

Determination of Terbutaline Enantiomers in Human Plasma by Coupled Achiral-Chiral High Performance Liquid Chromatography

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Achiral-chiral column switching HPLC assay was developed to allow the separation and quantification of the enantiomers of terbutaline in human plasma by means of fluorescence detection. Plasma samples were prepared by solid-phase extraction with sep-pak silica, followed by HPLC assay. The enantiomers of terbutaline and the internal standard were separated from the biological matrix on a silica column, and the two enantiomers were resolved and quantified on a Sumichiral OA-4900 column. The two columns were connected by a switching valve equipped with silica trap column. The trap column was used to concentrate the terbutaline in the eluent from the achiral column before back flushing onto the chiral phase. For each enantiomers, the assay was linear between 2.5-125 ng/ml ($r=0.9999$) and detection limit was 1.0 ng/ml.

Key words: Coupled column chromatography, Terbutaline, Chiral separation

INTRODUCTION

Analytical and preparative chromatographic and electrophoretic methods (mainly HPLC and capillary electrophoresis) are required to study the biological action and activity of enantiomeric drugs (Arens *et al.*, 1971 and Ferretti *et al.*, 1998). Among the methods currently used to achieve chiral separation of racemic mixtures, direct high-performance liquid chromatographic separations on columns packed with chiral stationary phases (CSPs) is used routinely for the resolution of racemic mixtures. In so doing the drawbacks arising from optically impure reagents or different rates of formation of the diastereoisomers are avoided.

Terbutaline, 1-(3,5-dihydroxyphenyl)-2-(*tert*-butylamino) ethanol, is a selective β_2 -receptor agonist widely used in the treatment of asthma (Jeppson *et al.*, 1984). The drug has an asymmetric center at the α -carbon (Fig. 1) and is usually administered as a racemate. However, it is a well established fact that single enantiomers are often much more potent and have reduced side effects compared to their racemates (Ariens *et al.*, 1992). For many other β_2 -agonist, the active isomer is the (R)-(-)-terbutaline, which

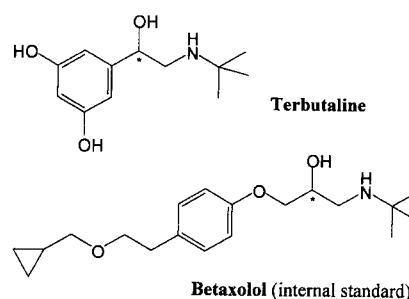


Fig. 1. Structures of terbutaline and betaxolol (internal standard). Chiral centers in these molecules are indicated by an asterisk.

is 200 times more potent than (S)-(+)-terbutaline (Ahuja *et al.*, 1990).

The literature reports that both α_1 -acid glycoprotein and β -cyclodextrin LC chiral stationary phases can be used to resolve the enantiomers of terbutaline (Bergguist *et al.*, 1983). However, its application to biological samples is not straightforward, because of several expected complications such as interferences from endogenous compounds and sensitivity limits.

In recent years, capillary electrophoresis (CE) has proved particularly useful for enantiomeric separation, and Boer *et al* (1998), Aturki *et al* (1994) and Sheppard *et al* (1995) have reported a CE method for the enantiomers of terbutaline using a cyclodextrin chiral selector added to the electrophoresis buffer. The main drawback of these CE

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methods has been the lack of sensitivity. With lower limits of quantification in the low ng/ml range, they have only been suitable for defining the pharmacokinetics of terbutaline after oral dosing (Joyce *et al.*, 1998).

Coupled column chromatography has been shown to be the most useful in the determination of drugs in biological materials. Coupled column chromatography based methods have been reported for the determination of terbutaline enantiomers in human plasma and intestinal juice (Walshagen *et al.*, 1989). The authors used β -cyclodextrin chiral column and C_{18} achiral column for coupled column chromatography. While a validated method was used to determine the ratio of each enantiomer of terbutaline in plasma and intestinal juice, determination of the total concentration of terbutaline in plasma samples demanded gas chromatography-mass spectrometry.

In this paper, we describe a coupled achiral-chiral HPLC method to separate terbutaline from a biological matrix on a silica achiral column, which is followed by chiral resolution of the corresponding enantiomers on a Sumichiral OA-4900 column. The method is shown to be selective, accurate and precise.

