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Short communication

Sensitive quantification of ranolazine in human plasma by liquid chromatography–tandem mass spectrometry with positive electrospray ionization

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Abstract

A rapid, selective and sensitive liquid chromatography–tandem mass spectrometry (LC–MS–MS) method with positive electrospray ionization (ESI) was developed for the quantification of ranolazine in human plasma. After liquid–liquid extraction of ranolazine and internal standard (ISTD) phenoprolamine from a 100 μl specimen of plasma, HPLC separation was achieved on a Nova-Pak C_{18} column, using acetonitrile–water–formic acid–10% *n*-butylamine (70:30:0.5:0.08, v/v/v/v) as the mobile phase. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using the transition *m/z* 428.5 → *m/z* 279.1 for ranolazine and *m/z* 344.3 → *m/z* 165.1 for the internal standard, respectively. Linear calibration curves were obtained in the concentration range of 5–4000 ng/ml, with a lower limit of quantitation (LLOQ) of 5 ng/ml. The intra- and inter-day precision values were below 3.7% and accuracy was within ±3.2% at all three quality control (QC) levels. This method was found suitable for the analysis of plasma samples collected during the phase I pharmacokinetic studies of ranolazine performed in 28 healthy volunteers after single oral doses from 200 mg to 800 mg.

Keywords: Ranolazine; Liquid chromatography; Tandem mass spectrometry

1. Introduction

Ranolazine, (±)-*N*-(2,6-dimethylphenyl)-4-[2-hydroxy-3-(2-methoxyphenoxy)propyl]-1-piperazine acetamide, is an interesting anti-anginal and anti-ischemic agent in clinical development. Unlike existing anti-ischemic agents, ranolazine has been shown to modulate the metabolism of ischemia myocardial cells and improve the efficiency of oxygen use, by increasing myocardial glucose oxidation and decreasing fatty acid oxidation [1,2].

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 [3]. 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 [3–5].

Since ranolazine lacks strong characteristic UV absorption, a HPLC–UV detection method does not provide suitable sensitivity and selectivity for the determination of ranolazine in biological samples [6]. Herron et al. [5] developed a LC–MS strategy with solid-phase extraction (SPE) procedure for estimation of ranolazine and its metabolites in human plasma, but it was not sensitive enough for pharmacokinetic studies and did not provide a detailed description of the method. Recently, two LC–MS methods with selected ion monitoring (SIM) have been published [7,8]; both of them allowed the quantitation of ranolazine in rat plasma with the lower limit of quantitation (LLOQ) above 20 ng/ml and much longer HPLC/MS analysis time (4 min or 7 min per sample).

Electrospray liquid chromatography–tandem mass spectrometry (LC–MS–MS) is currently gaining widespread acceptance among pharmaceutical scientists for the quantitation of drugs and their metabolites in biological matrices. The aim of this paper was to develop a fast and sensitive LC–MS–MS method for the determination of ranolazine in human plasma with positive electrospray ionization ((ESI(+)) in multiple reaction monitoring (MRM) mode. Following validation, this method

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was successfully applied to phase I pharmacokinetic studies of ranolazine performed in 28 healthy volunteers after single oral doses from 200 mg to 800 mg, using 0.1 ml plasma sample.

2. Experimental

2.1. Materials and reagents

Ranolazine hydrochloride was provided by Harbin Pharmaceutical Factory (Harbin, China) with the purity above 99%. The internal standard (ISTD) phenoprolamine hydrochloride (1-(2,6-dimethlphenoxy)-2-(3,4-dimethoxyphenylethylamino)propane hydrochloride) was obtained from China Pharmaceutical University (Nanjing, China) and its purity was also above 99%.

HPLC grade acetonitrile, methanol, and methyl-*tert* butyl ether were all obtained from Fisher (Fair Lawn, NJ, USA). Formic acid (98%) was purchased from Fluka (Buchs, Switzerland) and *n*-butylamine from Aldrich (Milwaukee, USA). All the reagents were used without any further purification. Deionized water was generated in-house with a Milli-Q Gradient system (Millipore, Bedford, MA, USA) and was used throughout the study.

For the validation of the method, blood samples from healthy volunteers were collected in heparinized tubes and plasma was obtained after centrifugation. Pooled drug-free plasma samples were frozen at −20 °C and used throughout the study for the preparation of calibration standards and quality control (QC) samples.

