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# Determination of ranolazine in human plasma by LC–MS/MS and its application in bioequivalence study

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ABSTRACT

A simple, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for quantification of ranolazine in human plasma. The analytical method consists in the precipitation of plasma sample with methanol, followed by the determination of ranolazine by an LC–MS/MS. The analyte was separated on a Peerless Cyano column (33 mm × 4.6 mm, 3 μm) an isocratic mobile phase of methanol–water containing formic acid (1.0%, v/v) (65:35, v/v) at a flow rate of 1.0 ml/min. Protonated ions formed by a turbo ionspray in positive mode were used to detect analyte and internal standard (IS). The MS/MS detection was made by monitoring the fragmentation of $m/z$ 428.20 → 279.50 for ranolazine and $m/z$ 448.30 → 285.20 for internal standard on a triple quadrupole mass spectrometer. The method was validated over the concentration range of 5–2000 ng/ml for ranolazine in human plasma with correlation coefficient of 0.9937 (S.D.: ±0.00367, range: 0.9895–0.9963). The accuracy and precision values obtained from six different sets of quality control samples analyzed in separate occasions ranged from 94.53 to 117.86 and 0.14% to 4.56%, respectively. Mean extraction recovery was 82.36–94.25% for three quality control (QC) samples and 88.37% for IS. Plasma samples were stable for three freeze–thaw cycles, or 24 h ambient storage, or 1 and 3 months storage at −20 °C. Processed samples (ready for injection) were stable up to 72 h at autosampler (4 °C). The developed method was successfully applied for analyzing ranolazine in plasma samples for a bioequivalence study with 12 healthy volunteers.



## 1. Introduction

Ranolazine, (±)-N-(2,6-dimethylphenyl)-4-[2-hydroxy-3-(2-methoxyphenoxy)propyl]-1-piperazine acetamide (Fig. 1) is an interesting anti-anginal and anti-ischemic agent [1]. In January 2006, the U.S. Food and Drug Administration (FDA) approved ranolazine for use in chronic stable angina following completion of a study mandated the FDA's special protocol assessment process, with the reported data in Journal of the American College of Cardiology (JACC) [2]. Ranolazine is the first approved agents from a new class of anti-anginal drug in almost 25 years. Ranolazine is believed to have its effects via altering the trans-cellular late sodium current. It acts by altering the intracellular sodium level that ranolazine affects the sodium-dependent calcium channels during myocardial ischemia [3]. Thus, ranolazine indirectly prevents the calcium overload that causes cardiac ischemia [4].

Ranolazine also modulates the metabolism of ischemia myocardial cells and improves the efficiency of oxygen use, by increasing myocardial glucose oxidation and decreasing fatty acid oxidation [5,6]. Ranolazine is extensively metabolized in the liver by the cytochrome P450 (CYP) 3A and 2D6 enzymes, with 5–10% being excreted unchanged by the kidneys [7]. Three major metabolites of ranolazine are produced by dearylation, O-demethylation and N-dealkylation, which are all at levels greater than 10% of the parent drug [7,8].

Since ranolazine lacks strong characteristic UV absorption, a HPLC-UV detection method is not sensitive and selective for the determination of ranolazine in biological samples [9]. Herron et al. developed a LC–MS method with solid-phase extraction (SPE) procedure for quantification of ranolazine and its metabolites in human plasma, but the method was not sensitive enough for pharmacokinetic studies and did not provide a detailed description of the method [10]. Recently, two LC–MS methods with selected ion monitoring (SIM) have been published for the quantitation of ranolazine in rat plasma, but the lower limit of quantitation (LLOQ) was above 20 ng/ml [11,12]. Hence the main objective of this work was to develop a simple one-step precipitation, sensitive, accurate and reliable mass spectrometry (LC–MS/MS) method for the quantification of ranolazine in human plasma.

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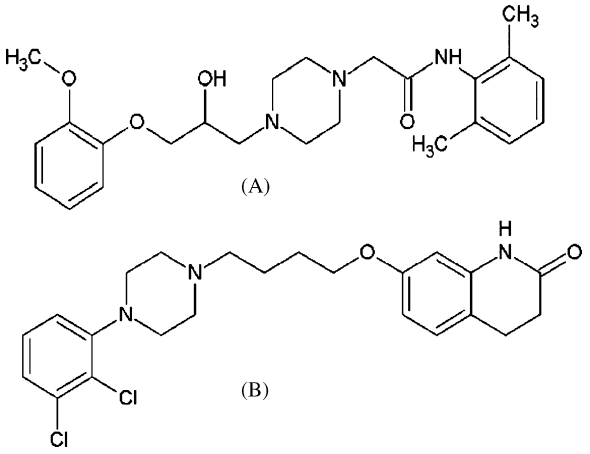