MATERIALS AND METHODS

Materials and equipment

Terbutaline sulfate and the internal standard, betaxolol hydrochloride were obtained from Aldrich (Milwaukee, WI, USA). (S)-(+)-terbutaline and (R)-(-)-terbutaline were prepared from racemic terbutaline by semi-preparative chiral high-performance liquid chromatography. All substances used were of analytical grade, all solvents were HPLC grade. *n*-Hexane, methanol, 1,2-dichloroethane, ethyl acetate and acetonitrile were obtained from Duksan Pure Chemicals Co. (Ansan, Kyeonggi, Korea). Trifluoroacetic acid was obtained from Aldrich (Milwaukee, WI, USA).

The HPLC instrument (Shimadzu, Kyoto, Japan) consisted of two LC-9A pump, two RF-10AXL fluorescence detector, and a FCV-2AH six port switching valve driven and time controlled by a SCL-6B controller. The chromatograms and integration were obtained using a C-R4A integrator (Shimadzu, Kyoto, Japan).

Chromatographic conditions

The achiral mobile phase (MP 1) consisted of a mixture of *n*-hexane-ethyl acetate-1,2-dichloroethane-methanol-trifluoroacetic acid (240:220:180:25:1, v/v/v/v/v). The chiral mobile phase (MP 2) was a mixture of *n*-hexane-ethyl acetate-1,2-dichloroethane-methanol-trifluoroacetic acid (240:220:220:35:1, v/v/v/v/v). Each mobile phase was delivered at a flow rate of 1.0 ml/min.

Separations were achieved on a Inertsil SIL-100 (250 \times 4.6 mm I.D., 5 μ m, GL science, Tokyo, Japan) protected

by a silica guard column (10 \times 4 mm I.D., 5 μ m, Shimadzu, Kyoto, Japan), and on a Sumichiral OA-4900 (250 \times 4.6 mm I.D., 5 μ m, SCAS, Osaka, Japan). A Phenomenex silica guard column (30 \times 4.6 mm I.D., 5 μ m, Torrance, CA, USA) also was chosen as the trap column.

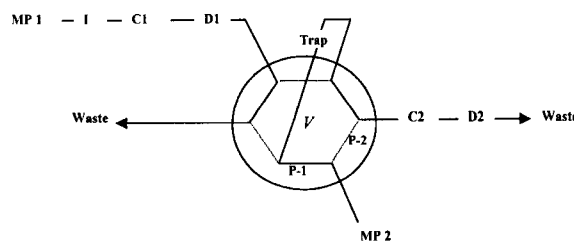
The sample injection volume was 200 μ l. The eluent was monitored with a fluorescence detector operating at an excitation wavelength of 276 nm and an emission wavelength of 306 nm.

Switching valve programming

A schematic diagram of the column-switching HPLC system and a time program of the system controller are shown in Fig. 2. At the beginning of the analysis (time zero), the valve was placed in position 1 (P-1, Fig. 2), and 200 μ l of a plasma sample was injected directly from Rheodyne 7725i injector. The injected sample dissolved in achiral mobile phase and passed first through the silica analytical column (C1, Fig. 2). Here, terbutaline and the internal standard, betaxolol, are pre-separated from many interferences in the plasma sample. When the peak containing terbutaline and the internal standard eluted from the silica analytical column, it was temporarily switched onto a silica guard column (Trap column, Fig. 2), then eluted onto Sumichiral OA-4900 chiral column and the enantiomers were separated using chiral mobile phase (MP 2, Fig. 2).

Sample preparation

For the preparation of standard samples the solution containing terbutaline was diluted with an appropriate amount of water. To achieve calibration concentrations of 2.5 ng/ml to 125 ng/ml, appropriate quantities of the



Valve Position	Time (minute)	Function
P-1	0.00-24.00	Waste & Data acquisition from achiral column
P-2	24.01-26.00	Load terbutaline and betaxolol on silica trap column
P-1	26.01-40.00	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: Inertsil SIL-100 silica column, C2: Sumichiral OA-4900 column, D1 and D2: fluorescence detector, Trap: Phenomenex silica guard column, V: switching valve.

various diluted solutions were added to blank plasma. The solutions were mixed on a vortex mixer for 10 s. Next, 1 ml of plasma was mixed on a vortex mixer for 10 s with 25 μ l of internal standard solution (1 μ l/ml betaxolol hydrochloride in water). The mixture was subjected to solid phase extraction. Prior to solid phase extraction, silica sep-pak cartridge (100 mg, Waters, MA, USA) was rinsed with 2.0 ml of acetonitrile, followed by rinsing with 2.0 ml of water. Next, 1.0 ml of the plasma samples was transferred onto the cartridge. The cartridges were then washed with 1.0 ml of acetonitrile. Elution of the absorbed analyte was performed with 5 ml of methanol. The elute was concentrated to dryness, and the residue was reconstituted in 400 μ l of achiral mobile phase, of which 200 μ l was injected into the achiral HPLC system.

