Rapid and Sensitive Method for the Determination of Sibutramine Active Metabolites in Human Plasma by Reversed-Phase Liquid Chromatography–Tandem Mass Spectroscopy

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Abstract

A new, rapid, and sensitive liquid chromatography–tandem mass spectrometry method is developed and validated to quantitate the sibutramine active metabolites mono desmethyl sibutramine (M_1) and di-desmethyl sibutramine (M_2) using imipramine as the internal standard in human plasma samples for routine bioequivalence studies. The method involves rapid solid-phase extraction from plasma, eliminating the drying and reconstitution steps. The analytes are chromatographed on a C_8 reversed-phase chromatographic column and analyzed by mass spectrometry in the multiple reaction monitoring mode, which enables a quantitation limit at the sub-nanogram level. The method has a chromatographic run time of 2.8 min. The proposed method is validated with a linear range of 0.1–8.0 and 0.2–16.0 ng/mL for M_1 and M_2, respectively, with a correlation coefficient of regression ≥ 0.9990. The method is sensitive and reproducible, having intra- and inter-assay precision at the lower limit of quantitation (0.1 ng/mL for M_1 and 0.2 ng/mL for M_2) < 10.0%. The overall recovery for M_1 and M_2 is 93.5% and 77.9%, respectively. The method has been applied to a bioequivalence clinical study with great success.

Introduction

Sibutramine is an orally administered agent for the treatment of obesity, and the chemically active ingredient is a racemic mixure of the (+) and (–) enantiomers of cyclobutanemethanamine,1-(4-chlorophenyl)-*N*,*N*-dimethyl-α-(2-methylpropyl)-hydrochloride monohydrate. Sibutramine is rapidly absorbed from the gastro intestinal tract following oral administration and undergoes extensive first-pass metabolism in the liver to form the pharmacologically active metabolites mono-desmethyl sibutramine (M_1) and di-desmethyl sibutramine (M_2) (1,2). Sibutramine pharmacokinetics in healthy young and elderly subjects are reported with low C_{max} values of M_1 and M_2 (3,4). Only a few methods have been reported for the determination of the active sibutramine metabolites M_1 and M_2. A liquid chromatography (LC)–mass spectrometric (MS) method has been reported with pseudo ion monitoring and a limit of quantitation (LOQ) of 0.5 ng/mL (3). Recently, an improved LC–MS–MS method was reported to enable the quantitation up to 0.328 ng/mL (5). The reported methods require laborious liquid–liquid extraction, high plasma volume, and long sample analysis time. The methods also have low precision values. Therefore, it was necessary to develop a sensitive, simple, specific, rapid, and reproducible quantitation method for the estimation of M_1 and M_2 in human plasma, which can be applied to the routine bioequivalence study.

This paper describes the development and validation of an LC–MS–MS method having an LOQ of 0.1 and 0.2 ng/mL for M_1 and M_2, respectively, with high precision values. The method involves solid-phase extraction (SPE) with reduced sample preparation and analysis time relative to other commonly

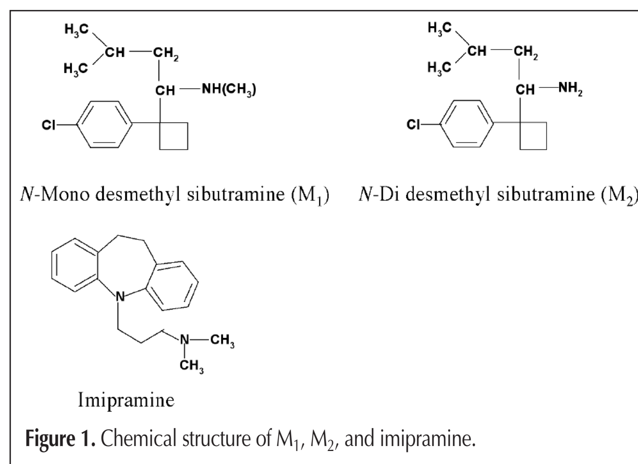

Figure 1. Chemical structure of M_1, M_2, and imipramine.

