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Determination of manidipine enantiomers in human serum using chiral chromatography and column-switching liquid chromatography

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ABSTRACT

A stereoselective and highly sensitive method using chiral chromatography and successive column-switching liquid chromatography is described for the determination of manidipine enantiomers in human serum. A human serum sample obtained after ingestion of manidipine was extracted twice with a mixture of *n*-hexane–diethyl ether under alkaline conditions. The enantiomers in the extract were separated on a chiral stationary phase column (Chiralcel OJ), and the effluents containing the respective enantiomers were collected. Each fraction was then analysed by column-switching liquid chromatography. The proposed stereoselective method offered high sensitivity: detection limits for both isomers were 0.2 ng/ml in human serum, both at a signal-to-noise ratio of 3. The method is suitable for the pharmacokinetic studies of manidipine enantiomers.

INTRODUCTION

Manidipine (MD), 2-[4-(diphenylmethyl)-1-piperazinyl]ethyl methyl (±)-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate (Fig. 1), is a new 1,4-dihydropyridine calcium antagonist with long-lasting activity [1]. The drug is a racemic mixture of *R*(−)- and *S*(+)-MD, because there is an asymmetric carbon in the dihydropyridine ring. It is known that the enantiomers of some calcium antagonists have different pharmacological activities, as demonstrated with verapamil [2], nimodipine [3] and nilvadipine [4]. The antihypertensive activity of *S*(+)-MD in rats is 30-fold more potent than that of *R*(−)-MD [5]. Therefore, it is necessary to develop a stereoselective determination method for MD in biological fluids for the purpose of pharmacokinetic studies. Chiral resolution of enantiomers by high-performance liquid chromatography (HPLC) is generally approached in three ways: (1) derivatization to corresponding diastereomers using a chiral reagent; (2) addition of a chiral reagent to the mobile phase; (3) use of a chiral stationary phase (CSP) column. Many CSP columns are now commercially available and they have rapidly become major tools in the analysis of enantiomers. After separation by CSP

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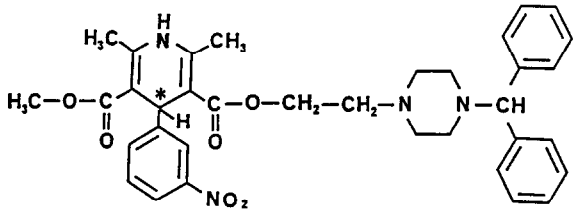

Fig. 1. Structure of manidipine: the chiral centre is marked with an asterisk.

HPLC, sensitive gas chromatographic (GC) determinations of drug enantiomers in human plasma have been carried out: nilvadipine by mass spectrometric (MS) detection [4] and felodipine by electron-capture detection (ECD) [6]. However, GC–MS and GC–ECD are not always suitable for routine work, and only a limited number of compounds can be detected sensitively. A sensitive non-stereoselective HPLC method for the determination of MD in human serum using column switching was previously reported by our laboratory [7,8]. In this paper, we describe a stereoselective and highly sensitive method for the determination of MD enantiomers in human serum using enantioseparation by CSP HPLC and following column-switching HPLC. The method is suitable for the pharmacokinetic studies of MD enantiomers.

EXPERIMENTAL

Reagents and materials

S(+)- and *R*(−)-MD · 2HCl, racemic MD and MD · 2HCl were synthesized in the Research and Development Division, Takeda Chemical Industries (Osaka, Japan). Acetonitrile, *n*-hexane, methanol, ethanol and 2-propanol were of HPLC grade (Wako, Osaka, Japan). All other reagents were of analytical-reagent grade and used without further purification.

Instruments and conditions

The HPLC system for the optical resolution of enantiomers consisted of an LC-6A pump, an SPD-6A UV detector, a CTO-6A column oven, an SIL-6A autosampler, an SCL-6A controller (all from Shimadzu, Kyoto, Japan) and a U-228 dual-pen recorder (Nippon Denshi Kagaku, Kyoto, Japan). The CSP column was Chiralcel OJ (10 μm particle size, 250 mm × 4.6 mm I.D.; Daicel Chemical, Tokyo, Japan). The mobile phase was *n*-hexane–ethanol–methanol (80:15:5, v/v). The operating temperature and the flow-rate were 50°C and 1.0 ml/min, respectively. UV detection was carried out at 230 nm.

