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Resolution of Salbutamol Enantiomers in Human Urine by Reversed-phase High Performance Liquid Chromatography after Derivatization with (S)-(-)-α-Methylbenzyl isocyanate

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A stereospecific HPLC method has been developed for the resolution of the enantiomers of salbutamol in human urine. After solid-phase extraction and derivatization with (S)-(-)-α-methylbenzyl isocyanate, the diastereomeric derivatives were resolved (R_s=1.59) on 5 μm octadecylsilan column using 47% methanol as a mobile phase with fluorescence detection. The detection limit of each enantiomer was 10 ng/ml (S/N=3).

Key words : Chiral separation, HPLC, Chiral derivatizing reagent, Salbutamol, Enantiomer, (S)-(-)-α-methylbenzyl isocyanate

INTRODUCTION

Salbutamol, 2-(tert-butylamino)-1-(4-hydroxy-3-hydroxymethylphenyl)-ethanol, also known as albuterol, is a relatively selective β_2-adrenergic bronchodilator, which has been used to relieve bronchospasm in patients with reversible obstructive airway diseases (Cullen *et al.*, 1969). It is used clinically as a recemic mixture of two optical isomers, R-(-)- and S-(+)- salbutamol. Even though both enantiomers show high selectivity for β-adrenoceptors in bronchial muscle compared to cardiac muscle, the drug's agonistic activity resides mainly in the R-(-) enantiomer (Brittain *et al.*, 1973; Hartley *et al.*, 1971; Hawkins *et al.*, 1973).

Stereoselective disposition of enantiomers can result in different pharmacological profiles owing to different rate of absorption or stereoselective presystemic metabolism, distribution or clearance. A few enatioselective assays for salbutamol have been reported. Tan *et al.* (1987) resolved salbutamol enantiomers in human urine using α_1-AGP column at 0°C. But the resolution was not satisfactory (R_s=1.06) and detection limit was high (259 ng/ml) because of the low sample capacity of the α_1-AGP column. Adams *et al.* (1993) and Boulton *et al.* (1995) determined salbutamol enantiomers in human blood using Pirkle-type chiral stationary phase with fluorescence detection. Though the assays were sensitive enough for the analysis of salbutamol enantiomers in human biological samples, prelimenary equilibrium between the nonpolar mobile phase and Pirkle-type chiral stationary phase required long time.

Seo *et al.* (1994) determined salbutamol enantiomers in human plasma using off-line achiral-chiral coupled chromatogrphy. Salbutamol racemate extracted from human plasma was first separated on the achiral silica column and fractionated. Secondly the salbutamol racemate was resolved into each enantiomer and quantitized on the Pirkle-type chiral stationary phase with fluorescence detection. The assay was sensitive enough but the procedure took long time.

This paper describes a simple method that involves the preparation of diastereomers of salbutamol with the chiral reagent (S)-(-)-α-methylbenzyl isocyanate (MBIC), followed by achiral reversed-phase high performance liquid chromatography with fluorescence detection.

MATERIALS AND METHODS

Materials and equipment

Salbutamol sulfate was acquired from Glaxo Korea (Ansan, Korea). Bamethane sulfate was obtained from Sigma Chemical Co. (St. Louis, MO, USA). S-(-)-α-methylbenzyl isocyanate (MBIC) and trifluoroacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC grade methanol, acetonitrile, n-hexane and 1,2-dichloroethane were obtained from J. T. Baker (Phillipsburg, NJ. USA). Water was purified with a Milli-RO 15 Water system (Nihon millipore, Japan)

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and was filtered through a 0.2 μm filter. All other reagents were of analytical reagent grade. The mobile phase was filtered through a 0.2 μm filter and degassed by sonication under vacuum before using it.

High performance liquid chromatograph was consisted of Shimadzu Model LC-9A pump, SPD-6AV spectrophotometric detector, RF-535 fluorescence detector, SCL-6B system controller, C-R4AD chromatopac (Kyoto, Japan). The separations were carried out on a Inertsil ODS-2 column, 4.6 mm I.D. × 150mm, with 5 μm particle size (GL Science Inc, Japan) at ambient temperature. Semi-preparative chiral chromatography was performed with Sumichiral OA-4700, 8.0 mm I.D.× 250 mm, with 5 μm particle size (Osaka, Japan). Bakerbond-spe (silica gel, 1ml) and vacuum manifold box which holds 12 solid-phase extraction cartridges were obtained from J. T. Baker Inc. (Phillipsburg, NJ, USA).

