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Chiral Separation of the Enantiomers of Metoprolol and Its Metabolites by High Performance Liquid Chromatography

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(1'R, 2R)-, (1'R, 2S)-, (1'S, 2R)- and (1'S, 2S)-α-hydroxymetoprolol; (2R)- and (2S)-*O*-desmethylmetoprolol; and (2R)- and (2S)-metoprolol acid are major metabolites of (2R)-and (2S)-metoprolol, β-adrenergic antagonist. The focus of most chiral separation methods until now has been on determination of the enantiomeric parent drug. However, it is just as important to be able to follow the metabolism of the enantiomers and their possible chiral metabolites. Therefore, for the study of stereoselective metabolism and pharmacokinetics of metoprolol, the chiral separation of the enantiomers of metoprolol and its metabolites has been investigated using four chiral stationary phases, i.e., Chiralcel OD, Chiral-AGP, Cyclobond I and Sumichiral OA-4900 columns. Metoprolol acid was resolved only by Sumichiral OA-4900. Chiralcel OD provided the highest separation factor and resolution value for metoprolol and O-desmethylmetoprolol and partially resolved the four stereoisomers of α-hydroxymetoprolol. Diastereomeric α-hydroxymetoprolols were resolved using the coupled column chromatographic system of two chiral stationary phases, Sumichiral OA-4900 column and Chiralcel OD column.

Key words: α-hydroxymetoprolol, *O*-desmethylmetoprolol, Metoprolol acid, Chiral stationary phase, Enantiomeric resolution

INTRODUCTION

Metoprolol is a β_1-selective aryloxypropanolamine adrenergic antagonist that is being used extensively in the treatment of a variety of cardiovascular disorders and administered as a racemic mixture (Baldwin *et al.*, 1988). Metoprolol enantiomers have been known to have different therapeutic effects, and (S)-(-)-metoprolol has been reported to have significantly greater α_1-adrenergic receptor affinity by>25-fold than (R)-(+)-metoprolol (Nathanson *et al.*, 1988). Enantioselective separation and the methods of determining these enantiomers in biological samples were needed due to this great difference in the pharmacological effect and pharmacokinetics between the two enantiomers. Development of the chiral stationary phases for metoprolol has allowed direct resolution of the metoprolol enantiomers without the need for prior derivatization. Ekelund *et al.* (1995) and Hermansson *et al.* (1995) reported the chiral resolution of metoprolol using β-cyclodextrin, α_1-acid glycoprotein and cellulose tris-3, 5-dimethylphenylcarbamate as the chiral stationary phase.

However, enantioselective metabolism of chiral drugs has been reported (Kim *et al.*, 1992 and Murthy *et al.*, 1990), and it became important to develop enantioselective separation methods of the parent drug and its chiral metabolites for studies on chiral pharmacokinetics and stereoselective metabolism.

Metoprolol is metabolized in the liver via three oxidative pathways: *O*-demethylation, α-hydroxylation and *N*-dealkylation. *O*-demethylation is the principal pathway of metoprolol metabolism in man, which accounts for about 65% of the dose, and α-hydroxylation of metoprolol, which produces a pharmacologically active metabolite, is a relatively minor pathway accounting for only about 10 % of the dose in humans (Borg *et al.*, 1975). *O*-desmethyl-

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metoprolol (H 105/22) and metoprolol acid (H 117/04) by *O*-demethylation contain one asymmetric carbon, and Kim et al (1992) reported that *O*-demethylation is R-enantioselective. α-Hydroxymetoprolol (H 119/66) of α-hydroxylation contains two asymmetric carbons with four possible stereoisomers (Fig. 1).

Previously, direct methods using a Chiralcel OD column were employed to study the chiral metabolism of metoprolol. Only the chromatographic resolutions of α-hydroxymetoprolol (Balmer *et al.*, 1991) and *O*-desmethylmetoprolol (Leloux, 1992), and indirect HPLC methods after derivatization with (-)-menthyl chloroformate (Li *et al.*, 1996 and Li *et al.*, 1995) were reported. The GC-MS method reported by Li *et al.* (1996) describes the development of a procedure for the simultaneous identification of metoprolol and its metabolites, and the HPLC method reported by Li *et al.* (1995) shows a limited utilization from a two-step derivatization. Until now, no study has been done to investigated the chiral separation of metoprolol and its metabolites at various chiral stationary phases.