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employed techniques. The structures of the analytes are shown in Figure 1.

Experimental

Materials and reagents

M_1 and M_2 were synthesized in the Torrent Research Centre (Ahmedabad, India). The internal standard, imipramine hydrochloride, was procured from Torrent Pharmaceuticals. High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and water were obtained from Ranbaxy (Mumbai, India). Suprapure formic acid and ortho-phosphoric acid were purchased from Merck (Darmstadt, Germany). The SPE cartridges (Oasis HLB, 1 cc/30 mg) were procured from Waters (Banglore, India). Drug-free human plasma samples were purchased from Green-cross hospital (Ahmedabad, India) and stored at –20°C prior to use.

Equipment

LC–MS–MS analysis was performed using a Surveyor HPLC system coupled with a TSQ Quantum (Thermo Finnigan triple stage quadrupole system, Manchester, UK) equipped with an electrospray ionization (ESI) unit (Thermo Finnigan). The HPLC column used was a Betabasic C8 (5-µ particle size, 4.6 × 100 mm) purchased from Thermo Electron.

Standard and quality control preparation

The individual stock solution of M_1, M_2, and imipramine were prepared in methanol at a concentration of 1.0 mg/mL each. A working solution of 10.0 µg/mL of M_1, M_2, and 40.0 ng/mL imipramine was prepared by appropriately diluting the respective stock solution. An M_1 and M_2 working solution was used to prepare the spiking stock solutions for preparation of nine-point calibration curves (0.1–8.0 ng/mL for M_1 and 0.2–16.0 ng/mL for M_2), and quality control samples were prepared at three concentration levels (0.3, 2.4, and 6.0 ng/mL for M_1 and 0.6, 4.8, and 12.0 ng/mL for M_2). All stock solutions were kept refrigerated (2–8°C) when not in use.

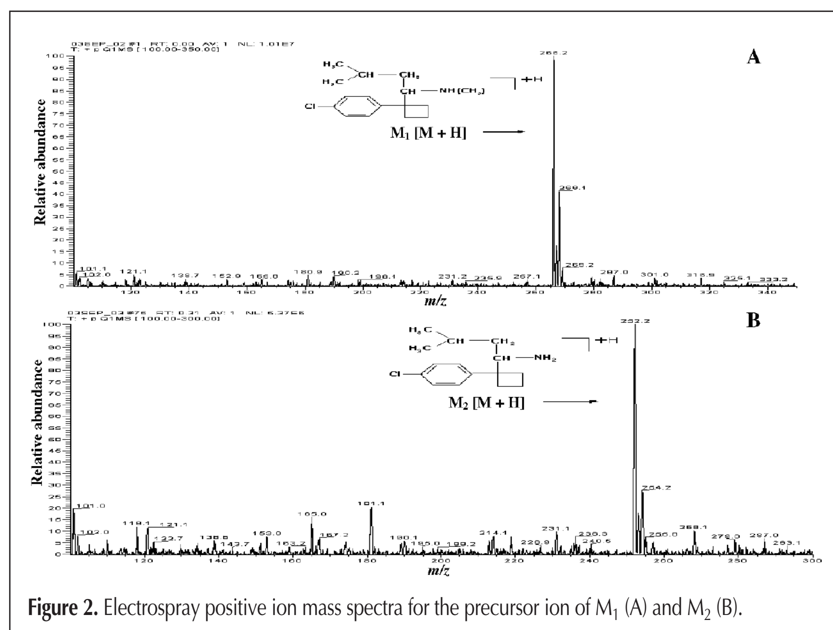

Figure 2. Electrospray positive ion mass spectra for the precursor ion of M_1 (A) and M_2 (B).

