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Stereoselective determination of *R,S*-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid and its taurine conjugates in dog urine by high-performance liquid chromatography

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Abstract

Two high-performance liquid chromatographic methods for the stereoselective determination of *R,S*-2-[4-(3-methyl-2-thienyl)-phenyl]propionic acid (*R,S*-MTPPA), a new anti-inflammatory agent, and its taurine conjugates (*R,S*-MTPPA-TAU) in dog urine have been developed and validated. The urine samples were subjected to solid extraction or TLC preparation, then *R,S*-MTPPA and *R,S*-MTPPA-TAU were separated on Chiralcel OD and Chiral AGP columns, respectively, with ultraviolet absorbance detection at 272 nm. The dose–response relationships for enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU were linear in the concentration ranges of 0.5–50 ($r>0.9993$) and 5–200 μg/ml ($r>0.9982$), respectively. Recoveries of all tested enantiomers from dog urine were roughly 90% within the above concentration ranges. Intra- and inter-day reproducibilities were sufficient for metabolic studies. These methods were applied to stereoselective determination of the enantiomers in dog urine after administration of either *S*- or *R*-MTPPA. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; *R,S*-2-[4-(3-Methyl-2-thienyl)phenyl]propionic acid

1. Introduction

R,S-2-[4-(3-Methyl-2-thienyl)phenyl]propionic acid (*R,S*-MTPPA) is a new, orally effective anti-inflammatory agent (Fig. 1). The *S*-isomer is pharmacologically active, while the *R*-isomer is inactive. The biological activities of *S*-MTPPA (code: M-5011) in animal models have been reported [1,2]. The metabolism of *R,S*-MTPPA is considered to involve oxidation of the thiophenyl moiety, glucuronidation and amino acid conjugation of the carboxylic group. The major urinary and fecal metabolite in dogs after oral administration of *S*-MTPPA was identified as the taurine conjugate of MTPPA (MTPPA-TAU) [3,4]. It is known that the enantiomers of 2-arylpropionic acid derivatives undergo chiral inversion from the inactive *R*-isomer to the active *S*-isomer [5–8] and the mechanism of chiral inversion is related to the mechanism of amino acid conjugation [9–12]. *R,S*-MTPPA was expected to undergo chiral inversion in the same way, but the optical form of the metabolite after dosing of each of *S*- and *R*-MTPPA is not known. A chiral separation method for *R,S*-MTPPA and its metabolite is needed to investigate the stereoselective metabolism and inversion of *R*- and *S*-MTPPA in animals. In this

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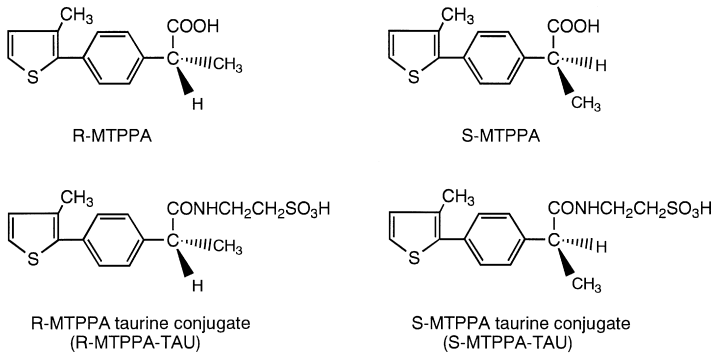

Fig. 1. Chemical structures of *R,S*-MTPPA and the taurine conjugates.

paper we report a stereoselective and quantitative assay for enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU in dog urine using high-performance liquid chromatography (HPLC). The method was employed to determine the enantiomers in dog urine samples after administration of *S*-MTPPA and *R*-MTPPA.

2. Experimental

2.1. Chemicals and reagents

The enantiomers of *R,S*-MTPPA and its taurine conjugate were prepared at the Maruho Kyoto Research Laboratory (Kyoto, Japan) as described in the previous paper [4]. The optical purities of *R*- and *S*-MTPPA determined by HPLC chiral separation analysis were 99.0 and 99.8%, respectively. Other solvents and chemicals used were of analytical or HPLC grade.

