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Short communication

Enantioselective determination of sibutramine and its active metabolites in human plasma

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ABSTRACT

Although racemic sibutramine has been widely used for the treatment of obesity, its enantioselective detection method has not been elucidated in human plasma. In this report we introduce a validated analytical method for the determination of sibutramine and its two active metabolites, desmethylsibutramines using LC–MS/MS. *R*- and *S*-isomers of those compounds in human plasma were extracted using diethyl ether–hexane (4:1, v/v) followed by an addition of NaOH solution. After removing the organic layer, the residue was reconstituted in the mobile phase 10 mM ammonium acetate solution adjusted to pH 4.0 with acetic acid–acetonitrile (94:6, v/v). Both isomers in the extract were separated on a Chiral-cel AGP chiral stationary-phase column and were quantified in a tandem mass spectrometry. The assay method was in accordance with FDA regulations for the validation of bioanalytical methods. This method was successfully used to profile the plasma concentrations of the stereoisomers of sibutramine and its two active metabolites with time in healthy volunteers.

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1. Introduction

Racemic sibutramine is widely used for the treatment of obesity due to the inhibition of serotonin and noradrenaline reuptake in synapse. Its mode of action results in enhancing satiety and energy expenditure [1]. It has been well known that sibutramine is mainly metabolized *in vivo* to mono- and di-desmethyl active metabolites [2,3], and a chiral chromatography method was recently introduced to separately determine each stereoisomer in rat plasma, and the time courses of plasma concentrations of both isomers of those three compounds were clearly elucidated for the first time in rat [4]. Interestingly, the systemic exposure of the *S*-isomers of desmethylsibutramines was much greater than that of the *R*-isomers representing much more potent compared to the *S*-enantiomers [4,5]. On the basis of the previous experience, we tried to develop an analytical method to determine both isomers of sibutramine, monodesmethylsibutramine (MDS) and didesmethylsibutramine (DDS) in human plasma. The present method was successfully applied to characterize the time courses of plasma concentrations of the stereoisomers of sibutramine as well as its two active metabolites in human, following an oral administration of racemic sibutramine commercially available.

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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 all other chemicals and solvents were of the highest analytical grade available. *R*- and *S*-isomers were separated at a Medicinal Chemistry Laboratory in College of Pharmacy, Catholic University of Daegu [6].

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 50 μl of each solution was added to 850 μl of drug-free plasma, to obtain final concentrations at 0.1, 0.25, 0.5, 1, 2.5, and 5 ng/ml for sibutramine and MDS; and at 0.25, 0.5, 1, 2.5, 10, and 25 ng/ml for 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 850 μl of blank human plasma by adding 50 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.