In this paper, we evaluated four different stationary phases, *i.e.*, Chiralcel OD, Chiral-AGP, Cyclobond I and Sumichiral OA-4900 columns, regarding their enantioselectivity towards metoprolol and its three metabolites, and investigated the optimum conditions of the chiral resolution for simultaneously determining the enantiomers of metoprolol and all of its metabolites in biological fluids. The Chiralcel OD column and Sumichiral OA-4900 column provided good enantioselective separations of metoprolol and its metabolites.

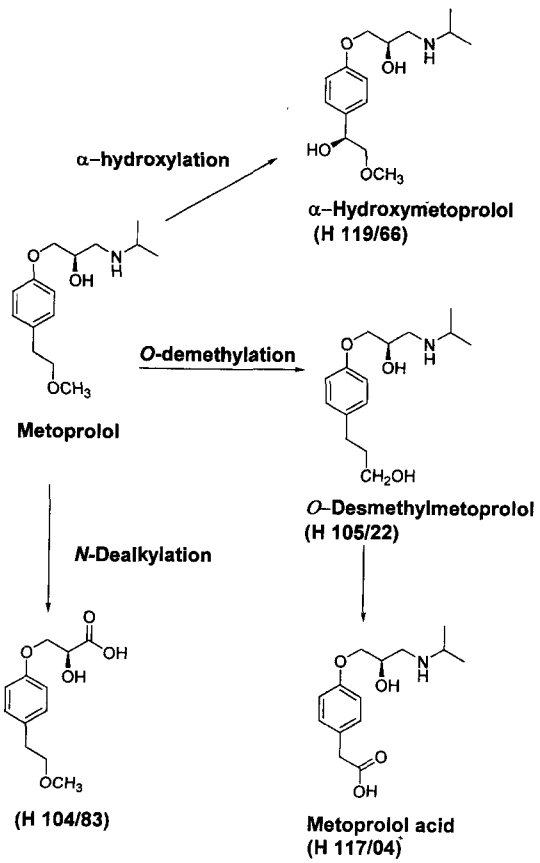

Fig. 1. Metabolism of metoprolol by oxidative pathways

MATERIALS AND METHODS

Chemicals

Racemic metoprolol tartarate was provided by Yuhan Corporation (Kunpo, Kyeonggi, Korea). Racemic O-desmethylmetoprolol and (S)-O-desmethylmetoprolol were obtained from the Department of Pharmacy, Chungnam National University (Chungnam, Korea). (S)-metoprolol and (R)-metoprolol were prepared by semi-preparative HPLC using a Chiralcel OD (250 × 10 mm I.D., 10 μm) chiral column and n-hexane-ethanol-2-propanol-diethylamine (90/5/5/0.25, v/v/v/v) as a mobile phase at the Department of Pharmacy, Kangwon National University (Kangwon, Korea) (Kim *et al.*, 1999). α-Hydroxymetoprolol and metoprolol acid were also isolated from human urine after administering racemic metoprolol tartarate to healthy volunteers. Molecular structures of these compounds are shown in Fig. 1.

Trifluoroacetic acid and triethylamine were purchased from Aldrich (Milkwaukee, WI, USA), diethylamine in analytical-reagent grade, from Junsei (Tokyo, Japan), and other solvents in HPCL-grade, from Duksan Pure Chemicals Co. (Ansan, Kyeonggi, Korea).

Solutions of each compound (ca. 5 μg/ml) were prepared in the relevant mobile phase. Aliquots of 5 μl or 20 μl of these solutions were injected.

Apparatus

The chromatographic system consisted of a Shimadzu LC-9A pump (Kyoto, Japan), a RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan) at excitation/emission wavelengths of 276/309 nm, a Rheodyne 7725*i* injector fitted with a 20 μl loop. All chromatograms were recorded on a C-R4A data processor (Shimadzu, Kyoto, Japan).