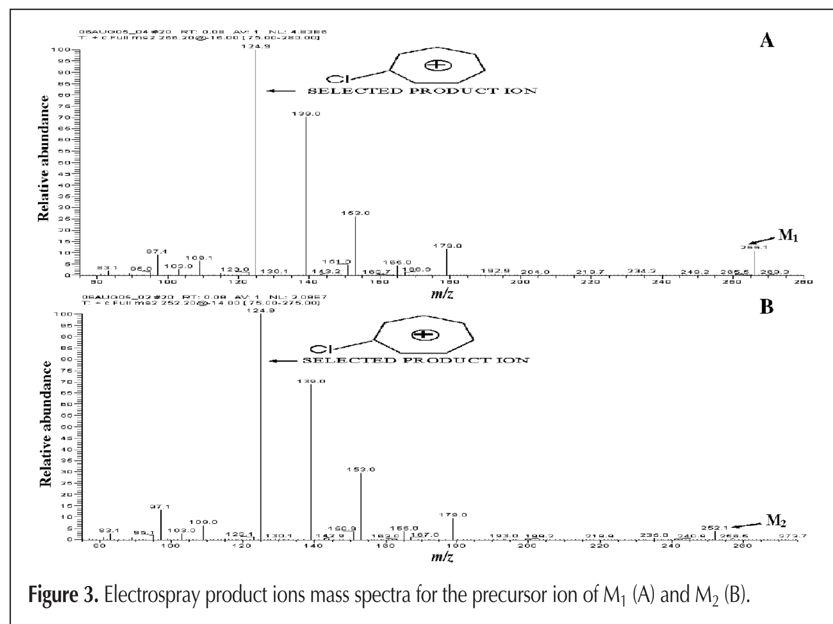

Figure 3. Electrospray product ions mass spectra for the precursor ion of M_1 (A) and M_2 (B).

Sample preparation

A 0.5-mL aliquot of plasma containing M_1 and M_2 was pipetted into an eppendorf microtube. A 25-µL addition of internal standard working solution (40.0 ng/mL imipramine) was made and vortex mixed. The sample was acidified by mixing 500 µL of 10% ortho-phosphoric acid. The sample mixture was loaded into an Oasis HLB extraction cartridge that was preconditioned with 1.0 mL methanol followed by 2.0 mL water. The extraction cartridge was washed with 1.0 mL of water followed by 1.0 mL of 30% methanol in water. The analytes were eluted from the cartridge with 0.8 mL acetonitrile and transferred to polypropylene autosampler vials. The extract (10 µL) was directly injected into the LC–MS–MS system.

Chromatographic and MS conditions

The analytes were chromatographically separated using reversed-phase chromatography with isocratic elution. The mobile phase consisted of acetonitrile–formic acid (0.1%) (80:20, v/v) at a flow rate of 0.5 mL/min. For all analysis, 10.0 µL of sample was injected. The total run time was 2.8 min. The MS was operated in the ESI mode with positive ion detection. The ion transfer capillary tube temperature was maintained at 350°C, and a voltage of 3500 V was applied to the sprayer needle. Nitrogen was used as the ion spray gas, and the collision energy for the M_1 and M_2 was 38 eV and for imipramine was 22 eV. The analytes were monitored by selected reaction monitoring

(SRM) of the collision-induced dissociation (CID) of the precursor ion to its corresponding product ion. The mass transition ion-pair was selected as 266.20 → 125.00, 252.20 → 125.00, and 281.20 → 86.10 for M_1, M_2, and imipramine, respectively.

Data processing and regression

The SRM chromatographic peaks were integrated using Xcalibur version 1.3, after which peak area ratios of M_1 and M_2 to imipramine versus the concentration were plotted, and a linear curve fit, weighted by 1/x (where x = concentration), was used to produce the regression line.

Validation

The method has been validated using selectivity, sensitivity, recovery, linearity, precision, and accuracy, stability, and dilution integrity parameters. Selectivity was performed by analyzing the blank plasma samples from different sources to test for interference at the retention time of M_1, M_2, and imipramine. Sensitivity was determined by analyzing five replicates of blank human plasma and plasma spiked with the analyte at the lowest level of the calibration curve. The accuracy and precision of the method was determined at lower limit of quantitation (LLOQ), low quality control (LQC), medium quality control (MQC), and high quality control (HQC) levels and calculated against the calibration curve. The intra- and inter-run precision and accuracy were determined by pooling all individual assay results of replicate (n = 5) quality control over the batch runs. Accuracy was defined as the percent relative error (%RE) and was calculated using the formula % RE = (E – T)(100 / T), where E is the experimentally determined concentration, and T is the theoretical concentration. Assay precision was calculated by using the formula % RSD = (SD/M) × (100), where M is the mean of the experimentally determined concentrations, and SD is the standard deviation of M.

