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Short communication

Analysis of enantiomers of sibutramine and its metabolites in rat plasma by liquid chromatography–mass spectrometry using a chiral stationary-phase column

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ABSTRACT

Sibutramine, a monoamine reuptake inhibitor, is used as a racemate, for the treatment of obesity. It is converted *in vivo* mainly to two desmethyl active metabolites, mono-desmethylsibutramine (MDS) and di-desmethylsibutramine (DDS). In the present study, we introduced a rapid and simple chromatographic method for separating the *R*(+)- and *S*(−)-isomers of sibutramine, MDS, and DDS, respectively. The stereoisomers of the three compounds were extracted from rat plasma using diethyl ether and n-hexane under alkaline conditions. After evaporating the organic layer, the residue was reconstituted in the mobile phase (10 mM ammonium acetate buffer adjusted to pH 4.03 with acetic acid:acetonitrile, 94:6, v/v). The enantiomers in the extract were separated on a Chiral-AGP stationary-phase column and were quantified in a tandem mass spectrometry. The accuracy and precision of the assay were in accordance with FDA regulations for the validation of bioanalytical methods. This method was used to measure the concentrations of the enantiomers of sibutramine, MDS, and DDS in plasma after a single oral dose of 10 mg/kg racemic sibutramine in rats.

1. Introduction

Sibutramine, a monoamine reuptake inhibitor, is currently used as a racemate, for the treatment of obesity. It is converted *in vivo* mainly to two desmethyl active metabolites, mono-desmethylsibutramine (MDS) and di-desmethylsibutramine (DDS) [1,2]. The enantioselective behaviors of sibutramine and its two major active metabolites have been of interest from a pharmacokinetic as well as a pharmacodynamic point of view. The selective effects of the enantiomers on pharmacological consequences have been well characterized, with the *R*(+)-enantiomer being 200-fold potent more than the *S*(−)-enantiomer [3]. However, their kinetic characteristics are still unclear.

To date, sibutramine and its metabolites in biological samples have been determined using gas or liquid chromatography/mass spectrometry (LC–MS) [4,5]. However, their chiral separation has been limited to pharmaceutical drug products [6,7]. Therefore, we developed a chiral chromatography method to determine the enantioselective pharmacokinetics of sibutramine, MDS, and DDS in rat plasma.

The chiral resolution of enantiomers has been carried out using a specific chiral stationary phase, a mobile phase with a chiral reagent, and a derivatization method [8,9]. In the present study, we introduce a rapid and simple chromatographic method for separating the *R*(+)- and *S*(−)-isomers of sibutramine as well as its two active metabolites. The method is based on the use of a chiral column and a tandem mass spectrometry. We successfully used the method to characterize the time course of changes in the plasma concentrations of the stereoisomers of sibutramine as well as those of its two active metabolites in rat plasma, following the oral administration of racemic sibutramine.

2. Experimental

2.1. Reagents and materials

Sibutramine, MDS, and DDS were kindly donated by Yuhan Pharmaceutical Co. (Seoul, Korea) and the purity of three compounds is more than 99.5%. Domperidone (internal standard, IS) was purchased from Sigma (Seoul, Korea), and n-hexane and diethyl ether

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were obtained from J.T. Baker (Seoul, Korea). All other chemicals and solvents were of the highest analytical grade available. *R*(+)- and *S*(−)-enantiomers were separated with dibenzoyl-D-tartaric acid at a medicinal chemistry laboratory in College of Pharmacy, Catholic University of Daegu, which was based on the literature previously published by Fang et al. [10].

2.2. Preparation of standards and quality controls

Sibutramine, MDS, DDS, and the IS were dissolved in methanol to obtain a concentration of 1.0 mg/ml. These solutions were diluted serially with the mobile phase (10 mM ammonium acetate buffer adjusted to pH 4.03 with acetic acid:acetonitrile, 94:6, v/v), and 5 μl of each solution was added to 85 μl of drug-free plasma, to obtain final concentrations of 1, 2, 5, 10, 20, and 50 ng/ml sibutramine; 1, 2, 5, 10, 50, and 100 ng/ml MDS; and 10, 50, 200, 1000, and 4000 ng/ml DDS. Using linear regression, six calibration graphs were derived from the ratio between the area under the peak of each compound and the IS.

Quality control samples were prepared in 85 μl of blank rat plasma by adding 5 μl of serially diluted solutions of each of the three racemates, to obtain low, intermediate, and high concentrations in control samples. These samples were used to evaluate the between days and within day precision and accuracy of the assay.