The cellulose tris-3,5-dimethylphenylcarbamate phase was a Chiralcel OD column (250 × 4.6 mm I.D., 10 μm) from Daicel Chemical Industries (Tokyo, Japan). The α_1-acid glycoprotein phase was a Chiral-AGP column (100 × 4.0 mm I.D., 5 μm) from ChromTech (Norsborg, Sweden). The β-cyclodextrin column was a Cyclobond I column (250 × 4.6 mm I.D., 5 μm) from ASTEC (Whippany, NI, USA). The Sumichiral OA-4900 column (250 × 4.6 mm I.D., 5 μm) was purchased from Sumika Chemicals Analysis Service (Osaka, Japan). The experiments were performed at ambient temperature and the chromatographic procedures used are described in the discussion section for the various stationary phases.

Isolation of α-hydroxymetoprolol and metoprolol acid from human urine

Metoprolol (100 μg metoprolol tartarate; Betaloc®) was administered orally to healthy volunteers and the total urine was collected for the period of 0~24 h after the administration of metoprolol.

α-hydroxymetoprolol, collected urine (400 ml) and sodium carbonate (50 ml, 1 M) were mixed with dichloromethane (800 ml) for 30 min. The mixture was allowed to stand at room temperature for 2 h. The organic phase was separated and then evaporated to dryness at a reduced pressure. α-Hydroxymetoprolol was obtained by semi-preparative HPLC from this extract on an Inertsil ODS (250 × 10 mm I.D., 10 μm, GL science, Tokyo, Japan) column with acetonitrile-methanol-0.5% triethylamine of pH 3.0 (4.5/0.5/95, v/v/v) at a flow rate of 4.5 ml/min. This product was identified by Mass spectrometry (VG-Trio 2000, VG ins, England) and was used without further purification (Mass spectrum (EI^+, 70 eV): *m/z* 283 (2.52), 268 (1.27), 123 (16.67), 116 (24.02), 72 (100.00)).

Metoprolol acid, collected urine (400 ml) and 0.02 M sodium dodecyl sulfate solution (40 ml, in 1 M phosphoric acid) were mixed, and then the mixture was extracted with 800 ml of dichloromethane-acetonitrile (75/25, v/v). The organic layer was evaporated at a reduced pressure. After performing ion-exchange column chromatography on Amberlite® IR-120 (Aldrich, WI, USA) using 0.1 M ammonia water, crude metoprolol acid was obtained. Metoprolol acid was purified by semi-preparative HPLC, and the chromatographic condition was Inertsil ODS (250 × 10 mm I.D., 10 μm) as a semi-preparative column and acetonitrile-methanol-0.5% triethylamine of pH 3.0 (7.2/0.8/92, v/v/v) as a mobile phase at a flow rate of 4.0 ml/min. The obtained product was identified by Mass spectrometry (Mass spectrum (EI^+, 70 eV): *m/z* 267 (2.88), 252 (4.47), 152 (8.17), 107 (32.37), 72 (100.00)).

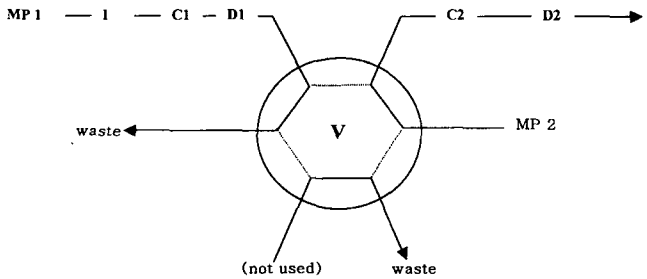

Fig. 2. Schematic diagram of the column-switching apparatus in the HPLC system. MP: mobile phase, I: injector, C1: Sumichiral OA-4900 column, C2: Chiralcel OD column, D1 and D2: fluorescence detector, V: switching valve.