The column-switching HPLC system for the determination of each enantiomer after optical resolution consisted of two LC-6A pumps, two SPD-6A UV detectors, two U-228 dual-pen recorders, an SIL-6A autosampler, an SCL-6A controller and an FCV-2AH six-port valve (Shimadzu). Two ODS columns were used: Develosil ODS-3K (3 μm particle size, 100 mm × 2.1 mm I.D.) for column 1 (C1) and Develosil ODS-5K (5 μm particle size, 100 mm × 2.1 mm I.D.) for column 2 (C2) (both from Nomura Chemical, Aichi, Japan). The mobile phase for C1 (MP1) was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (55:45, v/v) containing 5 m*M* sodium nonane sulphonate adjusted to pH 3.0 with 85% orthophosphoric acid. The mobile phase for C2 (MP2) was 0.02 *M* potassium dihydrogenphosphate–acetonitrile (55:45, v/v) adjusted to pH 3.0 with 85% orthophosphoric acid. The operating temperature and the flow-rate for both columns were 40°C and 0.3 ml/min, respectively. UV detection was carried out at 230 nm.

Extraction from serum

The extraction of MD from human serum was carried out according to the method described by Miyabayashi *et al.* [8]. To 1.0 ml of human serum in a 10-ml brown centrifuge tube was added 1.0 ml of 0.02 *M* disodium hydrogenphosphate (pH 10). The mixture was extracted twice with 5 ml of *n*-hexane–diethyl ether (2:1, v/v). The organic layer was evaporated to dryness under a nitrogen stream, the residue was reconstituted with 200 μl of ethanol, and an aliquot of 150 μl was injected into the CSP column.

Chiral separation of each enantiomer

Before analysis, the retention times of the MD enantiomers on the CSP column were checked

each day to determine the precise time programme for fractionation. The injected sample was separated on the CSP column and the eluate fractions containing the $R(-)$- and $S(+)$-enantiomers were collected separately. The solvent of each fraction was evaporated to dryness under a nitrogen stream, the residue was reconstituted with 200 μl of MP1, and an aliquot of 150 μl was injected into the analytical HPLC system.

Analytical HPLC system and procedure

The HPLC system and the procedure using column switching for the determination of each enantiomer were similar to those reported previously [8]. In summary, the injected sample was first separated on C1 and the eluate fraction containing the analyte was transferred to C2 by valve operation. The introduced eluate was further separated on C2 followed by UV detection at 230 nm.

Calibration graph and quantitation

Drug-free human serum samples spiked with known amounts of racemic MD were analysed according to the analytical procedure described above. The peak heights of MD enantiomers were plotted against the serum concentrations to give the calibration graphs. The percentage recoveries of MD enantiomers in human serum were calculated from the peak-height ratio of spiked standard samples relative to directly injected standard solutions. The concentrations in samples were calculated using calibration graphs from spiked standards, which were prepared by adding racemic MD to drug-free human serum or dog plasma.

Data analysis

The observed serum concentration–time data for each enantiomer were used to determine the maximum serum concentration (C_{max}) and the time to reach C_{max} (T_{max}). The serum concentration–time curve was fitted to a two-compartment open model by least-squares regression analysis to calculate the elimination half-lives ($T_{1/2}$). The area under the serum concentration–time curve (AUC) up to the last sampling point was calculated by a trapezoidal rule.

RESULTS AND DISCUSSION

Chiral separation

Chromatographic resolution of enantiomers is generally accomplished by direct separation on a CSP column, by addition of a chiral reagent to the mobile phase or by pre-column derivatization of the enantiomers with a chiral reagent followed by separation of the resulting diastereomers on an achiral phase. MD has no appropriate functional group to be derivatized to the corresponding diastereomers with a chiral reagent. Therefore, we tried to separate MD enantiomers directly on a CSP column. Several types of commercially available CSP columns were investigated. A Chiralcel OJ column, consisting of tris (4-methylbenzoate)-modified cellulose coated on silica, offered visibly discernible separation at room temperature with a mobile phase of *n*-hexane–2-propanol (8:2, v/v). However, the separation was not satisfactory for the present purpose.