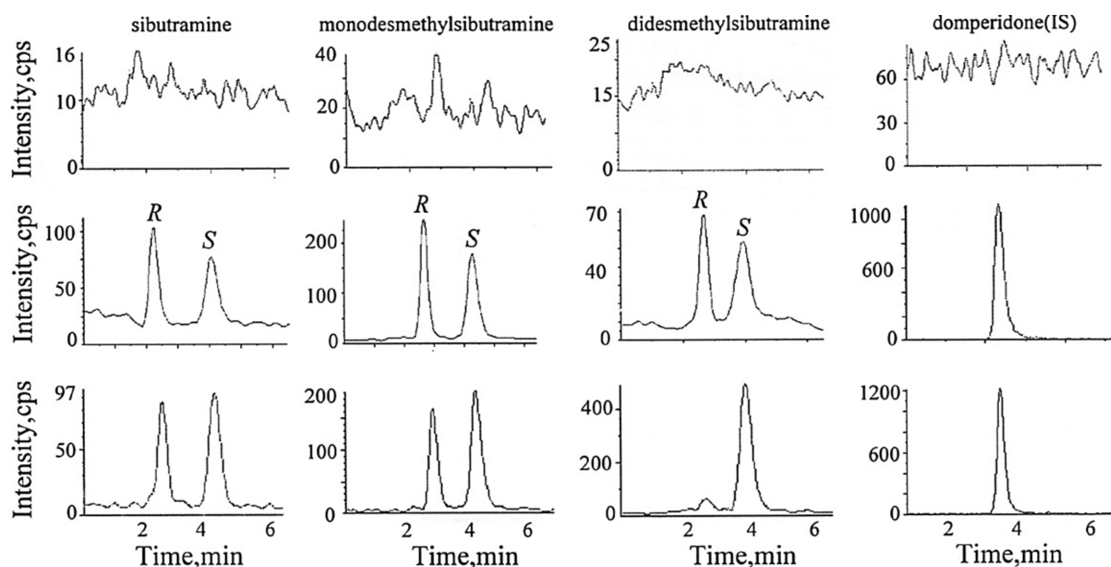

Fig. 1. Chromatograms of sibutramine, monodesmethylsibutramine, and didesmethylsibutramine enantiomers and domperidone. Top, double-blank plasma; middle, plasma spiked with 5 ng/ml sibutramine, monodesmethylsibutramine, and didesmethylsibutramine, and 100 ng/ml domperidone (IS); bottom, plasma sample of *R*- and *S*-isomers equivalent to 0.8 and 1.8 ng/ml for sibutramine, 0.6 and 3.2 ng/ml for monodesmethylsibutramine, and 0.0 and 4.8 ng/ml for didesmethylsibutramine, respectively, in a sample obtained from a volunteer 1 h after oral administration of 8.73 mg racemic sibutramine.

2.3. 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 (Chiralcel AGP, 100 mm × 2.0 mm inner diameter, 5-μm

Table 1
Precision and accuracy of the assay of sibutramine, MDS and DDS enantiomers ($n = 5$).

Quality control	Sibutramine				MDS				DDS			
	R-isomer		*S*-isomer		*R*-isomer		*S*-isomer		*R*-isomer		*S*-isomer	
	Accuracy[a]	RSD[b]	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD
Within day assay												
LOQ[c]	102.2 ± 7.6	7.3	104.6 ± 3.4	2.8	97.3 ± 11.4	11.7	101.0 ± 4.4	4.4	105.2 ± 8.6	8.1	101.1 ± 16.7	16.5
MOQ_1[d]	89.5 ± 4.5	5.0	93.6 ± 7.6	8.2	97.5 ± 6.4	6.6	98.0 ± 8.0	8.1	91.8 ± 4.6	5.3	95.1 ± 6.9	7.2
MOQ_2[e]	108.6 ± 5.1	4.7	106.5 ± 1.3	1.2	99.6 ± 2.2	2.2	101.3 ± 2.0	2.0	89.1 ± 4.8	5.4	87.9 ± 2.8	3.2
HOQ[f]	102.8 ± 7.0	6.8	102.4 ± 3.0	2.9	101.8 ± 1.1	1.1	104.1 ± 3.1	3.0	112.7 ± 1.5	1.4	110.9 ± 2.7	2.4
Between days assay												
LOQ[c]	101.9 ± 5.9	5.8	101.7 ± 3.3	3.3	102.5 ± 4.8	4.7	101.4 ± 5.5	5.5	105.6 ± 6.9	6.6	101.5 ± 2.6	2.5
MOQ_1[d]	92.8 ± 6.0	6.4	94.2 ± 3.7	3.9	94.3 ± 2.8	2.9	99.1 ± 2.8	2.8	90.6 ± 1.4	1.6	91.0 ± 4.0	4.4
MOQ_2[e]	104.0 ± 6.9	6.7	103.0 ± 3.0	2.9	95.5 ± 3.7	3.8	95.7 ± 5.1	5.3	90.2 ± 1.0	1.1	87.2 ± 0.9	1.0
HOQ[f]	101.4 ± 2.4	2.4	100.6 ± 3.4	3.4	104.1 ± 3.2	3.0	104.2 ± 1.1	1.1	111.1 ± 4.1	3.7	112.6 ± 1.5	1.3

[a] Mean % ± S.D.
[b] RSD (relative standard deviation, %) = S.D. × 100/mean.
[c] 0.1 ng/ml for sibutramine and MDS, 0.25 ng/ml for DDS.
[d] 0.5 ng/ml for sibutramine and MDS, 1.0 ng/ml for DDS.
[e] 1.0 ng/ml for sibutramine and MDS, 2.5 ng/ml for DDS.
[f] 5.0 ng/ml for sibutramine and MDS, 25.0 ng/ml for DDS.

