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Determination of the active metabolite of sibutramine by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A sensitive and specific method for the determination of the active primary amine metabolite of sibutramine, *N*-di-desmethylsibutramine (BTS 54 505), in human plasma was developed, based on high-performance liquid chromatography (HPLC)–electrospray ionization tandem mass spectrometry (MS–MS). The samples were extracted from plasma with methyl *tert.*-butyl ether, followed by separation and evaporation after addition of the internal standard, propranolol, and basification with sodium hydroxide. The residue was reconstituted in mobile phase and injected into the HPLC–MS–MS system. Chromatography was performed on an ODS MS column with a mobile phase consisting of acetonitrile (containing 0.1% trifluoroacetic acid, v/v)–0.1% trifluoroacetic acid (55:45, v/v) at a flow-rate of 0.3 ml/min. Multiple reaction monitoring using precursor→product ion combinations at *m/z* 252.00→125.00 and 260.00→115.70 was applied to determine BTS 54 505 and propranolol, respectively. Linearity was confirmed in the concentration range 0.328–32.8 ng/ml in human plasma and the imprecision of this assay was less than 19.90% over the entire concentration range. The method is sufficiently sensitive and repeatable to be used in pharmacokinetic studies.

Keywords: Sibutramine; *N*-Di-desmethylsibutramine

1. Introduction

Sibutramine HCl, *N*-{1-[1-(4-chlorophenyl)-cyclobutyl]-3-methylbutyl}-*N*,*N*-dimethylamine hydrochloride (BTS 54 524), is a novel monoamine-reuptake inhibitor involved in the regulation of food intake in humans [1]. Since sibutramine is rapidly metabolized into active primary and secondary amine metabolites, BTS 54 505 and BTS 54 354 (Fig. 1), it is not detectable due to its low concentration in plasma. These two metabolites have similar pharmacological profiles to sibutramine in vivo, but are up to 100-fold more active than sibutramine as monoamine uptake inhibitors in vitro [2]. In addition, the plasma concentration of BTS 54 354 is rather low and its half-life is rather short compared with BTS 54 505. It has been reported [3,4] that the in vivo effects of sibutramine are predominantly due to the actions of the primary metabolite BTS 54 505. Therefore, it is equally effective, but simple and convenient, to monitor BTS 54 505 alone instead of either sibutramine or BTS 54 354.

In humans, the plasma concentration of BTS 54 505 is also very low (<10 ng/ml) when sibutramine is administered orally at a dose of 15 mg/day.

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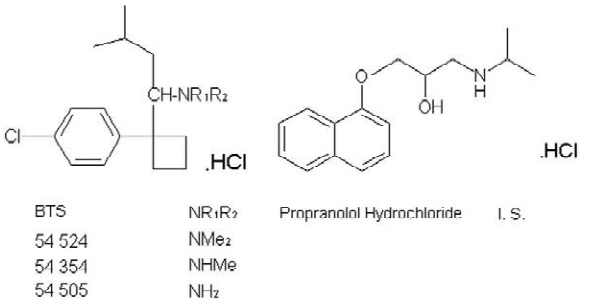

Fig. 1. Chemical structures of sibutramine hydrochloride, its active amine metabolites and the internal standard, propranolol hydrochloride.

Recently, an HPLC–MS method was applied to the determination of BTS 54 505 in human plasma [4]. However, the injection volume was very large (100 μl) due to its low sensitivity [4]. In our experience, an injection volume greater than 10 μl will cause significant contamination to the ionization interface and mass analyzer, therfore the ruggedness and selectivity of this method may be questioned. It is also not applicable to pharmacokinetics and bioavailability studies, because these studies are high-throughput quantitative analyses where ruggedness is always a concern.