The effect of increasing the column temperature on the separation of MD enantiomers with this column was then investigated, using the same mobile phase, and the separation was dramatically enhanced (Fig. 2). Although good separation was obtained when the chromatography was carried out at 50°C, the analysis time of 40 min was considered too long for the treatment of many samples. To solve this problem, the mobile phase was further investigated. The mobile phase of *n*-hexane–ethanol–methanol (80:15:5, v/v) was found to allow almost complete baseline resolution (α = 1.3, R_s = 1.2) in 15 min as shown in Fig. 3.

Intra- and inter-day variations for the retention times of the enantiomers under these conditions were satisfactory (Table I). The order of elution was confirmed by comparison with authentic samples of the enantiomers, and $R(-)$-MD was eluted faster than $S(+)$-MD with the CSP column.

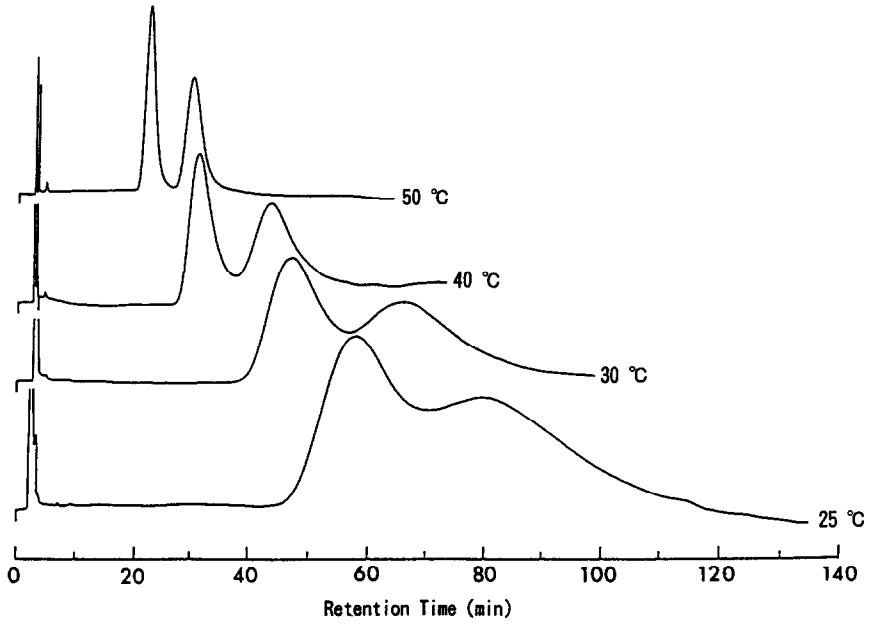

Fig. 2. Effect of the column temperature on the separation of MD enantiomers. Mobile phase, *n*-hexane–2-propanol (8:2, v/v); for other conditions, see text.

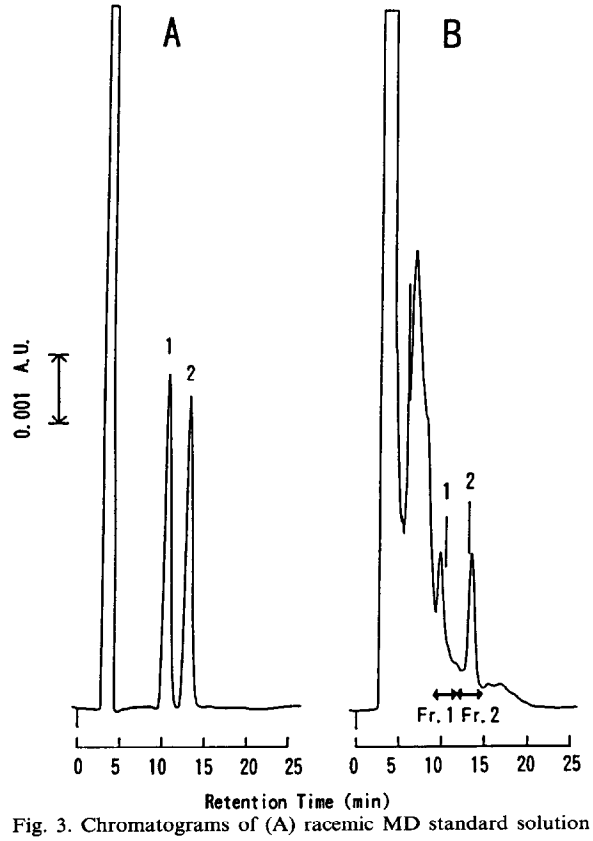

Fig. 3. Chromatograms of (A) racemic MD standard solution and (B) drug-free human serum, obtained with CSP column. Peaks: 1 = *R*(−)-MD; 2 = *S*(+)-MD. Fractions: Fr. 1 = fraction containing *R*(−)-MD; Fr. 2 = fraction containing *S*(+)-MD.

