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Determination of metoprolol enantiomers in human urine by coupled achiral–chiral chromatography

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Abstract

Achiral–chiral column switching HPLC assay was developed to allow the separation and quantitation of the enantiomers of metoprolol in human urine by means of fluorescence detection. Urine samples were prepared by liquid–liquid extraction, followed by HPLC. The racemic metoprolol and internal standard were separated from the interfering components in urine and quantified on the silica column, and the enantiomers were determined on a Chiralcel OD chiral stationary phase. The two columns were connected by a switching valve equipped with a silica trap column. Detection limit was 25 ng/ml for each enantiomer. The intra-day variation ranged between 0.38 and 4.94% in relation to the measured concentration and the inter-day variation was 0.15–3.13%. It has been applied to the determination of (*R*)-(+)-metoprolol and (*S*)-(−)-metoprolol in urine from healthy volunteers dosed with racemic metoprolol tartrate.

Keywords: Enantiomer; Coupled column chromatography; Column switching; Metoprolol

1. Introduction

Metoprolol is a β_1 selective aryloxypropanol amine adrenergic antagonist used extensively in the treatment of a variety of cardiovascular disorders and administered as a racemic mixture (Fig. 1). (*S*)-(−)-metoprolol has been reported to be significantly greater β_1 adrenergic receptor affinity by >25-fold than (*R*)-(+)-metoprolol [1,2]. And stereoselective receptor affinity, clearance, protein and tissue binding, metabolism and even drug interaction have been demonstrated for many agents [3]. Therefore, when racemic metoprolol is used clinically it is administered as two different drugs with different pharmacokinetic and potentially pharmacodynamic properties in select patient populations. The great difference in pharmacological effect and pharmacokinetics between the two enantiomeric forms has needed methods for enantioselective separation and determination in biological samples [4].

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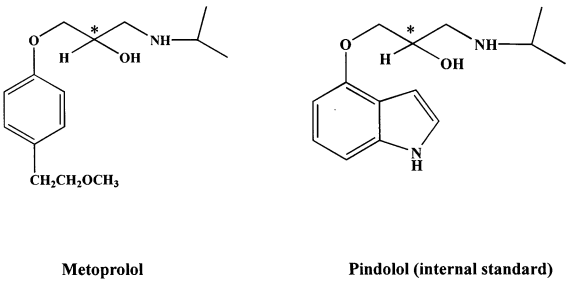


Fig. 1. Structures of metoprolol and pindolol (internal standard). Chiral centers in these molecules are indicated by an asterisk.

Column liquid chromatography can be employed for the resolution of the enantiomers of metoprolol using different approaches such as derivatization with a chiral reagent to form diastereomers (indirect method) [5,6], chemically bonded chiral stationary phases and chiral complexing or ion-pairing agents in the mobile phase (direct method) [7–9]. The methods used for determination in biological samples must beside good resolution also give high sensitivity, which can be achieved by techniques that combine high detectability with a small peak width [10].

A paper was published on the determination of metoprolol enantiomers in plasma with 2,3,4,5-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [11] and (*S*)-(+)-1-(1-naphthyl)-ethyl isocyanate (NEIC) [12] and in urine with (−)-methyl chloroformate [13]. But sample preparation can be rather laborious because of derivatization reaction.

Development of chiral stationary phases has allowed the direct resolution of metoprolol enantiomers without the need for prior derivatization. Persson et al. (1990) and Walhagen et al. (1989) employed α_1-acid glycoprotein as the CSP to separate metoprolol enantiomers [10,14]. Subsequently, determination methods of metoprolol enantiomers in human plasma or serum using Chiralcel OD column have been previously reported. However, in order to remove interfering peaks, a second liquid–liquid extraction or a solid phase extraction were added [4,15,16].