Standard curves and recovery

Plasma standards were prepared by spiking normal human drug-free plasma with solutions of racemic terbutaline to concentrations of 5, 20, 50, 100 and 250 ng/ml. Linearity was determined on two calibration curves for the two enantiomers of terbutaline. Calibration curves and analysis of linearity were performed for each enantiomers by using linear least-squares regression analysis and by plotting the ratios of the peak area of the separated enantiomer and the internal standard against the concentration of the enantiomer. The percent recovery and reproducibility were investigated using drug-free plasma samples spiked with 5, 20, 100 ng/ml racemic terbutaline with $n=6$ at each level.

RESULTS AND DISCUSSION

Chiral semi-preparative HPLC of (S)-(+)-terbutaline and (R)-(-)-terbutaline and determination of the elution order

Racemic terbutaline was injected into the semi-preparative chiral HPLC system and resolved into each enantiomer on the Sumichiral OA-4700 chiral column (250 \times 8.0 mm I.D., 5 μ m, SCAS, Osaka, Japan) by *n*-hexane-1,2-dichloroethane-methanol-trifluoroacetic acid (240:140:15:1, v/v/v/v) as a mobile phase at room temperature and flow rate of 4 ml/min monitored at 276 nm UV. Fractions containing single enantiomers were collected and evaporated to dryness under nitrogen stream. Optical purity was determined by the chiral HPLC using Sumichiral OA-4900 analytical column (250 \times 4.6 mm I.D., 5 μ m) and *n*-hexane-1,2-dichloroethane-methanol-trifluoroacetic acid (240:140:25:1, v/v/v/v) as a mobile phase. Optical purity of each enantiomer was not less than 99.9%. The direction of rotation (+/-) was determined using a Jasco DIP-1000 digital polarimeter (Kim *et al*, 2000). (S)-(+)-terbutaline was eluted first (Fig. 3). The retention time of (S)-(+)-terbutaline and (R)-(-)-terbutaline was 35 min and 41 min, respectively.

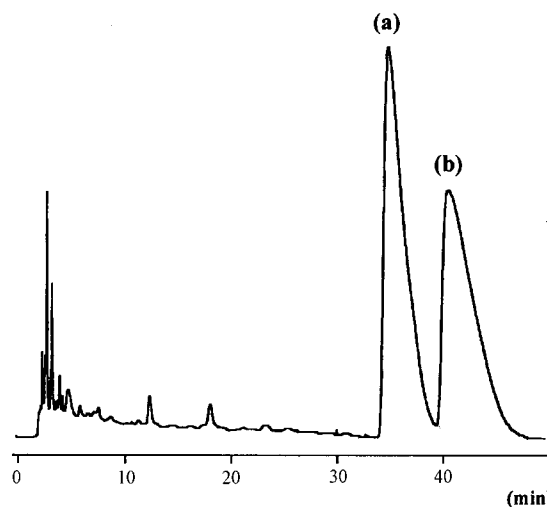


Fig. 3. Chiral semi-preparative HPLC chromatogram of terbutaline enantiomers. Peak a; (S)-(+)-terbutaline; Peak b; (R)-(-)-terbutaline. Column, Sumichiral OA-4900, 250 \times 8 mm I.D.; mobile phase, *n*-hexane-1,2-dichloroethane-methanol-trifluoroacetic acid (240:140:15:1, v/v/v/v); detector, UV 276 nm.

Chromatography

The mobile phase for the coupled system was limited to solvents compatible with the chiral Sumichiral OA-4900 column, i.e., *n*-hexane, ethyl acetate, 1,2-dichloroethane, methanol, and trifluoroacetic acid at all possible proportions because the solvent used for achiral HPLC could flow into the chiral HPLC system.