Column-switching chromatography

In spite of the good separation, the sensitive determination of each enantiomer in human serum by the CSP column was impossible owing to interferences at their retention times and the low efficiency of the CSP column (Fig. 3). Therefore, the fraction from CSP column containing each enantiomer was collected and analysed off-line on a reversed-phase ODS column. These columns are usually more efficient than CSP columns. A considerable clean-up effect was expected following the fractionation on the CSP column. However, sensitive determination of each enantiomer in the fraction was still impossible with a single ODS column system, owing to interferences (data not shown). Therefore, the previously reported column-switching method [8] was adopted after the fractionation by the CSP column. Fig. 4 shows typical chromatograms of serum samples after column switching obtained from the fraction containing *S*(+)-MD (fraction 2) on the CSP column. No interferences were observed at the retention time of *S*(+)-MD. Similar chromatograms were obtained from the fraction containing *R*(−)-MD (fraction 1) (data not shown). Determination of drug-free human se-

TABLE I

INTRA- AND INTER-DAY VARIATIONS OF RETENTION TIMES OF *R*(−)- AND *S*(+)-MD ON CSP COLUMN

Values in parentheses are coefficients of variation (%).

Day	*n*	Retention time (mean ± S.D.) (min)	
		R(−)-MD	*S*(+)-MD
1	3	10.48 ± 0.09 (0.85)	13.15 ± 0.04 (0.30)
2	3	10.75 ± 0.05 (0.47)	13.49 ± 0.05 (0.37)
3	3	10.61 ± 0.02 (0.19)	13.37 ± 0.02 (0.15)
4	3	10.62 ± 0.07 (0.66)	13.32 ± 0.00 (0.00)
Overall	12	10.62 ± 0.11 (1.04)	13.34 ± 0.13 (0.97)

rum spiked with each enantiomer (5 ng/ml) showed that the corresponding antipode was below the detection limit, which indicates that chiral inversion during the analytical procedures is negligible.

Linearity and recovery

The calibration graph was obtained by analysing drug-free human serum samples spiked with racemic MD in the concentration range 0.5–25 ng/ml as an enantiomer. Good linearity was observed between the peak heights and corresponding serum concentrations. The equations of the regression lines were $y = 19.99x - 0.234$ ($r = 0.999\,87$) for *R*(−)-MD and $y = 20.29x - 1.204$ ($r = 0.999\,94$) for *S*(+)-MD. The intra-assay recoveries of the enantiomers from spiked serum samples were 89.8% with a coefficient of varia-