To overcome the disadvantage of the direct method and indirect method, coupled column chromatography was developed. The principle of coupled column chromatography is transferring selective fraction of the mobile phase from the outlet of one column to the inlet of another column. The column-switching technique combines an automated clean-up and pre-concentration step with a separation step. When CSPs are used in biological sample, the problems in resolution and column stability can be overcome [17]. Mangani et al. (1997) reported column-switching method for the direct HPLC analysis of a number of cardiovascular drugs in serum. However, enantiomeric analysis only was performed on the pindolol [18]. We applied column-switching method for the separation of (*R*)- and (*S*)-metoprolol in human urine samples and for the separation of metoprolol enantiomers, the Chiralcel OD column provided good separation and was used. The assay also was applied to the stereoselective pharmacokinetic studies of metoprolol.

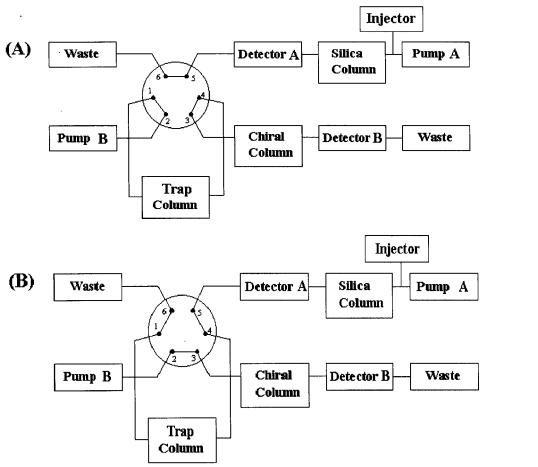
2. Experimental

2.1. Reagents

The metoprolol tartrate was provided by Yuhan Corp. (Kunpo, Kyeonggi, Korea). (*S*)-(−)-metoprolol, (*R*)-(+)-metoprolol, α-hydroxy metoprolol and *O*-desmethyl metoprolol were prepared at the Department of Pharmacy at Kangwon National University (Chunchon, Kangwon, Korea). Racemic pindolol hydrochloride was used as internal standard in the assay for metoprolol enantiomers and was purchased from Aldrich (Milkwaukee, WI, USA). Diethylamine was purchased from Junsei (Tokyo, Japan). Methanol, ethanol, 2-propanol, ethyl acetate and n-hexane as a HPLC grade and other reagents as a analytical grade were obtained from Duksan Pure Chemicals Co. (Ansan, Kyeonggi, Korea).

2.2. Apparatus

The achiral system was composed of a LC-9A pump (Shimadzu, Kyoto, Japan) with an achiral column. A Rheodyne 7725i injector fitted with a 20 μl loop and a RF-10AXL fluorescence detector



Position	Time(minute)	Function
A	0.00 – 20.00	Waste & Data acquisition from achiral column
B	20.01 – 21.50	Load metoprolol on silica trap column
A	21.50 – 55.00	Elute metoprolol from chiral column

Fig. 2. Schematic diagram of the coupled achiral–chiral column chromatographic system and positions and functions of switching.

(Shimadzu, Kyoto, Japan) with excitation/emission wavelengths of 276/309 nm were used. The chiral system was composed of a LC-9A pump with a chiral column and a RF-10AXL fluorescence detector with excitation/emission wavelengths of 276/309 nm. A phenomenex silica (250 × 4.6 mm I.D., Torrance, CA, USA) and a Chiralcel OD (250 × 4.6 mm I.D., Daicel, Tokyo, Japan) were used as the achiral column and chiral column, respectively. The two systems were connected with a FCV-2AH six-port valve equipped with trap column, Shim-pack silica guard column (10 × 4.6 mm I.D., Shimadzu, Kyoto, Japan). Switching of the valve was controlled with a SCL-6B controller. The acquisition of chromatograms and integration were obtained using a C-R4A integrator (Shimadzu, Kyoto, Japan).

2.3. Sample preparation

Urine (1.0 ml) was combined with 15 μl of internal standard solution (pindolol 20 μg/ml, in water) and 100 μl of 1 M sodium hydroxide solution, the mixture was shaken with 4.0 ml of dichloromethane for 10 min. After centrifuging (10 min, 3000 rpm), the organic layer was separated and was dissolved in the achiral mobile phase (500 μl) and an aliquot (10 μl) was injected into the HPLC system.

