Direct Injection Analysis of Atenolol Enantiomers in Plasma Using an Achiral/Chiral Coupled Column HPLC System

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A novel HPLC system was developed with an achiral/chiral coupled column for the direct injection analysis of atenolol (AT) enantiomers in plasma. The system consists of a size-exclusion column, an ODS silica column and a newly developed β-cyclodextrin perphenylcarbamate(ph-β-CD)-bonded silica column connected in a series *via* two switching valves. The neat plasma sample was directly injected onto the size-exclusion column, and the deproteinized fraction of AT enantiomers was concentrated on the ODS silica column. The enantiomers were then transferred and separated mutually on the ph-β-CD silica column. The calibration line for each of the AT enantiomers was linear in the range of plasma concentration of 10—200 ng/ml (r>0.9997) with good reproducibility (CV<9.7%, n=20). The recoveries from plasma were almost complete (>97.4%) for both enantiomers. One analysis finished within 30 min. The developed system was applied to the enantioselective determination of plasma concentration–time curve of AT after the oral administration of racemic AT to a healthy volunteer.

Keywords atenolol; high performance liquid chromatography; chiral separation; direct injection analysis

Atenolol (AT) is a cardioselective β-adrenoceptor blocking agent (β-blocker) used for the treatment of angina pectoris and hypertension. Like most of other β-blockers, the pharmacological activity of AT appears to reside in its (*S*)-(*l*)-enantiomer, while AT is clinically administered as a racemate. A few papers have dealt with the enantioselective pharmacokinetics of AT,[1,2] which involve contradictory results. Boyd *et al.* found no statistical difference in renal clearance between the enantiomers, but significant difference in *AUC* and urinary excretion, which they ascribed to enantioselective absorption.[1] On the other hand, Mehvar *et al.* reported that the renal clearance of (*S*)-AT was 8% higher than that of (*R*)-AT, and that the difference in *AUC* and urinary excretion might be caused by the enantioselective difference in the clearance.[2] This discrepancy may come from the small difference in the values of pharmacokinetic parameters between the enantiomers and in the relatively small number of subjects employed. Therefore, further investigation is necessary to clarify the enantioselectivity in the pharmacokinetics of AT. However, the lack of a precise analytical method to determine AT enantiomers in plasma has been delaying the progress in this study. So far, the determination of AT enantiomers in plasma has been achieved by the pretreatment of samples, through such methods as deproteinization and extraction, followed by derivation into diastereomers. The diastereomers are then separated on an achiral HPLC column.[3–6] These procedures may sometimes cause errors which arise from the incomplete extraction of the enantiomers, the optical impurity of the chiral reagent and/or the difference in the reaction rate between the enantiomers.[7] Recently, an α_1-acid glycoprotein immobilized silica column was found to be applicable to the chiral separation of AT.[7]

The direct injection analysis of plasma sample is a practical trend in HPLC study. Several HPLC columns, such as "restricted access" type columns, have been developed[8] for this purpose. These columns have a common feature in that they exclude plasma proteins but retain drugs of small molecular size, allowing repeated injections of untreated plasma. Thus they can be used both as a pretreatment column and an analytical column.

In the chiral separation of a drug in biological fluids such as plasma, it is often the case that the optical isomers cannot be separated from endogenous plasma components or from its metabolites on a single chiral HPLC column, even though their standard samples can be separated mutually. To improve the incomplete separation, an achiral/chiral coupled-column HPLC technique has been developed.[9] In this technique, the drug is first separated from endogenous plasma components or its metabolites on an achiral column, then the drug fraction of the eluent is transferred into a chiral column. This technique enhances the separation ability and, therefore, expands the applicability of a chiral column. However, a direct plasma injection analysis has not been achieved in the achiral/chiral HPLC system.

The present paper deals with a novel coupled column HPLC system for the direct injection analysis of AT enantiomers in plasma. The system consists of a hydrophilic size exclusion gel column for the deproteinization of plasma, an ODS silica column for the concentration of AT fractions and a new chiral column which has a perphenylcarbamate derivative of β-cyclodextrin (β-CD) as the chiral ligand for the enantioseparation. The validation tests were achieved to assure the reliability of the present system.