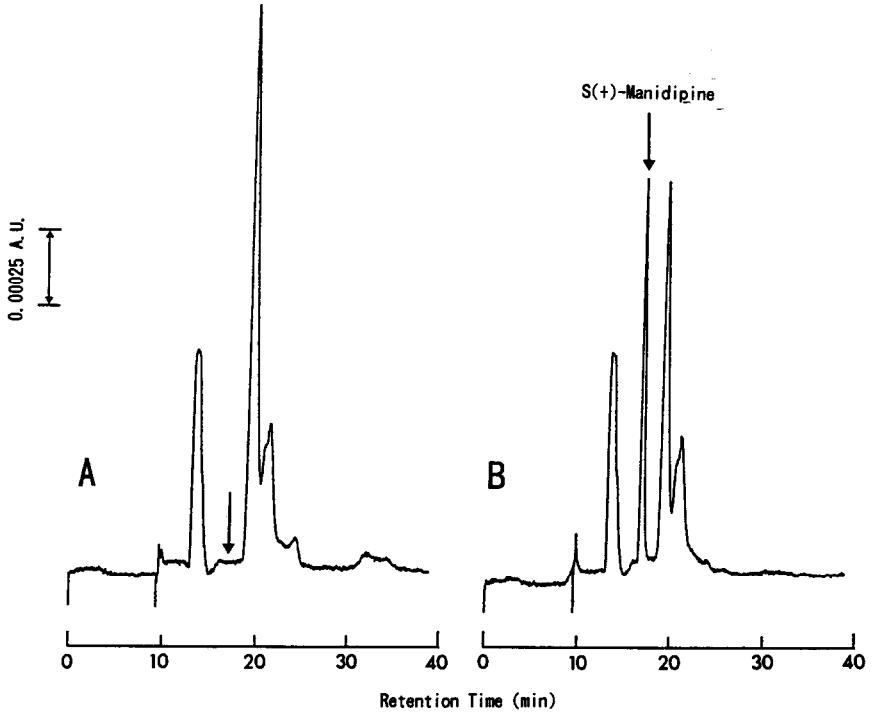

Fig. 4. Chromatograms of (A) drug-free human serum and (B) human serum spiked with racemic MD (5 ng/ml as an enantiomer), after column switching obtained from the fraction containing *S*(+)-MD (fraction 2) on the CSP column.

TABLE II

RECOVERY AND REPRODUCIBILITY IN THE DETERMINATION OF *R*(−)- AND *S*(+)-MD ADDED TO DRUG-FREE HUMAN SERUM

Values in parentheses are coefficients of variation (%).

Concentration spiked (ng/ml)	Mean recovery (%)			
	Intra-assay		Inter-assay	
	R(−)-MD	*S*(+)-MD	*R*(−)-MD	*S*(+)-MD
0.5	92.8	91.8	–	–
1.0	84.2	82.0	–	–
5.0 (n = 5)	89.8 (4.6)	90.1 (6.4)	98.0 (4.9)	96.3 (3.9)
10.0	92.7	98.0	–	–
25.0	95.7	97.4	–	–

tion (C.V.) of 4.6% for *R*(−)-MD, and 90.1% with a C.V. of 6.4% for *S*(+)-MD (both 5 ng/ml, n = 5). The inter-assay recoveries of the enantiomers from spiked serum samples were 98.0% with a C.V. of 4.9% for *R*(−)-MD, and 96.3% with a C.V. of 3.9% for *S*(+)-MD (both 5 ng/ml, n = 5). The recovery and reproducibility for the enantiomers in human serum are also shown in Table II. The lower limits of detection of both enantiomers were 0.2 ng/ml in human serum, using 1 ml of sample, both at a signal-to-noise ratio of 3.

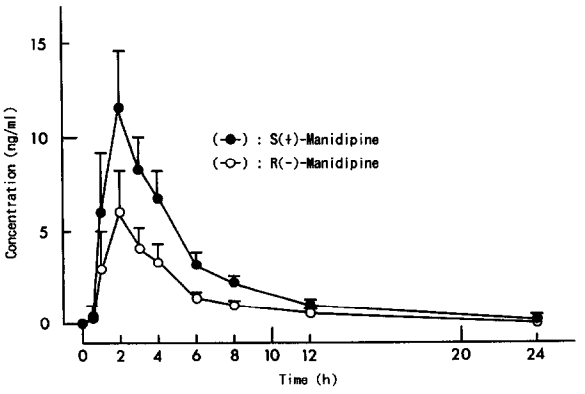

Fig. 5. Mean serum levels of *S*(+)- and *R*(−)-MD after oral administration of a 40-mg dose of racemic MD · 2HCl to humans. Each point and bar represent the mean (n = 4) and the standard deviation, respectively.

TABLE III

PHARMACOKINETIC PARAMETERS OF MD ENANTIOMERS IN HUMANS AFTER ORAL ADMINISTRATION OF 40 mg OF RACEMIC MD · 2HCl

Parameter	Value (mean ± S.D., n = 4)	
	R(−)-MD	*S*(+)-MD
C_{max} (ng/ml)	6.5 ± 1.6	11.6 ± 3.0
T_{max} (h)	1.5 ± 0.6	1.9 ± 0.3
AUC_{0-24} (ng·h/ml)	28.6 ± 2.8	57.4 ± 5.1
$T_{1/2\,\alpha}$ (h)	1.3 ± 0.5	1.2 ± 0.4
$T_{1/2\,\beta}$ (h)	11.8 ± 5.6	9.7 ± 4.3

TABLE IV

CONCENTRATIONS OF ENANTIOMERS AND ENANTIOMERIC MIXTURE IN HUMAN SERUM AFTER ORAL ADMINISTRATION OF 40 mg OF RACEMIC MD · 2HCl

Time (h)	Concentration (ng/ml)			
	R(−)-MD	*S*(+)-MD	Total[a]	Mixture[b]
1	4.3	6.3	10.6	9.3
2	6.7	12.5	19.2	18.4
3	3.6	8.7	12.3	14.3
4	3.9	7.8	11.7	11.3
6	1.5	3.3	4.7	4.3
8	0.9	2.0	2.9	2.8
12	0.4	0.9	1.3	1.3
24	<0.2	0.4	0.4	0.5

[a] Sum of *R*(−)-MD and *S*(+)-MD.