Above all, a Phenomenex silica column (250 \times 4.6 mm I.D., 5 μ m, Torrance, CA, USA) was chosen for the achiral analytical column. When a mixture of *n*-hexane-ethyl acetate-1,2-dichloroethane-methanol-trifluoroacetic acid (240:200:120:25:1, v/v/v/v/v) was used as an achiral mobile phase, terbutaline and the internal standard, betaxolol, were eluted as a single peak and a total fraction containing those was transferred to the chiral column. However, the retention time of interference peak was overlapped with that of (R)-(-)-terbutaline on the chiral column. Whatever the proportions of ethyl acetate and 1,2-dichloroethane in the chiral mobile phase, it was not possible to separate (R)-(-)-terbutaline from interference peak.

Thus, silica columns from various manufacturers were tested to remedy this problem and Inertsil SIL-100 (250 \times 4.6 mm I.D., 5 μ m, GL science, Tokyo, Japan) was found to be optimal. Terbutaline and the internal standard were eluted as a single peak, with the peak width of approximately 1.2 min by the use of mobile phase consisting of *n*-hexane-ethyl acetate-1,2-dichloroethane-methanol-trifluoroacetic acid (240:220:180:25:1, v/v/v/v/v). That peak was temporarily switched onto a silica guard column, and then was sent into Sumichiral OA-4900 chiral column.

The transferred terbutaline and the internal standard

were resolved each other on a Sumichiral OA-4900 column. Because peak sharpening effects generally can be obtained by enhancing the elution power (solvent strength) of the chiral mobile phase, the amount of methanol and 1,2-dichloroethane in the chiral mobile phase was increased. The use of *n*-hexane-ethyl acetate-1,2-dichloroethane-methanol-trifluoroacetic acid (240:220:220:35:1, v/v/v/v/v) as a chiral mobile phase resulted in baseline separation of terbutaline enantiomers, the internal standard and the interference peak.

The chromatograms obtained after extraction of a blank plasma and a plasma sample spiked with racemic terbutaline and the internal standard are presented in Fig. 4 and Fig. 5, respectively. The retention times of (S)-(+)-terbutaline, (R)-(-)-terbutaline and internal standard were 35.07, 36.51 and 32.51 min, respectively. For the terbutaline enantiomers, the stereoselectivity (α) was 1.23 and

the resolution factor (R_s) was 2.06. No interferences with either terbutaline enantiomers or the internal standard were detected from blank plasma.

Recovery and precision

Standard curves for (R)-(-)-terbutaline and (S)-(+)-terbutaline were linear over the range investigated. The equation of the calibration line obtained for (S)-(+)-terbutaline is: $Y=0.0133 X + 0.0263$, and for (R)-(-)-terbutaline is: $Y=0.0133 X + 0.0252$. The correlation coefficients of (S)-(+)-terbutaline and (R)-(-)-terbutaline were 0.9999.

The recovery test was carried out with plasma samples spiked with 5, 20, 100 ng/ml racemic terbutaline. The results are represented in Table I. The precision for each concentration was 1.6-5.9% for (S)-(+)-terbutaline and was 1.3-9.3% for (R)-(-)-terbutaline. The recovery, determined for each concentration, ranged from 96.1% to 101.0%. The limit of detection, estimated under the described condi-