Experimental

Reagents and Materials Atenolol and human plasma were provided by Taiyo Pharmaceutical Industry Co., Ltd. (Nagoya, Japan). Isopropanol and tetrahydrofuran of HPLC grade were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Kanto Chemical Co., Inc. (Tokyo, Japan), respectively. Ethanol, methanol and acetonitrile were purified by distilling the analytical grade of reagents obtained from Wako Pure Chemical Industries.

Apparatus A column switching system was set up with two LC-6A pumps (Shimadzu, Japan) and one Jasco Twincle pump (Japan Spectro-

TABLE I. HPLC Conditions

	Column	Mobile phase
Plasma pretreatment	Asahipak GS220M (10 cm × 7.6 mm i.d., Asahi Chemical Ind.) column temperature 21 °C	Phosphate buffer (pH 7.5, $I=0.01$) Flow rate 2.0 ml/min
Concentration	ODS column (5 cm × 4.6 mm i.d.)	Water Flow rate 1.0 ml/min
Chiral separation	β-CD derivative column (15 cm × 6 mm i.d., Shinwa Chemical Ind.) column temperature 21 °C	20 mM NaH_2PO_4 (pH 4.6) : ethanol = 90 : 10 (v/v) Flow rate 1.0 ml/min

scopic Co., Ltd.), an RT-353T HPLC fluorescence monitor (Shimadzu, Japan) and a Chromatopac C-R3A data analyzer (Shimadzu, Japan). A Rheodyne type 7125 injector with a 200 μl loop was used for sample injection. A six-port switching valve was the product of Japan Spectroscopic Co., Ltd. and a Rheodyne type 7000 valve was used as a four-port switching valve. Asahipak GS 220M column was purchased from Asahi Chemical Co. (Osaka, Japan) and a β-perphenylcarbamate-bonded silica column was supplied by Shinwa Chemical Industries, Ltd. The ODS column (5 cm × 4.6 mm i.d.) was packed in our laboratory with Chemcosorb 7-ODS-H (Chemco Scientific Co., Ltd. Osaka, Japan). The HPLC conditions are listed in Table I.

Stock solution was made up by dissolving a weighed amount of racemic atenolol in ethanol. Plasma samples were prepared by spiking human plasma with the stock solution to make 20—400 ng/ml.

A 150 μl portion of plasma sample was directly injected into the HPLC system.

Procedure of Solid-phase Extraction Solid phase extraction was employed as a reference method. An Adsorbex CN (100 mg, E. Merck, Darmstadt, F.R.G.) column was used as a solid phase extractor. The column was conditioned, just before use, by washing with 1.0 ml acetonitrile and 1.0 ml distilled water. The column was loaded with 1 ml of plasma sample and washed with 0.5 ml distilled water and 0.5 ml acetonitrile. AT was then eluted out three times with 0.25 ml of 0.05 M acetic acid–acetonitrile (7 : 3, v/v). The fractions were collected and the solvent was evaporated to dryness *in vacuo*. The residue was reconstituted with 0.4 ml of the mobile phase used for chiral separation. A 50 μl portion of the reconstituted solution was injected into the ph-β-CD silica column.

Results and Discussion

Selection of On-line Pretreatment Column Table II shows the elution time of AT from several restricted-access type HPLC columns which allow repeated direct injections of plasma samples. Because AT is a hydrophilic compound, the retention onto Hisep column and SPS column was not strong enough for the separation of AT from plasma components, whereas AT was well retained on Asahipak GS220M, which is a polymer-based size-exclusion HPLC column and has a weak cation-exchange property similar to Asahipack GS320 made of the same material.[10]

As shown in Fig. 1, AT was not only separated from plasma proteins, but also from other endogenous plasma components. The retention time (t_R) of AT on Asahipack GS220M was 12.7 min with satisfactory reproducibility (CV 0.64%, $n=5$). As shown in Table II, the higher ionic strength of the mobile phase gives rise to the faster elution of AT.