Determination of the order of elution

Salbutamol racemate 40 mg was dissolved in 10 ml of mobile phase. This solution was injected into the semi-preparative chiral HPLC system and resolved into each enantiomer on the Sumichiral OA-4700 column (5 μm, 8 mm×25 cm) by the n-hexane, 1,2-dichloroethane, methanol and trifluoroacetic acid (240:140:15:1) as a mobile phase at room temperature and flow rate of 4ml/min monitoring at 278 nm UV. Fractions containing single enantiomers were collected and evaporated to dryness under nitrogen stream. The direction of rotation (+/-) was determined using a Jasco DIP-1000 digital polarimeter. R-(-)-salbutamol and S-(+)-salbutamol were derivatized with MBIC at room temperature for 30 min, follwed by injected into the achiral HPLC system (column; Inertsil ODS-2, 5 μm, 4.6mm I.D.×150 mm, mobile phase; 47% methanol in water, flow rate; 1ml/min, detection; fluorescence 235 nm for excitation and 305 nm for emission) and the elution order of each isomer was confirmed.

Sample prepaation

1 ml aliquot of urine sample and 100 μl of internal standard solution of bamethane (1.2 μl/ml) were transferred to a prelabelled culture tube and mixed. The solid-phase (silica) extraction column was preconditioned by washing with 1 ml of acetonitrile followed by 1 ml of deionized water. The washing solvents was allowed to pass through with minimum vacuum (<50 mmHg) which was released immediately after the solvents eluted from all the cartridges. The previously mixed urine sample was transfered into the preconditioned column and minimum vacuum was applied. When the urine sample in the cartridge reservoir had been removed, the vacuum was increased to 500 mmHg for 2 min and then released. Each column was then washed with 1 ml of deionized water followed by 1 ml of acetonitrile under minimum vacuum until all the washing solvents eluted from the cartridges. The column was dried by full vacuum for an additional 5 min. Salbutamol and bamethane were then eluted from the silica adsorbent by rinsing with 3 ml of methanol under minimum vacuum until no effluent was observed. The effluent was evaporated to dryness under the nitrogen gas at room temperature.

To the dried extract, 100 μl of MBIC solution (1.2 μl/ml) was added and mixed well at room temperature for 30 min and then the solvent was removed under a stream of nitrogen at room temperature and residue was reconstituted in 200 μl of mobile phase and 100 μl was injected into the achiral HPLC system.

Standard curve samples were constituted by serial dilution of the working standard solution with the drug-free human urine to give final concentration of 50~1000 ng/ml.

Optimization of derivatization

A large volumn of solid-phase extract was prepared for the purpose of optimizing MBIC concentration by extracting miltiple drug-free human urines spiked with 1000 ng/ml of salbutamol enantiomers. Aliquots of the extract (3 ml) were evaporated to dryness to which 100 μl aliquots of MBIC solutions (0.075, 0.15, 0.3, 0.6, 1.2, 1.8, 2.4, 3.0 μl/ml) were added and stand for 30 min at room temperature. Following evaporation and dissolution of the residue in mobile phase, the resulting samples were injected into the achiral HPLC system and the peak areas quantitated.

The effects of time and temperature were investigated by preparing a 1000ng/ml extract, as outlined above. Aliquots of the extract (3 ml) were evaporated to dryness and incubated with 100 μl of 1.2 μl/ml MBIC solution at room temperature, 50°C, 70°C for 15 min, 30 min, 1 hr, 2 hr, 3 hr, or 4 hr. They were evaporated to dryness, the residue dissolved in the mobile phase and the resulting samples quantitated by achiral HPLC.

Assay validation and calculations

The accuracy and precision of the assay were determined by analyzing five replicates from the three samples spiked with 100, 500 and 1000 ng/ml of salbutamol enantiomers to drug-free human urine. Standard curves were constructed by plotting the concentration versus the peak area ratio (peak area of component/peak area of internal standard) and fitting a least squares linear regression analysis line to the data. Concentration of R-(-)-salbutamol and S-(+)-salbutamol in the samples were determined by extrapolating the peak area ratio to the least squares line of the standard curve.

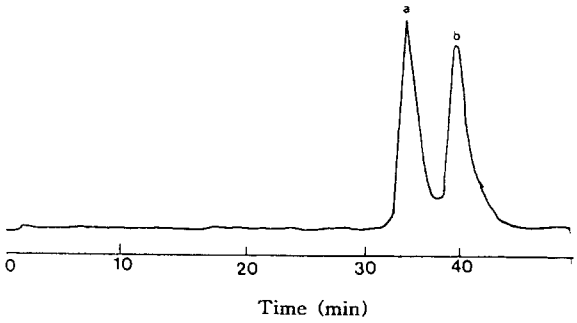

Fig. 1. Chiral semi-preparative HPLC chromatogram of salbutamol racemate. Column; Sumichiral OA-4700 (5 μm, 8× 250 mm), mobile phase; n-hexane:1,2-dichloethane:methanol:trifluoroacetic acid (240:140:15:1), flow rate; 4 ml/min, detection; UV at 278 nm. Peak (a); S-(+)-salbutamol, peak (b); R-(-)-salbutamol.