High-performance liquid chromatography (HPLC)–electrospray ionization tandem mass spectrometry (MS–MS) is a very specific and sensitive method when used for the determination of drugs and metabolites in biological fluids. Sample preparation may be simplified, and no, or very little, chromatographic separation is required. This paper describes a rapid, selective and sensitive method for the determination of BTS 54 505 in human plasma using HPLC–MS–MS. This method has been used successfully in clinical sibutramine pharmacokinetic studies.

2. Experimental

2.1. Reagents and materials

BTS 54 505 hydrochloride standard (99.48%) was supplied by the Drug Research Institute of the Chinese Academy of Science (Shanghai, PR China). The internal standard (I.S.) propranolol hydrochloride (99.8%, Fig. 1) was obtained from the Shanghai Institute for Drug Control (Shanghai, PR China). Trifluoroacetic acid (HPLC-grade reagent) was obtained from Merk (Germany). Methyl *tert.*-butyl ether (HPLC/Spectro) was purchased from Tedia (Fairfield, OH, USA). Sodium hydroxide (analytical reagent grade) was purchased from Shanghai Chemical Reagent Company (Shanghai, PR China). Double distilled water was purified using a Millipore Simplicity system (Millipore, Bedford, MA, USA). Drug-free human heparinized plasma was obtained from the Shanghai Blood Center (Shanghai, PR China). Nitrogen (99.999%) and argon were purchased from the Shanghai BOC Gas Industry (Shanghai, PR China).

2.2. Instrumentation

A Waters Xterra MS C_{18} 3.5 μm, 50×2.1 mm column was used for HPLC–MS–MS separation. The mobile phase was delivered by an Alliance 2690 HPLC system (Waters, Milford, MA, USA) consisting of a quaternary pump, a column heater, an auto-sampler, a 7725i injector and a Millennium chromatographic work station. Detection was performed by a Quattro-LC ion trap mass spectrometer (Micromass, Manchester, UK) using electrospray ionization (ESI) for ion production and a Masslynx work station.

2.3. Standard solutions

The stock standard solution of BTS 54 505 was prepared as follows: BTS 54 505 hydrochloride standard (4.7 mg, equal to 4.1 mg BTS 54 505) was first dissolved in 5 ml methanol, and then diluted with distilled water to 10 ml volume, with the concentration of BTS 54 505 finally being 4.1 mg/ml. This stock solution was further diluted with distilled water to obtain working standard solutions for validation and calibration. The stock I.S. solution was prepared as an aqueous propranolol hydrochloride solution (42.8 ng/ml). All stock solutions were stable for at least 2 months when stored at 4 °C.

2.4. Sample preparation

A 500 μl plasma sample was extracted with 3 ml methyl *tert.*-butyl ether after addition of 50 μl I.S.

solution and basification with sodium hydroxide (1 mol/l, 50 μl). After vortex mixing for 2 min, the supernatant was evaporated in a water bath (50 °C) under a nitrogen stream. The residue was then reconstituted in 100 μl mobile phase and a 5 μl aliquot was injected into the chromatographic system.

2.5. HPLC–ESI-MS–MS conditions

The HPLC separation was performed using a Waters Xterra MS C_{18} reversed-phase column at a column temperature of 20 °C. Acetonitrile (containing 0.1% trifluoroacetic acid, v/v)–0.1% trifluoroacetic acid (55:45, v/v) was used as mobile phase at a flow-rate of 0.3 ml/min. The temperature of the sample cooler in the autosampler was set at 10 °C. The analysis was complete within 8.0 min.

Electrospray ionization was performed in the positive ion mode with nitrogen as the nebulizer and drying gas. Collision-induced dissociation (CID) was achieved using argon as the collision gas. The exact source conditions were: drying gas temperature, 500 °C; drying gas flow, 435 l/h; nebulizer gas flow, 56 l/h; source block temperature, 100 °C; capillary voltage, 3.53 kV; cone voltage, 21 V; RF lens voltage, 0.11 V; gas cell pressure, $9.0 \cdot 10^{-4}$ mbar. The collision energies for both BTS 54 505 and the I.S. were 20.0 eV. The analyzer vacuum was set at $2.6 \cdot 10^{-5}$ mbar.