2.2. Instrumentation

An Agilent 1100 system (Wilmington, DE, USA) consisting of a vacuum degasser, a binary pump, a column oven and an autosampler was used for solvent and sample delivery. Chromatography was carried out using a Nova-Pak C_{18} column (150 mm × 3.9 mm, 5 μm, Waters, Milford, MA, USA), eluting isocratically at 1.2 ml/min with a mobile phase of acetonitrile–water–formic acid–10% *n*-butylamine (70:30:0.5:0.08, v/v/v/v). The effluent from the liquid chromatography was split post-column using a T connection in order to get a liquid flow to the TurboIonSpray interface of approximately 250 μl/min. The column temperature was maintained at 30 °C.

An Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 4000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray ionization (ESI) source was used for mass spectral analysis and the system was operated in positive mode. Optimisation of the MS conditions was carried out using a solution containing 200 ng/ml of ranolazine and the internal standard phenoprolamine, delivered via a Harvard syringe pump (Harvard Apparatus, SouthNatick, MA, USA) at a constant flow-rate of 5 μl/min. The nebulizer and TurboIonSpray gases (nitrogen) were set at 30 and 20 instrument units, respectively. The optimized TurboIonSpray voltage and temperature were set at 5000 V and 420 °C, respectively. Nitrogen was also used as curtain gas and collision cell gas, which were set at 30 and 6 instrument units, respectively. Quantitation was performed using the multiple reaction monitoring transition *m/z* 428.5 → *m/z* 279.1 for ranolazine and *m/z* 344.3 → *m/z* 165.1 for the internal standard, respectively, with a dwell time of 150 ms per transition. The optimized collision energy of 33 eV was used for the analyte and 29 eV for the internal standard. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3.

2.3. Preparation of standard and quality control solutions

Stock solutions of ranolazine and the internal standard were prepared by dissolving the accurately weighed standard compounds in methanol to give final concentrations of 1 mg/ml. Successive dilutions from this stock solution with purified water gave working standard solutions at concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 4000 ng/ml. The quality control working solutions were prepared at three different concentration levels, low level (10 ng/ml), middle level (200 ng/ml) and a high level (2000 ng/ml). The extraction solvent containing 10 ng/ml ISTD was prepared by diluting ISTD stock solution with methyl-*tert* butyl ether.

The working solutions (0.1 ml) were used to spike blank plasma (0.1 ml) either for calibration curves or for QC samples in prestudy validation and during the pharmacokinetic study.

All the solutions were stored at 4 °C and were brought to room temperature prior to use.

2.4. Sample preparation

An aliquot of plasma (0.1 ml) was mixed with 0.1 ml purified water and 0.2 ml of 0.5 M Na_2CO_3, then extracted with 2 ml methyl-*tert* butyl ether (containing 10 ng/ml ISTD) for 3 min. The organic and aqueous phases were separated by centrifugation at 3000 × *g* for 10 min. The upper organic phase was transferred to another glass tube and was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 400 μl of the mobile phase, a 10 μl aliquot was injected onto the LC–MS–MS system for analysis.

2.5. Data acquisition and analysis

Data were collected and analyzed by Analyst 1.3.1 software (Applied Biosystems MDS Sciex). Calibration of analyte was done by establishing a linear regression function after $1/x^2$ weighting of the analyte/ISTD peak area ratio versus analyte concentration relationship. Drug concentrations for the unknown and QC samples were calculated by interpolation from the calibration curves prepared in the same analysis run.

2.6. Method validation

The selectivity of the method was measured by analysis of six blank plasma samples of different origin for interference at the retention times of the analyte and ISTD. The selective determination of ranolazine was illustrated by analysis of two MRM transitions characteristic of the analyte and ISTD.

In order to assess the intra- and inter-day precision and accuracy, complete analytical runs were performed on the same day and on four consecutive days. Each analytical run consisted of a matrix blank, a set of calibration standards, six replicate LLOQ samples, and a set of low, medium and high concentration QC samples. Concentrations for the QC samples were calculated by reference to the calibration curve generated from the calibration standards. The LLOQ was defined as the concentration of the lowest concentration standard in the calibration curve that was analyzed with accuracy within ±15% and a precision ≤15%. During routine analysis each analytical run included a matrix blank, a set of calibration samples, a set of QC samples in duplicate and unknowns.

