Determination of Sibutramine and Its N-Desmethyl Metabolites in Human Plasma Using HPLC Coupled with Tandem Mass Spectroscopy

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Abstract

A sensitive and selective liquid chromatographic method coupled with tandem mass spectrometry has been developed and validated for the determination of Sibutramine and its N-desmethyl metabolites in human plasma. The analytes Sibutramine, N-monodesmethylsibutramine, N-N-didesmethylsibutramine and Bisoprolol (internal standard) have been separated on a reversed phase column (Merck, Purospher RP-C18, 30 × 4.0 (mm), 3 μm) using a mobile phase which consists of an aqueous solution 20 mM ammonium acetate pH 4.0 (adjustment by acetic acid) in water and acetonitrile (67:33 v/v (%)), flow rate 0.40 (mL/min). Detection has utilized a tandem MS/MS and the analytes have been ionized using an ESI source in the positive ion mode prior to detection by Multiple Reaction Monitoring (MRM) mode. The analytes were monitored at the following transitions (m/z) 280.20 → 125.20 for Sibutramine, (m/z) 268.10 → 126.9 and 268.10 → 141.00 for N-monodesmethylsibutramine, (m/z) 254.10 → 126.9 and 254.10 → 141.00 for N-N-didesmethylsibutramine and (m/z) 232.20 → 107.10 for Bisoprolol respectively. Sibutramine, N-monodesmethylsibutramine and N-N-didesmethylsibutramine linearity were demonstrated over the concentrations ranging from 0.10 to 11.00 (ng/mL). The developed method has been fully validated.

Keywords

Sibutramine, HPLC-MS/MS, Plasma, ESI Source, Positive Ion Mode, MRM Mode

1. Introduction

Sibutramine hydrochloride, N-{1-[1-(4-chlorophenyl) cyclobutyl]-3-methylbutyl}-N,N-dimethylamine hydrochloride...
ride, are a reuptake inhibitor of noradrenaline and 5-hydroxytryptamine [1]-[4], and have been used as an anti-obesity drug. Pre-clinical investigations in rodents indicate that sibutramine decreases bodyweight both by reducing food intake [5] [6], and by increasing energy expenditure via enhanced resting metabolic rate [7]. When being administered to animals and humans, sibutramine is rapidly metabolized to N-mono-desmethylsibutramine (metabolite 1) and N-di-desmethylsibutramine (metabolite 2), and the in vivo effects of this drug are mainly due to the actions of these two metabolites [7]. Hind et al. [8] reported an LC-MS method for determination of metabolites 1 and 2 of sibutramine in human plasma, in which the limit of quantitation (LOQ) for the two metabolites was 0.5 µg·l⁻¹. In fact, the LOQ of 0.5 µg·l⁻¹ in that study was not sensitive enough for pharmacokinetic research because the maximum plasma concentration (Cmax) of metabolite 1 was only about 3.2 µg·l⁻¹. And many of its plasma levels on the terminal elimination phase were below the LOQ. Radhakrishna et al. [9] has developed two HPLC methods for the purity estimation and quantitative determination of sibutramine hydrochloride. These methods offer a rapid and reliable analysis of sibutramine hydrochloride as bulk or in dosage forms.

Sibutramine Figure 1(a), N-monodesmethylsibutramine Figure 1(b), N-N-didesmethylsibutramine Figure 1(c), and the internal standard Bisoprolol Figure 1(d) were extracted from human plasma and analyzed using LC-MS/MS with ESI ionization and MRM detection mode.

The present work reports a selective, simple and sensitive HPLC-MS/MS bioanalytical method for the determination of Sibutramine and its N-desmethyl metabolites in human plasma.

2. Experimental

2.1. Chemicals and Reagents

In Saudia Arabia, SAJA Pharma has provided Sibutramine hydrochloride monohydrates. N-monodesmethyl-sibutramine and N-N-didesmethylsibutramine have been provided by LGC and BisoprololFumarate has been provided by JamjoomPharma, Saudia Arabia. HPLC acetonitrile gradient grade solvent, HPLC ter-butyl methyl ether TBME grade solvent and ammonium acetate have been purchased from Merck (Darmstadt-Germany). Reagents have been used without further purification. Blank human plasma samples have been obtained from the plasma of participants who volunteered in blood bank.