In the direct plasma injection analysis, the interaction between drug and plasma proteins such as albumin can interfere with the analysis. When protein binding of a drug is very strong and the mobile phase contains a low concentration of organic modifier, the release of the bound drug will not proceed rapidly, resulting not only in the broadening or splitting of the drug peak but also in incomplete recovery.[11-13] However, no influence of the protein binding was observed in this study, because the protein binding of AT is very weak. The bound AT fraction in plasma is reported as 3%.[14] Thus the recovery of AT from the Asahipak GS220M column was 99.5% at the concentration of 400 ng/ml.

TABLE II. Retention of Atenolol (AT) on the "Restricted-Access" Type HPLC Columns

Column	Retention time of AT (min)	Mobile phase	Flow rate (ml/min)
SPS C8 (Regis Co.) 15 cm × 4.6 mm i.d., 5 μm, 100 Å	6.27	M-1	0.9
SPS C18 (Regis Co.) 15 cm × 4.6 mm i.d., 5 μm, 100 Å	4.42	M-1	0.9
Hisep (Supelco) 15 cm × 4.6 mm i.d., 5 μm	3.60	M-1	0.9
Asahipak GS220M (Asahi Chemical Ind.) 10 cm × 7.6 mm i.d., 9 μm	3.98 12.7 22.8	M-1 M-2 M-3	2.0

M-1: sodium phosphate buffer (pH 7.5, $I=0.02$). M-2: sodium phosphate buffer (pH 7.5, $I=0.01$). M-3: sodium phosphate buffer (pH 7.5, $I=0.005$).

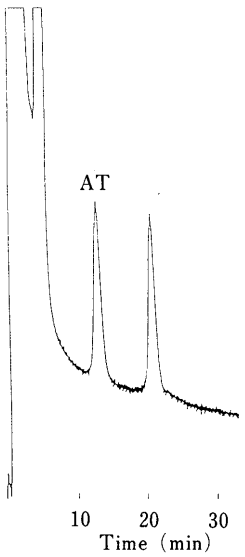

Fig. 1. Chromatogram of Atenolol (AT) in Human Plasma

HPLC conditions: column, Asahipak GS220M (10 cm × 7.6 mm i.d.), mobile phase, phosphate buffer (pH 7.5, $I=0.01$); flow rate, 2.0 ml/min; sample, racemic atenolol 400 ng/ml in plasma; injection volume, 125 μl; detection, λ_{ex} 279 nm, λ_{em} 305 nm; column temperature, 21 °C.

Chiral Separation Enquist and Hermansson reported that an α_1-acid glycoprotein (α_1-AGP) column could separate AT enantiomers without derivation, although the acetylation of AT enantiomers significantly improved their resolution.[7] Therefore, we first tried to incorporate an α_1-AGP column into our coupled-column system. Figure 2 shows the effects of pH and ionic strength of the mobile phase upon the capacity factors (k') and the separation factor (α) of AT enantiomers on an α_1-AGP column. The capacity factors and the separation factor decreased with increasing proton concentration, which agreed with the reported result.[15] The capacity factors increased in the low range of ionic strength, while enantioselectivity was unaffected.

Figure 3 shows the effect of the organic modifiers added

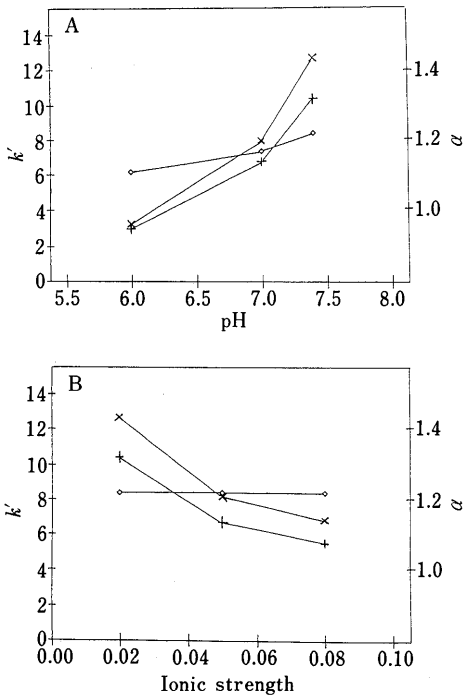

Fig. 2. Effect of pH (A) and Ionic Strength (B) on Capacity Factor k' of the (*R*)-Enantiomer (+) and the (*S*)-Enantiomer (×) and Separation Factor α (◇) of AT