The tandem-in-time MS was operated at unit resolution in the multiple reaction monitoring (MRM) mode. The software was Masslynx Version 3.1. The ions for MRM analysis of the two channels of BTS 54 505 and the I.S. were selected at m/z 252.00 and 260.00 as the precursor ions, and at m/z 125.00 and 115.70 as the product ions, respectively. The dwell time was 0.2 s.

2.6. Validation test

2.6.1. Linearity and calibration curve

To prepare calibration standards, 25 μl of each stock solution in water was added to 0.5 ml drug-free plasma. The spiked concentrations of the calibration standards were 0.328, 1.64, 6.56, 16.4 and 32.8 ng/ml. The samples were then processed as described in Section 2.4. Each concentration was analysed in triplicate. The calibration curves were constructed by plotting the peak-area ratio of BTS54505 to the internal standard versus the spiked concentration. The calibration curves were calculated by least-squares regression.

2.6.2. Specificity and interference

Chromatograms of the sample prepared with human blank plasma were inspected visually for peaks from endogenous sources which might correspond to the BTS 54 505 and I.S. peaks. The standard plasma sample extracts were dissolved in 100 μl mobile phase and a 5 μl aliquot was injected into the chromatographic system to determine the detection limit ($S/N = 3$).

2.6.3. Accuracy and precision

Samples at each of three concentrations (0.328, 3.28 and 16.4 ng/ml plasma, $n = 5$) were prepared and assayed to determine the intra-day accuracy expressed as the relative error (RE), and the precision as the relative standard deviation (RSD). The same method was used over 5 days for the inter-day assay.

2.6.4. Evaluation of matrix suppression effects

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence analyte ionization) was investigated by extracting ''blank'' biological fluids from five different sources, and reconstituting the final extract in mobile phase containing a known amount of the analyte. The reconstituted extracts were analyzed and the peak areas of the analytes were compared.

3. Result and discussion

3.1. ESI mass spectra and MS–MS conditions

Full-scan ESI-positive mass spectra of BTS 54 505 and propranolol are shown in Fig. 2. The pseudo-molecular ions $[M+H]^+$ were identified at m/z 252.00 and 260.00, respectively. The product ion spectra (Fig. 2) of the two compounds were acquired with these pseudo-molecular ions as precursors. The assay of BTS 54 505 shows the