2.3. Characterization of the product ions using tandem mass spectrometry

In brief, 10 ng/ml each of the sibutramine, MDS, DDS, and IS solutions were separately infused into the mass spectrometer at a flow rate of 10 μl/min, to characterize the product ions of each solution. The precursor ions $[M+H]^+$ and the pattern of fragmentation were monitored using the positive ion mode. The major peaks in the MS/MS scan were used to quantify sibutramine, MDS, DDS, and the IS.

2.4. Analytical system

Plasma concentrations of sibutramine, MDS, and DDS were quantified using an API 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface that was used in the positive ion mode ($[M+H]^+$).

The compounds were separated on a chiral stationary-phase column (Chiral-AGP, 100 mm × 2.0 mm internal diameter, 5-μm particle size; ChromTech Ltd., Congleton, Cheshire, UK) with a mobile phase that consisted of 10 mM ammonium acetate adjusted to pH 4.03 with acetic acid:acetonitrile (94:6, v/v). The column was heated to 22 °C, and the mobile phase was eluted at 0.2 ml/min using an HP 1100 series pump (Agilent, Wilmington, DE, USA). The Turboion spray interface was operated in the positive ion mode at 5500 V and 450 °C. Sibutramine, MDS, DDS, and domperidone (IS) produced mainly protonated molecules at *m*/*z* 280.2, 266.0, 252.1, and 427.2, respectively. The product ions were scanned in Q3 after collision with nitrogen in Q2 at *m*/*z* 125.2 for sibutramine, MDS, and DDS, and at *m*/*z* 175.1 for domperidone. Quantitation was performed by multiple reaction-monitoring (MRM) of the protonated precursor ions and the related product ions, using the ratio of the area under the peak for each solution and a weighting factor of $1/y^2$. The analytical data were processed with Analyst software (version 1.4.1, Applied Biosystems).

2.5. Sample preparation

One hundred microliters of the IS (100 ng/ml in mobile phase) and 0.01 ml of 10 N NaOH were added to 0.1 ml of rat plasma, followed by liquid–liquid extraction for 10 min with 1.5 ml of diethyl ether:n-hexane (4:1, v/v). The organic layer was separated and removed at 40 °C in a heated centrifugal evaporator (EYELA CVE-200D; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The residue was reconstituted in 50 μl of the mobile phase by vortex-mixing for 15 s, and 5 μl of this solution was injected onto the column.

2.6. Validation procedure

The validation parameters were selectivity, extraction recovery, precision, and accuracy. Blank plasma samples obtained from five rats were screened to determine specificity. The extraction recoveries of sibutramine, MDS, and DDS were calculated by comparing the peak area ratios measured for the standard solution, considering condensation, with those obtained for the plasma extracts after