2.2. Instrumentation

The HPLC system for chiral separation of *R*- and *S*-MTPPA consisted of a pump system (600E, Waters, Milford, MA, USA), an auto sample injector (715 ULTRA WISP, Waters), a chiral separation column (Chiralcel OD, 250 mm×4.6 mm I.D.; Daicel Chemical Industries, Tokyo, Japan), a column oven (Waters) and a variable-wavelength UV detector, Model 484 (Waters). For separation of *R*- and *S*-MTPPA-TAU, an isocratic solvent delivery pump (LC-6A, Shimadzu, Kyoto, Japan), an auto sample injector (SCL-6B, Shimadzu), a chiral separation column (Chiral AGP, 100×4.0 mm I.D.; Daicel), a column oven (CTO-6A, Shimadzu) and a UV detector (SPD-6A, Shimadzu) were used. The data recording system for the chiral separation of *R,S*-MTPPA and *R,S*-MTPPA-TAU consisted of a Waters personal computer with a Maxima 825 system and a Shimadzu integrator (C-R4A), respectively.

2.3. Chromatographic conditions

The mobile phase for separation of *R*- and *S*-MTPPA consisted of *n*-hexane–2-propanol–acetic acid (96:4:0.5, v/v) and was delivered at a flow-rate of 1.0 ml/min. For the separation of *R*- and *S*-MTPPA-TAU, acetonitrile: 10 m*M* sodium dihydrogenphosphate buffer adjusted to pH 6.0 by addition of 10 m*M* disodium hydrogenphosphate (3:97, v/v) was used and the flow-rate was 0.7 ml/min. The optimum wavelength for detection was determined by a UV scan of *R,S*-MTPPA and *R,S*-MTPPA-TAU in methanol; both compounds exhibit absorption maxima at 272 nm. The column temperature was set at 30°C for the separations.

2.4. Stock solutions

Stock solutions of *R,S*-MTPPA and *R,S*-MTPPA-TAU (1 mg/ml each) were prepared in 50% methanol and stored at 4°C. Working stock solutions were prepared by diluting primary stock solutions with 50% methanol to a suitable concentration. These stock solutions were stable for at least one month when stored at 4°C.

2.5. Preparation of dog urine standards

The standard solution of *R,S*-MTPPA or *R,S*-MTPPA-TAU (1 mg/ml each) was diluted with 50% methanol to obtain spiking solutions. Urine standards were prepared by spiking aliquots of the spiking solutions into blank dog urine to give the following final concentrations: 0.5, 1.0, 5.0, 10 and 50 μg/ml for *R,S*-MTPPA and 5, 10, 20, 50, 100 and 200 μg/ml for *R,S*-MTPPA-TAU.

2.6. Preparation of samples

Extraction of *R,S*-MTPPA from dog urine was carried out by solid–liquid extraction and liquid–liquid extraction with diethyl ether. Dog urine (1 ml) was treated with 2 ml of 0.2 *M* HCl and applied to a Sep-Pak C_{18} column (Waters), which had been pretreated with 10 ml of methanol and 10 ml of water. The column was washed with 3 ml each of water, 1% acetic acid and 1% acetic acid–methanol (6:4, v/v) in that order. Four ml of 1% acetic acid–methanol (1:9, v/v) was used to elute *R,S*-MTPPA. The eluate was evaporated under a nitrogen flow. The residue was taken up in 0.3 ml of 0.2 *M* HCl and 3 ml of diethyl ether and the mixture was shaken for 10 min, then centrifuged at 3000 rpm for 10 min. The supernatant was evaporated to dryness and the residue was redissolved in 100 μl of ethanol. A 20-μl aliquot of this solution was injected into the HPLC system. To extract *R,S*-MTPPA-TAU from dog urine, 0.1 ml of urine was subjected to thin-layer chromatography (TLC) (Silica gel 60 F_{254}, 20×20 cm, Merck, Darmstadt, Germany) and developed with ethyl acetate–acetone–water–acetic acid (8:2:1:1, v/v). The band at R_F 0.15–0.20 was scraped off and the metabolite was extracted with 3 ml of methanol. The extract was evaporated under nitrogen and the residue was dissolved in 100 μl of methanol. A 10-μl aliquot of this solution was injected into the HPLC system.

2.7. Method validation

Calibration curves were obtained by plotting peak area of *R*- and *S*-MTPPA against concentration. Injections of a series of dog urine standards containing *R*- and *S*-MTPPA at concentrations ranging from 0.5 to 50 μg/ml were performed. The absolute recoveries from dog urine were determined by comparing peak areas for dog urine samples spiked with standards with those for the standards in methanol solution at concentrations of 0.5, 1.0, 5.0, 10 and 50 μg/ml. The precision and accuracy were obtained by determining five urine samples spiked with *R*- and *S*-MTPPA at 0.5, 1.0, 5.0, 10 and 50 μg/ml. Validation of the method for *R*- and *S*-MTPPA-TAU was carried out in the same way at concentrations of 5, 10, 20, 50, 100 and 200 μg/ml.

