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Determination of the *R,R*- and *S,S*-enantiomers of vamicamide in human serum and urine by high-performance liquid chromatography on a Chiral-AGP column

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ABSTRACT

An enantioselective liquid chromatographic assay for the determinations of the *R,R*- and *S,S*-enantiomers of vamicamide, a potent anticholinergic drug, in human serum and urine is described. Racemic vamicamide and internal standard were purified from biological fluids using a two-step extraction procedure involving diethyl ether and 0.1% phosphoric acid. The overall recoveries of racemic vamicamide and internal standard were greater than 80%. The purified samples were measured by high-performance liquid chromatography on a Chiral-AGP column with ultraviolet absorbance detection at 260 nm. The standard curves for the analytes were linear from 10 to 200 ng/ml in serum and from 0.25 to 50 μg/ml in urine. The quantification limit of both enantiomers was 10 ng/ml for serum and 250 ng/ml for urine. Both intra-day and inter-day accuracy and precision data showed good reproducibility of the method. The assay has been applied for the analysis of vamicamide enantiomers in serum and urine samples from a healthy volunteer.

INTRODUCTION

Vamicamide, 4-dimethylamino-2-phenyl-2-(2-pyridyl)valeramide (Fig. 1), is now under clinical investigation as a new anticholinergic agent. It is a chiral compound having two chiral centres, and it is administered as a racemic mixture of *R,R*- and *S,S*-vamicamide. However, enantiomers of many drugs possessing a chiral carbon centre show different pharmacological activities [1–3], pharmacokinetic properties [4–6] or metabolism [7,8]. Studies of the effect on acetylcholine-induced contractions of isolated guinea pig ileum suggest that the *R,R*-enantiomer possess 80 times more anticholinergic activity than *S,S*-vamicamide (unpublished data). Although the stereoselective pharmacokinetics of vamicamide is important, the disposition kinetics of individual vamicamide isomers in human or animals has not been investigated. Investigation of possible stereoselective differences in pharmacokinetics and metabolism of vamicamide requires a method for quantitation of its enantiomers in biological samples. For pharmacokinetic studies of vamicamide enantiomers, a quantification limit of at least 20 ng/ml racemic vamicamide in serum would be required. The separation of enantiomers by high-performance liquid chromatography (HPLC) can be divided into three major principles: the addition of an appropriate chiral additive to the mobile phase [9], the separation of the enantiomers on chiral stationary phases (CSPs)

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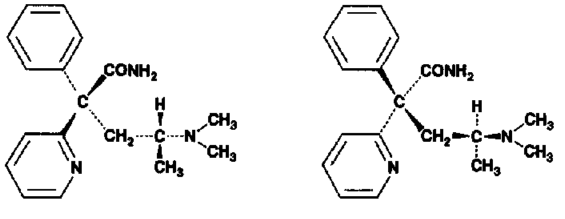
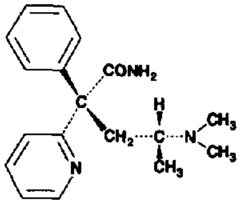


R,R-vamicamide S,S-vamicamide



S,R-vamicamide (FR071741)

Fig. 1. Structures of *R,R*-vamicamide, *S,S*-vamicamide and the internal standard (*S,R*-vamicamide).

[10] and the derivatization of the enantiomers to their diastereomers followed by an achiral separation [11]. Protein-based chiral stationary phases have become one of the most widely used CSPs for the direct analysis of drug enantiomers because of their broad applicability in spite of poor column stability [12,13]. The second-generation α_1-acid glycoprotein-based chiral column, Chiral-AGP, exhibits an improved stability and is becoming especially widely used in pharmaceutical analyses. Many different types of drug enantiomers have been separated on Chiral-AGP [14–17].

This paper describes a method for the simultaneous determination of vamicamide enantiomers in serum and urine samples by HPLC on the Chiral-AGP with UV detection. Data on the concentration of the drug in serum and excretion in urine from one healthy volunteer after oral administration of racemic vamicamide are presented.

EXPERIMENTAL

Chemicals

Racemic vamicamide, *R,R*-vamicamide, *S,S*-vamicamide and the internal standard *S,R*-vamicamide (FR071741, I.S.) were synthesized in the New Drug Research Laboratories, Fujisawa Pharmaceutical (Osaka, Japan). The structures of these compounds are shown in Fig. 1. Acetonitrile (HPLC grade), diethyl ether (high purity grade) and phosphoric acid (85%, analytical grade) were all obtained from Wako (Osaka, Japan). The other chemicals used were of analytical grade. All reagents were used without further purification. Drug-free human serum was obtained from male healthy volunteers. Drug-free human urine was obtained from male healthy volunteers and diluted ten times with water.