The extraction recoveries of ranolazine were determined at three QC levels by comparing the analyte/ISTD peak area ratios in spiked samples with the peak area ratios of samples that had the analyte spiked post-extraction. The internal standards were added to both sets of samples post-extraction.

Stability tests were performed for analyte-spiked plasma samples under various conditions (four freeze-thaw cycles; storage at room temperature for 24 h) by analyzing six replicates at low, medium and high QC concentrations.

3. Results and discussion

3.1. LC–MS–MS

The fast HPLC separation was achieved in a total runtime of 2.0 min on a Nova-Pak C_{18} column, using a mobile phase of acetonitrile–water–formic acid–10% *n*-butylamine (70:30:0.5:0.08, v/v/v/v). The addition of the two modifiers was a critical factor in achieving good chromatographic peak shape and keeping the analyte and the internal standard at suitable retention time (1.1 min and 1.4 min, respectively) (Fig. 1). It was found that the presence of formic acid in the mobile phase improved the intensity of the analyte response under ESI conditions. The addition of *n*-butylamine effected the retention time of the analyte and the internal standard significantly, we observed a shift in the retention time of ranolazine from 5.5 min to 1.1 min by the addition of 80 μl 10% *n*-butylamine in 100 ml mobile phase.

APCI was also investigated in the analysis but provided no sensitivity advantages over electrospray. The positive ion electrospray mass spectra of ranolazine and the internal standard in the full scan Q1 mode both showed the protonated molecular ion $[M+H]^+$ as the base peak, *m/z* 428.5 for ranolazine and *m/z* 344.3 for the internal standard. By increasing the collision energy, the fragmentation patterns of the protonated molecular ions were observed. The product ion mass spectra of ranolazine and the internal standard were shown in Fig. 2, in which the most intense product ions were observed at *m/z* 279.1 for ranolazine and *m/z* 165.1 for the internal standard. These fragmentation schemes are shown in Fig. 2.

3.2. Method validation

3.2.1. Assay selectivity

Interference from endogenous substances was investigated by measurement of six blank plasma of different origin and this interference was minimized by the combination of sample preparation, HPLC separation and MS/MS detection. Assay