Coupled column chromatographic system and operating conditions

Fig. 2 shows a schematic diagram of the column switching apparatus in the HPLC system. This system consisted of Shimadzu LC-9A HPLC system equipped with two pumps (LC-9A), a six-port switching valve (FCV-2AH), a system controller (SCL-6B), a data processor (C-R4A) and two fluorescence detectors (RF 10-AXL).

The column switching HPLC system was performed with two chiral columns. α-Hydroxymetoprolol was injected onto a Sumichiral OA-4900 column (250 × 4.6 mm I.D., 5 μm), and the column was eluted with a mobile phase consisting of *n*-hexane-dichloromethane-methanol-trifluoroacetic acid (240/400/10/1, v/v/v/v) at a flow rate of 1 ml/min. Either from 26 to 28 min or from 28 to 30 min, the flow path was switched to the second column, Chiralcel OD (250 × 4.6 mm I.D., 10 μm), by switching the positoin of the valve. Consequently, the heart-cut fraction containing either (2R)-α-hydroxymetoprolol or (2S)-α-hydroxymetoprolol was transferred. After the valve was switched back to the initial position, the final chiral separation of (2R)-α-hydroxymetoprolol and (2S)-α-hydroxymetoprolol was achieved on Chiralcel OD by using a mixture of *n*-hexane-ethanol-diethylamine (90/10/0.3, v/v/v) as a mobile phase at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Chiralcel OD

Table I gives the chromatographic results obtained using the Chiralcel OD column and the mobile phases are listed. The choice of the initial mobile phase used with each different column was based on literature data and on information given by the manufacturer, concerning the enantioseparation of other substances. The mobile

Table I. Chromatographic results from the separation of metoprolol and its metabolites on the Chiralcel OD stationary phase

Compound	Eluent[a]	k' (1)	k' (2)	α	R_S
Metoprolol	A	0.703	1.391	1.978	2.865
O-desmethylmetoprolol	A	1.814	2.780	2.780	2.211
Metoprolol acid	B	6.620	6.620	6.620	-

Compound	Eluent[a]	k' (1)	k' (2)	k' (3)	k' (4)	α_{12}	α_{23}	α_{34}
α-hydroxymetoprolol	C	11.442	13.865	15.414	16.552	1.212	1.112	1.074

[a]Eluents: A=n-hexane-ethanol-diethylamine (85/15/0.1, v/v); B=*n*-hexane-ethanol-diethylamine (45/55/0.1, v/v); C=*n*-hexane-ethanol-diethylamine (95/5/0.1, v/v). Flow rate: 1.0 ml/min

phase chosen for the Chiralcel OD stationary phase was various mixtures of *n*-hexane and ethanol with the addition of 0.1% diethylamine. It appears from the results that this column is capable of separating metoprolol and *O*-desmethylmetoprolol. Metoprolol acid was eluted as a single peak and the four isomers of α-hydroxymetoprolol were not separated satisfactorily. Chromatograms of metoprolol, *O*-desmethylmetoprolol and α-hydroxymetoprolol