2.4. Chromatographic procedures

The HPLC system shown in Fig. 2 was programmed to operate automatically. Pump A delivered mobile phase A [n-hexane–ethanol–2-propanol–diethylamine (90/5/5/0.05, v/v/v/v)] and was used to separate the racemic metoprolol and internal standard (pindolol) on the silica analytical column. Mobile phase B was n-hexane–ethanol–2-propanol–diethylamine (85/7.5/7.5/0.05, v/v/v/v) and was used to separated the enantiomers of metoprolol on the Chiralcel OD column. Mobile phases A and B was pumped continuously at a flow-rate of 1.0 ml/min.

To summarize the operation of the column switching system, at the beginning of the analysis the valve was placed in position A (Fig. 2) and 10 μl of a urine sample was injected into achiral column. The injected sample was dissolved in achiral mobile phase. Here, racemic metoprolol and internal standard (pindolol) were separated from other biological materials and were monitored on fluorescence detector. After 20 min, the valve was switched to position B and racemic metoprolol was temporarily sent into the trap column. After 1.5 min, the valve was switched back to position A. Metoprolol was sent into the chiral column by back flushing with mobile phase B using pump B and the enantiomers were separated.

2.5. Validation studies

Spiked urine samples were prepared by adding known amounts of racemic metoprolol and internal standard (pindolol) to drug-free urine at five concentration levels (0.2, 0.5, 1, 2 and 4 μg/ml) and used for evaluation of the linearity, accuracy and precision.

The precision of the method was assessed by determining the intra- and inter-assay coefficients of variation (C.V.) of the analysis ($n = 6$) of spiked urine samples. The accuracy of the assay was expressed as [(determined concentration)/(spiked concentration)] × 100. The inter-assay coefficients of variation were determined at 0.2, 1 and 4 μg/ml every day over a 3-day period.

2.6. Method application

Metoprolol enantiomers in human urine were assayed by this method. Racemic metoprolol tartrate (100 mg) was administered once orally to a 24-year-old healthy male volunteer. Urine was collected at hourly intervals for 38 h and samples were stored at −20°C until analysis.

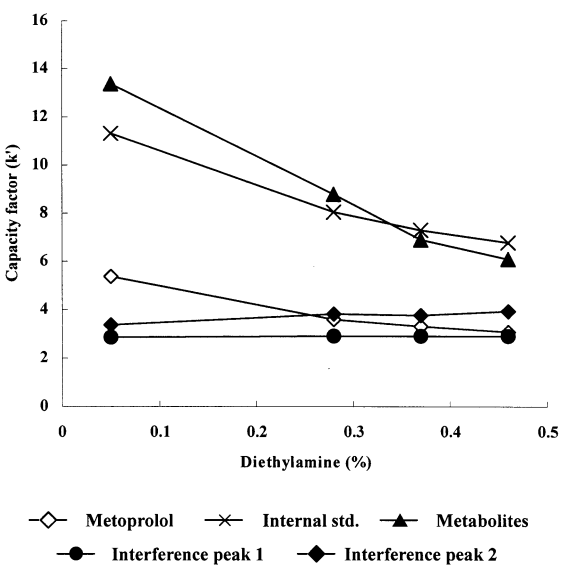


Fig. 3. The effect of diethylamine concentration in the achiral mobile phase on the capacity factors of metoprolol, pindolol (internal standard) and interference peaks in the achiral HPLC system. Column, phenomenex silica, 250 × 4.6 mm I.D.; mobile phase, the indicated volume percent of diethylamine was added to a mobile phase consisting of n-hexane–ethanol–2-propanol (90/5/5, v/v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm.