2.2. Instrumentation

The LC-MS/MS system consisted of a High Performance Liquid Chromatography (Agilent 1200 series, Agilent Technologies, Germany) coupled with a, Sciex Triple Quadrupole Mass Spectrometer (API 5000, MDS, Sciex, Ontario-Canada) and equipped with an electrospray ionization (ESI) (Applied Biosystems). Data acquisition and processing have been controlled by Applied Biosystems/MSD SCIEX Analyst software (version 1.4.2).

2.3. Chromatographic Conditions and MS/MS Instrumental Settings

Chromatographic separations were performed using Merck, Purospher RP-18e column (30 mm × 4.0 mm, 3 µm) thermostated at 30°C. The mobile phase was an aqueous solution 20 mM ammonium acetate pH 4.0 (adjustment by acetic acid) in water and acetonitrile (67:33 v/v (%)). The separation has been performed under isocratic conditions set at a constant flow rate of 0.40 (mL/min.). The injection volume has been fixed at 30 µL.

LC-MS/MS experimental conditions have utilized the Multiple Reaction Monitoring (MRM) for both S

![Figure 1](image)
ibutramine, N-monodesmethylsibutramine, N-N-didesmethylsibutramine and the internal standard (Bisoprolol). The detection was performed in the positive ESI mode for each of the analytes respective ions [M + H]^+. Instrument settings of the MS/MS are summarized in Table 1. The full scan product ion spectrum of [M + H]^+ of Sibutramine, N-desmethyl metabolites and Bisoprolol is illustrated in Figure 2.

2.4. Preparation of Stock and Working Standard Solutions

A Sibutramine stock standard solution has been prepared by dissolving an equivalent amount of 20.27 mg Sibutramine in a 100.00 mL methanol to make up the concentration of 202.7 μg Sibutramine/mL. Two working standard solutions concentration of 0.10 and 0.02 (μg/mL) have also been prepared. N-monodesmethylsibutramine stock standard solution has been prepared by dissolving an equivalent amount of 17.61 mg N-monodesmethylsibutramine in a 50.00 mL methanol to make up the concentration of 352.20 μg N-monodesmethylsibutramine/mL. Two working standard solutions concentration of 0.10 and 0.02 (μg/mL) have also been prepared. N-N-didesmethylsibutramine stock standard solution has been prepared by dissolving an equivalent amount of 17.25 mg N-N-didesmethylsibutramine in a 50.00 mL methanol to make up the concentration of 345.0 μg N-N-didesmethylsibutramine/mL. Two working standard solutions concentration of 0.10 and 0.02 (μg/mL) have also been prepared. The internal standard Bisoprolol stock solution was prepared by dissolving an equivalent amount of 20.00 mg Bisoprolol with 100.00 mL Acetonitrile to make up a stock standard solution containing 200.00 μg Bisoprolol/mL. One working standard solutions concentration of 3.80 (μg/mL) was also prepared.

2.5. Preparation of Matrix Based Calibrators and Quality Control Samples

Sibutramine, N-monodesmethylsibutramine and N-N didesmethylsibutramine matrix based calibrators have been prepared from the above working standard solutions of Sibutramine N-monodesmethylsibutramine and N-N-didesmethylsibutramine in 5.0 mL plasma to produce the following calibrators: 0.10, 0.60, 1.20, 2.40, 4.00, 7.00, 9.00 and 11.00 (ng/mL). Additionally 10.0 mL standard solutions of each of the quality control (QC) samples have also been prepared in blank plasma to make up the concentrations of: 0.30, 5.50, and 8.80 (ng/mL). Prior to analysis each sample (500 µL) was spiked with a 3.80 µg aliquot of Bisoprolol.

2.6. Sample Preparation

Into the appropriate tubes (6.0 mL capacity), a 50 μL of Bisoprolol solution 3.80 μg/mL has been added to each