Chromatographic conditions

The HPLC system consisted of a Waters Model 510 pump, a Waters WISP Model 712 autoinjector and a Waters Lambda-Max Model 481 ultraviolet absorbance detector (Waters, Millipore, Milford, MA, USA). The peak areas were measured with a Shimadzu Chromatopack C-R6A integrator (Shimadzu, Kyoto, Japan). The chiral columns were a 100 mm × 4 mm I.D. Chiral-AGP column equipped with a 10 mm × 3 mm I.D. Chiral-AGP guard column (ChromTech, Norsborg, Sweden). The mobile phase consisted of 0.02 *M* phosphate buffer (pH 6.3)–acetonitrile (20:1, v/v). The flow-rate of the mobile phase was 0.9 ml/min, the separation was performed at room temperature and the absorbance was monitored at 260 nm.

Standard solutions

Stock solutions of vamicamide and the I.S. were prepared by dissolving each compound in 0.1 *M* HCl–0.1 *M* $NaHCO_3$ (1:1.2, v/v) to a final concentration of 200 μg/ml. The solutions were stored at 4°C. Working solutions were obtained by further dilution of the stock solutions with water.

Sample preparation

To 1.0 ml of serum or ten-fold diluted urine in a glass-stoppered glass centrifuge tube, 100 μl of the internal standard solution (5 μg/ml I.S. for serum or 10 μg/ml I.S. for urine), 100 μl of distilled water and 1.0 ml of 0.1 *M* sodium hydroxide were added. The mixture was extracted with 5.0 ml of diethyl ether on a mechanical horizontal shaker for 10 min at 250 rpm. After centrifugation for 5 min at 1900 *g*, the organic phase was transferred to a conical glass tube and 200 μl of 0.1% phosphoric acid were added. The tube was shaken vigorously for 5 min, and then centrifuged at 1900 *g* for 5 min. The organic layer was removed by aspiration and a 150-μl aliquot of the clear aqueous layer was transferred to a 200-μl autosampler tube for HPLC and neutralized by the addition of 1 *M* NaOH (3.5 μl for serum or 4.0 μl for urine). A 100-μl sample was injected into the column.

Determination of recovery

The recovery of extraction efficiency of the serum and urine extraction procedure for both analytes and the I.S. was determined by five samples. The data of extracted serum and urine samples were compared with known standards. The known standards were prepared by direct solution to account for any loss, dilution or concentration effects of the extraction procedure.

Calibration curves

Spiked serum and urine samples were prepared by adding 100 μl of working solution containing racemic vamicamide instead of distilled water to blank serum and diluted urine. The concentrations of the spiked samples were 20, 50, 100, 200 and 400 ng/ml for serum and 0.05, 0.2, 0.5, 2.0, 5.0 and 10.0 μg/ml for diluted urine. The samples were then processed as described above. The peak-area ratios of the enantiomers to internal standard were plotted against the concentrations of *R,R*-vamicamide and *S,S*-vamicamide. The calibration curves for both enantiomers were calculated by least-squares linear regression analysis.

Application

Permission to perform the study was obtained from the local ethics committee. One healthy male volunteer was given an 18-mg oral dose of vamicamide. Blood samples were collected into tubes at 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 h post dose, and the serum was separated immediately after collection. Urine was collected into plastic containers at intervals 0–2, 2–4, 4–6, 6–8, 8–12 and 12–24 h after vamicamide administration. The volume of collected urine was measured, and a 1.0-ml aliquot was diluted ten times with water. Serum and urine samples were stored frozen at −20°C until analysed.

RESULTS AND DISCUSSION

Chromatography

Representative chromatograms of vamicamide enantiomers in human serum and urine are shown in Fig. 2, in which retention times of *S,S*-vamicamide, *R,R*-vamicamide and internal standard are 5.9 min, 8.6 min and 10.4 min, respectively. The resolution (R_s) between the peaks of *S,S*-vamicamide and *R,R*-vamicamide was 2.7.

