Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 848–852

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Determination of tropisetron in human plasma by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 16 September 2008
Received in revised form 19 December 2008
Accepted 26 December 2008
Available online 8 January 2009

Keywords:
Tropisetron
Liquid chromatography–tandem mass spectrometry
Pharmacokinetics
Liquid–liquid extraction
Human plasma

ABSTRACT

A sensitive and selective liquid chromatography–tandem mass spectrometry method (LC–MS/MS) for the determination of tropisetron in human plasma was developed and validated over the concentration range of 0.100–100 ng/mL. Diphenhydramine was used as the internal standard (IS). The tropisetron and the IS were extracted from alkalized plasma samples into diethyl ether–dichloromethane (2:1, v/v) and the LC separation was performed by a Diamonsil C_{18} column (150 mm × 4.6 mm, i.d., 5 μm). The mobile phase was methanol:water (80:20, v/v) containing 0.2% formic acid delivered at a flow rate of 0.5 mL/min. The total chromatographic run time was 4.5 min. The MS data acquisition was accomplished by selected reaction monitoring (SRM) mode with positive atmospheric pressure chemical ionization (APCI) interface. The lower limit of quantification (LLOQ) achieved was 0.100 ng/mL with precision (RSD) of 3.1% and accuracy (RE) of −0.7%. For both inter-batch and intra-batch tests, the precision (RSD) for the entire validation was less than 6.0%, and the accuracy (RE) was within the −0.5% to 0.2% range. This validated LC–MS/MS method was later used to characterize the pharmacokinetics as well as the bioequivalence of tropisetron formulations.

1. Introduction

Tropisetron is a potent and selective 5-hydroxytryptamine (5-HT_3) receptor antagonist. It is used to treat patients with chemotherapy-induced or postoperative nausea and vomiting [1]. Tropisetron was extensively metabolized by oxidative hydroxylation of the indole ring with subsequent conjugation [2,3], and these metabolites are all inactive [3]. It has been reported that in human plasma $AUC_{0-24\,h}$ pools, the 5-glucuronide was the major metabolite, followed by the 6-hydroxy tropisetron and its sulfate conjugate, and the proportion of unchanged tropisetron of the total radioactivity in plasma AUC was 21% after the 20 mg dose of [^{14}C] tropisetron [2]. The oxidation was primarily mediated by the polymorphic CYP2D6 and CYP1A2 [4]. This can cause a significant variability for different individuals in their pharmacokinetics and clinical response after taking tropisetron. The pharmacogenetic study and the plasma drug levels monitoring serve as tools to determine the optimal dosage for each patient. As a result, there is a strong demand to develop fast, simple and sensitive quantitation methods for the determination of tropisetron in human plasma during the drug treatment.

Several methods have been reported for the quantification of tropisetron in biological samples. These methods were all based on high performance liquid chromatography (HPLC) separation with fluorescence detection [3], MS detection [5] or more frequently, ultraviolet (UV) detection [6–9]. Among these methods, only two reports [7,9] provided complete validation results. All other methods simply described either a pharmacokinetic study [3,6,8] or a drug metabolism study [5]. Kees et al. [3] reported a pharmacokinetic study of tropisetron using a HPLC-fluorescence method, the LLOQ reported with this method was 0.075 ng/mL. This was accomplished with long chromatographic run time (about 9.5 min) and large volume of biological sample consumption (1.5 mL human plasma). The LLOQ reported of all other methods were all higher than 1.25 ng/mL.

The high sensitivity and selectivity of tandem mass spectrometry (MS/MS) have led to a growing trend of developing fast analytical methods. These methods require very small sample volume and short chromatographic cycle time. In this paper, a sensitive LC–MS/MS method for the determination of tropisetron in human plasma using one-step liquid–liquid extraction (LLE) was developed and validated. This robust method allowed tropisetron in plasma level to be determined with acceptable accuracy and precision to support a variety of pharmacokinetic and bioequivalence studies of tropisetron.