| Parameter                  | Unit | Sibutramine | N-monodesmethylsibutramine | N-N-didesmethylsibutramine | Bisoprolol (IS) |
|----------------------------|------|-------------|---------------------------|---------------------------|-----------------|
| Source temperature         | °C   | 600.0       |                           |                           |                 |
| Nebulizer gas              | psi  | 65          |                           |                           |                 |
| Turbolon gas               | psi  | 65          |                           |                           |                 |
| Curtain gas                | psi  | 35          |                           |                           |                 |
| Collision gas              | psi  | 12          |                           |                           |                 |
| Ion spray voltage          | V    | 2500        |                           |                           |                 |
| Dwell time per transition  | ms   | 150         |                           |                           |                 |
| Entrance potential         | V    | 7           | 15                        | 10                        | 15              |
| MRM transition             | amu  | 280.20 – 125.20 | 268.10 – 126.90 | 254.10 – 126.90 | 326.15 – 107.10 |
| Collision energy           | V    | 40          | 20                        | 50                        | 20              |
| Declustering potential     | V    | 125         | 50                        | 25                        | 90              |
| Collision cell exist potential | V  | 40          | 15                        | 10                        | 15              |
Figure 2. Full scan product ion spectrum of [M + H]^+ of Sibutramine and its N-desmethyl metabolites.
plasma aliquot (5000 µL). Then the samples were vortexed (5 s); a 4.0 mL of ter-butyl-methy ether TBME was added, and then the samples were shaken for 25 minutes and centrifuged (4000 rpm per min) for 5 minutes. A 2.50 mL aliquot of the organic supernatant has been transferred to a 10 ml glass tube then the sample has been evaporated under N2. The dry sample has been reconstituted with 0.30 mL mobile phase. The sample was vortexed (30 s) before it was transferred to the well plate of the autosampler (250 µL). A 30 µL volume has been injected onto the equilibrated LC-MS/MS system.

3. Result and Discussions

3.1. Method Validation

The developed method has been fully validated with respect to the following parameters: selectivity, stability, linearity and linear working range, limit of detection and as well as of lower and upper Limits of quantitation, sensitivity, recovery, accuracy, and precision.

3.1.1. Selectivity

All samples have been extracted and analyzed using the developed and optimized method, no interferences have been observed at the retention time of Sibutramine and its N-desmethyl metabolites or the IS. Analytical signals from blank plasma sample extracts and from spiked samples at the lower limit of quantitation (0.10 ng/mL) are illustrated in Figure 3. In addition, chromatograms obtained from incurred samples after 3 h dosing with (15 mg/capsule Sibutramine) are illustrated in Figure 3. A high selectivity with no matrix effects has been observed, and this is attributed to the high dilution factor (20) of the extracted matrix. The use of a highly sensitive MS/MS (API 5000) facilitated the recording of a high signal. Therefore, a simple highly selective and highly sensitive method has been concluded.

3.1.2. Response Function, Linearity, Lower and Upper Limits of Quantitation

Response functions (Area ratios) plotted against nominal Sibutramine and its N-desmethyl metabolite concentrations in the dynamic range 0.10 to 11.00 (ng/mL) have been measured. Different statistical calibration models have been evaluated; lowest residuals have been demonstrated using a weighted regression model with a 1/x² as a statistical weight. The model gave a correlation coefficient of 0.9977 and a resulted in this linear equation: 

\[ y = 0.0852X - 0.0002 \] for sibutramine, 
\[ y = 0.3320X + 0.0003 \] for N-monodesmethysibutramine with correlation coefficient of 0.9979 and 
\[ y = 0.0886X + 0.0032 \] for N-N didesmethysibutramine with correlation coefficient of 0.9980.

3.1.3. Precision and Accuracy

Six replicate measurements of each quality control contain QC L 0.30 (ng/mL), QC M 5.50 (ng/mL) and QC H 8.80 (ng/mL) matrix based standards of Sibutramine and N-desmethyl metabolites that have been chromatographed to evaluate instrument precision, as well as method, inter-day, and intra-day precision and accuracy. The results summarized in Table 2. Results indicate that the bioanalytical method id precise CV% not exceeding 15%.

3.1.4. Stability

Stability has been investigated during sampling and sample storage, processing and analysis. Stability data have been evaluated with respect to analytical signals obtained from freshly prepared QC samples and compared to those samples measured after stressed conditions. Stability experiments have extended throughout the analysis duration until assay of the last harvested sample. Concerning short term stability studies, quality control samples in plasma that include QC L (0.30 ng/mL), QC M (5.50 ng/mL) and QC H (8.80 ng/mL) have been thawed and kept un-treated at room temperature for 6h. Autosampler stability has been evaluated over 24 h; freeze and thaw stabilities have covered three cycles of freeze and thaw cycles. Long term matrix based solution stability has been investigated under prolonged storage condition (216 days, −80°C). Table 3 which summarizes stability data demonstrates that Sibutramine and N-desmethyl metabolites have been stable under the investigated experimental conditions.

