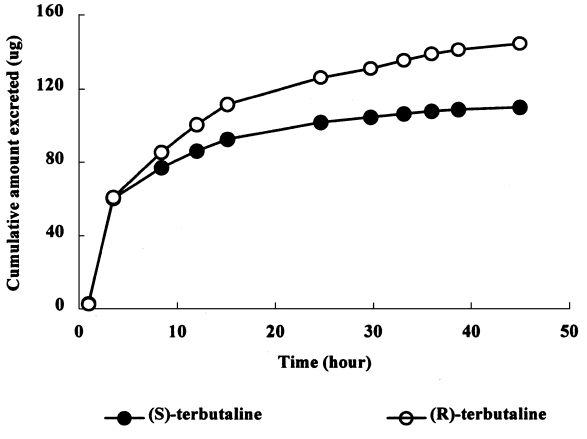


Fig. 8. Cumulative urinary excretion of terbutaline enantiomers following a single oral dose of 5 mg terbutaline sulfate.

Acknowledgements

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