Table 2
Stability of sibutramine, MDS and DDS enantiomers.

Stability condition	Sibutramine				MDS				DDS			
	R-isomer		*S*-isomer		*R*-isomer		*S*-isomer		*R*-isomer		*S*-isomer	
	0.5 ng/ml	5 ng/ml	0.5 ng/ml	5 ng/ml	0.5 ng/ml	5 ng/ml	0.5 ng/ml	5 ng/ml	0.5 ng/ml	5 ng/ml	0.5 ng/ml	5 ng/ml
Room temp. (12 h)	87.1 ± 2.5[a]	97.5 ± 7.0	88.3 ± 3.3	93.7 ± 5.2	97.0 ± 3.3	99.8 ± 6.6	99.5 ± 4.8	99.2 ± 5.2	88.9 ± 3.2	89.3 ± 3.1	90.5 ± 2.0	89.6 ± 2.4
Post-extraction (12 h)	90.5 ± 3.9	96.6 ± 6.4	89.3 ± 2.5	97.3 ± 2.5	85.7 ± 1.5	93.1 ± 3.7	88.2 ± 1.2	91.4 ± 4.9	89.3 ± 4.2	91.1 ± 4.0	89.6 ± 4.2	90.4±3.2
Freeze–thaw	90.7 ± 3.1	104.3 ± 1.6	88.3 ± 2.5	98.2 ± 2.3	90.4 ± 3.2	97.5 ± 0.7	85.4 ± 0.2	100.2 ± 2.0	106.0 ± 3.5	99.0 ± 3.3	89.2 ± 2.1	96.3 ± 5.1
Long-term (4-week)	90.6 ± 2.1	88.6 ± 3.6	87.2 ± 1.5	94.8 ± 3.1	92.6 ± 1.5	92.0 ± 3.5	91.2 ± 3.4	90.8 ± 2.0	88.0 ± 2.5	85.9 ± 0.8	94.7 ± 2.7	90.8 ± 1.6

[a] Mean % ± S.D. ($n = 3$).

Table 3
Pharmacokinetic parameters of sibutramine, MDS and DDS enantiomers after a single oral administration of 8.37 mg *rac*-sibutramine. Data are mean ± S.D. (n = 5).

Parameter	Sibutramine		MDS		DDS	
	R-isomer	*S*-isomer	*R*-isomer	*S*-isomer	*R*-isomer	*S*-isomer
C_{max} (ng/ml)	0.7 ± 0.3	1.6 ± 1.1	0.9 ± 0.2	1.6 ± 1.0	0.8 ± 0.2	11.9 ± 2.1
T_{max} (h)	1.0 ± 0.0	1.0 ± 0.0	4.2 ± 2.2	2.6 ± 1.5	5.8 ± 1.5	3.6 ± 1.8
AUC_t (ng h/ml)	2.0 ± 0.5	4.4 ± 2.2	7.7 ± 3.3	10.4 ± 2.5	8.5 ± 2.4	163.3 ± 48.8
$T_{1/2}$ (h)	1.5 ± 0.2	1.9 ± 0.8	6.2 ± 2.8	6.1 ± 3.5	5.9 ± 1.4	10.4 ± 3.4

particle size; ChromTech Ltd., Congleton, Cheshire, UK). The column temperature was 22 °C, and the mobile phase was eluted at 0.2 ml/min using an HP 1100 series pump (Agilent, Wilmington, DE, USA). 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 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.

2.4. Sample preparation

One-hundred microliters of the IS (100 ng/ml in mobile phase) and 0.1 ml of 10 M NaOH aq. solution were added to 1 ml of human plasma, followed by liquid–liquid extraction with 5 ml of diethylether:n-hexane (4:1 v/v) for 10 min. 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 100 μl of the mobile phase by vortex-mixing for 15 s, and 5 μl of this solution was injected onto the column.