HPLC conditions: column, Chiral AGP (10 cm × 4 mm i.d.); flow rate, 0.9 ml/min; detection, UV, 225 nm; column temperature, ambient; mobile phase, phosphate buffer (I=0.02) for (A) and phosphate buffer (pH 7.4) for (B).

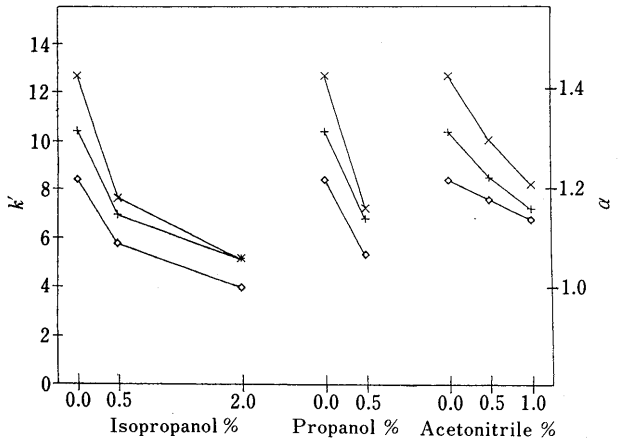

Fig. 3. Effect of Organic Modifiers on Capacity Factor k' of the (*R*)-Enantiomer (+) and the (*S*)-Enantiomer (×) and Separation Factor α (◇) of AT

HPLC conditions: column, chiral AGP (10 cm × 4 mm i.d.); flow rate, 0.9 ml/min detection, UV, 225 nm; column temperature, ambient; mobile phase, phosphate buffer (pH 7.4, I=0.02) with organic modifiers.

to the phosphate buffer (pH 7.4, I=0.02). The pH and the ionic strength were selected as to give the largest capacity factors. The capacity factors of the enantiomers quickly decreased in the presence of a low concentration of an organic modifier (isopropanol, *n*-propanol or acetonitrile), and the enantioseparation also decreased.

Therefore, use of an organic modifier was not recommendable. This is a critical limitation which prevents an α_1-AGP column from being coupled with the pretreatment column. Because there is no difference in the eluotropic strength between the mobile phase for the

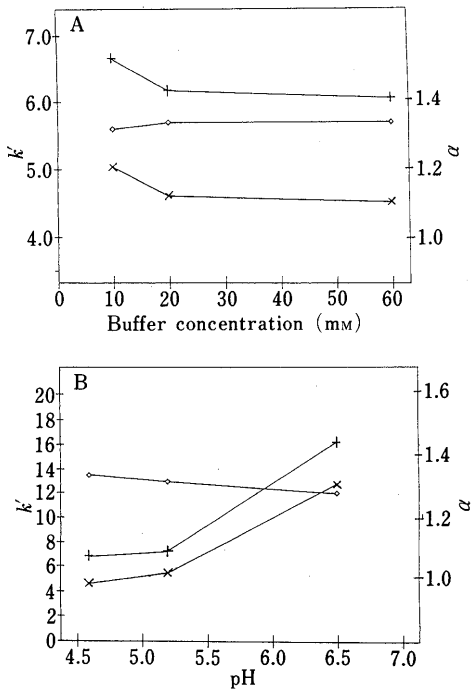

Fig. 4. Effect of Buffer Concentration (A) and pH (B) on Capacity Factors k' of the (*R*)-Enantiomer (+) and the (*S*)-Enantiomer (×) and Separation Factor α (◇) of AT

HPLC conditions: column, β-CD derivative column (15 cm × 6 mm i.d.); flow rate, 1.0 ml/min; detection, fluorescence (λ_{ex} 279 nm, λ_{em} 305 nm); injection volume, 20 μl; column temperature 21 °C; mobile phase, 10—60 mM KH_2PO_4–ethanol (9 : 1, v/v) for (A) and pH 4.6—6.5 phosphate buffer–ethanol (9 : 1, v/v) for (B).