**Fig. 1.** Chemical structure of: (A) ranolazine and (B) aripiprazole (IS).

## 2. Experimental

### 2.1. Materials and reagents

Ranolazine dihydrochloride (>99%) was obtained from Ravenbhel Healthcare Pvt. Ltd. (Jammu, India). Aripiprazole (Fig. 1) (>99%) used as internal standard (IS) was supplied by Sun Pharmaceutical Industries Ltd. (Mumbai, India). Formic acid (98%) (analytical-reagent grade) and methanols (HPLC grade) were purchased from Merck Pvt. Ltd. (Mumbai, India). HPLC grade water (resistivity of 18 M cm) generated from Milli Q water purification system was used throughout the analysis. The blank human plasma with EDTA-$K_3$ anticoagulant was collected from Clinical Pharmacological Unit (CPU) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

### 2.2. LC–MS/MS

The liquid chromatographic (LC) system consists of LC-20AD pump, SIL-20AC autosampler and CTO-10ASvp column oven (Shimadzu, Kyoto, Japan). The LC–MS/MS system consisting of turbo spray and atmospheric pressure ionization source (API-2000) with triple quadrupole tandem mass spectrometer (AB Sciex Instruments, Foster, CA) was used for quantitative determination of ranolazine in plasma. Data integration was performed with Analyst 1.4.1 software version (AB Sciex Instruments, Foster, CA).

LC separation was performed on a Peerless Cyano column (33 mm × 4.6 mm, 3 μm) from Chromatopak, Mumbai, India. The mobile phase consists of a mixture of methanol–water containing formic acid (1.0%, v/v) (65:35, v/v). The LC system was operated isocratically at 1 ml/min. The column eluent was split and approximately 400 μl was introduced in the mass spectrometer.

A triple quadrupole mass spectrometer (MS–MS) was used with API source and channel electron multiplier (CEM) detector in positive ion detection mode. The collision energy (CE) and other parameters for the analyte and IS were optimized by infusing each compound solution with a concentration of 500 ng/ml in water. A high voltage of 5.5 kV was applied to the spray needle. The source temperature was set at 550 °C, using nitrogen (5.0 grade) at 7 l/min as auxiliary gas and zero grade air as nebulizer gas at a pressure of 80 psi. The setting of curtain gas and collision gas flow at instrument was 15 and 12 (arbitrary scale), respectively. Multiple reaction-monitoring (MRM) mode was used for scanning throughout this study. The transitions selected were $m/z$ 428.20 → 279.50 and $m/z$ 448.30 → 285.20 for ranolazine and IS, respectively, with a dwell time of 200 ms per transition.

### 2.3. Preparation of standard and quality control samples

The stock solutions of analyte and IS were prepared by dissolving the accurately weighted standard compound in water to give final concentration of 1 mg/ml. The working solutions of 50, 250, 1000, 2500, 5000, 10,000 and 20,000 ng/ml of the analyte and 2500 ng/ml of IS were prepared from the stock solution. These working solutions of analyte and IS were used to prepare the calibration and quality control (QC) samples. A seven-point standard curve was prepared by spiking the 0.1 ml of working solution of analyte and IS into the 0.8 ml blank plasma to obtain final concentrations of 5, 25, 100, 250, 500, 1000 and 2000 ng/ml for the analyte and 250 ng/ml for IS. All stock solutions and working standard solutions were stored in polypropylene vials at −20 °C freezer.

The linear regression of the peak area ratio of analyte/IS vs. concentration using a weighted 1/concentration$^2$ was used to obtain calibration curve. The regression equation of the calibration curve was then used to calculate the plasma concentration. The back calculated values of the concentrations were statistically evaluated.

QC samples were made using the stock solution. Four levels of QC samples in plasma were 5.0 (lower limit of quantitation), 15 ng/ml (low), 1000 ng/ml (medium), and 1600 ng/ml (high) for the analyte. QC samples were prepared in a 50-ml pool, then aliquoted into pre-labeled 2 ml polypropylene vials and stored at −20 °C.

### 2.4. Sample preparation

The samples were prepared by taking 0.9 ml of plasma sample in polypropylene tube. Then 0.1 ml of IS working solution (2500 ng/ml) was added to each tube and all the samples were vortex mixed for 30 s. Then 1.0 ml of methanol was added and mixed for 15 min by cyclo mixer. All the samples were centrifuged for 15 min at 5000 rpm. 1.0 ml supernatant clear solution was separated and filtered through 0.2 μm membrane filter. The resulting samples were transferred into a 1.0 ml glass vial, which was loaded into autosampler cabinet, and 25 μl aliquot was injected into the LC–MS/MS system.