2.5. Validation procedure

The validation parameters were selectivity, extraction recovery, precision, and accuracy. Blank plasma samples obtained from five volunteers 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 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.

2.6. Stability

Drug-free control plasma samples were spiked with 1 and 10 ng/ml sibutramine, MDS and DDS to study their stability in human plasma. Short- and long-term stabilities were assessed after 12 h and 4 weeks of storage at room temperature, and −80 °C, respectively. The stability of three compounds in plasma samples was also tested after three freeze–thaw cycles (−80 °C to room temperature). The stability in extracts was examined after 12 h of storage at 4 °C.

2.7. Clinical application

Five male healthy volunteers aged between 21 and 25 years, who gave written informed consent, participated in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were the exclusion criteria. This study was approved by the Institutional Review Board of Catholic University of Daegu, Korea. After an overnight fast, all volunteers were given a single oral dose of 8.37 mg *rac*-sibutramine commercially available.

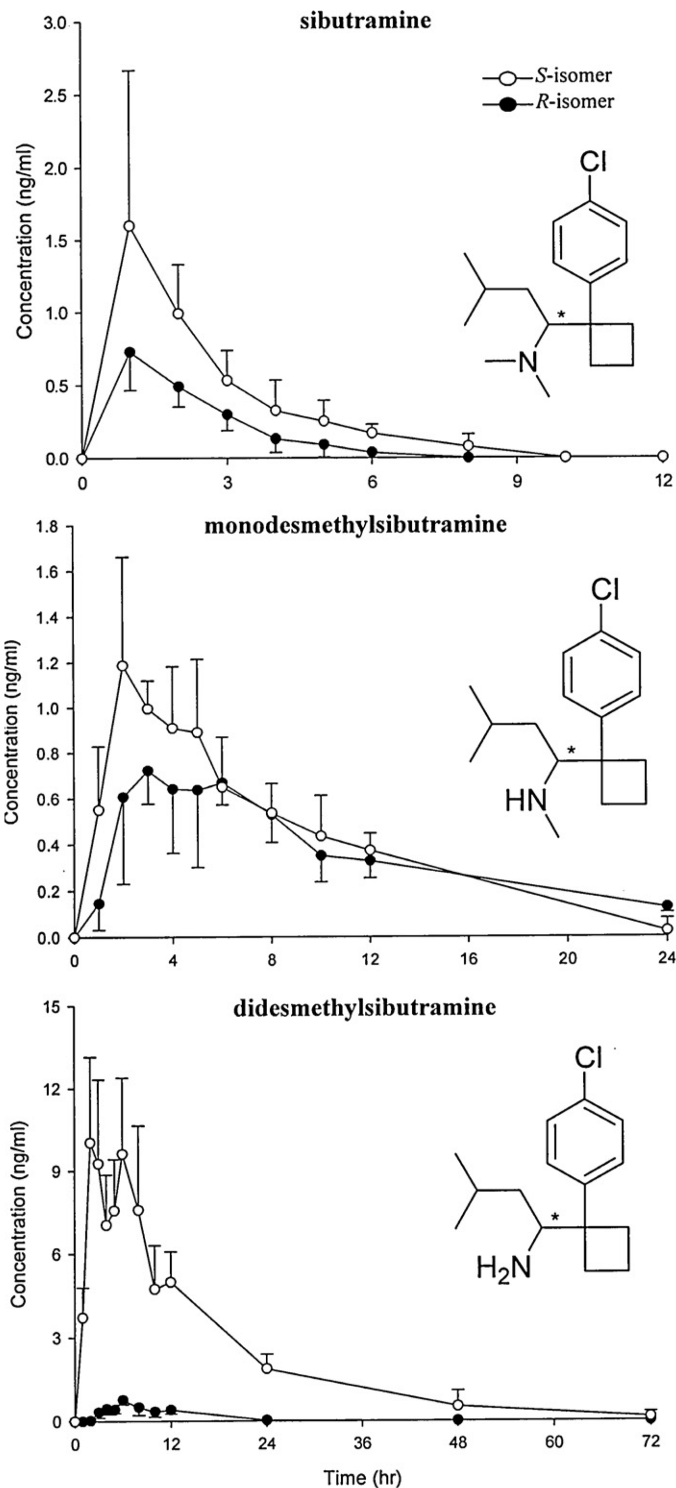

Fig. 2. Time courses of the plasma concentrations of sibutramine, monodesmethylsibutramine, and didesmethylsibutramine stereoisomers in healthy volunteers after a single oral dose of 8.37 mg *rac*-sibutramine (n = 5). Each point represents the mean ± S.D.

Heparinized blood (5 ml) was taken up to 72 h after drug administration. Plasma was separated and 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 as previously used [4]. 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 real human plasma sample (bottom). The stereoisomers of three compounds were clearly separated without interference from endogenous compounds from the matrix; *R*- and *S*-isomers were eluted at 2.4 and 4.1 min for sibutramine, 2.8 and 4.1 min for MDS, and 2.8 and 3.9 min for DDS, with satisfactory resolution, respectively; the IS was eluted at 3.7 min.

3.2. Validation of the method and stability