RESULTS AND DISCUSSION

Chiral semi-preparative HPLC of R-(-)- and S-(+)-salbutamol and dermination of elution order of the derivatives

On the chiral semi-preparative HPLC system, S-(+)-salbutamol was eluted first (Fig. 1). The retention times

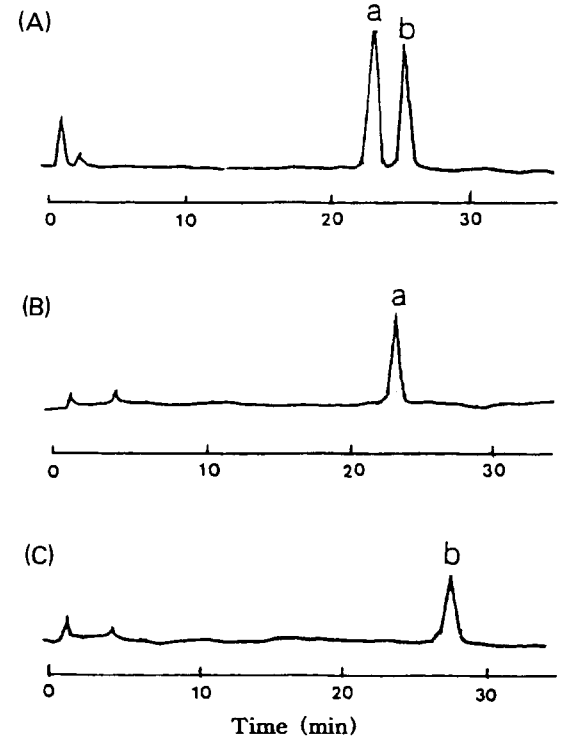

Fig. 2. HPLC chromatograms of (A) salbutamol racemate, (B) S-(+)-salbutamol and (C) R-(-)-salbutamol after fractionation from the chiral semi-preparative HPLC and dervatization with MBIC. Column; Inertsil ODS 2 (5 μm, 4.6×250 mm), mobile phase; 47% methanol in water, flow rate; 1 ml/min, detection; fluorescence 235 nm for excitation and 305 nm for emission. Peak (a); derivative of S-(+)-salbutamol, peak (b); derivative of R-(-)-salbutamol.

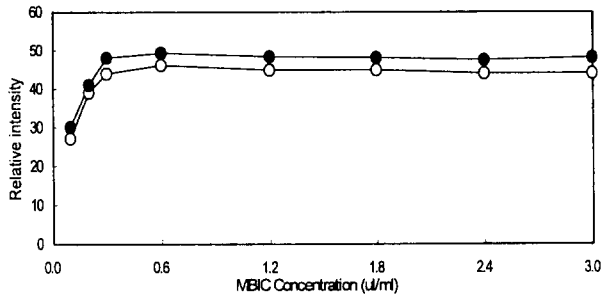

Fig. 3. The effect of MBIC concentration on the peak areas of the derivatives of salbutamol enantiomers. ●; R-(-)-salbutamol, ○; S-(+)-salbutamol.

of each enantiomer were 33.49 and 38.15 min, respectively. After fractionation of the eluent containing each enantiomer and derivatization with MBIC, derivative of S-(+)-salbutamol was found to be eluted faster than that of R-(-)-salbutamol on the achiral reversed-phase HPLC system. During derivatization racemization was not occured (Fig. 2).

Optimization of derivatization

As shown in Fig. 3, the maximum peak area response for the enantiomers was seen at the MBIC concrntration of 0.6 μl/ml. In the final analytical conditions, the MBIC concentration was doubled to 1.2 μl/ml to provide an adequate excess of reagent.

As the reaction temperature was increased from room temperature to 50°C or 70°C, the peak areas of enantiomers were decreased and other impurity peaks supposed to emerge from side reaction were increased. The effect of reaction time at room temperature was shown in Fig. 4. The formation of the derivatives of enantiomers increased with the reaction time up to 20 min and reached a plateau.