[b] Concentrations obtained by the achiral method [8].

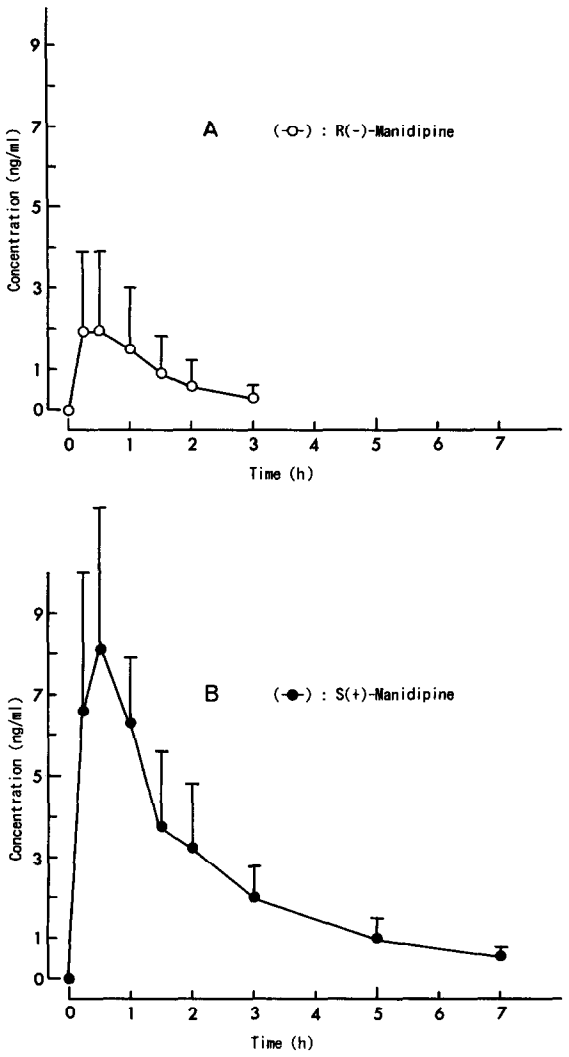

Fig. 6. Mean plasma levels of $S(+)$- and $R(-)$-MD after oral administration of a 0.5 mg/kg dose of (A) $R(-)$-MD · 2HCl or (B) $S(+)$-MD · 2HCl to dogs. Each point and bar represent the mean (n = 4) and the standard deviation, respectively.

Application of the method

The proposed method was applied to the enantioselective determination of MD in human serum and dog plasma. The mean serum concentration–time profiles of $R(-)$- and $S(+)$-enantiomers in humans after oral administration of a single dose of 40 mg of racemic MD·2HCl are shown in Fig. 5. The serum concentrations of $S(+)$-MD were always higher than those of $R(-)$-MD. Mean values of the pharmacokinetic parameters are presented in Table III. The mean concentrations of $R(-)$- and $S(+)$-enantiomers showed peaks of 6.5 and 11.6 ng/ml at 1.5 and 1.9 h after administration, respectively. The area under the curve (AUC) of $S(+)$-MD was almost double that of $R(-)$-MD.

The validity of the present stereoselective assay was confirmed by comparison with the previously reported non-stereoselective method [8] (Table IV). The mean plasma concentration–time profiles of each enantiomer in dogs after oral administration of a single dose of 0.5 mg/kg $R(-)$- or $S(+)$-MD · 2HCl are shown in Fig. 6. The plasma concentrations of $S(+)$-MD were higher than those of $R(-)$-MD, for reasons that are unclear. The antipode was not detected in plasma after oral administration of each isomer to dogs which indicates that enantiomeric inversion does not occur in dogs.

In conclusion, a stereoselective and highly sensitive method for the determination of MD in serum was established by combination of CSP and column-switching HPLC. This method was suitable for the pharmacokinetic studies of MD enantiomers after oral administration of MD · 2HCl to human and dog.

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