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doi:10.1016/j.jpba.2008.12.038

2. Experimental

2.1. Materials

Tropisetron hydrochloride (99.9% purity) was supplied by Xi'an Guantong Technology Co., Ltd. (Shaanxi, China). Diphenhydramine hydrochloride (99.7% purity) purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) was used as the internal standard. Commercial available tropisetron hydrochloride capsules (4.44 mg base per capsule) were manufactured by Beijing Shuanglu Pharmaceutical Co., Ltd. (Beijing, China). Acetonitrile and methanol (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Formic acid was obtained from Tedia (Fairfield, OH, USA). All the other chemicals used were of analytical grade. Blank heparinized (drug free) human plasma was obtained from Shanghai Shuguang Hospital (Shanghai, China).

2.2. Preparation of standard and quality control (QC) samples

Two stock solutions of tropisetron were prepared separately by dissolving accurately weighed reference standard in methanol to give a final concentration of 400 μg/mL (calculated as the free base). These two stock solutions were used for calibration standards and QC standards, respectively. The solutions were serially diluted with methanol:water (50:50, v/v) to obtain the working solutions at concentrations over 1.25–1250 ng/mL. A stock solution of diphenhydramine (calculated as the free base) at 400 μg/mL was also prepared in methanol and then diluted with methanol:water (50:50, v/v) to obtain a working solution at 80.0 ng/mL. All of the solutions were stored at 4 °C up to 1 month and were brought to room temperature before use.

The analytical standard and QC samples were prepared by spiking blank heparinized human plasma (250 μL) with standard working solutions (20 μL) during validation and during each experiment for the pharmacokinetic study. Calibration samples were made at concentrations of 0.100, 0.200, 0.800, 3.00, 8.00, 20.0, 50.0 and 100 ng/mL. QC samples were prepared at the concentrations of 0.200, 8.00 and 80.0 ng/mL. The analytical standards and QC samples were stored at −20 °C.

2.3. Sample preparation

Frozen plasma samples were thawed at room temperature and vortexed thoroughly. To a 250 μL aliquot of the plasma sample, 20 μL of methanol:water (50:50, v/v) solution, 100 μL of internal standard working solution (80.0 ng/mL diphenhydramine) and 500 μL of phosphate buffer (10 mM, pH 10) were added. This sample solution was extracted with 3 mL of diethyl ether–dichloromethane (2:1, v/v) by vortex mixing for 1 min and shaking for 10 min. After centrifugation at 2000 × *g* for 5 min, the upper organic layer was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of air. The residue was then dissolved with 250 μL of mobile phase and vortexed briefly. A 20 μL aliquot of the resulting solution was injected onto the LC–MS/MS system for analysis.

2.4. Liquid chromatographic and mass spectrometric conditions

A Shimadzu LC-10AD pump (Kyoto, Japan) was used for solvent delivery. Chromatographic separation was accomplished with a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Dikma, Beijing, China). A SecurityGuard C_{18} column (4 mm × 3.0 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA) was used as the guard column. The analytical separation was performed at ambient temperature (~25 °C). The separation was carried out under an isocratic condition with a mobile phase of methanol:water (80:20, v/v) containing 0.2% formic acid at a flow rate of 0.5 mL/min. A Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA, USA) was used as the detector with an atmospheric pressure chemical ionization (APCI) source. The mass spectrometer was operated in the positive ion detection mode. The corona discharge current was set at 4.0 μA. Nitrogen was used as the sheath gas (35 Arb) and auxiliary gas (8 Arb). The temperatures of the vaporizer and the heated capillary were 450 and 300 °C, respectively. For collision-induced dissociation (CID), argon was used as a collision gas at a pressure of 1.0 mTorr. Quantitation was performed using selected reaction monitoring (SRM) of the transitions of m/z 285 → m/z 124 for tropisetron and m/z 256 → m/z 167 for the IS, with a scan time of 0.2 s per transition. The collision energy (CE) of 30 eV was used for both tropisetron and IS.