2.8. Pharmacological application

The present chiral HPLC methods were used to evaluate the concentration of enantiomers in urine after administration of either *S*- or *R*-MTPPA to dogs (male beagles, weighing 11–14 kg). Three dogs each received *S*- or *R*-MTPPA orally at a dosage of 10 mg/kg. Urine specimens were collected up to 24 h after administration.

3. Results and discussion

3.1. Chromatograms

Two methods have been reported for the chiral separation of enantiomers of 2-arylpropionic acid derivatives, one is a direct method with chiral stationary phase and the other is an indirect method using chiral derivatization techniques [13]. We selected the direct method for the present assay of enantiomers of MTPPA to avoid the possibility of stereochemical conversion of enantiomers induced by derivatization. Adequate separation of enantiomers of MTPPA was obtained by using a chiral separation column packed with silica modified with a cellulose carbamate derivative. Direct injection of dog urine was impossible because of interfering peaks, so clean-up was conducted by solid–liquid extraction with Sep-Pak C_{18} and liquid–liquid extraction with diethyl ether. Typical chromatograms of enantiomers of *R,S*-MTPPA are shown in Fig. 2. Fig. 2A shows a chromatogram of extracted blank dog urine and Fig. 2B shows a chromatogram of blank urine spiked with 10 μg/ml each of *R*- and *S*-MTPPA. The separation of enantiomers was suffi-

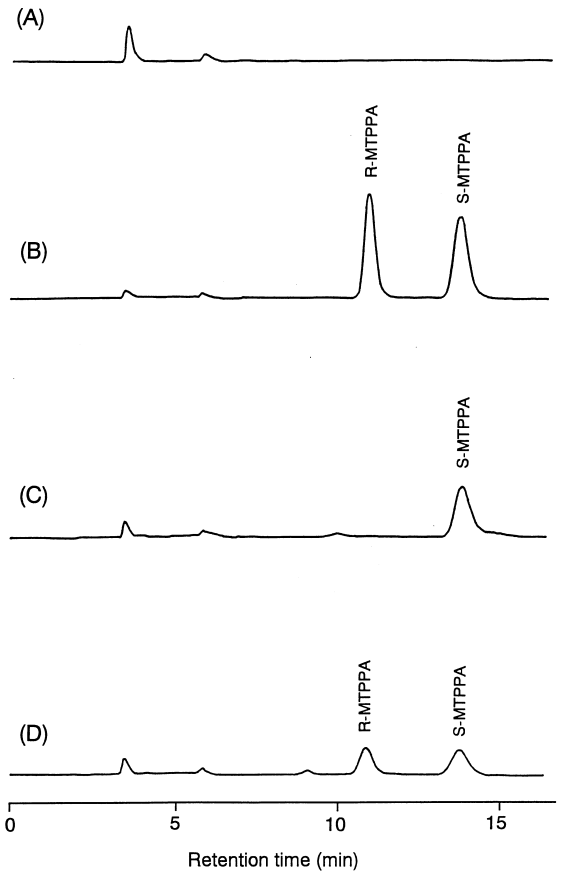

Fig. 2. Chromatograms of *R,S*-MTPPA. (A) Blank dog urine; (B) blank dog urine spiked with 10 μg/ml each of *R*- and *S*-MTPPA; 24-h dog urine sample after administration of (C) *S*-MTPPA or (D) *R*-MTPPA at a dose of 10 mg/kg. *S*-MTPPA in (C) was estimated 6.1 μg/ml. *S*- and *R*-MTPPA in (D) were estimated 2.7 and 3.7 μg/ml, respectively. The absorbance unit (full scale=0.16 AU) was employed at 272 nm.

cient and there was no interfering peak on the chromatogram of the blank sample. The enantiomers were eluted within 15 min.

In the case of the taurine conjugate, the enantiomeric analysis of the chiral component of the conjugate is usually conducted after hydrolysis with a strong acid [14]. In this study, good separation of *R*- and *S*-enantiomers was obtained by using a chiral separation column packed with silica modified with an α-acidic glycoprotein without hydrolysis of the taurine conjugate. The mobile phase was a mixture of sodium phosphate buffer and acetonitrile, which was optimized with respect to the salt concentration and pH of the buffer. In the case of analytical procedure for *R,S*-MTPPA-TAU from dog urine, clean-up was conducted by TLC before HPLC analysis. Fig. 3A shows a chromatogram of blank dog urine. The enantiomers of *R*- and *S*-MTPPA-TAU in the urine sample of dog dosed with *R*-MTPPA were resolved completely without interference (Fig. 3B). The retention times of *S*- and *R*-MTPPA-TAU were at 21 and 25 min, respectively.