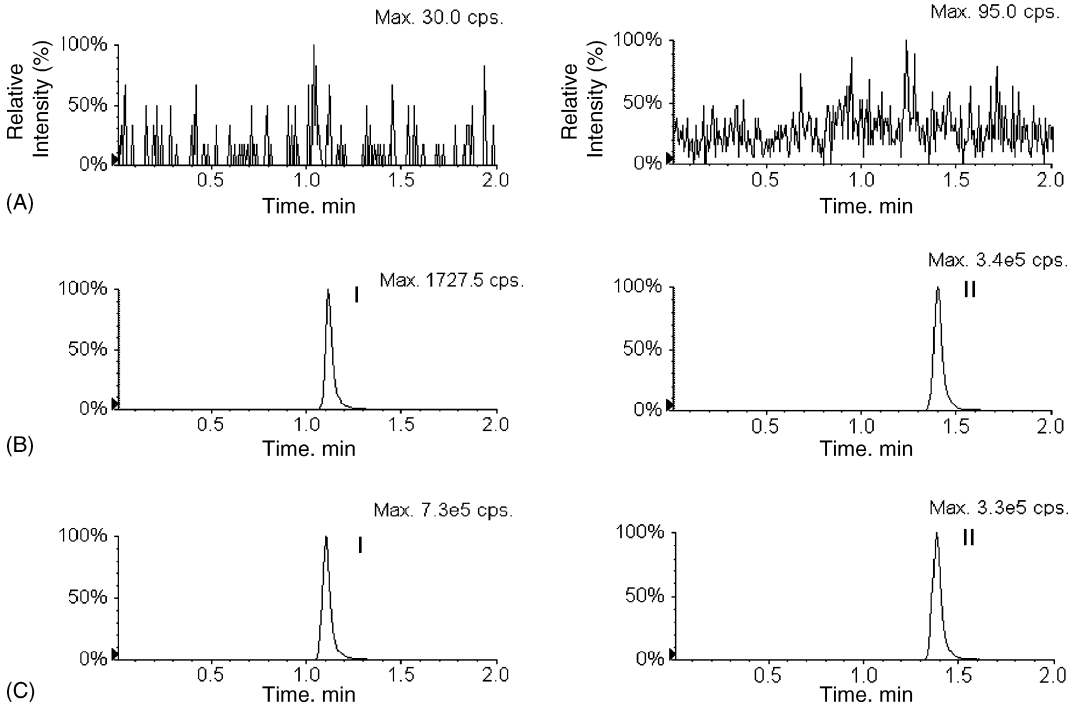

Fig. 1. Representative MRM chromatograms of (A) blank plasma sample; (B) blank plasma sample spiked with 5 ng/ml ranolazine and 200 ng/ml internal standard; and (C) a plasma sample about 1000 ng/ml from a volunteer after oral administration of 400 mg ranolazine. Peaks I and II refer to ranolazine and the internal standard, respectively.

Table 1
Back-calculated concentrations from calibration curves for LC–MS–MS determination of ranolazine

Added *C* (ng/ml)										
	5.0	10.0	20.0	50.0	100.0	200.0	500.0	1000.0	2000.0	4000.0
Back-calculated *C* (ng/ml)	5.1	9.9	19.1	52.5	96.9	218.6	518.5	1015.4	1938.0	3652.9
	5.1	10.1	18.5	47.4	91.7	205.7	491.8	1085.9	2077.2	4169.0
	5.2	9.6	19.0	48.6	99.1	203.4	523.6	1038.1	1960.7	4041.1
	5.3	9.2	19.0	50.5	98.8	205.0	512.0	1057.4	1940.8	4011.2
	5.2	9.7	18.4	48.6	99.8	214.9	498.7	1088.8	2041.3	3678.7
Mean (ng/ml)	5.2	9.7	18.8	49.5	97.3	209.5	508.9	1057.1	1991.6	3910.6
S.D. (ng/ml)	0.1	0.3	0.3	2.0	3.3	6.8	13.4	31.4	63.7	231.4
R.S.D. (%)	1.4	3.4	1.8	4.0	3.4	3.2	2.6	3.0	3.2	5.9
Recovery (%)	103.0	97.1	94.0	99.1	97.3	104.8	101.8	105.7	99.6	97.8

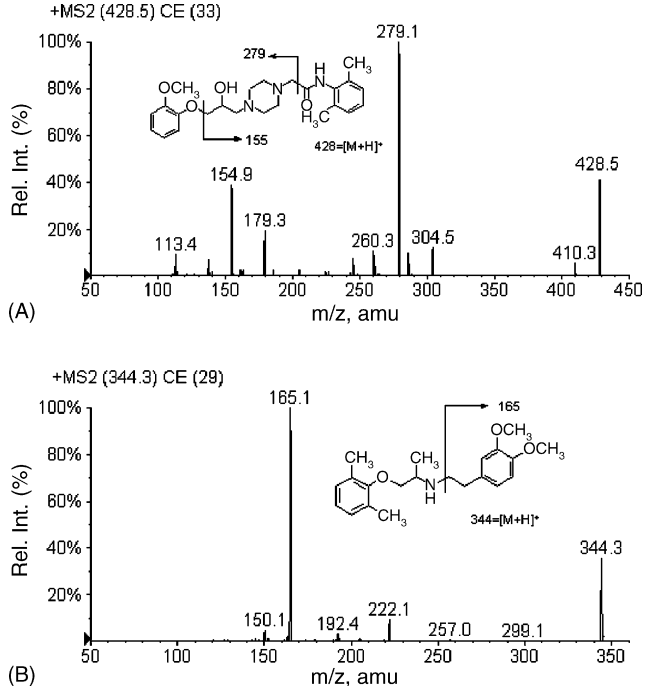

Fig. 2. Positive ion ESI mass spectra of (A) ranolazine and (B) phenoprolamine (ISTD) with each protonated molecule $[M+H]^+$ as precursor ion.

selectivity was confirmed by the absence of interfering peaks at the retention times of ranolazine and the internal standard (Fig. 1).

Ion suppression due to co-eluting substances was also investigated by comparing the peak areas from standards added to six extracted blank plasma samples of different origin with those of the corresponding standard solutions. No significant matrix effect was observed for ranolazine and the internal standard.