Table III. Effect of Organic Solvent on Capacity Factors (k') and Separation Factor (α) of AT

	EtOH 5%	EtOH 10%	MeOH 10%	MeCN 10%	IPA 10%	THF 10%
k'_1 (*S*-AT)	16.0	4.61	16.0	3.60	5.25	1.42
k'_2 (*R*-AT)	21.0	6.18	20.3	4.00	6.25	1.42
α	1.31	1.34	1.27	1.11	1.19	1.00

HPLC conditions: column, β-CD derivative column (15 cm × 6 mm i.d.); flow rate, 1.0 ml/min; detection, fluorescence (λ_{ex} 279 nm, λ_{em} 305 nm); injection volume, 20 μl; column temperature 21 °C; mobile phase, 20 mM NaH_2PO_4 (pH 4.6) with organic solvent, where EtOH denotes ethanol, MeOH methanol, MeCN acetonitrile, IPA isopropanol and THF tetrahydrofuran.

pretreatment column and that for α_1-AGP column, it is hard to incorporate the pre-concentration column between them. When the AT fraction eluted from the pretreatment column was directly transferred into an α_1-AGP column, the enantioseparation ability of this column completely deteriorated, because the heart-cut volume was as large as 10 ml.

Then, we investigated the applicability of a newly developed β-CD derivative silica column. This packing material is a phenylcarbamate derivative of the available OH groups on β-CD as a chiral ligand. Figure 4 shows the effect of buffer concentration and pH of the mobile phase upon the capacity factors and separation factor of AT enantiomers. In every case, (*S*)-AT was eluted faster than (*R*)-AT. As the buffer salt concentration increased, the capacity factor decreased, but the separation factor did not change. With increasing pH, the capacity factor increased, and the separation factor decreased. At pH 7, the enantio-

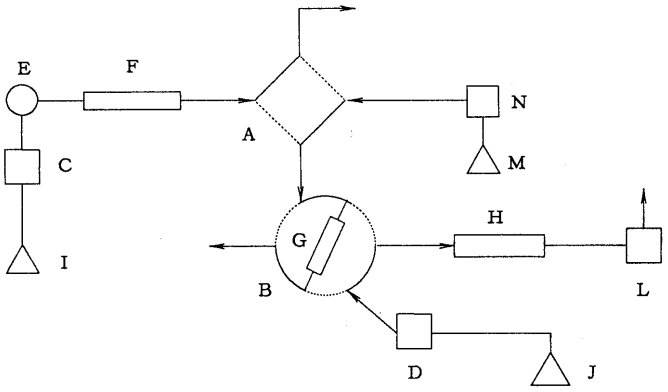

Fig. 5. Schematic Diagram of the On-line Achiral/Chiral Column Switching System

Key: (A) four-way switching valve; (B) six-way switching valve; (C and D) pump; (E) sample injector; (F) column for plasma pretreatment; (G) column for concentration; (H) column for chiral separation; (I) mobile phase for plasma pretreatment; (J) mobile phase for chiral separation; (L) fluorescence detector; (M) distilled water to wash concentration column; (N) pump.

TABLE IV. Recoveries from Asahipak and ODS Column

	Recovery (%)	CV (%)	n
(*R*)-AT	99.38	1.6	3
(*S*)-AT	99.35	0.5	3

Recovery from Asahipak and ODS column is calculated from the peak area by injecting standard sample of racemic AT 400 ng/ml in phosphate buffer (pH 7.4, $I=0.17$) to the β-CD derivative column directly and injecting the same standard sample into the column switching system. (peak area from column switching system/peak area from direct injection to the β-CD derivative column). For HPLC conditions, see Table I.

mers were not eluted within 1 h.

Table III shows the effect of organic modifiers. The capacity factors of the enantiomers observed using several organic modifiers in the mobile phase were in the order of tetrahydrofuran (THF) < MeCN < EtOH < isopropanol (IPA) < MeOH at the concentration of 10%. When the EtOH content was decreased from 10% to 5%, the capacity factors increased, but enantioseparation did not improve. The separation factor was largest when 10% EtOH was used.