No chromatographic peaks at retention times of vamicamide enantiomers or internal standard were observed in blank human serum and urine. In addition, blank rat serum, rat urine, dog se-

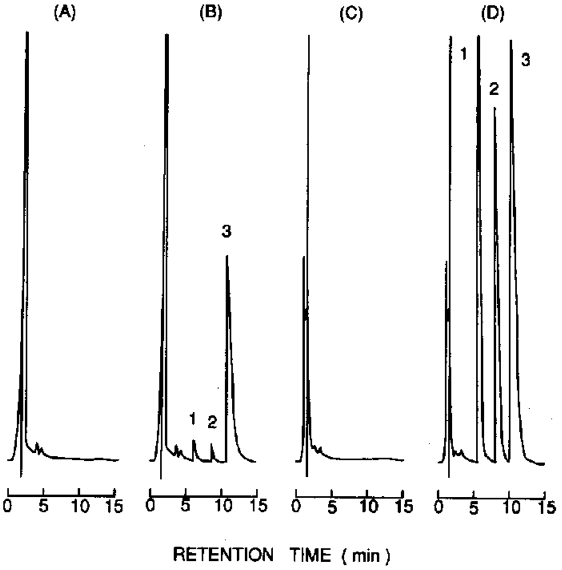


Fig. 2. Chromatograms of (A) blank serum, (B) a volunteer's plasma sample 2 h after oral administration of 18 mg of racemic vamicamide, (C) blank urine and (D) a volunteer's urine sample 0–2 h after oral administration of 18 mg of racemic vamicamide. Peaks: 1 = *S,S*-vamicamide; 2 = *R,R*-vamicamide; 3 = I.S.

TABLE I

ANALYTICAL RECOVERIES OF THE ENANTIOMERS OF VAMICAMIDE AND I.S. FROM SERUM AND DILUTED URINE

Compound	Serum ($n = 5$)		Diluted urine ($n = 5$)	
	Added (ng/ml)	Recovery (mean ± S.D.) (%)	Added (ng/ml)	Recovery (mean ± S.D.) (%)
R,R-Vamicamide	10	86.1 ± 6.2	25	86.6 ± 4.5
	50	82.7 ± 3.7	1000	93.0 ± 1.6
	200	85.4 ± 4.0	5000	94.5 ± 1.1
S,S-Vamicamide	10	93.2 ± 9.9	25	80.4 ± 3.3
	50	83.5 ± 4.2	1000	95.4 ± 1.6
	200	85.1 ± 3.2	5000	95.6 ± 1.1
I.S.	500	85.1 ± 3.5	1000	94.5 ± 3.4

rum and dog urine did not show any interfering peaks, suggesting that this method may also be applicable to these samples.

Recovery

The recovery or extraction efficiency of the analytes was performed at three concentrations. The I.S. recovery was determined at the concentrations used in the assay procedure. The mean analytical recoveries of the analytes and the internal standard from serum and urine ranged from 82.7% to 93.2% and from 80.4% to 95.6%, respectively (Table I).

Calibration curves

The calibration curves were linear over the concentration range used for both *R,R*- and *S,S*-vamicamide in human serum and urine. The correlation coefficient values (r) were always shown to exceed 0.9998 for both enantiomers.

TABLE II

ANALYTICAL ACCURACY AND PRECISION FOR DETERMINING ENANTIOMERS OF VAMICAMIDE IN SERUM

Compound	Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)
Intra-day ($n = 5$)			
R,R-Vamicamide	10	9.8 ± 0.8	8.5
	50	49.7 ± 0.7	1.4
	200	199.7 ± 2.7	1.4
S,S-Vamicamide	10	11.0 ± 1.1	9.7
	50	49.6 ± 0.8	1.7
	200	195.2 ± 3.0	1.5
Inter-day ($n = 15$)			
R,R-Vamicamide	10	9.9 ± 0.8	7.8
	50	48.8 ± 2.0	4.2
	200	198.6 ± 5.3	2.7
S,S-Vamicamide	10	10.5 ± 0.9	8.9
	50	50.0 ± 2.3	4.6
	200	197.2 ± 3.7	1.9

TABLE III

ANALYTICAL ACCURACY AND PRECISION FOR DETERMINING ENANTIOMERS OF VAMICAMIDE IN DILUTED URINE

Compound	Concentration added	Concentration found (mean ± S.D.)	C.V. (%)
Intra-day (n = 5)			
R,R-Vamicamide	25 ng/ml	25.5 ± 1.4	5.6
	1.0 μg/ml	1.00 ± 0.01	0.6
	5.0 μg/ml	4.97 ± 0.06	1.2
S,S-Vamicamide	25 ng/ml	23.2 ± 0.8	3.6
	1.0 μg/ml	1.01 ± 0.00	0.3
	5.0 μg/ml	4.96 ± 0.06	1.3
Inter-day (n = 15)			
R,R-Vamicamide	25 ng/ml	24.1 ± 1.8	7.6
	1.0 μg/ml	1.01 ± 0.01	0.6
	5.0 μg/ml	4.98 ± 0.04	0.9
S,S-Vamicamide	25 ng/ml	24.0 ± 1.4	5.7
	1.0 μg/ml	1.01 ± 0.01	0.7
	5.0 μg/ml	4.97 ± 0.04	0.9