The recovery of M_1, M_2, and imipramine was evaluated by comparing the peak area response of extracted analytes at three concentrations (low, medium, and high quality control samples) with unextracted standards that represent 100% recovery. Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. Five replicates, each at a concentration two and four times the upper most concentration, were prepared and diluted to 2- and 4-fold with blank plasma and processed.

As a part of method validation, stability was evaluated. The stock solution stability was evaluated at room temperature and 2–8°C by comparing with freshly prepared stock solution. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with samples re-injected after storing in the autosampler at 5°C for 24 h. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 6 h and compared with freshly prepared extracted samples. The freeze-thaw stability was conducted by comparing the stability of samples that had been frozen and thawed three times with freshly prepared calibration standards and quality control samples. The long-term stability was conducted by analyzing low, medium, and high quality control samples stored at –70°C for 52 days with freshly prepared calibration standards and quality control samples. All stability evaluations were based on back-calculated concentrations.

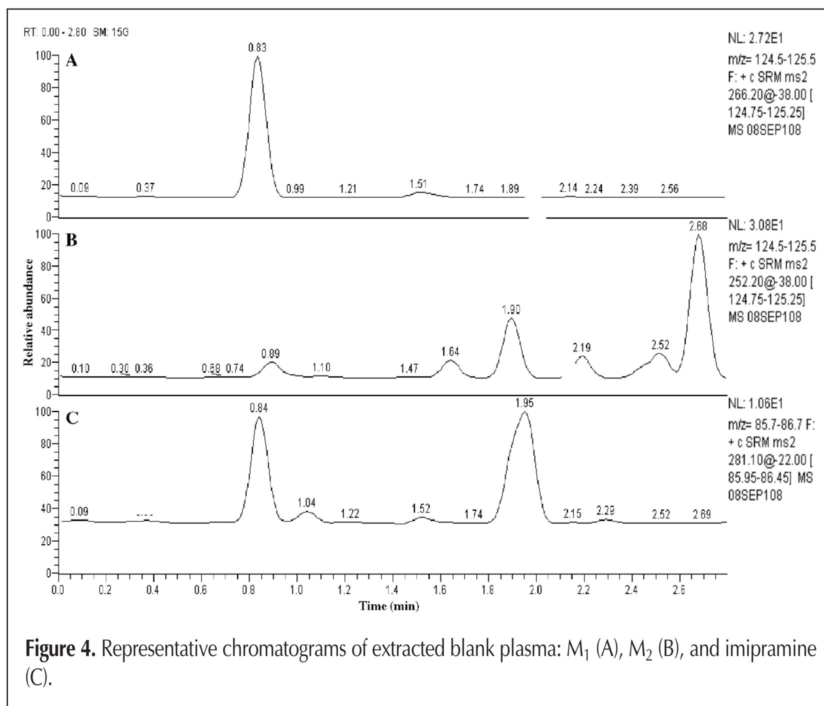

Figure 4. Representative chromatograms of extracted blank plasma: M_1 (A), M_2 (B), and imipramine (C).