From these results, the mobile phase condition for the chiral separation was optimized as 20 mM NaH_2PO_4 (pH4.6) : EtOH = 90 : 10 (v/v).

Determination of AT Enantiomers by the Coupled Column HPLC System The coupled column HPLC system was constructed as shown in Fig. 5.

After injection of a 150 μl-portion of the plasma sample directly to Asahipak GS220M, 10 ml of the eluate of the AT peak fraction was heart-cut and transferred to the ODS column by switching the four-port valve from the solid line to the broken line shown in Fig. 5. The heart-cut volume was determined to be large enough to transfer the total quantity of AT enantiomers. AT was completely adsorbed onto the ODS column, then eluted out into the chiral separation column by switching the six-port valve from the solid line to the broken line. The recoveries of AT before chiral separation exceeded 99% for both enantiomers with small variation (see Table IV).

During the chiral separation, the eluate remaining in the ODS column was washed with water. The time schedule of the valve switching is summarized in Fig. 6. One analysis

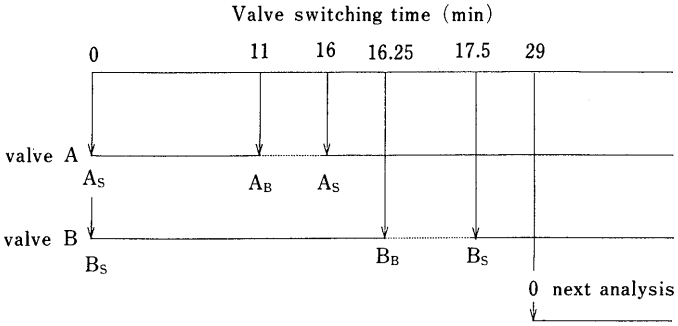

Fig. 6. Column Switching Time Program

A_S = valve A in solid line; A_B = valve A in broken line; B_S = valve B in solid line; B_B = valve B in broken line.

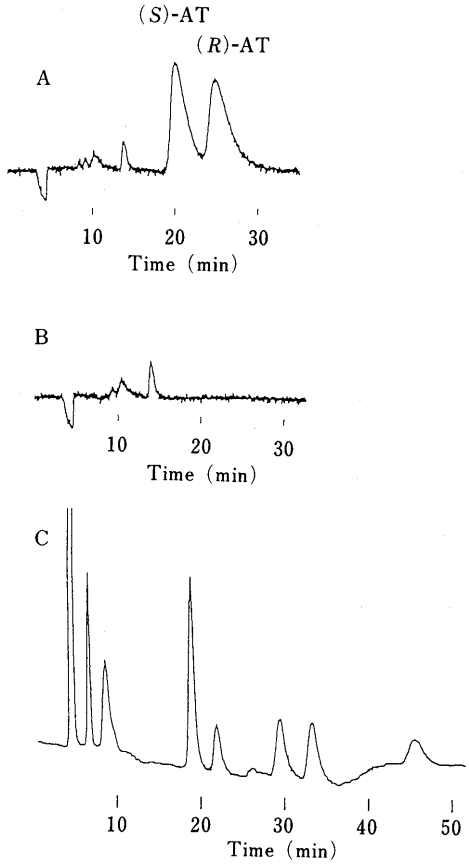

Fig. 7. Chromatogram of Human Plasma Spiked with Racemic AT (400 ng/ml) (A) and without AT (B) on the Achiral/Chiral Coupling HPLC System and Chromatogram of Human Plasma on the β-CD Derivative Column after Solid-Extraction Pretreatment (C)

Achiral/chiral coupling HPLC system, see Fig. 5; HPLC conditions for (A) and (B), see Table I; injection volume, 150 μl. HPLC conditions for (C) are as "chiral separation" shown in Table I; injection volume, 50 μl.

required about 30 min.