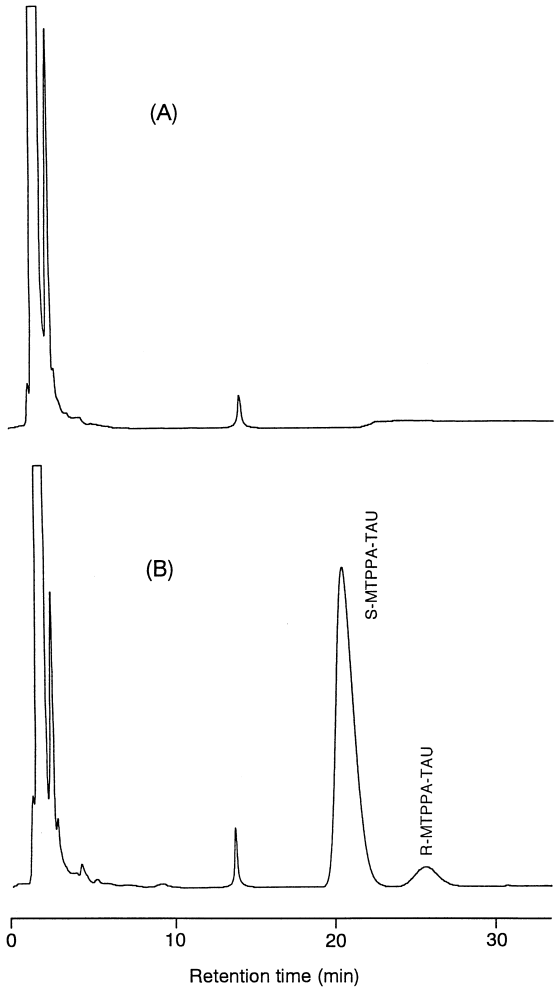

Fig. 3. Chromatograms of *R,S*-MTPPA-TAU. (A) Blank dog urine, (B) 24-h dog urine sample after administration of *R*-MTPPA at the dose of 10 mg/kg. *S*- and *R*-MTPPA-TAU in (B) were estimated 131.5 and 10.4 μg/ml, respectively. The absorbance unit (full scale=0.08 AU) was employed at 272 nm.

Table 1
Recoveries of enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU from dog urine at various concentrations

Spiked concentration (μg/ml each)	Recovery (%)	
	R	*S*
R,S-MTPPA		
0.5	89.6±4.8	90.4±5.2
1.0	89.5±6.0	90.3±5.5
5.0	93.9±3.4	94.0±3.5
10.0	90.9±13.0	90.8±12.9
50.0	97.8±6.7	97.9±6.7
R,S-MTPPA-TAU		
5	90.0±19.5	89.5±17.9
10	91.7±14.3	99.2±13.3
20	96.7±13.8	95.1±11.9
50	90.0±6.0	90.6±6.3
100	87.8±4.1	87.0±3.9
200	93.4±6.3	93.0±6.0

Each value is the mean±S.D. of five experiments.

3.2. Standard curves and assay validation

The calibration curves of enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU were linear over the ranges of 0.5–50 ($r>0.9993$) and 5–200 μg/ml ($r>0.9982$), respectively. The limits of detection ($S/N=3$) were 0.2 and 1 μg/ml for *R,S*-MTPPA and *R,S*-MTPPA-TAU, respectively. The absolute recoveries of enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU from dog urine were 89.6–97.9 and 87.0–99.2% over the concentration ranges of 0.5–50 and 5–200 μg/ml, respectively, as shown in Table 1. The drug concentrations were calculated from calibration curves obtained using blank dog urine spiked with appropriate standards. The limits of quantitation were 0.5 and 5 μg/ml for *R,S*-MTPPA and *R,S*-MTPPA-TAU, respectively. The intra- and inter-day reproducibilities for enantiomers of *R,S*-MTPPA are shown in Table 2 in terms of the coefficient of variation (C.V.). Although the C.V. values of intra-day precision at the concentration of 10 μg/ml were approximately 14%, those at other concentrations and all C.V. values of inter-day precision were less than 8.4%. In the case of *R,S*-MTPPA-TAU (Table 3), the C.V. values of inter-day precision at the concentrations of 5, 10 and 20 μg/ml were fairly high, but they are acceptable for practical use. Although an internal standard was not used for the present methods, accuracy was confirmed to be sufficient as the value within ±20% except only one sample at 5 μg/ml of *R*-MTPPA-TAU intra-day reproducibility.