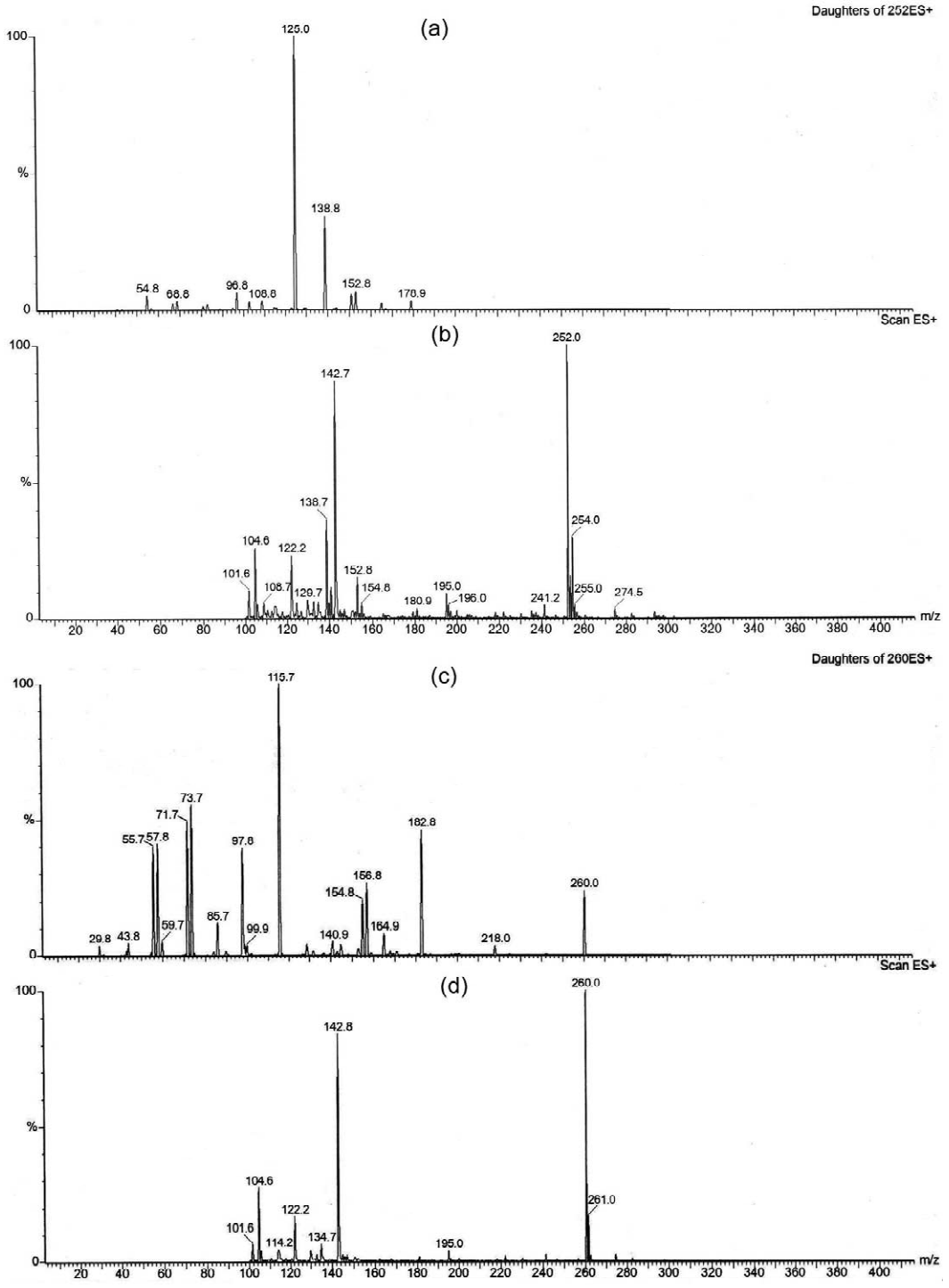

Fig. 2. ESI mass spectra of BTS 54 505 (b) and I.S. (d) showing m/z 252.0 and 260.0 as the pseudo-molecular ions. Product-ion spectra of the two pseudo-molecular ions (a, c) showing m/z 125.0 and m/z 115.7 as their predominant fragment ions, respectively.