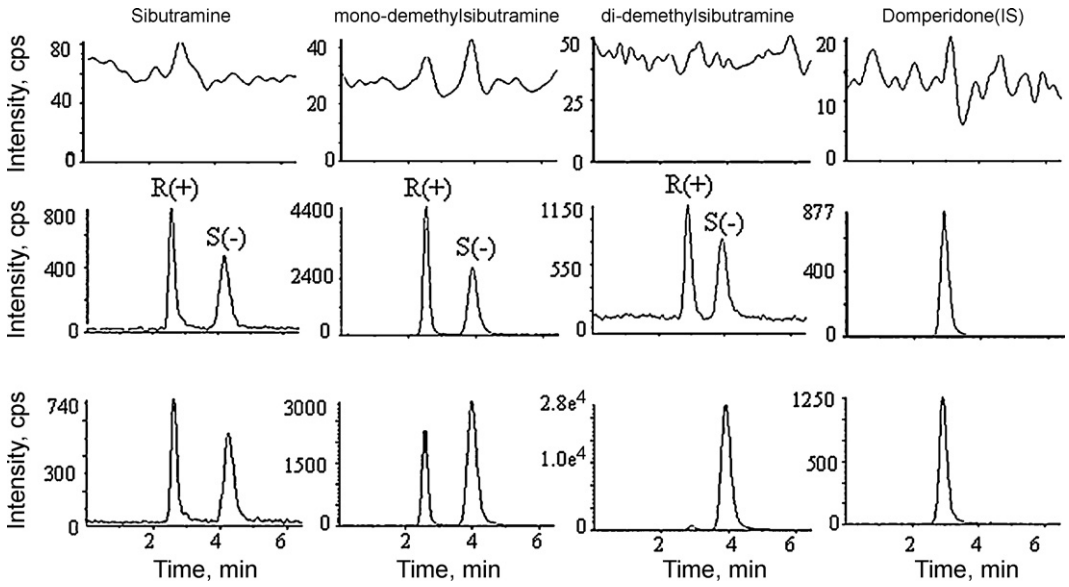

Fig. 1. Chromatograms of sibutramine, MDS, and DDS enantiomers and domperidone. Top, double-blank plasma; middle, plasma spiked with 5 ng/ml sibutramine, MDS, and DDS, and 100 ng/ml domperidone (IS); bottom, plasma sample of *S*(−)- and *R*(+)-isomers equivalent to 7.5 and 5.8 ng/ml for sibutramine, 9.5 and 3.8 ng/ml for MDS, and 308.0 and 6.9 ng/ml for DDS, respectively, in a sample obtained from a rat 30 min after oral administration of 10 mg/kg sibutramine.

the extraction procedure. The within day and between days assay precision and accuracy were estimated using a calibration curve to predict the concentration of the quality controls.

2.7. In vivo application

Five Sprague–Dawley rats were included in the study. Rats with health problems or abnormalities in standard laboratory screening tests were excluded. This study was approved by the Institutional Review Board of the College of Pharmacy (Kyoungsan, Korea). After an overnight fast, all rats were given a single oral dose of 10 mg/kg sibutramine. Blood (0.3 ml) was withdrawn before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, and 54 h after drug administration. Plasma was separated after centrifugation at 3000 × *g* for 10 min and was stored at −70 °C until analyzed.

3. Results and discussion

3.1. Chiral separation

The chromatographic resolution of the sibutramine, MDS, and DDS enantiomers was accomplished directly on the Chiral-AGP analytical column. The chiral stationary-phase column is based on bonding to α_1-acid glycoprotein (AGP). Through a patented process, α1-AGP has been immobilized on porous, spherical silica particles (5 μm) [6].

Three compounds were equally separated into their enantiomers representing the same intensity in peak area. In order to confirm the elution order both sibutramine isomers resolved with dibenzoyl-D-tartaric acid were individually chromatographied, and *R*(+)-isomer eluted prior to *S*(−)-isomer. The *R*(+)- and *S*(−)-isomers, respectively, were eluted at 2.6 (*k*′, capacity factor, 0.88) and 4.2 (*k*′, 2.04) min for sibutramine (*α*, selectivity, 2.31; *R*, resolution, 2.13), 2.5 (*k*′, 0.81) and 3.9 (*k*′, 1.83) min for MDS (*α*, 2.25; *R*, 2.15), and 2.9 (*k*′, 1.10) and 3.9 (*k*′, 1.83) min for DDS (*α*, 1.66; *R*, 1.54), with satisfactory resolution; the IS was eluted at 2.9 min (Fig. 1). Note that the capacity factor was calculated using the retention time (1.38 min) of acetonitrile which was separately injected onto the column, because the solvent peak was not seen as shown in Fig. 2.

3.2. Quantification of sibutramine, MDS, and DDS, and validation of the method

There were no interfering peaks at the elution times for either of the isomers of sibutramine, MDS, and DDS, or for the IS. Fig. 1 presents typical chromatograms for the blank plasma (top), plasma spiked with 5 ng/ml of each isomer of sibutramine, MDS, and DDS plus 100 ng/ml IS (middle), and a rat plasma sample (bottom). The calibration curves provided a reliable response for sibutramine (1–50 ng/ml, $r^2 = 0.996$), MDS (1–100 ng/ml, $r^2 = 0.998$), and DDS (10–4000 ng/ml, $r^2 = 0.998$). The ratio of the peak area of each isomer of sibutramine and its two metabolites relative to that of the IS was correlated with the corresponding plasma concentration, and good linearity was observed. The detection limit for both isomers of sibutramine, MDS, and DDS was 0.1 ng/ml at a signal-to-noise (S/N) ratio of 5. The estimates of the within day and between days precision and accuracy of the assay are presented in Tables 1 and 2, respectively. The relative standard deviations of the within day assay precision were less than 11.2 and 11.3% for *R*(+)- and *S*(−)-sibutramine, 11.4 and 8.8% for *R*(+)- and *S*(−)-MDS, and 9.5 and 11.4% for *R*(+)- and *S*(−)-DDS, respectively. The within day assay accuracy of the respective *R*(+)- and *S*(−)-isomers were 99.6–106.2 and 95.2–106.2% for sibutramine, 98.5–105.1 and 99.6–105.6% for MDS, and 95.2–112.7 and 98.5–110.8% for DDS. The relative standard deviations of the between days assay precision were less than 6.2 and 6.6% for *R*(+)- and *S*(−)-sibutramine, 8.3 and 7.9% for *R*(+)- and *S*(−)-MDS, and 4.9 and 5.4% for *R*(+)- and *S*(−)-DDS, respectively. The between days assay accuracy of the respective *R*(+)- and *S*(−)-isomers were 98.2–104.7 and 97.3–106.9% for sibutramine, 99.5–105.1 and 100.8–104.8% for MDS, and 97.6–110.9 and 99.4–110.5% for DDS. The mean recovery of both isomers for all sibutramine, MDS, and DDS concentrations exceeded 90% for both the within day and between days assays.