3.2. Method Application

The validated bioanalytical method is recommended to apply and evaluate the comparative bioavailability
Figure 3. Representative chromatograms of (A) Sibutramine and IS (Bisoprolol, B) in human plasma samples. (I) Blank plasma sample; (II) Blank plasma sample spiked with Sibutramine at BLLOQ 0.10 ng/mL and IS; and (III) Plasma sample from a volunteer 3 hours after administration of 15 mg/capsule Sibutramine.
Table 2. Summary of instrument, inter-day, intra-day precision and accuracy data for sibutramine.

(a) Sibutramine

| Nominal concentrations of QC (ng/mL) | Instrument Method | Intra-day (ranges of 3 days) | Inter-day |
|-----------------------------------|------------------|-----------------------------|-----------|
|                                   | Precision (CV%)  | Precision (RE%) | Precision (CV%) | Precision (RE%) | Precision (CV%) | Precision (RE%) |
| QC, 0.30 ng/mL                   | 9.09             | 3.03             | 10.00            | 3.03 to 3.70   | −10.00 to 10.00 | 6.90            | −3.33          |
| QC,M 5.50 ng/mL                  | 0.88             | 4.55             | −0.18            | 3.51 to 6.53   | −0.18 to 8.55   | 6.82             | 1.27           |
| QC,H 8.80 ng/mL                  | 2.03             | 2.40             | 4.20             | 1.88 to 4.30   | 4.20 to 9.09    | 8.86             | 3.86           |

(b) N-monodesmethylsibutramine

| Nominal concentrations of QC (ng/mL) | Instrument Method | Intra-day (ranges of 3 days) | Inter-day |
|-------------------------------------|------------------|-----------------------------|-----------|
|                                    | Precision (CV%)  | Precision (RE%) | Precision (CV%) | Precision (RE%) | Precision (CV%) | Precision (RE%) |
| QC, 0.30 ng/mL                     | 2.78             | 3.70             | −10.00          | 3.70 to 10.71  | −10.00 to −6.67 | 7.41            | −10.00         |
| QC,M 5.50 ng/mL                    | 0.87             | 4.24             | −10.00          | 2.05 to 4.24   | −10.00 to 8.36  | 5.69             | 2.18           |
| QC,H 8.80 ng/mL                    | 1.33             | 1.92             | −5.11           | 1.59 to 2.47   | −5.11 to 10.34  | 9.07             | 2.73           |

(c) N-N-didesmethylsibutramine

| Nominal concentrations of QC (ng/mL) | Instrument Method | Intra-day (ranges of 3 days) | Inter-day |
|-------------------------------------|------------------|-----------------------------|-----------|
|                                    | Precision (CV%)  | Precision (RE%) | Precision (CV%) | Precision (RE%) | Precision (CV%) | Precision (RE%) |
| QC, 0.30 ng/mL                     | 0.00             | 6.25             | 6.67             | 3.33 to 7.14   | −6.67 to 6.67   | 10.34            | −3.33          |
| QC,M 5.50 ng/mL                    | 0.54             | 6.30             | −4.73           | 2.50 to 6.30   | −4.73 to 10.00  | 6.51             | 3.27           |
| QC,H 8.80 ng/mL                    | 1.85             | 2.06             | −0.68           | 2.06 to 2.92   | −0.68 to 10.11  | 7.23             | 3.75           |

QC,: Quality control sample of low concentration; QC,M: Quality control sample of medium concentration; QC,H: Quality control sample of high concentration.

Table 3. Summary of stability data pertaining.

(a) Sibutramine

| Storage condition                  | Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Recovery (%) |
|------------------------------------|-------------------------------|--------------------------------|--------------|
| Short term stability (6 h in plasma) | 0.30                          | 29381*                         | 101.82       |
|                                    | 5.50                          | -                              | -            |
|                                    | 8.80                          | 937065*                        | 94.13        |
|                                    | 0.30                          | 0.32                           | 94.12        |
| Autosampler stability after 65 h   | 5.50                          | -                              | -            |
|                                    | 8.80                          | 9.48                           | 97.43        |
|                                    | 0.30                          | 0.29                           | 96.67        |
| Freeze-thaw cycles (N = 5)         | 5.50                          | 5.84                           | 106.18       |
|                                    | 8.80                          | 9.69                           | 110.11       |
|                                    | 0.30                          | 0.29                           | 96.67        |
| Long term stability (198 day, −80°C) | 5.50                          | 5.57                           | 101.30       |
|                                    | 8.80                          | 9.14                           | 103.87       |
### 4. Conclusion