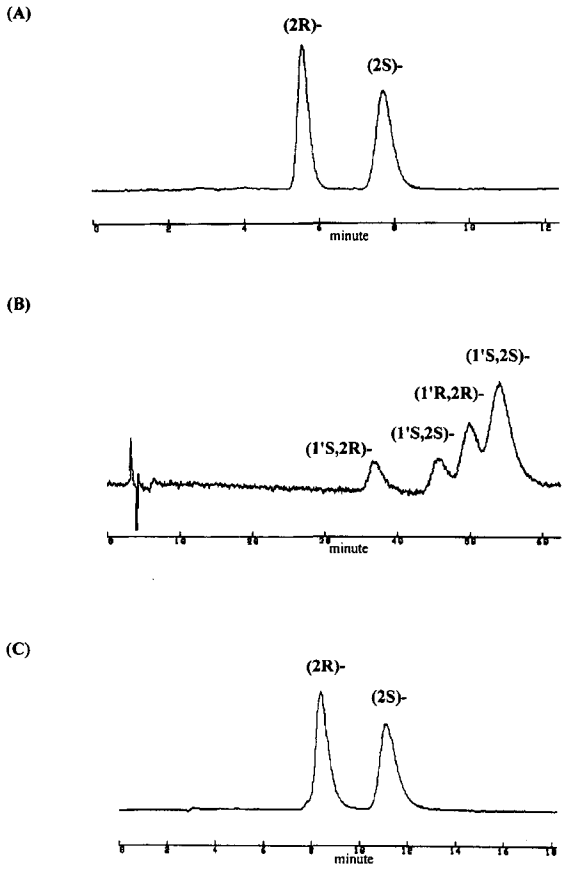

Fig. 3. Chiral separation of (A) metoprolol, (B) α-hydroxymetoprolol and (C) O-desmethylmetoprolol on Chiralcel OD. [Column, Chiralcel OD, 250 4.6 mm I.D.; mobile phase, (A) and (C), n-hexane-ethanol-diethylamine (85/15/0.1, v/v), (B), *n*-hexane-ethanol-diethylamine (95/5/0.1, v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm.]

are shown in Fig. 3. For α-hydroxymetoprolol altered capacity factors and retention orders were achieved by the addition of the organic modifiers, 1-propanol, 2-propanol, 1-butanol and ethanol, to the mobile phase. But the use of these modifiers did not offer any improvement.

The enantiomeric elution order was determined by injecting each enantiomer of metoprolol and *O*-desmethylmetoprolol separately under the same chromatographic conditions. For metoprolol and *O*-desmethylmetoprolol, the (2R)-form was eluted before the (2S)-form. The elution order of diastereomeric α-hydroxymetoprolols is given in the discussion section for the coupled column chromatographic system to resolve α-hydroxymetoprolol.

Chiral-AGP

In the present investigation, 20 mM aqueous phosphate buffer was used as the mobile phase for the separation of metoprolol and its metabolites, and the influence of pH on retention behavior and enantioselectivity was briefly studied. The retention times of the metoprolol, α-hydroxymetoprolol and *O*-desmethylmetoprolol were increased by increasing the pH of the mobile phase. Metoprolol was separated using 20 mM phosphate buffer (pH 7.0) as a mobile phase, resulting in Rs=1.79 (see Table II). O-desmethylmetoprolol was slightly resolved (α=1.09) and α-hydroxymeoprolol was not resolved. Fig. 4 shows a chromatogram of metoprolol on the Chiral-AGP. Metoprolol acid did not show an affinity for Chiral-AGP column. By comparing (2S)-metoprolol with (2R)-metoprolol, we found that (2R)-metoprolol has a shorter retention time than (2S)-metoprolol.

Cyclobond I

This column has been used with medium-polarity mobile phase, consisting of the mixture of acetonitrile, methanol, acetic acid and triethylamine; the separation of some β-blocking substances has been reported (Ekelund *et al.*, 1995 and Pharm-Hui *et al.*, 1995). Therefore, the separation of the metoprolol and its metabolites was attempted using various compositions of the above-mentioned mobile phase. Ekelund et al. reported that the resolution of metoprolol was achieved on Cyclobond I

Table II. Chromatographic results from the separation of metoprolol and its metabolites on the Chiral-AGP and Cyclobond I stationary phase

Compound	Chiral-AGP[a]				Cyclobond[b]			
	k' (1)	k' (2)	α	R_S	k' (1)	k' (2)	α	R_S
Metoprolol	2.082	2.735	1.313	1.792	2.855	2.855	1.000	-
O-desmethylmetoprolol	1.829	2.000	1.093	-	6.607	7.180	1.087	0.809
Metoprolol acid	0	-	-	-	Did not elute from the column			
α-hydroxymetoprolol	0.396	0.396	1.000	-	4.382	4.382	1.000	-

[a]mobile phase: 20 mM phosphate buffer (pH 7.0); flow rate: 0.7 ml/min
[b]mobile phase: acetonitrile-methanol-acetic acid-triethylamine (99/1/0.6/0.6, v/v); flow rate: 1.0 ml/min