Figure 7A shows a chromatogram of the chiral separation of AT in plasma. The retention times were 24.3 and 19.8 min for (*R*)- and (*S*)-AT, respectively, with good reproducibility (CV, 0.29% and 0.44% at $n=10$, respectively). Figure 7B is the chromatogram of a plasma blank. No interference by the plasma components was observed. It is interesting to note that when AT in plasma was analyzed by using the chiral separation column alone, after "off-line" pretreatment with the disposable solid-extraction kit (for procedure, see experimental section),

Table V. Calibration Lines and Recoveries of Plasma Samples

	Slope[a]	Intercept[a]	R[b]	Conc. (ng/ml)	Recovery[c] (%)
(*R*)-AT	1.35	−2.05	0.9998	10—200	98.8
(*S*)-AT	1.34	1.81	0.9998	10—200	97.4

a) Area = slope × concentration + intercept. *b*) Correlation coefficient. *c*) Ratio of the slope of the calibration line (plasma sample/standard solution).

Table VI. Reproducibility of Peak Area of Atenolol Enantiomer in Plasma Sample

	Concentration (ng/ml)	Within-run CV% ($n=10$)	Day-to-day CV% ($n=20$)
(*R*)-AT	200	2.4	2.6
	25	9.7	9.6
(*S*)-AT	200	1.3	2.1
	25	7.8	7.1

there appeared several interfering peaks on the chromatogram (see Fig. 7C).

Table V lists the calibration lines and the recoveries of AT enantiomers in human plasma. Good linearity was observed within the clinical plasma concentration range (10 ng/ml to 200 ng/ml for each enantiomer). The recovery, calculated as the ratio of the slope of the calibration line for AT in plasma to that for AT in a buffer solution, indicated almost 100% for each enantiomer.

Table VI lists the results of the validation study. The CV% of the within-run ($n=10$) and day-to-day ($n=20$) analyses of each enantiomer was 2.6% or less at a high concentration (200 ng/ml) and was 9.7% or less at a low concentration (25 ng/ml).

The present system was applied to the enantioselective determination of plasma concentration–time curve following oral administration of a tablet containing 50 mg racemic AT to a healthy volunteer. Figure 8 is a chromatogram obtained at 2 h after the administration. Figure 9 shows the time course curve. The plasma concentration of (*R*)-AT was obviously a little higher than that of (*S*)-AT, which agrees with the reported results.[1,2] The enantioselective pharmacokinetics of AT will be reported elsewhere. These results clearly indicate the applicability of this system to the real plasma samples.

Conclusion

The present achiral/chiral coupled-column system allows direct sample analysis of AT enantiomers in plasma. The results of the validation tests indicate good reproducibility and reliability. This system is beneficial to the enantioselective pharmacokinetic study of AT.

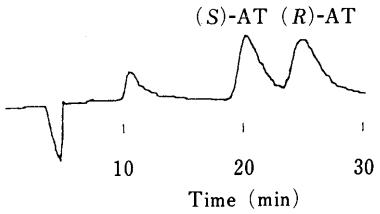

Fig. 8. Chromatogram of Human Plasma Obtained at 2 h after Oral Administration of a Tablet Containing 50 mg Racemic AT to a Healthy Volunteer

For HPLC conditions see Table I.

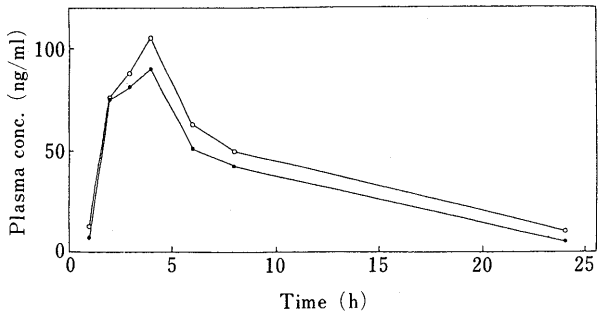

Fig. 9. The Plasma Concentration–Time Curve of AT after *p.o.* Administration of a Tablet Containing 50 mg Racemic AT to a Healthy Volunteer

(○) indicates the concentrations of (*R*)-AT, (●) indicates the concentrations of (*S*)-AT.

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