Determination of Ranolazine in Human Plasma by Liquid Chromatographic–Tandem Mass Spectrometric Assay

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Abstract

A highly sensitive liquid chromatographic–tandem mass spectrometric method (LC–MS–MS) is developed to quantitate ranolazine in human plasma. The analyte and internal standard tramadol are extracted from plasma by liquid–liquid extraction using diethyl ether–dichloromethane (60:40 v/v), and separated on a Zorbax extend C_{18} column using methanol–10mM ammonium acetate (60:40 v/v, pH 4.0) at a flow of 1.0 mL/min. Detection is carried out by multiple reaction monitoring on a QtrapTM LC–MS–MS system with an electrospray ionization interface. The assay is linear over the range 10–5000 ng/mL with a limit of quantitation of 10 ng/mL and a lower limit of detection (S/N > 3) of 1 ng/mL. Intra- and inter-day precision are < 3.1% and < 2.8%, respectively, and the accuracy is in the range 96.7–101.6%. The validated method is successfully used to analyze the drug in samples of human plasma for pharmacokinetic studies.

Introduction

Ranolazine (RS-43285;*N*-[2,6-dimethylphenyl]-2-[4-{2-hydroxy-3-2-methoxyphenoxy}propyl]piperazin-1-yl]acetamide) is a novel anti-anginal drug. Clinical trials show that ranolazine reduces the frequency of anginal attacks and increases exercise capacity in patients with chronic angina (1). The pharmacokinetic profile of ranolazine is best described as 1-compartment, first-order absorption and linear saturable elimination. Ranolazine has an oral bioavailability of 30% to 50%, and is extensively metabolized in the liver by the cytochrome P450 system, with less than 5% of the dose excreted unchanged by the kidneys (2). Recent results indicate that ranolazine is an inhibitor of late sodium channel current (late INa) in cardiac myocytes. Selective inhibition of late INa relative to peak INa by ranolazine reduces sodium entry into myocytes during the action potential plateau, thereby reducing Na^+/Ca^+ exchange and attenuating the effect of ischemia resulting in an elevation of the intracellular calcium concentration (3–5).

A recent survey revealed that many quantitation methods were available for the determination of ranolazine in biological samples. In 1995, Herron et al. reported a method using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry for the estimation of ranolazine and its metabolites (6), but it required a more complex solid-phase extraction procedure and a large volume of plasma, and its sensitivity was insufficient for pharmacokinetic studies. To overcome these problems, two new methods have been published for the analysis of ranolazine in biological samples, based on liquid chromatography (LC) coupled with atmospheric pressure chemical ionization mass spectrometry (MS) and LC coupled with electrospray ionization (ESI) tandem MS with improved specificity, efficiency, and sensitivity (7,8). Although both were sensitive, the analysis times (4.2 min or 5.8 min per sample) were much longer. Recently, Tian et al. developed an LC–MS–MS method for the determination of ranolazine in human plasma, in which the lower limit of quantitation was 5 ng/mL and the analysis time was 2.0 min (9). To our knowledge, no report has been mentioned in the literature for the stability of ranolazine during sample preparation. In our study, we found that ranolazine is unstable after being evaporated to dryness following liquid–liquid extraction.

The present study reports the development and validation of a simple, sensitive, and specific LC–MS–MS method for the determination of ranolazine in human plasma. The method includes an improved procedure for liquid–liquid extraction to make the results more reliable. At the same time, this method is efficient for analyzing the large number of plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies after administering therapeutic doses of ranolazine.

Experimental

Chemicals and reagents

Ranolazine hydrochloride (purity > 99.0%) and tramadol hydrochloride [internal standard (IS), purity > 99.0%] were pur-

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chased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ), and heparinized blank (drug-free) human plasma was obtained from Changchun Blood Donor Service (Changchun, China). All other chemicals were analytical grade and used without further purification. Distilled, demineralized water was produced by a Milli-Q Reagent Water System (Milford, MA).

Preparation of standard solutions

Stock solutions of ranolazine and tramadol (both 1 mg/mL) were separately prepared in 10-mL volumetric flasks with methanol and stored at 4ºC. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol–water (50:50, v/v). The I.S. working solution (10 ng/mL) was prepared by diluting its stock solution with methanol–water (50:50, v/v). Calibration curves were prepared using blank plasma spiked at concentrations of 10, 30, 100, 300, 1000, 2000, and 5000 ng/mL. Low, medium, and high quality control (QC) samples (30, 300, and 4000 ng/mL) were prepared in a similar way by a separate weighing.

Instrumentation and conditions

The LC–MS–MS system consisted of an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) coupled to an Applied Biosystems Sciex Q-trap MS (Applied Biosystems Sciex, Ontario, Canada) equipped with a TurboIonSpray source for ion production.