2.5. Method validation

The selectivity of the method was evaluated by analyzing six samples of blank plasma and six samples of spiked plasma at LLOQ level from different sources. The SRM chromatograms of blank plasma samples were compared with those at LLOQ. Peak area of endogenous compounds coeluting with the analyte needs to be less than 20% of the peak area of the LLOQ standard.

To evaluate the matrix effect, six different lots of blank plasma were extracted and then spiked with the analyte at 0.200 and 80.0 ng/mL. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the absolute matrix effect (ME). The assessment of the relative ME was made by a direct comparison of the analyte peak-area values between different lots of plasma. The variability in the values, expressed as RSD (%), is a measure of the relative ME for the analyte. The matrix effect of IS was evaluated at the working concentration (80.0 ng/mL) in the same manner.

Calibration curves were constructed by analyzing spiked calibration samples (each concentration in duplicate) on three separate days. Peak area ratios of tropisetron to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) in the concentration range of 0.100–100 ng/mL. Concentrations were calculated using the ratio of the peak area of the analyte to that of IS.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (low, 0.200 ng/mL; medium, 8.00 ng/mL; high, 80.0 ng/mL) in three validation days. The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE). The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%, except at the lower limit of quantification (LLOQ), where precision should be below 20% and accuracy within ±20%.

Extraction recoveries for tropisetron at three QC levels were determined by comparing the peak area ratios of the analyte to IS in plasma samples that had been spiked with the analyte prior to extraction with plasma samples, to which the analyte had been added post-extraction. The extraction recovery of the IS was determined in a similar way using medium QC as a reference.

The stabilities of tropisetron in human plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at the concentrations of 0.200, 8.00 and 80.0 ng/mL, which were exposed to different conditions (time and temperature). The analyte was considered stable when 85–115% of the initial concentration was found. The short-term stability was determined after the exposure of the spiked samples at room temperature for 2 h, and the ready-to-inject samples (after extraction) in the HPLC autosampler at room temperature for 24 h. The freeze/thaw stability was evaluated after three

complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at −20 °C for 30 days.

2.6. Application of the method

The validated LC–MS/MS method was used in pharmacokinetic and bioequivalence studies for a variety of tropisetron formulations. In this paper, the applicability of the method was demonstrated in a pharmacokinetic study of tropisetron after a single oral dose of 5 mg tropisetron hydrochloride capsule (4.44 mg base per capsule) to 20 healthy Chinese male volunteers. The pharmacokinetic study was approved by the Medical Ethics Committee of People's Hospital of Liaoning Province. Venous plasma samples (3 mL) were collected in heparinized tubes before and at 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h after dose. Plasma samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Mass spectrometry and solvent adduct ions

In our previous study, a chiral LC–MS/MS method was developed to determine ondansetron enantiomers in human plasma using tropisetron as the IS [10]. An electrospray (ESI) LC/MS interface was used for that study and high chemical background noise was observed for tropisetron. For the present study, an APCI source was used and it showed good reproducibility. Previous studies have already indicated that APCI is much less susceptible to ME compared with ESI [11,12].

The mass spectrum of tropisetron was studied under different mobile phases with positive APCI source. It was found that the organic solvent used in LC mobile phase had an effect on the formation of solvent adduct ions, which further affected the intensity of the $[M+H]^+$ ion. When the full spectrum scan of tropisetron was recorded across the range of 50–400 Da using a LC flow composed of methanol–water–formic acid, a clear response is observed at m/z 285 corresponding to the protonated molecule, $[M+H]^+$, and no adduct ions were observed. Compared with methanol, using acetonitrile as organic component in the mobile phase showed a stronger tendency to form solvent adduct ion $[M+H+CH_3CN]^+$ at m/z 326 for tropisetron.