Resolution of salbutamol enantiomers in urine

Fig. 5. depicts representative chromatograms of drug-free blank human urine and urine containing 100 ng/ml of R-(-)-salbutamol and S-(+)-salbutamol analyzed

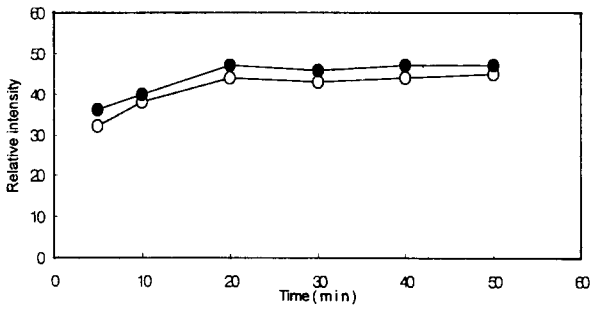

Fig. 4. The effect of reaction time on the peak areas of the derivatives of salbutamol enantiomers at room temperature. ●; R-(-)-salbutamol, ○; S-(+)-salbutamol.

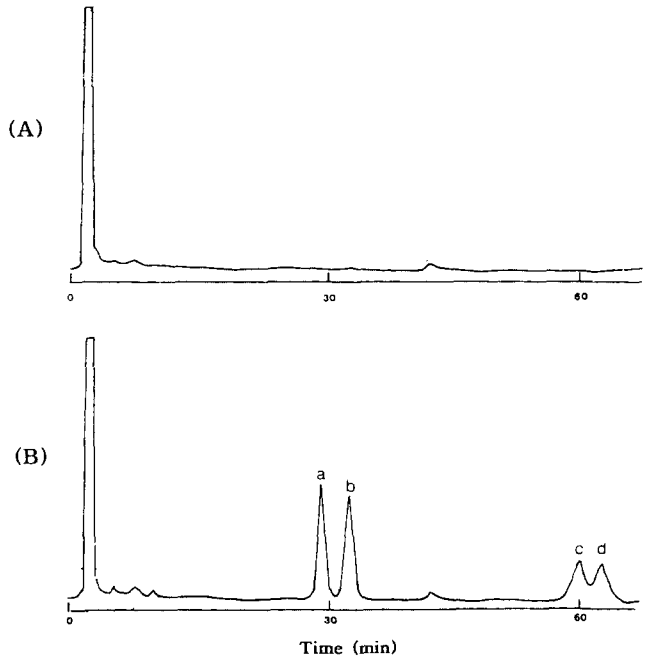

Fig. 5. Typical chromatograms of (A) extract of blank human urine; (B) extract of 100 ng/ml stantard salbutamol enantiomers spiked to human urine. Column; Inertsil ODS-2 (5 μm, 4.6×150 mm), mobile phase; 47% methanol in water, flow rate; 1 ml/min, detection; fluorescence 235 nm for excitation and 305 nm for emission. Peak (a); derivative of S-(+)-salbutamol, peak (b); derivative of R-(-)-salbutamol, peak (c), (d); derivatives of bamethane enantiomers.

by the procedure described above. The peaks corresponding to derivatives of salbutamol enantiomers and derivatives of bamethane enantiomers, internal standard, were easily resolved from coeluting endogenous substances. The retention times of the diastereomeric derivatives of S-(+)-salbutamol and R-(-)-salbutamol were 29.67 and 32.79 min (R_s=1.59). The retention times of the derivatives of bamethane enantiomers were approximately 60.52 and 61.02 min, respectively and their orders of elution were not determined.

The values determined for the precision and accuracy of the assay in human urine are presented in Table I. The accuracy was good and the reproducibility was within 10%. The standard curves for the enantiomers gave a linear response with correlation coefficients greater than 0.999.

Table I. Recovery test for the R-(-)-salbutamol and S-(+)-salbutamol in human urine (n=5)

Amount added (ng/ml)	Component	Amount recoverd (ng/ml)	Recovery (%)	Precision (C.V.)
100	R-(-)-salbutamol	98.6	98.0	9.3
	S-(+)-salbutamol	92.0	92.0	9.8
500	R-(-)-salbutamol	490.7	98.1	6.8
	S-(+)-salbutamol	490.6	98.1	3.8
1000	R-(-)-salbutamol	1005.0	100.5	4.8
	S-(+)-salbutamol	1004.9	100.5	1.5

In conclusion, it was found that the derivatization of salbutamol with MBIC was an useful technique for the separation of the enantiomers by reversed phase HPLC. The procedure has several major advantage over previously described techniques: the derivatization is simple and rapid, the chiral reagent (MBIC) is commercially available and inexpensive and commercial inexpensive reversed-phase chromatographic column can be used. And good separation of the diastereomeric derivatives was obtained.

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