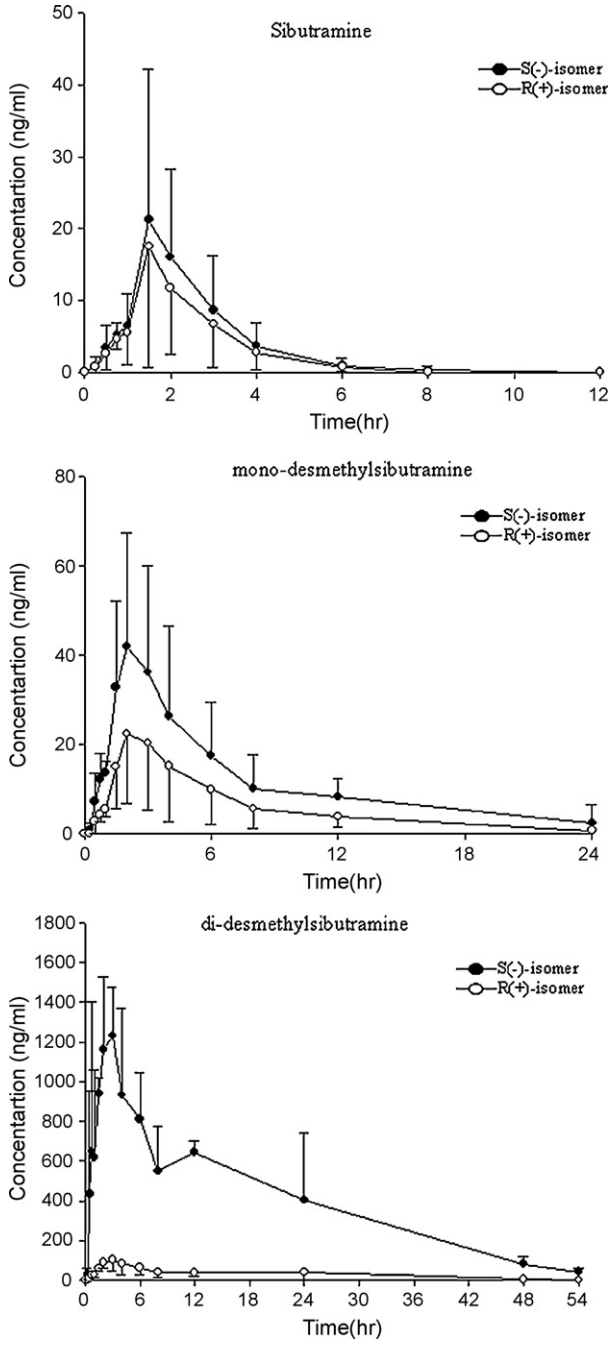

Fig. 2. Time course of the concentrations of sibutramine, MDS, and DDS enantiomers in plasma samples obtained from rats ($n = 3$) after a single oral dose of 10 mg/kg sibutramine. Each point represents the mean ± S.D.

3.3. Application of the method

The validated method (described above) was used to evaluate the enantioselective pharmacokinetics of sibutramine, MDS, and

Table 1
Precision and accuracy of the within day assay of sibutramine, mono-desmethylsibutramine (MDS) and di-desmethylsibutramine (DDS) enantiomers ($n = 3$).