3. Results and discussion

3.1. Chromatography

The chromatographic conditions used for the determination of racemic metoprolol in achiral column were investigated. Diethylamine content in the achiral mobile phase had a profound effect on the separation factors of the metoprolol, its metabolites, pindolol and interfering substance peaks, as shown in Fig. 3. As the content of diethylamine decreased, the capacity factors of the metoprolol, its metabolites and pindolol increased but capacity factors of interfering substances did not change. Thus, metoprolol, its metabolites, pindolol and interfering substances peaks were resolved by using n-hexane–ethanol–2-propanol–diethylamine (90/5/5/0.05, v/v/v/v) as a achiral mobile phase.

Three chiral stationary phases were investigated for their usefulness in achiral–chiral column switching system and for the separation of metoprolol enantiomers. The phases were Chiralcel OD, Chiral-AGP and Cyclobond I. The Cyclobond I column did not provide sufficiently good separation. In the case of Chiral-AGP column, stereoselective resolution factor of 1.79 and asymmetry factor of 1.70 for (*R*)-(+)-metoprolol were obtained. On the Chiralcel OD column stereoselective resolution factor and asymmetry factor for (*R*)-(+)-metoprolol were 3.41 and 1.17, respectively. The Chiralcel OD column provided excellent separation with sufficiently good peak shapes and with reasonable time of analysis and was very stable for periods of analysis. Thus, a Chiralcel OD column was used as the chiral column. The chiral mobile phase was modified for the increase of compression effect in the chiral column. The difference of diethylamine content between achiral mobile phase and chiral mobile phase resulted in great solvent distortion. Therefore, a chiral mobile phase was used with n-hexane–ethanol–2-propanol–diethylamine (85/7.5/7.5/0.05, v/v/v/v) and good stereoselective resolution factor of 2.99 and stereoselectivity of 2.30 were obtained.

Typical chromatograms for the chiral separation of metoprolol enantiomers are presented in

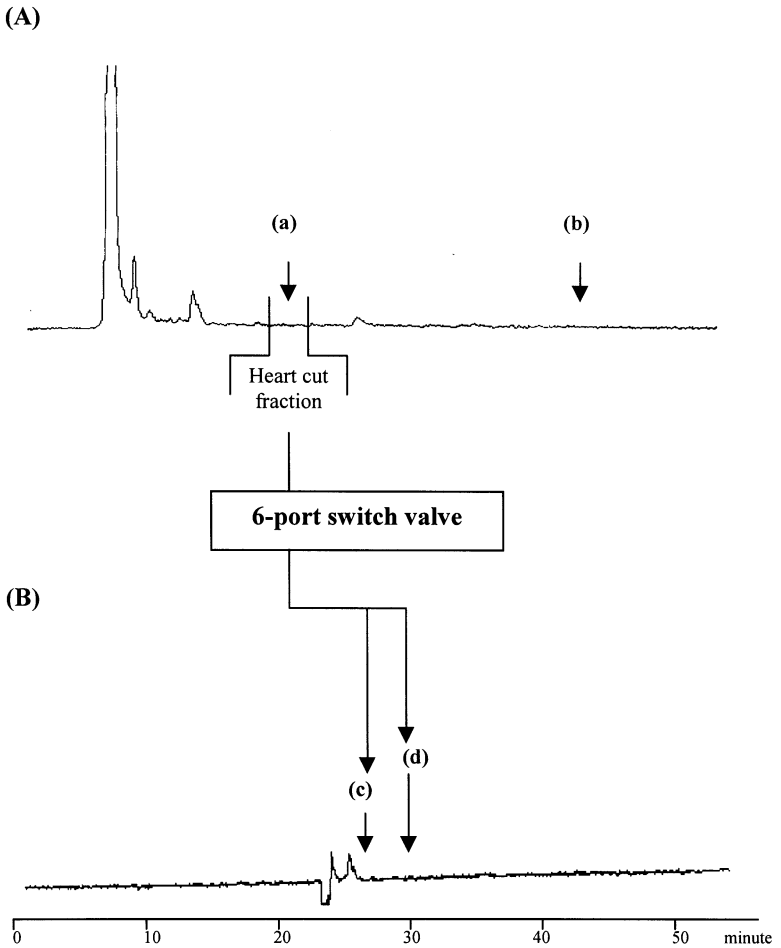


Fig. 4. Representative chromatograms of blank urine sample on the coupled achiral–chiral chromatographic system (A) achiral chromatogram; (B) chiral chromatogram after column switching. Peak a, racemic metoprolol; Peak b, internal standard (pindolol); Peak c, (*R*)-(+)-metoprolol; Peak d, (*S*)-(−)-metoprolol.