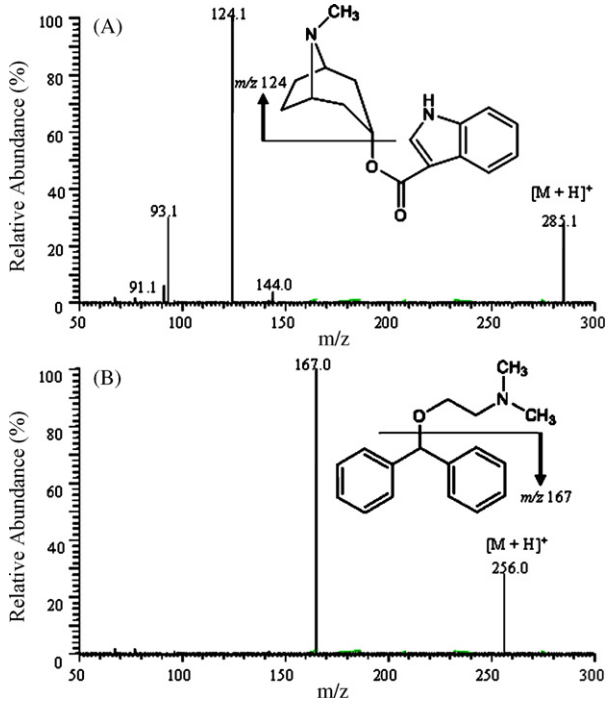

Fig. 1. Full scan product ion spectra of $[M+H]^+$ of tropisetron (A) and diphenhydramine (IS, B) dissolved in the mobile phase.

The differences in the solvent adduct ion formation of acetonitrile and methanol to form solvent adducts ions is related to their different proton affinities. Acetonitrile has a stronger proton affinity (PA = 8.2 eV) than methanol (PA = 7.9 eV) [13], and thus has higher tendency to form solvent adduct ions than methanol. This relationship is in agreement with Field's rule [14]. Understanding of the relationship between proton affinity of an organic solvent and its tendency to form solvent adduct ions is helpful in selecting appropriate LC solvent in LC–MS/MS studies. Since the formation of solvent adduct ions could decrease the abundance of $[M+H]^+$ ion, methanol was chosen over acetonitrile as organic solvent in the mobile phase in the present study.

The collision behavior of $[M+H]^+$ of tropisetron is strongly dependent on the CE, and the optimal CE for the analyte was determined by observing the response of the obtained fragment ion peaks. A major fragment ion at m/z 124 was formed at the CE of 25–30 eV (Fig. 1A), while $[M+H]^+$ ion intensity was reduced by less than 50%. At higher CE, the intensity of $[M+H]^+$ ion was reduced further and more fragment ions were formed, resulting in a weaker MS response. Thus, the SRM was performed by monitoring the transition of m/z 285 → m/z 124 for tropisetron at 30 eV. The fragment ion at m/z 124 was derived from the loss of indole-3-carboxylic acid moiety by breaking the single carbon–oxygen bond. Using similar procedures, the optimum SRM transition of the internal standard was chosen to be m/z 256 → m/z 167 (Fig. 1B).

3.2. Liquid chromatography

The chromatographic conditions were optimized to achieve maximum peak response with short chromatography run time without interfering with the ionization process of the analyte in the APCI source. Best results were obtained with a mobile phase consisting of methanol:water (80:20, v/v), and a great improvement of the peak shape and retention time was seen when 0.2% formic acid was added. A short HPLC column offers short separation time, but it also offers less separation efficiency. In addition, after multiple injections, distorted chromatographic peaks were observed with increased column back-pressure. This resulted in required column flushing routine to be applied after no more than 80 injections every day.

To improve the method ruggedness, a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm) was selected, which had much longer column life. Under the present chromatography conditions, the retention times were 3.0 and 3.8 min for tropisetron and IS, respectively, with a total run time of 4.5 min. Moreover, at least 2500 injections of prepared plasma samples were injected onto the single column without any column regeneration, and peak shape as well as retention time remained unchanged.