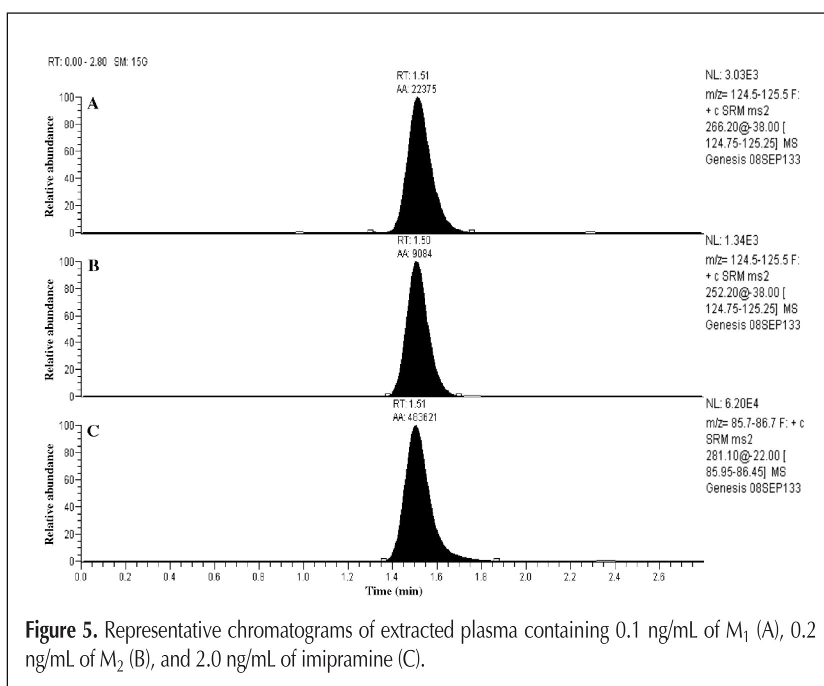

Figure 5. Representative chromatograms of extracted plasma containing 0.1 ng/mL of M_1 (A), 0.2 ng/mL of M_2 (B), and 2.0 ng/mL of imipramine (C).

Results and Discussion

Sample preparation and LC–MS–MS conditions

In this assay, the plasma sample was treated with 10% ortho-phosphoric acid and loaded onto the HLB SPE cartridge. The hydrophilic and lipophilic balance of the HLB SPE cartridge enables the interaction with M_1 and M_2, and the strong binding of the analytes to the copolymer of the SPE cartridge enabled sufficient clean up. However, the analytes were easily eluted by 0.8 mL acetonitrile and injected directly into the system. The extraction procedure does not involve the evaporation and reconstitution steps. The analytes ionized by the ESI method were analyzed with positive-ion detection because of the efficiency of ionization of analytes. In general, positive-ion detection is selective and highly sensitive to compounds, which readily accept the proton. It was considered that M_1 and M_2 analytes accepted the proton in an acidic mobile phase and produced a protonated precursor ion ($[M+H]^+$) at *m/z* 266.2 and 252.2, respectively, (Figure 2). The strongest fragment of each compound, as indicated in Figure 3, was selected and used as the product ion to be monitored for better sensitivity. A significant difference in response was observed with a change in the column. The Beta-basic C8 column gave good peak symmetry with less column bleeding.

Table I. Summary of Calibration Curve Parameters for M_1 and M_2

Calibration curve	Slope	Intercept	Correlation coefficient
M_1			
1	0.4406	–0.0011	0.9995
2	0.4343	0.0099	0.9999
3	0.4496	0.0029	0.9998
4	0.4611	0.0048	0.9998
5	0.4616	0.0062	0.9999
M_2			
1	0.1026	–0.0026	0.9990
2	0.1116	0.0008	0.9998
3	0.1088	–0.0008	0.9996
4	0.1170	–0.0023	0.9997
5	0.1182	–0.0014	0.9996

Table II. Interrun Precision and Accuracy of Plasma Calibration Standard (n = 5) for M_1 and M_2

Standard (ng/mL)	Mean (ng/mL)	%RSD	%RE
M_1			
0.100	0.103	2.5	2.6
0.200	0.198	3.9	–1.2
0.400	0.399	3.1	–0.2
0.800	0.798	4.7	–0.3
1.600	1.584	1.9	–1.0
3.200	3.198	2.1	–0.8
4.800	4.746	0.6	–1.1
6.400	6.481	1.1	1.3
8.000	7.992	0.9	–0.1
M_2			
0.200	0.210	5.7	4.8
0.400	0.400	3.4	–0.0
0.800	0.779	4.1	–2.6
1.600	1.584	3.0	–1.0
3.200	3.165	2.0	–1.1
6.400	6.445	2.1	0.7
9.600	9.310	3.0	–3.0
12.800	13.047	1.7	1.9
16.000	16.061	1.5	0.4

Selectivity

A representative chromatogram obtained from blank human plasma and plasma spiked with M_1, M_2, and imipramine are shown in Figures 4 and 5. There was no interference peak of endogenous compounds in the chromatograms obtained from the blank human plasma of six different lots of lipemic and heamolyzed plasma at the retention time of the analytes. Therefore, it was expected that the assay for clinical samples would be prevented by the interference peak in this method. Under the chromatographic conditions described, the retention time of M_1, M_2, and the internal standard was 1.51 min.