3.2.2. Linearity of calibration curve and lower limit of quantitation

Linear calibration curves with correlation coefficients greater than 0.995 were obtained over the concentration range of 5–4000 ng/ml for ranolazine in human plasma. Results of five representative calibration curves for determination of ranolazine are given in Table 1. The current assay had a lower limit of quantitation of 5 ng/ml ($n=6$) with signal-to-noise ratio above 100.

Despite the success of electrospray for quantitative analysis, the technique does have certain limitations. One such fundamental problem is limited dynamic range. In this study, it was found that the response no longer increased in a linear fashion with concentration above 6000 ng/ml. Experiments by Bruins indicated that the limited dynamic range was caused by an inefficiency of ionization of droplet being converted to gas-phase ions [9].

3.2.3. Precision, accuracy and extraction recovery

Data for intra- and inter-day precision and accuracy of the assay are summarized in Table 2. The intra- and inter-day precision were less than 4% for each QC level of ranolazine. The accuracy, expressed in the relative error (RE), was within ±3% at all three QC levels.

The mean extraction recoveries of ranolazine were 72.5 ± 1.4%, 69.1 ± 0.9%, and 75.8 ± 2.5% at concentrations of 10 ng/ml, 200 ng/ml, and 2000 ng/ml, respectively. The extraction recovery of the internal standard was 70.6 ± 6.4%.

3.2.4. Stability

Plasma samples spiked with ranolazine extracted and allowed to stand in reconstituted solutions at room temperature for 24 h showed no sign of degradation when compared with freshly prepared extracts. The analyte was also shown to be stable after four

Table 2
Precision, accuracy and LLOQ results for the determination of ranolazine in human plasma ($n=5$ day, six replicates per day)

Added *C* (ng/ml)	Found *C* (ng/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
10.0	9.7	3.4	3.6	−3.2
200.0	202.7	2.5	3.5	1.8
2000.0	1945.0	2.8	3.7	−2.8
5.0	4.9	5.2	6.3	−2.6

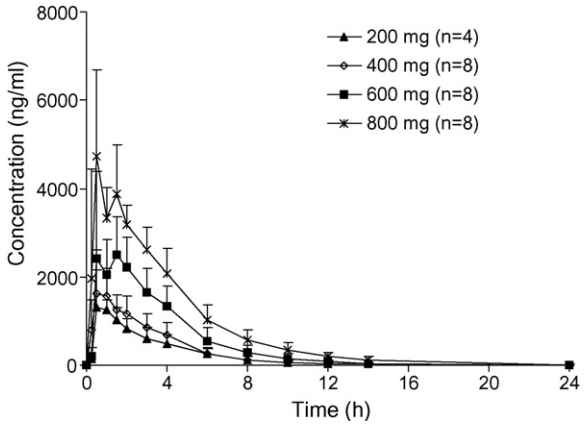

Fig. 3. Mean plasma concentration–time profiles of ranolazine after a single oral dose of 200 mg, 400 mg, 600 mg or 800 mg of ranolazine hydrochloride to healthy volunteers.

freeze-thaw cycles. The REs for the three QC levels were ranged from −1.6% to 3.8%.

3.3. Assay application

The method described above was successfully applied to phase I pharmacokinetic studies of ranolazine performed in 28 healthy subjects after single oral doses from 200 mg to 800 mg. Plasma concentration-time curves of ranolazine after administration (Fig. 3) show a peak plasma concentration (C_{max}) was achieved after 1 h and the plasma elimination half-life varied from 2.5 h to 3.1 h for the four dosage groups. There was no detectable difference in dose-normalized C_{max} and $AUC_{0–24}$, which indicated dose proportionality of ranolazine in the dosage levels of 200–800 mg.

4. Conclusion

This research outlines a sensitive, selective and reproducible LC–MS–MS method that has been validated for the determination of ranolazine in human plasma with a lower limit of quantitation of 5 ng/ml. The fast analysis has a total run time only 2 min per sample. This approach shows much higher throughput than previous reports [6], and is amenable for high-throughput analysis of large sample batches. The method described has been shown to be successfully applied to phase I pharmacokinetic studies in healthy subjects.

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