Isocratic chromatography was carried out on a 150 × 4.6 mm, 5 µm Zorbax extend C_{18} column maintained at 25ºC using a mobile phase of methanol–10mM ammonium acetate (60:40, v/v) adjusted to pH 4.0 using formic acid. The column effluent was split so that approximately 0.5 mL/min entered the MS. The electrospray interface heater was set to "on" mode and the IonSpray Voltage was set at 1300 V for positive ionization. The nitrogen curtain gas was adjusted to a constant value of 20 units, and Gas 1 and Gas 2 were set to 35 and 75 units, respectively. The MS parameters were optimized to obtain maximum sensitivity without compromising the selectivity at unit resolution. The multiple reaction monitoring (MRM) experiments were conducted by monitoring the precursor ion to product ion transitions in the positive ion mode for ranolazine from *m/z* 428.3 (Q1) to *m/z* 98.1 (Q3) with a declustering potential (DP) of 45 eV and a collision energy (CE) of 50 eV, and for tramadol from *m/z* 264.1 (Q1) to *m/z* 58.0 (Q3) with a DP of 29 eV and a CE of 40 eV. The dwell time was set at 200 ms. Data acquisition and integration were controlled by Applied Biosystems Analyst Software 1.3.

Sample preparation

Liquid–liquid extraction of mixtures of thawed plasma (50 µL) and I.S. working solution (50 µL) was carried out by shaking with 2 mL diethyl ether–dichloromethane (60:40 v/v) for 10 min. After centrifugation at 2000 g for 5 min, the organic phase was transferred to another tube containing 120 µL of 10mM ammonium acetate with addition of 1% formic acid and then evaporated at 40ºC under a gentle stream of nitrogen. After the solution became clear, demonstrating that the organic phase was evaporated completely, 180 µL of methanol were added. After a vortexing period of 1 min, a 10 µL aliquot of solution was injected into the LC–MS–MS system for analysis.

Assay validation

The method validation assays were performed according to the currently accepted US Food and Drug Administration bioanalytical method validation guide (10). To evaluate the linearity, the calibration curves were generated using the analyte to I.S. peak area ratios by weighted (1/x2) least-squares linear regression on three consecutive days. QC samples at three concentration levels (30, 300, and 4000 ng/mL) were analyzed to assess the accuracy and precision of the method. Again, the assays were performed on three separate days, and on each day six replicates of the QC samples at each concentration level were analyzed. The assay accuracy was calculated as relative error. The assay precision for each QC level was determined as the relative standard deviation (RSD) of the measured concentrations. The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ± 15%, except at the limit of quantitation (LOQ), where precision should be below 20% and accuracy within ± 20%. The lower limit of detection was determined as the concentration with a signal-to-noise ratio of 3. The recovery of ranolazine was evaluated by comparing peak area ratios of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts. The matrix effect (ME) of the assay was evaluated by comparing the peak area ratios of analytes resolved in the

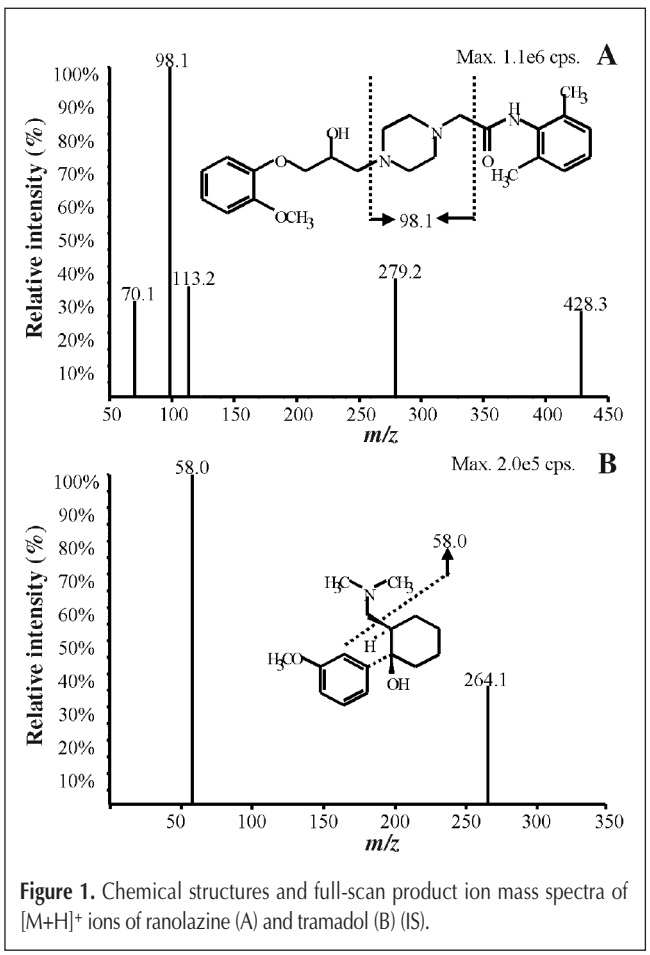

Figure 1. Chemical structures and full-scan product ion mass spectra of $[M+H]^+$ ions of ranolazine (A) and tramadol (B) (IS).