### 2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, recovery and stability according to the principles of the Food and Drug Administration (FDA) industry guidance [13]. Three validation batches were processed on three separate days. Each batch included two set of calibration standards and six replicates of LLOQ, low-, medium-, and high-concentrations of QC samples.

Accuracy and precision were determined by analyzing six replicates of three QC samples (low-, medium-, and high-concentration). Accuracy (DEV) was determined as percent difference between the mean observed concentration and the nominal concentration [14]:

$$\mathrm{DEV}\,(\%) = \frac{\text{nominal} - \text{observed}}{\text{nominal}} \times 100$$

The precision of the assay was assessed by the between-run and within-run precision. The between-run precision (BRP) was defined as

$$\mathrm{BRP}\,(\%) = \frac{\sqrt{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}}{\mathrm{GM}} \times 100$$

The within-run precision (WRP) was calculated as

$$\mathrm{WRP}\,(\%) = \frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \times 100$$

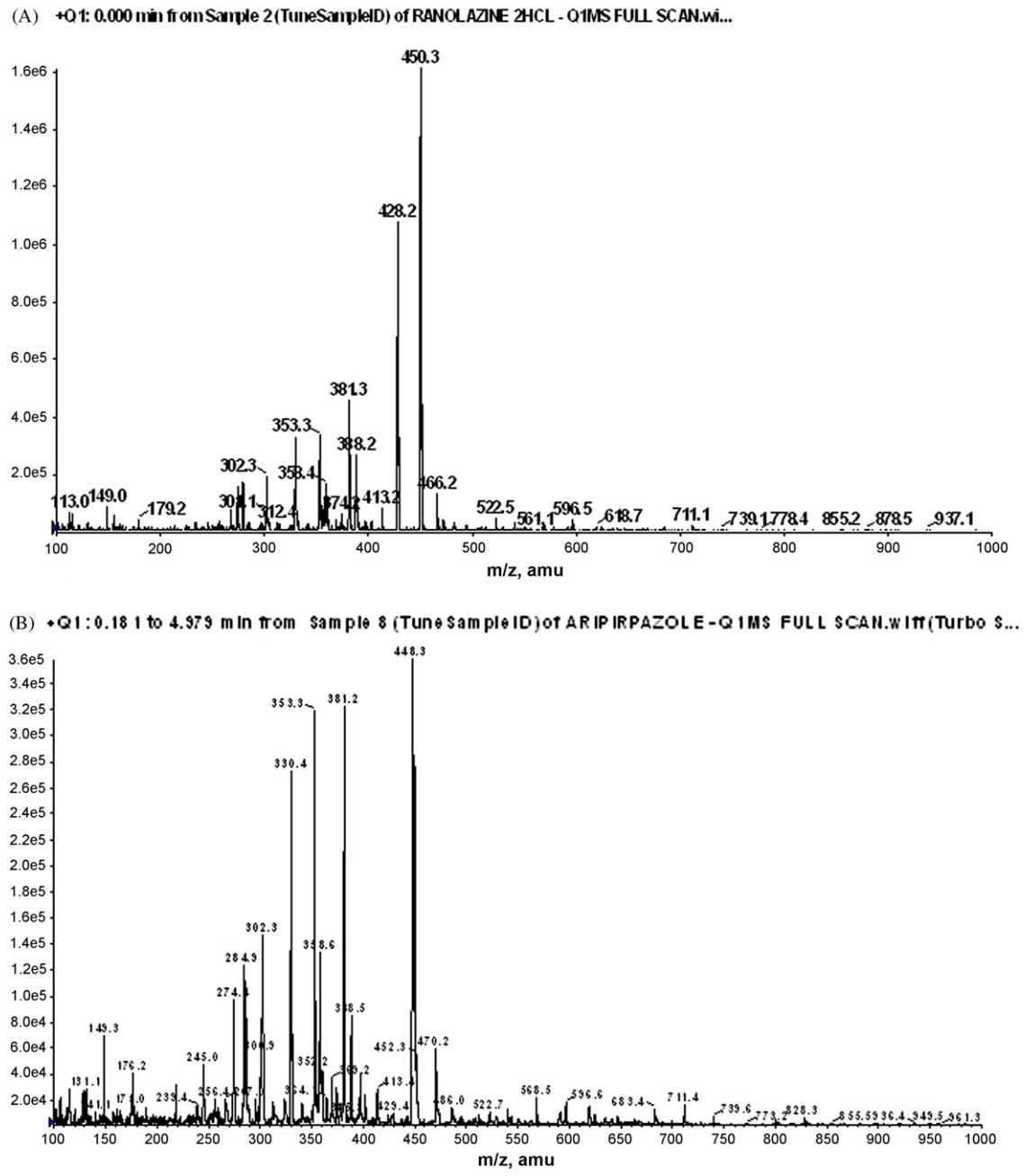


**Fig. 2.** Parent ion mass spectra of: (A) ranolazine ($m/z$ 428.20) and (B) IS ($m/z$ 448.30).

where $MS_{bet}$ is the between-groups mean square, $MS_{wit}$ is the within-groups mean square, GM is the grand mean and $n$ represents the number of replicates within each validation run.

The selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This test was performed by analyzing the blank plasma samples from six different sources (or doners) to test for interference at the retention time of ranolazine and aripiprazole (IS).

The extraction recovery of ranolazine (low-, medium- and high-QC) from human plasma was evaluated by comparing the peak area responses from plasma samples spiked with particular standard working solution of analyte before extraction with those from drug-free plasma samples extracted and spiked with same concentration of analyte after extraction. The recovery of IS at a single concentration of 250 ng/ml was determined in the same way. The number of replicates for each concentration was six.

The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post-extracted spiked QC samples with the response of analytes from neat samples at equivalent concentrations. Matrix effect was determined at same concentration of analyte and IS as in recovery experiment. The matrix effect (ME) was calculated by using the following equation:

$$\mathrm{ME} = \left(1 - \left(\frac{\text{response for post-extraction spiked drug}}{\text{response in pure solvent}}\right)\right) \times 100$$

Stability study was evaluated as part of the method validation. The processed sample stability was evaluated by comparing the precipitated samples that were injected immediately (time 0), with the

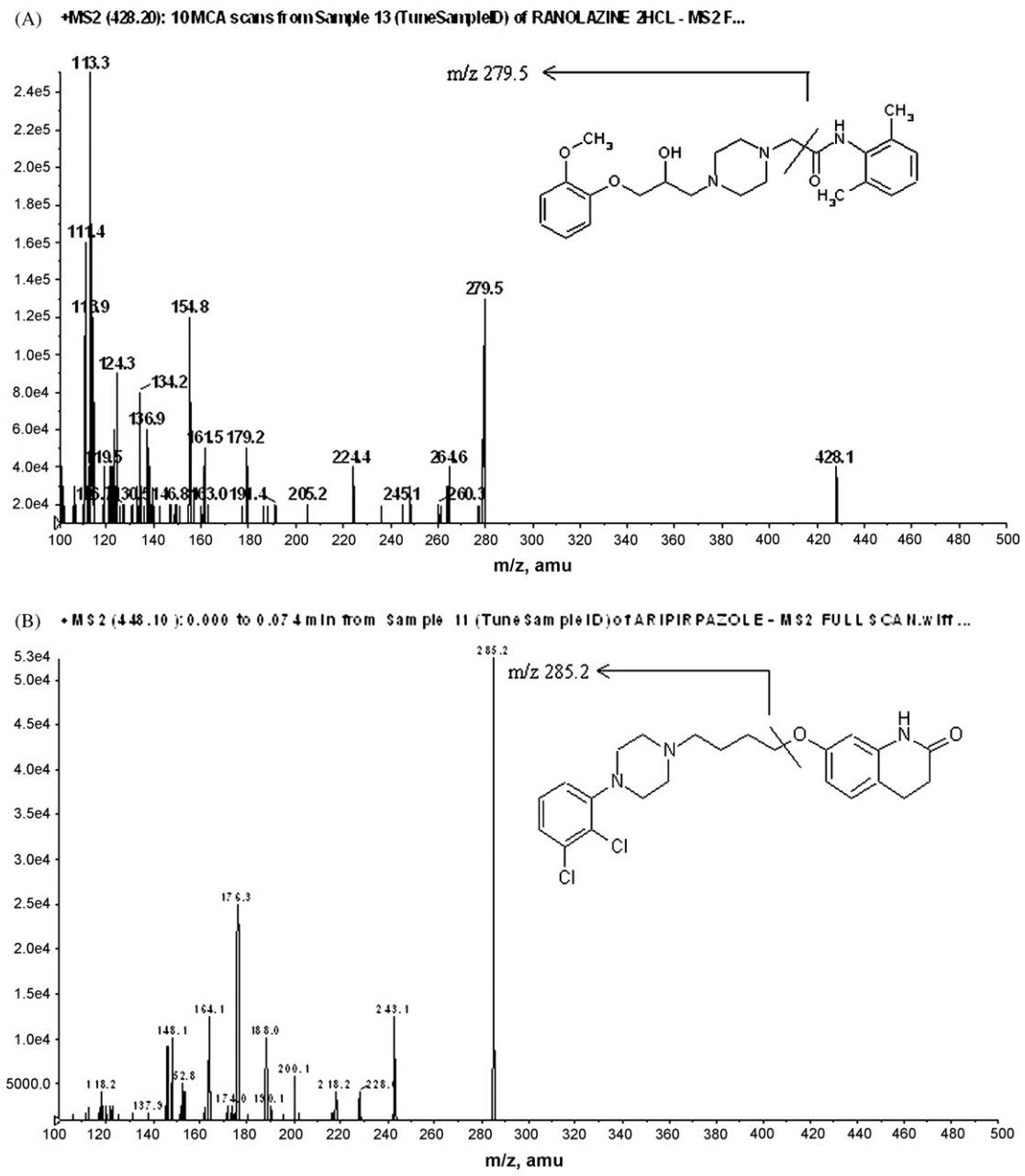