Figs. 4 and 5. Racemic metoprolol and the internal standard (pindolol) were separated from the achiral system and a fraction of racemic metoprolol was transferred to the chiral column by six-port switching valve. The transferred racemic metoprolol was resolved on a Chiralcel OD column. Under the described achiral chromatographic conditions, metoprolol and the internal standard eluted at 20.78 and 43.42 min, respectively. The metabolites of metoprolol (α-hydroxy metoprolol and *O*-desmethyl metoprolol) were eluted at 51.32 min, which were not resolved. Under the described chiral chromatographic conditions, retention times of (*S*)-(−)-metoprolol and (*R*)-(+)-metoprolol were 30.18 and 27.70 min, respectively.

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

3.2. Linearity and limit of quantitation

The calibration curves were obtained by analyzing spiked urine samples. Calibration curves for each enantiomer showed good linearity in the

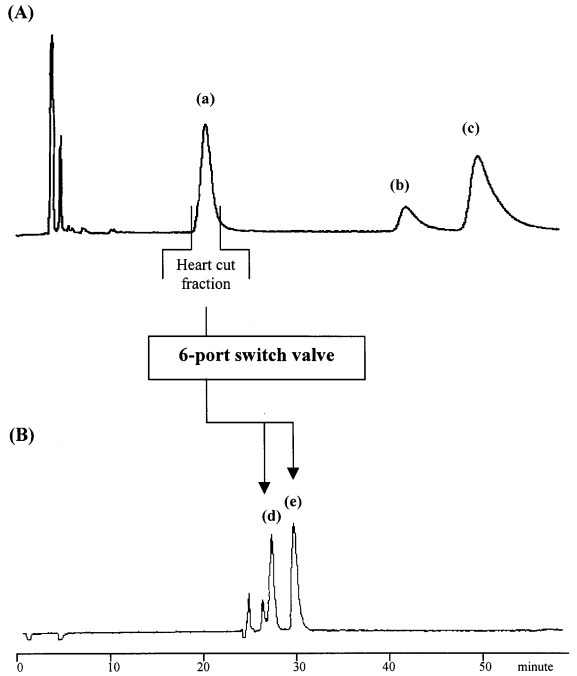


Fig. 5. Representative chromatograms of 2 h urine sample after oral administration of 100 mg metoprolol tartrate on the coupled achiral–chiral chromatographic system (A) achiral chromatogram; (B) chiral chromatogram after column switching. Peak a, racemic metoprolol; Peak b, internal standard (pindolol); Peak c, metabolites; Peak d, (*R*)-(+)-metoprolol; Peak e, (*S*)-(−)-metoprolol. Achiral column, phenomenex silica, 250 × 4.6 mm I.D.; chiral column, Chiralcel OD, 250 × 4.6 mm I.D.; achiral mobile phase, n-hexane–ethanol–2-propanol–diethylamine (90/5/5/0.05, v/v/v/v); chiral mobile phase, n-hexane–ethanol–2-propanol–diethylamine (85/7.5/7.5/0.05, v/v/v/v); flow rate 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm.

concentration range 0.1–2 μg/ml in urine. The equation of the calibration line obtained for (*S*)-(−)-metoprolol is $y = 0.6978x - 0.0118$ and for (*R*)-(+)-metoprolol $y = 0.6903x - 0.0099$. The correlation coefficients of *S*-(−)-metoprolol and *R*-(+)-metoprolol were 0.9999. The limits of quantitation were 25 ng/ml for (*R*)-(+)-metoprolol and (*S*)-(−)-metoprolol.