Accuracy and precision

Both intra-day ($n = 5$) and inter-day ($n = 15$) accuracy and precision of the method were determined at three concentrations, 10, 50 and 200 ng/ml for serum and 0.025, 1.0 and 5.0 μg/ml for diluted urine; the results are summarized in Tables II and III. These data suggest that the method is accurate and precise for routine assays of samples from clinical trials. The quantification limit of both enantiomers was 10 ng/ml for serum and 250 ng/ml for urine; these limits are sufficient for pharmacokinetic studies.

Stability

The reference solutions of racemic vamicamide, its two enantiomers, *R,R*-vamicamide and *S,S*-vamicamide, and the internal standard were stable for at least nine months if stored at 4°C. No inversion of the enantiomers occurred. In serum and urine samples both enantiomers were stable for at least two months if stored at −20°C.

Application

Fig. 3 shows serum concentration–time curves of the vamicamide enantiomers in one healthy subject after a single oral dose of 18 mg of racemic vamicamide. The $AUC_{0-\infty}$ (area under the serum concentration–time curve) of the *R,R*-enantiomer was 638.0 ng · h/ml, whereas that of

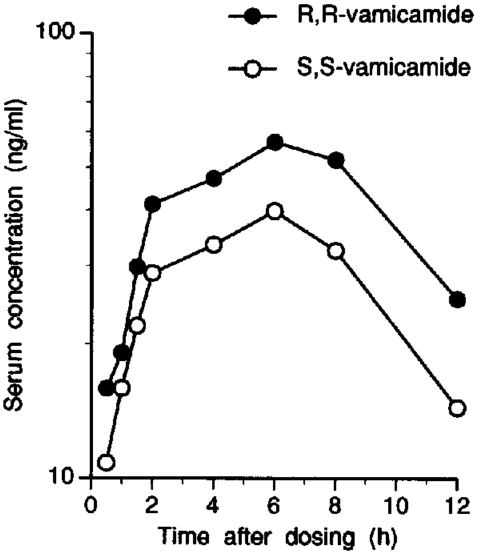


Fig. 3. Serum concentration–time curves of *R,R*-vamicamide and *S,S*-vamicamide in a healthy subject after a single oral administration of 18 mg of racemic vamicamide.

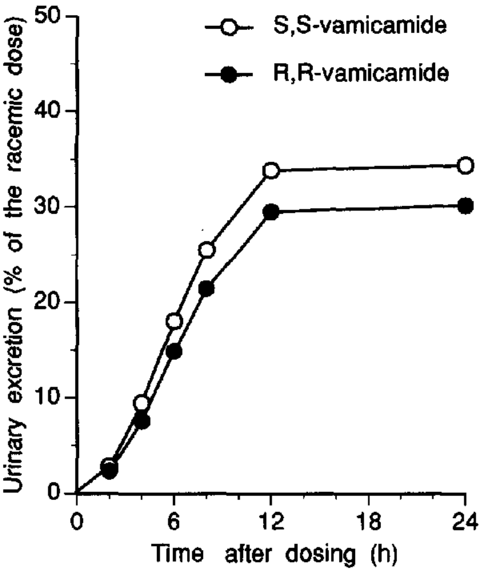


Fig. 4. Cumulative urinary excretion–time curves of *R,R*-vamicamide and *S,S*-vamicamide in a healthy subject after a single oral administration of 18 mg of racemic vamicamide.

the *S,S*-enantiomer was 403.7 ng · h/ml. Serum concentrations of *R,R*-enantiomer were about 1.4 times higher than those of *S,S*-enantiomer throughout the 12-h sampling period. The apparent oral clearances, calculated as dose divided by $AUC_{0-\infty}$, were 4.52 ml/min/kg for *R,R*-vamicamide and 7.14 ml/min/kg for *S,S*-vamicamide.

Fig. 4 shows the cumulative urinary excretion–time curves of the vamicamide enantiomers after oral administration of 18 mg of racemic vamicamide to a healthy subject. More than 64% of the dose was excreted in the urine as unchanged vamicamide. The unchanged vamicamide was excreted into the 24-h urine at 30.2% for *R,R*-enantiomer and 34.4% for *S,S*-enantiomer.

CONCLUSION

The column liquid chromatographic method reported is highly sensitive and selective for the determination of vamicamide enantiomers in human serum and urine. The applicability of the assay for pharmacokinetic studies of vamicamide enantiomers in humans is demonstrated.

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