Table III. Intrarun Precision and Accuracy (n = 5) of M_1 and M_2 in Human Plasma

Analyte	Spiked conc. (ng/mL)	Mean calculated conc. (ng/mL)	%RSD	%RE
M_1	0.100	0.096	4.5	–4.0
	0.300	0.309	5.0	3.0
	2.400	2.500	1.1	4.2
	6.000	6.081	2.3	1.4
M_2	0.200	0.199	8.4	–0.5
	0.600	0.626	6.1	4.3
	4.800	5.268	3.7	9.8
	12.000	12.113	2.0	0.9

Table IV. Interrun Precision and Accuracy (n = 3) of M_1 and M_2 in Human Plasma

Analyte	Spiked conc. (ng/mL)	Mean calculated conc. (ng/mL)	%RSD	%RE
M_1	0.100	0.106	7.8	6.0
	0.300	0.313	4.1	4.3
	2.400	2.481	2.2	3.4
	6.000	6.043	3.6	0.7
M_2	0.200	0.204	10.0	2.0
	0.600	0.617	6.6	2.8
	4.800	5.267	2.8	9.7
	12.000	12.314	5.2	2.6

Linearity

Representative calibration curve data of M_1 and M_2 is listed in Tables I and II. The calibration curves of M_1 and M_2 were linear over the range of 0.1 to 8.0 ng/mL and 0.2 to 16.0 ng/mL, respectively. The mean correlation coefficients of M_1 and M_2 (n = 5) were ≥ 0.9995 and ≥ 0.9990.

Sensitivity (LLOQ)

The sensitivity experiment was carried out at the LLOQ level. The mean intrarun accuracy with deviation from the nominal concentration was 4.0% for M_1 and 0.5% for M_2, and the intrarun precision was 4.5% for M_1 and 8.4% for M_2. These data are tabulated in Table III for M_1 and M_2.

Precision and accuracy

Interrun precision and accuracy for M_1 and M_2 plasma calibration standards are summarized in Table II. The interrun precision for calibration standards was ≤ 4.7% and ≤ 5.7% for M_1 and M_2, respectively. The interrun accuracy for calibration standards was ≤ 2.6% and ≤ 4.8% for M_1 and M_2, respectively. The intra- and interrun precision and accuracy for quality control samples are enumerated in Tables III and IV, respectively. The intrarun precision was ≤ 5.0% for M_1 and ≤ 8.4% for M_2. The intrarun accuracy was ≤ 4.2% for M_1 and ≤ 9.8% for M_2. The interrun precision was ≤ 7.8% for M_1 and ≤ 10.0% for M_2. The interrun accuracy was ≤ 6.0% for M_1 and ≤ 9.7% for M_2.

Recovery

Peak areas from unextracted analyte with those of extracted analyte determined the recovery. The mean absolute recovery of M_1 and M_2 at LQC, MQC, and HQC were 93.54% and 77.94%, respectively. The recovery of imipramine was found to be 89.51%.

Stability

An analysis of stock solution stability was performed at a concentration of 1.0 mg/mL for M_1 and M_2. After storage for 26 days at 2–8°C and at room temperature for 6 h, more than 98.0% of M_1 and M_2 remained unchanged, based on their peak areas in comparison with freshly prepared solutions of M_1 and M_2. This suggests that the M_1 and M_2 in standard solutions were stable for at least 26 days when stored at 2–8°C and 6 h at room temperature.