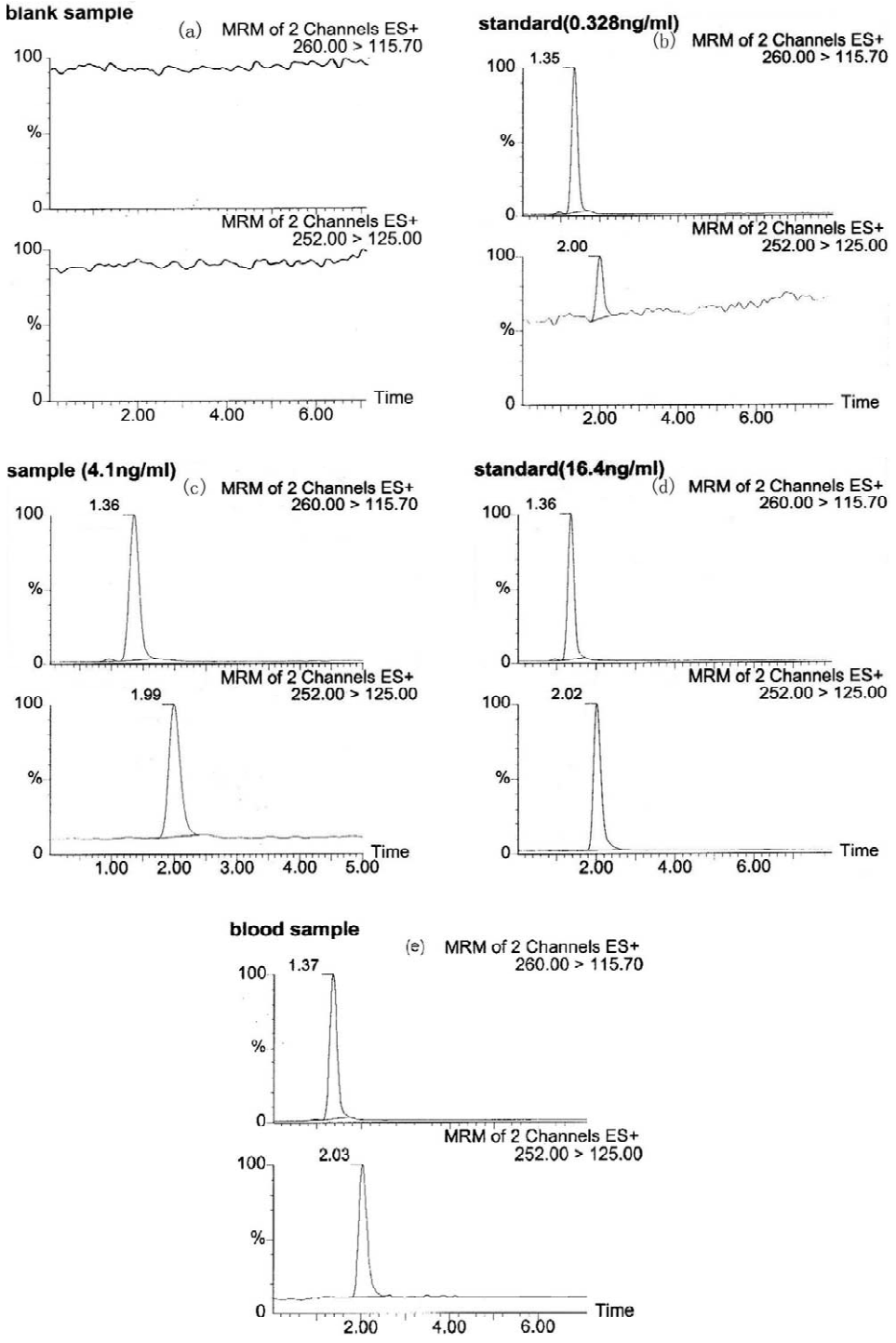

Fig. 3. Representative HPLC–ESI-MS–MS chromatograms of blank plasma (a), spiked plasma with I.S. and 0.328 (b), 4.1 (c) or 16.4 ng/ml (d) BTS 54 505 and that of a subject after sibutramine intake (e).

Table 1
Inter-day precision in the slope and intercept of standard curves ($r = 0.9916–0.9955$)

Day	Slope	Intercept	Correlation
1	0.0260	0.0154	0.9936
2	0.0269	0.0061	0.9952
3	0.0275	0.0129	0.9917
Mean±SD	0.0268±0.0008	0.0115±0.0048	0.9935±0.0018
RSD (%)	2.99	41.74	0.18

predominant fragment ion at m/z 125.00, and for I.S. at m/z 115.70.

3.2. Selectivity and specificity

High selectivity was found in MRM mode for the determination of drugs in plasma samples. Representative MRM chromatograms of blank plasma and spiked plasma samples (0.328, 4.1 and 16.4 ng/ml) are shown in Fig. 3. No endogenous sources of interference were observed at the retention time of the analyte. The chromatogram of a sample from a subject after sibutramine intake is also shown in Fig. 3. The detection limit was 0.082 ng/ml ($S/N = 3$).