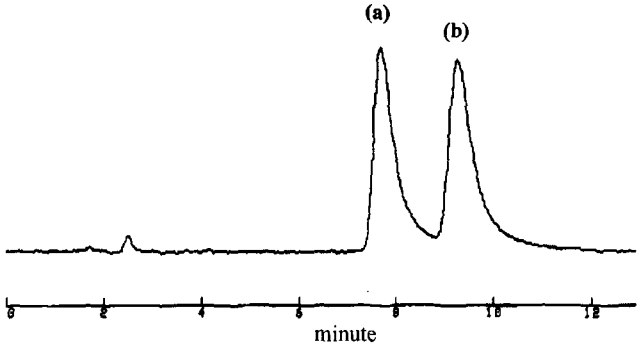

Fig. 4. Chiral separation of racemic metoprolol on Chiral-AGP. Peak (a), (2R)-metoprolol; Peak (b), (2S)-metoprolol. [Column, Chiral-AGP, 100 × 4.0 mm I.D.; mobile phase, 20 mM phosphate buffer, pH 7.0; flow rate, 0.7 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm.]

Fig. 5. Chiral separation of racemic *O*-desmethylmetoprolol on Cyclobond . Peak (a), (2S)-*O*-desmethylmetoprolol; Peak (b), (2R)-*O*-desmethylmetoprolol. Column, Cyclobond, 250 × 4.6 mm I.D.; mobile phase, acetonitrile-methanol-acetic acid-triethylamine (99/1/0.6/0.6, v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm.]

(Rs=1.90, α=1.11) using acetonitrile-methanol-acetic acid-triethylamine (97/3/0.24/0.36) as a mobile phase. When the same mobile phase was used, metoprolol and α-hydroxymetoprolol were not chirally separated, even though they showed high affinity for this stationary phase. And metoprolol acid did not elute from the column under these conditions.

For *O*-desmethylmetoprolol, an improved separation could be obtained using acetonitrile-methanol-acetic acid-triethylamine (99/1/0.6/0.6, v/v/v/v), but could not be obtained with baseline separation. The results obtained are given in Table II. Fig. 5 shows a chromatogram for the separation of *O*-desmethylmetoprolol.

(2S)-O-desmethylmetoprolol was analyzed under the same chromatographic conditions and (2S)-*O*-desmethylmetoprolol was eluted prior to (2R)-O-desmethylmetoprolol. The order of retention is reversed compared with the Chiralcel OD stationary phase.

Sumichiral OA-4900

Phenomex company reported that metoprolol was almost baseline separated on exactly the same HPLC column called Chirex 3022 as Sumichiral OA-4900 using n-hexane-dichloromethane-ethanol-trifluoroacetic acid (60/35/5/0.25, v/v/v/v) as a mobile phase. No work has been published which indicated that this stationary phase could provide the separation of metabolites of metoprolol for analytical purpose. However, we evaluated from this study that *O*-desmethylmetoprolol and metoprolol acid could be chirally separated on this type of column under the mobile phase conditions, and α-hydroxymetoprolol was resolved on two peaks, i.e. (2R)-α-hydroxymetoprolol and (2S)-α-hydroxymetoprolol. The mobile phases were various mixtures of n-hexane, dichloromethane, methanol and trifluoroacetic acid. Table III shows the results of the enantioselectivity of Sumichiral OA-4900 under these conditions. Retention and enantioselectivity can be controlled by changing the concentration and the type of halogenated hydrocarbon in the mobile phase. When dichloromethane was used as a halogenated hydrocarbon, an improved separation could be obtained for all the compounds (see Table III). The retention and enantioselectivity of the compounds increased with the increase in the content of dichloromethane.