reconstituted solution of the blank plasma extracts (A) with those resolved in the mobile phase (B). The ME was calculated by using the formula ME (%) = A/B × 100%.

Stability in plasma was assessed in the autosampler at room temperature for 12 h and on storage at –20ºC for 30 days. The effect of three freeze-thaw cycles was also investigated.

Results and Discussion

MS

Tandem MS with ESI source detection was used to provide a sensitive and selective assay for ranolazine and tramadol in human plasma. The structures and positive ESI mass spectra of ranolazine and tramadol are shown in Figure 1. MRM was performed at unit resolution using the mass transition ion-pairs *m/z* 428.3 → 98.1 for ranolazine and *m/z* 264.1 → 58.0 for tramadol, respectively.

Chromatographic conditions

HPLC conditions were optimized to improve the HPLC separation and enhance sensitivity. The inclusion of 10mM ammonium acetate instead of pure water reduced matrix effects without decreasing response. Peak shape was improved by using formic acid to adjust the mobile phase pH to 4.0. Further improvement in peak shape with reduced cycle time was achieved by increasing the flow rate. With a flow rate of 1.0 mL/min (split), the cycle time was 2.5 min.

Sample preparation

In our study, we found that ranolazine was unstable if the extraction solvent diethyl ether–dichloromethane (60:40 v/v) was completely dried by a stream of nitrogen. Approximately 5 min after evaporating to dryness, approximately 20% of the ranolazine was lost. It was also difficult to consistently control the time required to evaporate each sample to dryness. To overcome these problems, 120 µL 10mM ammonium acetate with 1% formic acid were added to the extract to prevent loss of ranolazine during evaporation. After the organic phase was evaporated completely, the residues (aqueous solution) was diluted with 180 µL methanol, resulting in a composition of the sample which is closer to the composition of the mobile phase, thereby avoiding solvent focusing effects upon injection for MS analysis without affecting peak symmetry and retention.

Assay specificity, precision, and accuracy

As shown in Figure 2, no endogenous peaks were observed at the retention times of the analyte and the I.S. in the chromatogram of blank plasma, demonstrating the selectivity and specificity of the MRM technique. The assay was linear over the concentration range 10–5000 ng/mL (r > 0.996). Intra- and inter-day precision were 1.9–3.1% and 1.8–2.8%, respectively, and accuracy was –3.27–1.6% (Table I).

Extraction recovery and matrix effect

The recoveries of ranolazine at 30, 300, and 4000 ng/mL were 71.6%, 67.0%, and 69.5%, respectively. The recovery of the I.S. was 88.5%. The matrix effect of the assay was evaluated at 30, 300, and 4000 ng/mL for ranolazine. Three samples at each level were analyzed. The percent nominal concentrations determined were 96.9 ± 5.6%, 95.7 ± 6.2%, and 99.9 ± 4.3% at each concentration level. The same evaluation was performed for the I.S. and the percent nominal concentration was 96.0 ± 7.4%. The results indicate that ion suppression or enhancement from the plasma matrix was negligible for this analytical method.

Processed sample stability

Table II summarizes the data from the short-term, freeze/thaw, and long-term stability tests for ranolazine. The short-term test indicated reliable stability behavior under the experimental conditions of the analytical runs. The results of the freeze/thaw stability test indicated that the analytes were stable in human plasma for three cycles when stored at –20°C and thawed to room temperature. The findings from the long-term test indicate that storage of plasma samples containing