Quality control	Sibutramine				Mono-desmethylsibutramine				Di-desmethylsibutramine			
	R(+)-isomer		*S*(−)-isomer		*R*(+)-isomer		*S*(−)-isomer		*R*(+)-isomer		*S*(−)-isomer	
	Accuracy[a]	R.S.D.[b]	Accuracy	R.S.D.	Accuracy	R.S.D.	Accuracy	R.S.D.	Accuracy	R.S.D.	Accuracy	R.S.D.
Lowest[c]	100.9 ± 6.3	6.2	105.3 ± 1.1	1.0	102.0 ± 5.5	5.4	101.4 ± 2.9	2.8	101.9 ± 1.8	1.8	104.1 ± 3.5	3.4
Middle$_1$[d]	106.2 ± 6.9	6.5	95.2 ± 5.3	5.6	105.1 ± 0.5	0.4	102.1 ± 5.5	5.4	95.2 ± 9.1	9.5	98.5 ± 11.3	11.4
Middle$_2$[e]	99.6 ± 10.6	10.6	98.1 ± 11.1	11.3	98.5 ± 8.1	8.2	99.6 ± 5.6	5.6	99.8 ± 8.2	8.2	101.5 ± 10.9	10.7
Highest[f]	101.7 ± 11.2	11.2	106.2 ± 11.8	11.1	103.4 ± 11.8	11.4	105.6 ± 9.3	8.8	112.7 ± 2.2	1.9	110.8 ± 3.8	3.4

[a] Mean ± S.D.
[b] R.S.D. (relative standard deviation, %) = S.D. × 100/mean.
[c] 0.5 ng/ml sibutramine and MDS, respectively; 10 ng/ml DDS.
[d] 2.5ng/ml sibutramine and MDS, respectively; 25 ng/ml DDS.
[e] 5 ng/ml sibutramine and MDS, respectively; 50 ng/ml DDS.
[f] 25 ng/ml sibutramine, 50 ng/ml MDS and 4000 ng/ml DDS.

Table 2
Precision and accuracy of the between days assay of sibutramine, mono-desmethylsibutramine (MDS) and di-desmethylsibutramine (DDS) enantiomers ($n = 3$).

Quality control	Sibutramine				Mono-desmethylsibutramine				Di-desmethylsibutramine			
	R(+)-isomer		*S*(−)-isomer		*R*(+)-isomer		*S*(−)-isomer		*R*(+)-isomer		*S*(−)-isomer	
	Accuracy[a]	R.S.D.[b]	Accuracy	R.S.D.	Accuracy	R.S.D.	Accuracy[a]	R.S.D.[b]	Accuracy	R.S.D.	Accuracy	R.S.D.
Lowest[c]	101.7 ± 6.3	6.2	100.4 ± 6.7	6.6	101.6 ± 2.0	2.0	100.8 ± 1.5	1.5	103.8 ± 1.9	1.8	104.0 ± 2.3	2.2
Middle$_1$[d]	104.7 ± 5.3	5.1	98.0 ± 3.4	3.5	105.1 ± 4.7	4.5	101.6 ± 0.4	0.4	97.6 ± 2.2	2.3	99.4 ± 1.0	1.0
Middle$_2$[e]	98.2 ± 5.7	5.8	97.3 ± 6.7	6.9	99.5 ± 8.3	8.3	101.3 ± 8.0	7.9	100.7 ± 4.9	4.9	101.0 ± 5.5	5.4
Highest[f]	103.8 ± 3.0	2.9	106.9 ± 4.4	4.1	102.9 ± 2.2	2.2	104.8 ± 2.1	2.0	110.9 ± 3.3	2.9	110.5 ± 0.5	0.5

[a] Mean ± S.D.
[b] R.S.D. (relative standard deviation, %) = S.D. × 100/mean.
[c] 0.5 ng/ml sibutramine and MDS, respectively; 10 ng/ml DDS.
[d] 2.5ng/ml sibutramine and MDS, respectively; 25 ng/ml DDS.
[e] 5 ng/ml sibutramine and MDS, respectively; 50 ng/ml DDS.
[f] 25 ng/ml sibutramine, 50 ng/ml MDS and 4000 ng/ml DDS.

DDS in rats. Fig. 2 shows the mean plasma concentrations of the *R*(+)- and *S*(−)-isomers of sibutramine, MDS, and DDS after a single oral dose of 10 mg/kg sibutramine in rats. To our knowledge, the present study reveals for the first time the enantioselective pharmacokinetics of sibutramine and its two active metabolites in rats. The higher concentrations of the *S*(−)-enantiomers of sibutramine, MDS, and DDS in rats observed in the present study may be attributable to prior stereoselective metabolism of the *R*(+)-isomers by microsomal enzymes. The competitive inhibition of the metabolism of the different enantiomers of these three compounds in humans is under investigation.

In conclusion, a stereoselective and sensitive method for simultaneously determining the pharmacokinetics of the enantiomers of sibutramine and its two active metabolites in rat plasma was developed by combining separation via a chiral stationary-phase column with MS/MS. This method is suitable for pharmacokinetic studies of sibutramine, MDS, and DDS after the oral administration of a sibutramine racemate in rats.

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