For metoprolol and O-desmethylmetoprolol, we determined that the (2R)-form elutes before the (2S)-form by the injection of each enantiomer of metoprolol. The elution orders were determined from the known enantioselectivity of metabolism as expressed by enantiomeric excess found in human urine. With slight 2R<2S enantioselectivity in the α-hydroxylation process, the 2R/2S ratio of 0.85, slight 2R>2S enantioselectivity for *O*-demethy-

Table III. Chromatographic results from the separation of metoprolol and its metabolites on the Sumichiral OA-4900 stationary phase

Compound	Eluent[a]	k' (1)	k' (2)	α	R_S
Metoprolol	A	6.783	7.367	1.086	1.381
O-desmethylmetoprolol	B	3.508	3.952	1.127	1.906
Metoprolol acid	B	7.117	8.000	1.124	2.048
α-hydroxymetoprolol	B	2.850	3.200	1.123	1.278

[a]Eluents: A=n-hexane-dichloromethane-methanol-trifluoroacetic acid (240/600/5/1, v/v); B=n-hexane-dichloromethane-methanol-trifluoroacetic acid (240/600/20/1, v/v); flow rate: 1.0 ml/min

lation, and the reported 2R/2S ratio of 1.15, (Murthy *et al.*, 1990), (1'R, 2R)-α-hydroxymetoprolol, (1'S, 2R)-α-hydroxymetoprolol, (2R)-*O*-desmetoprolol and (2R)-metoprolol acid were found to be eluted faster than their antipodes. (Fig. 6).

Coupled column chromatographic system to resolve α-hydroxymetoprolol

Online chiral-chiral column switching technique was investigated to separate the four isomers of α-hydroxymetoprolol. Chromatograms for the chiral separation of α-hydroxymetoprolol are presented in Fig. 7.

(2R)-α-hydroxymetoprolol and (2S)-α-hydroxymetoprolol were separated on the Sumichiral OA-4900 column using n-hexane-dichloromethane-methanol-trifluoroacetic acid (240/400/10/1, v/v/v/v) as a mobile phase; and each fraction containing (2R)-form and (2S)-form was transferred to the Chiralcel OD column by a six-port switching valve and was separated to (1S)-form and (1R)-form using *n*-hexane-ethanol-diethylamine (90/10/0.3, v/v/v) as a mobile phase. Under the described conditions, the stereoselective resolution and stereoselectivity factor of (1'S, 2R)-α-hydroxymetoprolol and (1'R, 2R)-α-hydroxymetoprolol were 1.77 and 1.35, respectively, and those of (1'R, 2S)-α-hydroxymetoprolol and (1'R, 2S)-α-hydroxymetoprolol were 1.08 and 1.17, respectively. When a poor separation of α-hydroxymetoprolol was observed using a single chiral column, a coupled chiral-chiral HPLC system was used to overcome this problem.

The elution order of the four isomers of α-hydroxymetoprolol could be explained using the stereoselective ratio of α-hydroxymetoprolol in human urine. Murthy *et al.* (1990) reported that the α-hydroxylation process generates a (1R)-α-hydroxymetoprolol over a (1S)-α-hydroxymetoprolol by 3-folds. Therefore, we identified that (1'S,