Bench top stability and process stability of M_1 and M_2 were investigated at LQC and HQC levels. This revealed that the M_1 and M_2 in plasma were stable for at least 6 h at room temperature and 24 h in the autosampler, with a mean % change of ≤ 3.3% and ≤ 4.0% for M_1 and ≤ 1.9% and ≤ 2.5% for M_2, respectively. It was confirmed that repeated freeze-and-thaw cycles (n = 3) of plasma samples spiked with M_1 and M_2 at LQC and HQC levels did not affect the stability of M_1 and M_2, with a mean % change of ≤ 6.8% for M_1 and ≤ 6.1% for M_2. Long-term stability of the M_1 and M_2 in plasma at –70°C was also performed for 52 days at LQC, MQC, and HQC levels with a mean % change of ≤ 3.1% for M_1 and ≤ 4.5% for M_2. The results of the stability studies are enumerated in Table V.

Dilution integrity

The upper concentration limits of M_1 and M_2 can be extended to 16.0 ng/mL for M_1 and 32.0 ng/mL for M_2, with an acceptable precision and accuracy of 15% by a 2- or 4-fold dilution with blank human plasma. The summarized results demonstrate a precision of ≤ 2.7% for M_1 and ≤ 1.9% for M_2, and the mean percentage deviation from the nominal concentration was ≤ 5.7% for M_1 and ≤ 6.0% for M_2.

Application of method

The proposed method was applied for the determination of M_1 and M_2 in plasma samples from an ongoing project for the development conventional formulation. Plasma samples were periodically collected up to 72 h after oral administration of a 15.0 mg tablet to 40 healthy male volunteers. The mean maximum plasma concentration obtained for M_1 in test and reference formulation was 3.3 ± 1.5 and 3.2 ± 1.7 ng/mL, respectively, and for M_2 in test and reference formulations was 10.5 ± 3.1 and 10.4 ± 3.3 g/mL, respectively. The area under the plasma concentration-time curve (AUC) measured from 0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from zero time to infinity, which

Table V. Sample Stability Results for M_1 and M_2

Stability	Analyte	Spiked conc. (ng/mL)	Mean calculated comparison sample conc. (ng/mL)	Mean calculated stability sample conc. (ng/mL)	%RSD	Mean % change
Process*	M_1	0.300	0.315	0.309	2.6	–1.9
		6.000	5.879	5.850	2.0	–0.5
	M_2	0.600	0.626	0.642	4.4	2.5
		12.000	12.113	11.927	8.4	–1.5
Bench top†	M_1	0.300	0.315	0.313	4.0	–0.9
		6.000	6.169	6.373	3.0	3.3
	M_2	0.600	0.627	0.651	3.3	3.8
		12.000	12.710	13.215	1.0	4.0
Freeze and thaw‡	M_1	0.300	0.315	0.294	1.8	–6.8
		6.000	5.879	5.636	1.1	–4.1
	M_2	0.600	0.626	0.588	2.9	–6.1
		12.000	12.113	11.471	3.1	–5.3
Long-term§	M_1	0.300	0.294	0.303	1.2	3.1
		2.400	2.333	2.375	1.8	1.8
		6.000	5.871	5.827	1.3	–0.8
	M_2	0.600	0.575	0.601	5.7	4.5
		4.800	5.075	5.276	2.5	4.0
		12.000	12.506	12.287	7.5	–1.8

* After 24 h in autosampler at 5°C.
† After 6 h at room temperature.
‡ After three freeze and thaw cycles at –70°C.
§ At –70°C for 52 days.

indicated a suitability of the analytical method for pharmacokinetic studies.

Conclusion

A rapid and sensitive LC–MS–MS method for the determination of M_1 and M_2 in human plasma has been developed and validated, with a lower quantitation limit of 0.1 and 0.2 ng/mL, respectively. The precision at an LOQ of < 10% has been obtained; therefore, the LOQ level of the proposed method can be extended to still lower concentrations. The run time of only 2.8 min was adequate to achieve the required chromatographic separation of M_1 and M_2 from other components in plasma. Validation experiments have shown that the assay method has good precision, accuracy, specificity, and recovery for M_1 and M_2. The method is simple and reproducible in terms of chromatographic conditions, mass detection, and sample preparation.

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