**Fig. 3.** Product ion mass spectra of: (A) ranolazine (*m*/*z* 279.50) and (B) IS (*m*/*z* 285.20).

samples that were re-injected after loading into autosampler at 4 °C for 72 h. Stability of analytes in the biomatrix after 8 h exposure in bench top was determined at all QC samples in six replicates. QC samples were kept at ambient temperature for 24 h and analyzed against freshly spiked standard curve and QC samples for short-time stability. The long-term stability of spiked human plasma stored at −20 °C was evaluated by analyzing all QC samples that were stored at −20 °C for 1 and 3 months together with freshly spiked standard curve and QC samples. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with the plasma samples thawed once. Three aliquots of each QC samples were used for freeze–thaw stability evaluation.

### 2.6. Application

The above-mentioned validated method was successfully used to analyze plasma sample for a bioequivalence study of ranolazine.

**Table 1**
Tandem mass spectrometric parameters of ranolazine and aripiprazole (IS).

| Compound | Molecular weight | Protonated ion | Fragment | CE (eV) | DP (V) | EP (V) | FP (V) | CXP (V) | Dwell time (ms) |
|---|---|---|---|---|---|---|---|---|---|
| Ranolazine | 427.53 | 428.20 | 279.50 | 34.89 | 90.00 | 8.00 | 360 | 9.00 | 200 |
| Aripiprazole | 448.39 | 448.30 | 285.20 | 37.60 | 65.00 | 10.0 | 385 | 10.00 | 200 |

eV, electron volt; V, volt; CE, collision energy; DP, declustering potential; EP, entrance potential; FP, focusing potential; CXP, collision cell exit potential; ms, milliseconds.