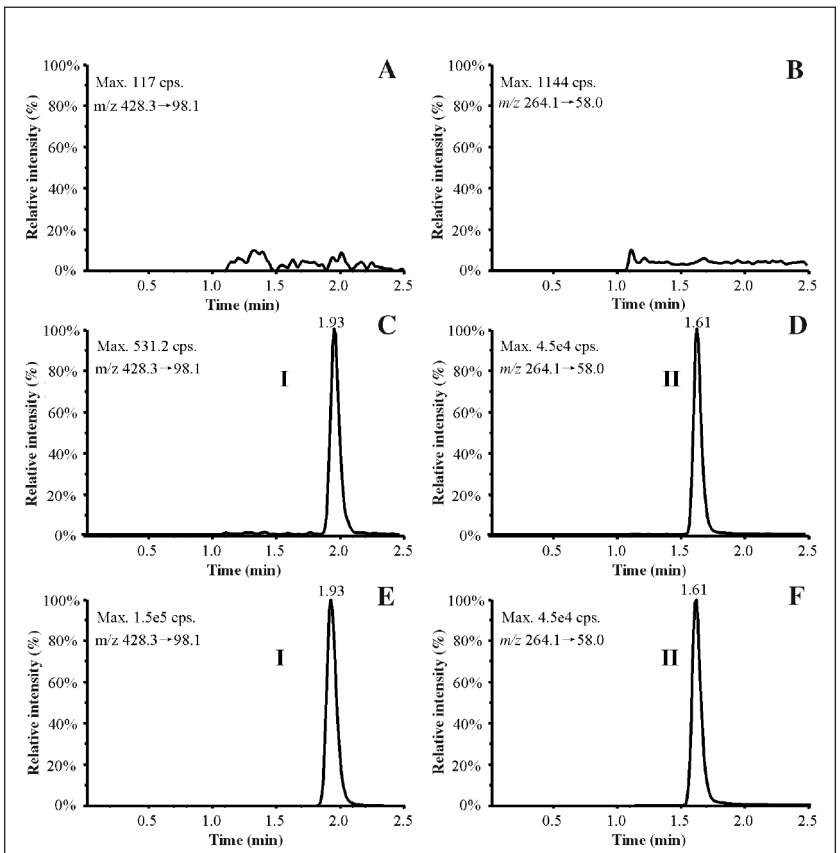

Figure 2. Representative LC–MRM chromatograms for ranolazine (I) and tramadol (I.S., II) in human plasma samples: a blank plasma sample (A,B); a blank plasma sample spiked with ranolazine (10 ng/mL) (C) and I.S. (10 ng/mL) (D); and a human plasma sample 1.0 h after an oral administration of ranolazine 400 mg (E,F).

ranolazine at –20°C is adequate when maintained for 30 days. Thus, no stability-related problems are expected during routine analyses for the PK study.

Application

The validated method has been successfully used to quantitate the ranolazine concentration in the human plasma samples after the administration of a single 400 mg oral dose of ranolazine. The representative concentration vs. time profiles of six healthy volunteers receiving a single dose of ranolazine are presented in Figure 3.

Table I. Precision and Accuracy for the Determination of Ranolazine in Human Plasma*

Nominal conc. (ng/mL)	Mean found conc. (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Relative error (%)
30	30.5	3.13	1.81	1.61
300	301.8	2.60	2.82	0.59
4000	3869	1.91	2.70	–3.27

* Data are based on assay of 6 replicates on 3 different days.

Table II. Stability Data of Ranolazine in Human Plasma*

Storage conditions	Nominal conc. (ng/mL)	Mean found conc. (ng/mL)	Relative error (%)
Freezing for 30 days at –20°C	30	30.8	2.6
	300	298.3	–0.6
	4000	3827	–4.3
Three freeze/thaw cycles	30	32.8	4.8
	300	300.3	0.1
	4000	3753	–6.2
Autosampler stability at room temperature for 12 h (after extraction and reconstitution)	30	29.4	–4.3
	300	292	–4.6
	4000	3771	–3.1

* Three samples each concentration.

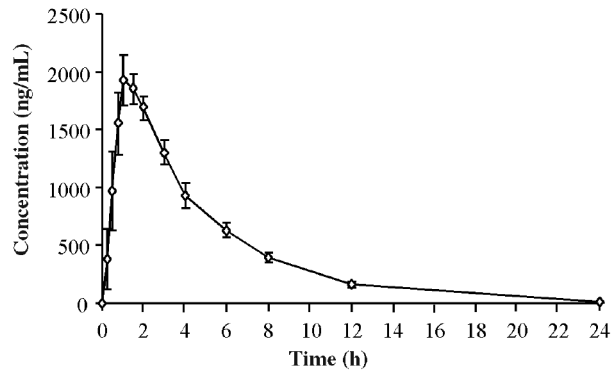

Figure 3. Plasma concentration-time profile of ranolazine after administration of a single oral 400 mg dose of ranolazine. Data are mean ± S.D. for 6 healthy volunteers.

Conclusions

A stable, high throughput, and sensitive LC–MS–MS method is reported for the determination of ranolazine in human plasma. Acceptable precision and accuracy were obtained for concentrations within the standard curve range of 10–5000 ng/mL. The method is flexible and requires only 50 µL of plasma, making it suitable for pharmacokinetics studies of ranolazine.

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