3.3. Urinary excretion study of enantiomers in dog

To investigate the stereoselectivity of metabolism of *R,S*-MTPPA in dogs, the enantiomers of *R,S*-MTPPA and its major metabolite (the taurine conjugate) in the urine after dosing of the animals with

Table 2
Reproducibility of assay for enantiomers of *R,S*-MTPPA at various concentrations

Spiked concentration (μg/ml each)	Found concentration (μg/ml)		C.V. (%)	
	R	*S*	*R*	*S*
Intra-day ($n=5$)				
0.5	0.56±0.02	0.56±0.03	4.4	4.8
1.0	1.0±0.1	1.0±0.1	6.1	5.5
5.0	4.9±0.2	4.9±0.2	3.5	3.6
10.0	9.5±1.3	9.4±1.3	14.2	14.0
50.0	49.2±3.4	49.3±3.4	6.8	6.9
Inter-day ($n=5$)				
0.5	0.49±0.04	0.50±0.04	7.8	7.4
1.0	1.0±0.1	1.0±0.1	8.4	7.9
5.0	4.9±0.3	4.9±0.4	6.9	7.4
10.0	10.0±0.8	10.0±0.8	8.0	8.0
50.0	49.0±2.4	50.1±2.9	4.8	5.8

Table 3
Reproducibility of assay for enantiomers of *R,S*-MTPPA-TAU at various concentrations

Spiked concentration (μg/ml each)	Found concentration (μg/ml)		C.V. (%)	
	R	*S*	*R*	*S*
Intra-day (n=5)				
5	6.1±1.0	5.9±1.0	15.7	16.4
10	10.7±1.4	10.8±1.3	13.1	12.1
20	20.7±2.7	21.0±2.5	13.1	11.8
50	49.8±3.2	50.1±3.4	6.5	6.8
100	96.8±4.5	97.5±4.3	4.6	4.4
200	196.0±13.1	195.6±12.5	6.7	6.4
Inter-day (n=5)				
5	5.0±0.4	5.0±0.3	7.9	6.2
10	10.1±0.5	10.0±0.6	5.3	5.9
20	19.8±0.9	19.9±0.7	4.5	3.5
50	50.9±3.1	51.1±3.2	6.2	6.2
100	100.1±5.1	100.6±5.2	5.1	5.2
200	198.0±5.6	197.1±5.7	2.8	2.9

each enantiomer were determined by the present chiral HPLC methods. Typical chromatograms obtained are shown in Fig. 2C,D. There was a single peak of unchanged *S*-MTPPA after administration of the *S*-isomer, which both *R*- and *S*-MTPPA were detected after administration of the *R*-isomer. The peaks of *R*- and *S*-MTPPA-TAU were also detected after administration of the *R*-isomer (Fig. 3B). Urinary excretion of the enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU up to 24 h after oral administration of *S*- or *R*-MTPPA at the dose of 10 mg/kg to male dogs is summarized in Table 4. The urinary excretion of *S*-MTPPA-TAU after *S*- and *R*-isomer administration amounted to 7.61±5.72 and 8.71±2.02% of the dose, respectively. *R*-MTPPA-TAU excretion in urine was 0.52±0.30% of the dose after *R*-isomer administration. Thus, chiral inversion from *R*-MTPPA to *S*-MTPPA occurs in dogs.

In this study, we have developed an assay which allows quantitative analysis of the enantiomers of *R,S*-MTPPA and the taurine conjugate in dog urine. However, we have recognized that hydroxylated metabolites of the thiophenyl moiety were also excreted in the urine of dogs dosed with MTPPA [3,4]. A chiral separation method for the hydroxylated metabolites of *R,S*-MTPPA and *R,S*-MTPPA-TAU is under investigation.

4. Conclusion

Our newly developed chiral HPLC method for assay of *R,S*-MTPPA and its taurine conjugate is sensitive and accurate enough for the analysis of stereoselective pharmacokinetics in dogs.

Table 4
Urinary excretion of enantiomers of *R,S*-MTPPA and *R,S*-MTPPA-TAU up to 24 h after oral administration of *S*-MTPPA or *R*-MTPPA at a dose of 10 mg/kg to male dogs

Administered enantiomer	Urinary excretion (% of dose)			
	R-MTPPA	*S*-MTPPA	*R*-MTPPA-TAU	*S*-MTPPA-TAU
S-MTPPA	N.D.	0.44±0.24	N.D.	7.61±5.72
R-MTPPA	0.22±0.05	0.34±0.08	0.52±0.30	8.71±2.02

Each value is the mean±S.D. of three animals.
N.D.=not detected.

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