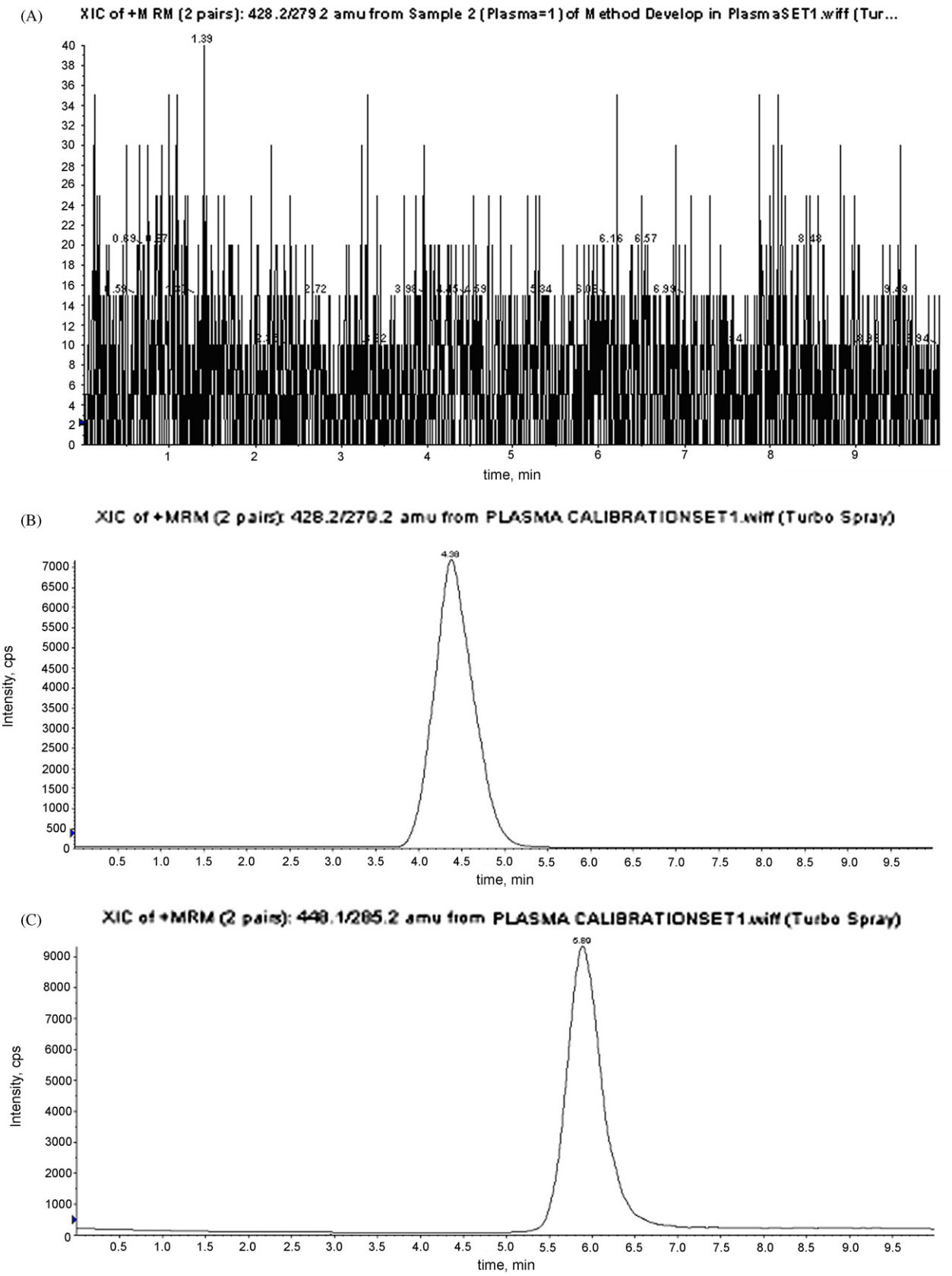


**Fig. 4.** Representative MRM chromatograms of: (A) blank human plasma, (B) blank plasma spiked with ranolazine and (C) blank plasma spiked with IS.

**Table 2**
Summary of calibration standards.

| Conc. added (ng/ml) | Mean conc. found (ng/ml) | S.D. | CV (%) | RE (%) | *n* |
|---|---|---|---|---|---|
| 5 | 5.31 | 0.14 | 2.66 | +6.17 | 6 |
| 25 | 25.92 | 0.24 | 0.94 | +3.69 | 6 |
| 100 | 101.68 | 1.18 | 1.16 | +1.68 | 6 |
| 250 | 254.05 | 2.09 | 0.82 | +1.62 | 6 |
| 500 | 490.16 | 3.91 | 0.80 | −1.97 | 6 |
| 1000 | 988.97 | 2.35 | 0.24 | −1.10 | 6 |
| 2000 | 2023.37 | 3.75 | 0.19 | +1.17 | 6 |

S.D., standard deviation; CV (%), coefficient of variation [(S.D./mean) × 100]; RE (%), relative error [{(Conc. found − Conc. added)/Conc. added} × 100]; *n*, number of replicates.

The study was approved by the ethics committee of Jadavpur University, Kolkata, India. It was an open, randomized crossover study to determine relative bioavailability of ranolazine in 12 healthy male volunteers with mean age between 28.33 ± 2.71 years and mean weight between 64.83 ± 5.89 kg following single dose administration of ranolazine 500 mg SR tablet. Test preparation was ranolazine 500 mg SR tablet manufactured by Ravenbhel Healthcare Pvt. Ltd., Jammu, India. Tablet Ranozex ER containing 500 mg of ranolazine, manufactured by Sun Pharmaceutical Industries Ltd., Mumbai, India was used as reference preparation.

## 3. Results and discussion

### 3.1. Mass spectrometry

LC–MS/MS for the determination of ranolazine in human plasma was investigated. Positive electrospray mass spectra of ranolazine shows an intense $[M+H]^+$ ion at *m*/*z* 428.20 (Fig. 2). Another intense $[M+H]^+$ ion at *m*/*z* 448.30 is shown for aripiprazole (Fig. 2). When these molecular ions undergo fragmentation in the collision cell, the product ion mass spectra shown in Fig. 3 are generated. With the experimental conditions used in these experiments, ranolazine and aripiprazole show an intense product ion at *m*/*z* 279.50 and 285.20, respectively. The collision energies and other optimized parameters used for analyte and IS are presented in Table 1.