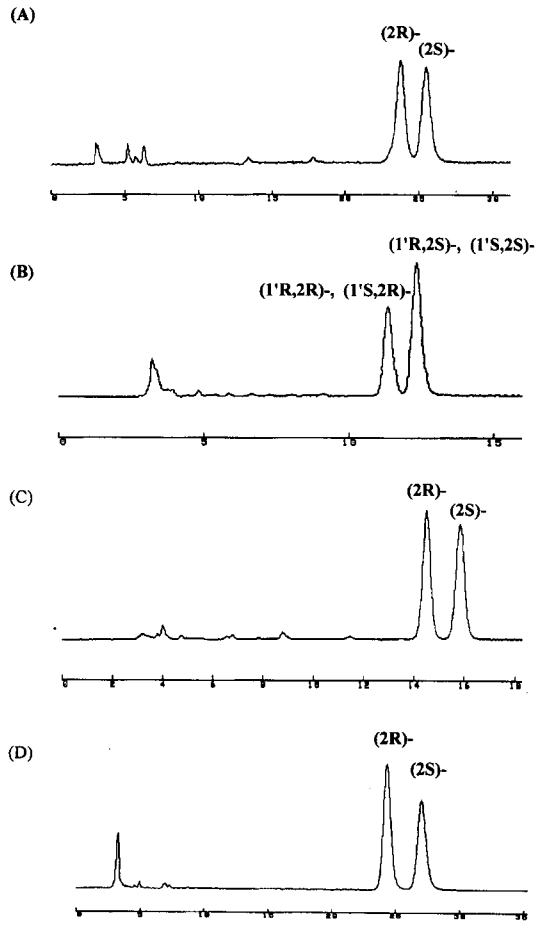

Fig. 6. Chiral separation of (A) metoprolol, (B) α-hydroxymetoprolol, (C) *O*-desmethylmetoprolol and (D) metoprolol acid on Sumichiral OA-4900. [Column, Sumichiral OA-4900, 250×4.6 mm I.D.; mobile phase, (A), *n*-hexane-dichloromethane-methanol-trifluoroacetic acid (240/600/5/1, v/v), (B), (C) and (D), *n*-hexane-dichloromethane-methanol-trifluoroacetic acid (240/600/20/1, v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm

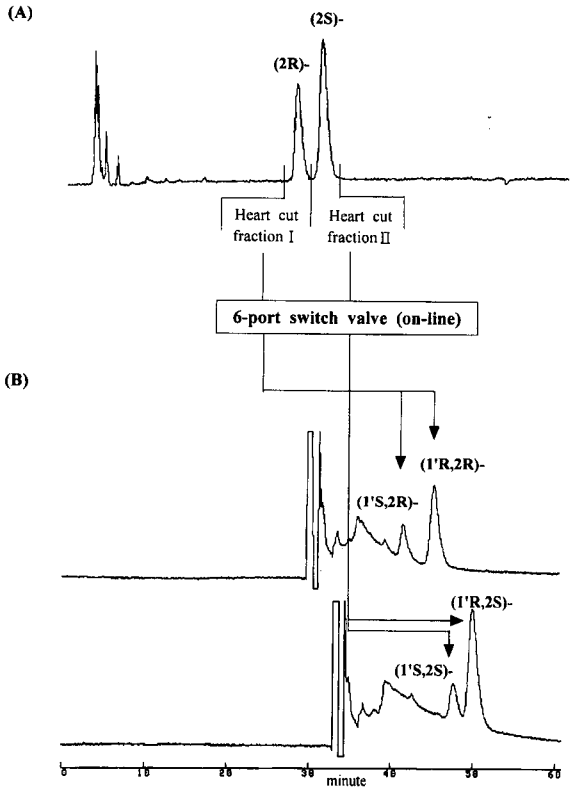

Fig. 7. Chiral separation of α-hydroxymetoprolol on the coupled column chromatographic system. (A) Chromatogram of α-hydroxymetoprolol on Sumichiral OA-4900, mobile phase, *n*-hexane-dichloromethane-methanol-trifluoroacetic acid (240/400/10/1, v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm (B) chromatogram of α-hydroxymetoprolol on Chiralcel OD after column switching, mobile phase, *n*-hexane-ethanol-diethylamine (90/10/0.3, v/v); flow rate, 1.0 ml/min; fluorescence detector, excitation wavelength 276 nm, emission wavelength 309 nm

2R)-form of the (1'S, 2R)- and (1'R, 2R)-α-hydroxymetoprolol and (1'S, 2S)-form of the (1'S, 2S)- and (1'R, 2S)-α-hydroxymetoprolol elute prior to the (1R, 2R)- and (1R, 2S)-forms, respectively; this elution order was the same as reported by Balmer *et al.*(1991).

CONCLUSIONS

The chiral separation of the enantiomers of metoprolol and its metabolites has been investigated using four chiral stationary phases, Chiralcel OD, Chiral-AGP, Cyclobond I and Sumichiral OA-4900. The enantiomers of metoprolol and O-desmethylmetoprolol were resolved on Chiralcel OD, Chiral-AGP and Sumichiral OA-4900 column and (2R)- and (2S)-metoprolol acid were resolved only on Sumichiral OA-4900 column. Simultaneous direct resolution of all of the four stereoisomers of α-hydroxymetoprolols was not achieved on the four chiral stationary phases but was achieved by a coupled column chromatography using a switching valve. This study could provide preliminarily information of chiral separation to develop the method of simultaneously identifying the enantiomers of metoprolol and its metabolites in biological fluids.

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