The calibration curves provided a reliable response for sibutramine (0.1–5 ng/ml, r^2 = 0.999), MDS (0.1–5 ng/ml, r^2 = 0.999), and DDS (0.25–25 ng/ml, r^2 = 0.999). 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 quantification limit for both isomers of sibutramine, MDS, and DDS was 0.1, 0.1, 0.25 ng/ml, respectively, 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 Table 1. The relative standard deviations of the within day assay precision were less than 8.2 and 7.3% for *S*- and *R*-sibutramine, 8.1 and 11.7% for *S*-and *R*-MDS, and 16.5 and 8.1% for *S*- and *R*-DDS, respectively. The within day assay accuracy of the respective *S*- and *R*-isomers were 93.6–106.5 and 89.5–108.6% for sibutramine, 98.0–104.1 and 97.3–101.8% for MDS, and 87.9–110.9 and 89.1–112.7% for DDS. The relative standard deviations of the between days assay precision were less than 3.9 and 6.7% for S- and *R*-sibutramine, 5.5 and 4.7% for *S*- and *R*-MDS, and 4.4 and 6.6% for *S*- and *R*-DDS, respectively. The between days assay accuracy of the respective *S*- and *R*-isomers were 94.2–103.0 and 92.8–104.0% for sibutramine, 95.7–104.2 and 94.3–104.1% for MDS, and 87.2–112.6 and 90.2–111.1% for DDS. The mean recovery of both isomers for all sibutramine, MDS, and DDS concentrations ranged 90–95% for both the within day and between days assays.

To evaluate stability of enantiomers of sibutramine, MDS and DDS in human plasma, drug-free plasma samples were spiked at 1 and 10 ng/ml. Samples were found to be stable in plasma for up to 12 h at room temperature, 4 °C in the autosampler and remained stable for up to 4 weeks at −80 °C, with no observable degradation even after three freeze–thaw cycles (Table 2).

3.3. Clinical application

Fig. 2 shows the mean plasma concentrations of the *R*- and *S*-isomers of sibutramine, MDS, and DDS after a single oral dose of 8.37 mg *rac*-sibutramine in human volunteers and the pharmacokinetic parameters are listed in Table 3. To our knowledge, the present study reveals for the first time the enantioselective pharmacokinetics of sibutramine and its two active metabolites in human. The higher concentrations of the *S*-isomers of sibutramine, MDS, and DDS in human may be attributable to further metabolism [7] and/or prior stereoselective metabolism of the *R*-isomers by Phase I and II microsomal enzymes.

In conclusion, the stereoisomers of sibutramine, MDS and DDS are determined in human plasma using LC–MS/MS with chiral stationary-phase column. The present validated method was successfully applied to characterize the enantioselective pharmacokinetics of sibutramine and its two active metabolites after a single oral administration of *rac*-sibutramine in healthy volunteers.

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