### 3.2. Separation and specificity

Typical MRM chromatograms from the study of ranolazine and aripiprazole in human plasma are shown in Fig. 4. Retention time of ranolazine and IS are at 4.38 and 5.80 min, respectively. The total chromatographic run time was 10 min. No interference peak was found in the MRM profiles for blank plasma samples (Fig. 4).

### 3.3. Limit of quantitation, linearity

Lower limit of quantitation was established as 5 ng/ml, its precision (coefficient of variance: %CV) and accuracy (relative error: %RE) values being 2.66% and +6.17%, respectively. The equation of the calibration curve was obtained by least-squares linear regression analysis of the peak-area ratios of ranolazine to internal standard vs. concentration. The curve was linear in the concentration range 5.0–2000 ng/ml with regression coefficient of 0.9937 (S.D.: ±0.00367, range: 0.9895–0.9963). The calibration equation shows with the average slope and intercept 0.0031 (S.D.: ±0.000361, range 0.0028–0.0035) and 0.0927 (S.D.: ±0.000265, range: 0.0924–0.0929), respectively. All back calculated values showed excellent accuracy and precision (Table 2). No single calibration standard point was dropped during the validation.

**Table 3**
Assessment of accuracy and precision from quality-control sample.

| Nominal (ng/ml) | GM (ng/ml) | S.D. (ng/ml) | DEV (%) | WRP (%) | BRP (%) | *N* |
|---|---|---|---|---|---|---|
| 15.00 | 16.33 | 0.76 | −8.87 | 4.56 | 1.12 | 18.00 |
| 1000.00 | 962.84 | 9.79 | 3.72 | 1.01 | 0.17 | 18.00 |
| 1600.00 | 1630.27 | 7.86 | −1.89 | 0.47 | 0.14 | 18.00 |

GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; *N*, total number of replicate observation.

### 3.4. Precision and accuracy

The precision and accuracy of three QC samples are presented in Table 3. The between-run and within-run precision for the various concentrations ranged from 0.14% to 1.12% and 0.47% to 4.56%, respectively. At the same concentrations, the values for accuracy were between −10.86% and +5.46%.

### 3.5. Recovery

Six replicates of three QC samples were prepared for recovery determination. Mean (%CV) extraction recovery for ranolazine at 15, 1000 and 1600 ng/ml was 82.34% (6.32), 86.21% (1.87) and 93.25% (1.06), respectively. Mean (%CV) recovery of internal standard (250 ng/ml) was 84.72% (3.84).

### 3.6. Matrix effect

In our study, the matrix effect was evaluated by analyzing three batches of QC samples (QC-low, QC-medium and QC-high) for all the three analytes. Average matrix effect values obtained were <8% for all the three analytes and IS. No significant peak area differences were observed.

### 3.7. Stability

Each stability test included six replicates of three levels of QC samples. All stability results, as well as the linear regression correlation coefficients of calibration curves generated from each stability test run for the analyte are presented in Table 4. QC samples undergoing three freeze–thaw cycles gave %CV ≤5.21 and an

**Table 4**
Short-term and long-term stability data[a].

| Storage condition | Low-QC (15 ng/ml) | Medium-QC (1000 ng/ml) | High-QC (1600 ng/ml) | $r^2$ |
|---|---|---|---|---|
| 3 freeze/thaw cycle | 93.11 (5.21) | 94.15 (2.87) | 98.07 (4.15) | 0.9982 |
| 24 h ambient | 92.81 (6.18) | 97.08 (4.72) | 98.14 (3.09) | 0.9979 |
| 72 h at autosampler | 96.24 (4.13) | 98.02 (3.28) | 99.28 (3.22) | 0.9984 |
| 8 h bench top | 95.62 (5.47) | 98.43 (2.65) | 99.73 (2.35) | 0.9996 |
| 1 month frozen (−20 °C) | 93.82 (5.54) | 98.18 (3.79) | 99.57 (2.07) | 0.9992 |
| 3 month frozen (−20 °C) | 93.49 (4.27) | 95.87 (3.26) | 98.83 (3.41) | 0.9967 |

[a] The data presented in this table is the percentage of measured value vs. theoretical value with CV in parentheses ($n = 6$). $r^2$ is the linearity of the calibration curve used for this treatment.