A highly sensitive, simple and fast RP-LC-ESI-MS/MS method for the determination of Sibutramine and its \(N\)-desmethyl metabolites in human plasma has been developed and fully validated according to the current FDA guidance. This method involves a single step liquid-liquid extraction, using Bisoprolol, a commercially available substance, as internal standard. The short run time of 3.5 min and the relatively low flow rate (0.4 mL/min) allows the analysis of a large number of samples with less mobile phase consumption. Validation results show that the optimized RP-LC-ESI-MS/MS method possesses specificity, accuracy, precision, sensitivity, linearity, recovery, and stability over the entire range of significant therapeutic plasma concentrations.

### References

[1] Heal, D.J., Aspley, S., Jackson, H.C., Martin, K.F. and Cheetham, S.C. (1998) Sibutramine: A Novel Anti-Obesity...
Drug. A Review of the Pharmacological Evidence to Differentiate It from d-Amphetamine and d-Fenfluramine. International Journal of Obesity, 22, S18-S28.

[2] Luscombe, G.P., Hopcroft, R.H., Thomas, P.C. and Buckett, W.R. (1989) The Contribution of Metabolites to the Rapid and Potent Down-Regulation of Rat Cortical Beta-Adrenoceptors by the Putative Antidepressant Sibutramine Hydrochloride. Neuropharmacology, 28, 129-134. http://dx.doi.org/10.1016/0028-3908(89)90048-8

[3] Balcioglu, A. and Wurtman, R.J. (2000) Sibutramine, a Serotonin Uptake Inhibitor, Increases Dopamine Concentrations in Rat Striatal and Hypothalamic Extracellular Fluid. Neuropharmacology, 39, 2352-2359. http://dx.doi.org/10.1016/S0028-3908(00)00083-6

[4] Gundlah, C., Martin, K.F., Heal, D.J., et al. (1997) In Vivo Criteria to Differentiate Monoamine Reuptake Inhibitors from Releasing Agents: Sibutramine Is a Reuptake Inhibitor. Journal of Pharmacology and Experimental Therapeutics, 283, 581-591.

[5] Jackson, H.C., Bearham, M.C., Hutchins, L.J., Mazurkiewicz, S.E., Needham, A.M. and Heal, D.J. (1997) Investigation of the Mechanisms Underlying the Hypophagic Effects of the 5-HT and Noradrenaline Reuptake Inhibitor, Sibutramine, in the Rat. British Journal of Pharmacology, 121, 1613-1618. http://dx.doi.org/10.1038/sj.bjp.0701311

[6] Jackson, H.C., Needham, A.M., Hutchins, L.J., Mazurkiewicz, S.E. and Heal, D.J. (1997) Comparison of the Effects of Sibutramine and Other Monoamine Reuptake Inhibitors on Food Intake in the Rat. British Journal of Pharmacology, 121, 1758-1763. http://dx.doi.org/10.1038/sj.bjp.0701312

[7] Connoley, I.P., Liu, Y.L., Frost, I., Reckless, I.P., Heal, D.J. and Stock, M.J. (1999) Thermogenic Effects of Sibutramine and Its Metabolites. British Journal of Pharmacology, 126, 1487-1495. http://dx.doi.org/10.1038/sj.bjp.0702446

[8] Hind, I.D., Mangham, J.E., Ghani, S.P., Haddock, R.E., Garratt, C.J. and Jones, R.W. (1999) Sibutramine Pharmacokinetics in Young and Elderly Healthy Subjects. European Journal of Clinical Pharmacology, 54, 847-849. http://dx.doi.org/10.1007/s002280050565

[9] Radhakrishna, T., Narayana, C.L., Rao, D.S., Vyas, K. and Reddy, G.O. (2000) LC Method for the Determination of Assay and Purity of Sibutramine Hydrochloride and Its Enantiomers by Chiral Chromatography. Journal of Pharmaceutical and Biomedical Analysis, 22, 627-639. http://dx.doi.org/10.1016/S0731-7085(99)00303-9
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