3.3. Linearity and calibration curves

Acceptable linearity was observed over the concentration range 0.328–32.8 ng/ml plasma ($r = 0.9916–0.9955$). The RSD ($n = 3$) of the slope calculated with calibration curve data was 2.99%, showing good repeatability (Table 1). The stability of the sample solution in the autosampler at 10 °C was also assessed. BTS 54 505 in sample solutions was stable for approximately 24 h, since the concentrations of analytes determined were within 95.7–104.3% of the initial concentrations.

3.4. Accuracy and precision

The intra- and inter-day precision and accuracy are shown in Table 2. The RSD of BTS 54 505 ranged from 9.04 to 13.99% for intra-day and from 12.52 to 19.90% for inter-day, respectively. The RE for BTS 54 505 ranged from −0.6 to 7.95 for intra-day and from −2.6 to 16.5% for inter-day, respectively.

3.5. Matrix suppression effects

No matrix effect for BTS 54 505 (RSD 3.72%) was observed to influence the ionization of analytes for five different plasma pools. This indicates that the extracts were ''clean'' with little or no detectable co-eluting compounds that could influence ionization of the analytes.

4. Application

The present HPLC–ESI-MS–MS method for the analysis of BTS 54 505 was employed to determine

Table 2
Intra- and inter-day precision and accuracy for the analysis of BTS 54 505 from sibutramine spiked in human plasma by LC–MS–MS ($n = 5$)

	Actual conc. (ng/ml)	Detected conc. (mean±SD) (ng/ml)	Precision (RSD, %)	Accuracy (error, %)
Intra-day	0.328	0.354±0.032	9.04	7.9
	3.28	3.36±0.47	13.99	2.4
	16.4	16.3±1.6	9.82	−0.6
Inter-day	0.328	0.382±0.076	19.90	16.5
	3.28	3.48±0.49	14.08	6.1
	16.4	15.97±2.0	12.52	−2.6

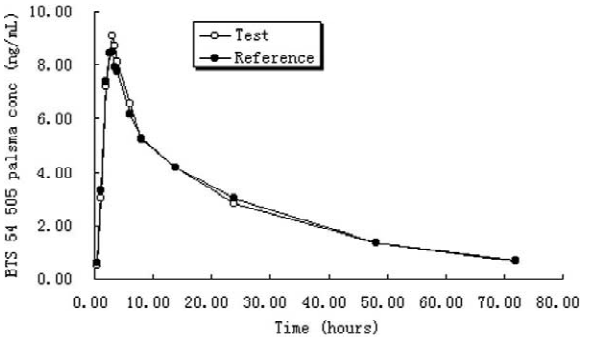

Fig. 4. Mean BTS 54 505 plasma concentration curves after oral administration of 20 mg sibutramine hydrochloride. ($n = 20$).

the pharmacokinetic parameters of sibutramine in plasma samples from volunteers in a clinical study. After a single oral dose of 20 mg sibutramine per treatment phase to 20 healthy volunteers, concentration versus time profiles were constructed for up to 72 h for BTS 54 505. The mean maximum BTS 54 505 plasma concentration was 10.51 ng/ml, T_{max} was 2.90 h, $AUC_{0-72\ h}$ was 188.07 ng·h/ml, $AUC_{0-\infty}$ was 211.22 ng·h/ml, and $t_{1/2}$ in the terminal elimination phase was 23.18 h. Overall, this method has been shown to be very suitable and convenient for determining plasma BTS 54 505 over a wide range of concentrations (Fig. 4).

5. Discussion and conclusion

A highly sensitive and specific method for the determination of BTS 54 505 was developed using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The analysis was completed within 8 min. In addition, only 0.5 ml of plasma was required for each determination of BTS 54 505, and thus the stress to volunteers or patients in clinical studies is greatly reduced. This method is very suitable and convenient for pharmacokinetic and bioavailability studies of the drug sibutramine.

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