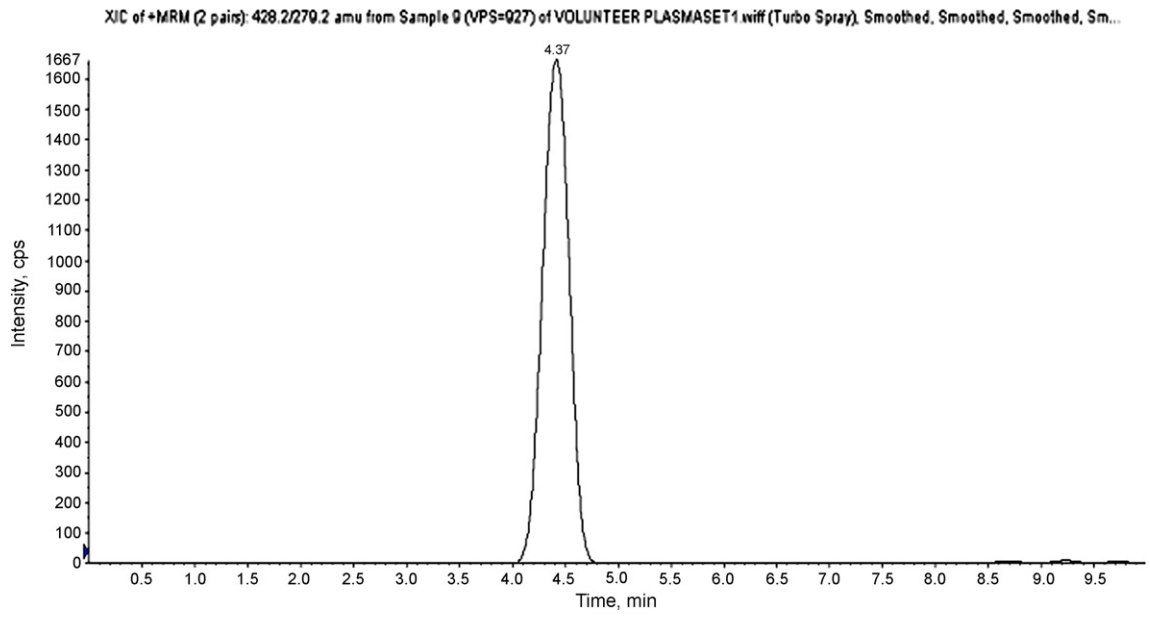


**Fig. 5.** Representative chromatogram of ranolazine in volunteer plasma sample from bioequivalence study.

accuracy of 93.11–98.07%. QC samples storing at ambient for 24 h gave ≤6.18%CV and an accuracy of 92.81–98.14%. Processed samples (ready for injection) were found to be stable for at least 72 h at 4 °C in the autosampler with %CV of ≤4.13% and accuracy of 96.24–99.28%.

Long-term frozen storage stability was tested at 1 and 3 months after QC sample pools were prepared and stored at −20 °C. The 1-month stability data of all three QC samples showed an accuracy of 93.82–99.57% with %CV ≤5.54 in comparison with their theoretical values in plasma samples. The 3-month stability data of all three QC samples had an accuracy of 93.49–98.83% with %CV ≤4.27 in plasma.

### 3.8. Application

The above-mentioned LC–MS/MS method was used in the plasma sample analysis for a bioequivalence study of ranolazine as described in Section 2.6 (Fig. 5). The mean (±S.D.) plasma level of ranolazine for test and reference preparation after the oral administration of a single dose 500 mg of ranolazine in 12 healthy human volunteers is shown in Fig. 6.

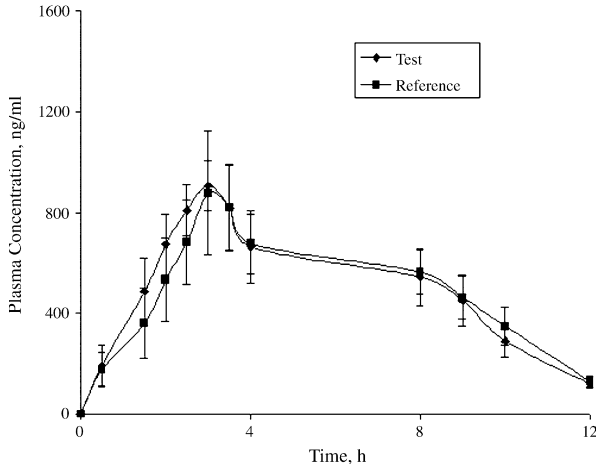


**Fig. 6.** Mean (±S.D.) plasma concentration of ranolazine following 500 mg oral dose of test and reference preparation to 12 healthy human volunteers.

## 4. Conclusions

The LC–MS/MS method described here has significant advantages over the other techniques already described in the literature [9,10]. The method has proved to be very simple, sensitive, reliable and one-step precipitation and successfully applied for the pharmacokinetic study in human plasma. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The major advantage of this method is the simple one-step precipitation for sample preparation. This new method will be extremely helpful for comparative pharmacokinetic as well as bioequivalence study of drug formulations.

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