3.3. Accuracy and precision

Accuracy and precision of the method were determined by replicate analysis of blank human urine spiked with five concentrations of metoprolol enantiomer within the range 0.1–2 μg/ml. Six replicates of each concentration were analyzed on each of the three separate days. The results obtained are shown in Tables 1 and 2. The intra-day precision for each concentration was 0.38–4.90% for (*R*)-(+)-metoprolol and was 0.79–4.94% for (*S*)-(−)-metoprolol. The inter-day precision was 0.15–3.13% for (*R*)-(+)-metoprolol and was 0.27–2.05% for (*S*)-(−)-metoprolol. The accuracy, determined for each concentration, ranged from 95.46 to 101.46%.

3.4. Application

The method was applied to the analysis of urine samples from a study performed on human healthy volunteer treated with single oral dose of metoprolol tartrate (100 mg). A representative chromatogram obtained from volunteer before and after administration is reported in Figs. 4 and

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

Added conc. (μg/ml)	(*R*)-(+)-metoprolol			(*S*)-(−)-metoprolol		
	Measured conc. (μg/ml)	Accuracy (%)	C.V. (%)	Measured conc. (μg/ml)	Accuracy (%)	C.V. (%)
0.10	0.099	98.72	4.90	0.100	99.86	4.63
0.25	0.242	96.72	4.25	0.239	95.46	4.94
0.50	0.507	101.46	0.38	0.502	100.44	0.79
1.25	1.257	100.59	1.91	1.263	101.07	2.98
2.00	1.995	99.73	2.01	1.990	99.51	2.19

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

Added conc. (μg.ml)	*R*-(+)-metoprolol			*S*-(−)-metoprolol		
	Measured conc. (μg/ml)	Accuracy (%)	C.V. (%)	Measured conc. (μg/ml)	Accuracy (%)	C.V. (%)
0.1	0.098	97.93	3.13	0.098	97.92	2.05
0.5	0.505	100.91	0.88	0.503	100.67	0.34
2.0	1.998	99.89	0.15	1.996	99.81	0.27

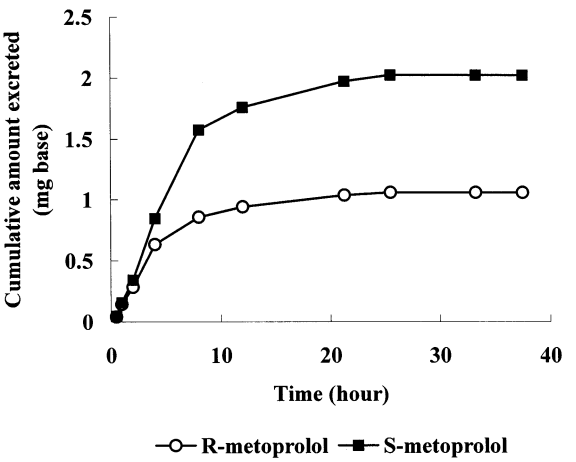


Fig. 6. Cumulative urinary excretion of metoprolol following a single oral dose of 100 mg metoprolol tartrate.

5, respectively. The urinary excretion data given in Fig. 6 indicates that the present method is suitable for the assay of metoprolol enantiomer after administration of metoprolol. From the 0–38 h urine the amounts of metoprolol enantiomers were determined. Unchanged metoprolol accounted for 3.96% of the dose and ((*R*)-metoprolol)/((*S*)-metoprolol) ratio was 0.52. The (*S*)-(−)-metoprolol had a higher excretion rate than (*R*)-(+)-metoprolol. Our results for enantiomeric metoprolol excretion rates in human urine was similar to the findings previously reported [1,11].

4. Conclusions

When CSPs are used in bioanalysis, separation problems are frequently encountered due to the restricted choice of solvent conditions available. Also 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. The coupled achiral–chiral system using column-switching technique was developed for the stereoselective assay of metoprolol in human urine and was applied to the determination of metoprolol enantiomers in urine after oral administration of metoprolol tartrate to human. The present method is convenient and simple and has been fully validated and proved to be suitable for the stereoselective pharmacokinetic studies of metoprolol.

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