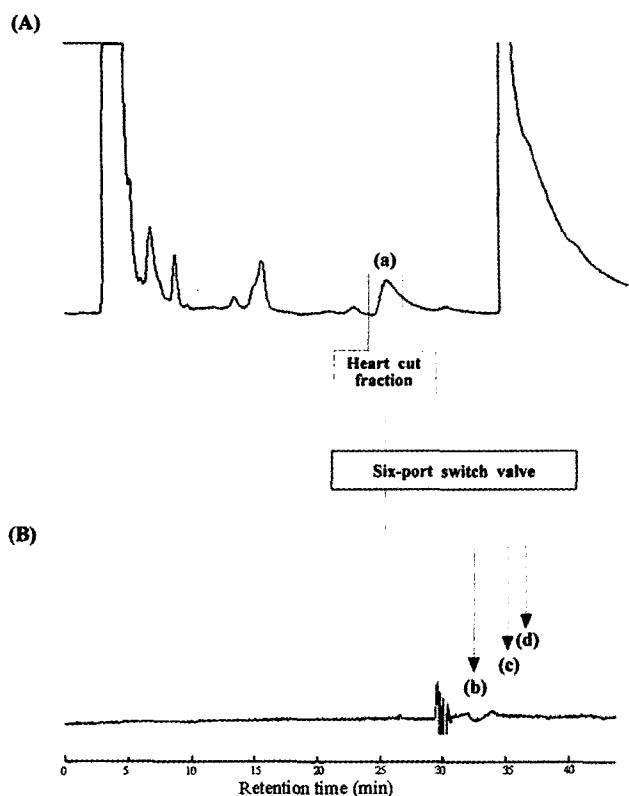


Fig. 4. Representative chromatograms of blank plasma sample on the coupled achiral-chiral chromatographic system. (A) achiral chromatogram (B) chiral chromatogram after column switching, Peak a; racemic terbutaline and internal standard (betaxolol), Peak b; internal standard (betaxolol), Peak c; (S)-(+)-terbutaline, Peak d; (R)-(-)-terbutaline. Achiral column, Inertsil SIL-100, 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 (240:220:180:25:1, v/v/v/v/v); chiral mobile phase, *n*-hexane-ethyl acetate-1,2-dichloroethane-methanol-trifluoroacetic acid (240:220:220:35:1, v/v/v/v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 306 nm.

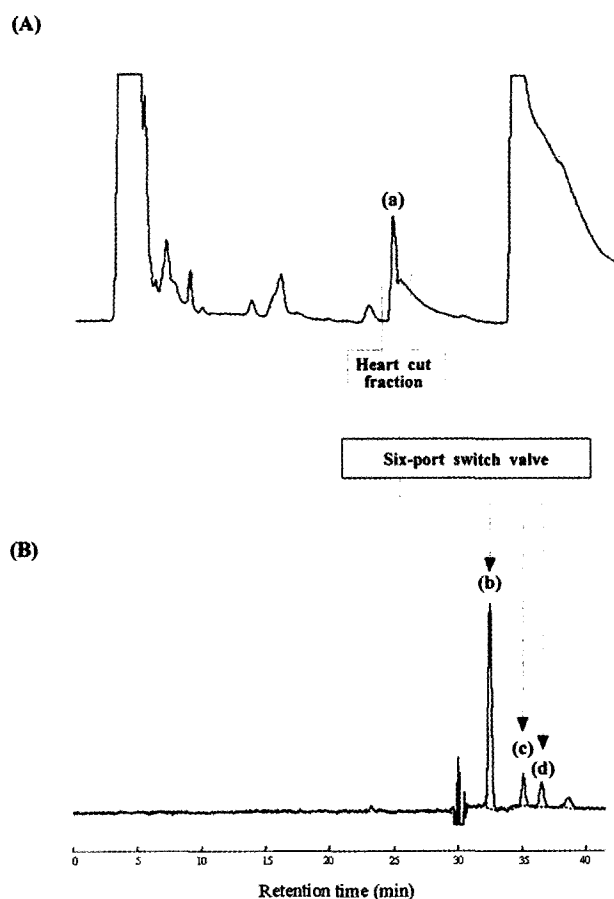


Fig. 5. Representative chromatograms of plasma sample spiked with 20 ng/ml racemic terbutaline on the coupled achiral-chiral chromatographic system. (A) achiral chromatogram (B) chiral chromatogram after column switching, Peak a; racemic terbutaline and internal standard (betaxolol), Peak b; internal standard (betaxolol), Peak c; (S)-(+)-terbutaline, Peak d; (R)-(-)-terbutaline. Conditions as in Fig. 4.

Table I. Recovery test for the (S)-(+)-terbutaline and (R)-(-)-terbutaline in human plasma (n=6)

Added conc. (ng/ml)	Component	Measured conc. (ng/ml)	Recovery (%)	Precision (C.V.)
2.5	(S)-(+)-terbutaline	2.45	98.08	5.72
	(R)-(-)-terbutaline	2.46	98.32	9.33
10	(S)-(+)-terbutaline	9.61	96.10	5.86
	(R)-(-)-terbutaline	9.81	98.12	5.40
50	(S)-(+)-terbutaline	50.14	100.28	1.60
	(R)-(-)-terbutaline	50.29	100.58	1.29

tions, was 1 ng/ml (S/N=3).

CONCLUSION

One of the major problems encountered in the application on HPLC chiral stationary phase to the analysis of biological samples is the coelution of the enantiomers and interfering compounds from the matrix because of the low sample capacity. This problem could be solved by the use of achiral-chiral coupled column systems, in which the enantiomers are separated from interferences in the biological matrix on the achiral phase and then switched to the chiral stationary. A rapid and convenient method for the determination of terbutaline enantiomers in human plasma has been developed. The method was shown to be selective, accurate and precise. Therefore, the assay described in this paper can be used for the pharmacokinetic study of terbutaline enantiomer.

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