3.3. Sample preparation

In order to obtain a clean extract with high analyte recovery, LLE was used for the sample preparation in this work. One of the key factors for achieving high and reproducible recovery in a LLE process is the neutralization of the analyte by adjusting sample pH. Tropisetron is a weak organic base with an amino group in its structure. The pKa values for amines typically range from 8.0 to 11.0. Therefore the pH of the sample can be adjusted from 10.0 to 13.0 to allow conversion of tropisetron to its unionized form [15]. Different LLE conditions were evaluated by varying pH of the sample

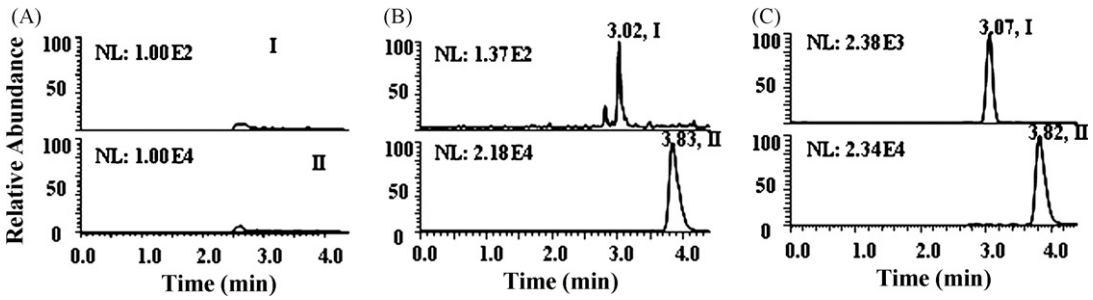

Fig. 2. Typical SRM chromatograms of (A) a blank human plasma sample; (B) a blank human plasma sample spiked with tropisetron at LLOQ (0.100 ng/mL) and IS at 80 ng/mL; (C) a human plasma sample obtained 2.0 h after a single oral administration of 5 mg tropisetron hydrochloride (4.44 mg base). Peak I, tropisetron; Peak II, IS.

using phosphate buffers, ammonia, or sodium hydroxide solution. Results showed that 10 mM phosphate buffer at pH 10 offered best reproducibility and extraction recovery.

In order to develop a single step LLE procedure with sufficient recovery, several commonly used extraction solvents, such as diethyl ether, dichloromethane, hexane, used alone or in combination at different proportions were investigated. As a different result from prior works reported by Yu et al. [9], the best extraction recovery of tropisetron after single step extraction from plasma was obtained by using relative small volume of diethyl ether:dichloromethane (2:1, v/v) (3 mL extraction solvent versus 250 μL plasma). In the end, diethyl ether:dichloromethane (2:1, v/v) extraction following pH 10 phosphate buffer solution (10 mM) alkalization was used in the sample preparation.

3.4. Method validation

3.4.1. Assay selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma samples. Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with tropisetron (at the LLOQ) and IS, and a plasma sample obtained at 2.0 h after a single oral administration of 5.0 mg tropisetron hydrochloride (4.44 mg base) to a volunteer. No interfering endogenous substances were observed at the retention times of the analyte and IS. The chromatograms presented in Fig. 2 clearly demonstrated the selectivity of this method.

The absolute ME for tropisetron at concentrations of 0.200 and 80.0 ng/mL were measured to be 102 ± 7.5 and 97.4 ± 4.7%, respectively. The relative ME for tropisetron at concentrations of 0.200 and 80.0 ng/mL were measured to be 7.3 and 4.8%, respectively. The absolute and relative ME for IS (80.0 ng/mL) were 104 ± 7.1 and 6.8%, respectively. As a result, ME from plasma was negligible in this method.

3.4.2. Linearity of calibration curve and lower limit of quantification

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 0.100–100 ng/mL for tropisetron in human plasma. Typical equation of the calibration curve was: $y = 1.36 \times 10^{-2}x + 1.02 \times 10^{-3}$, $r^2 = 0.9923$, where y represents the ratios of tropisetron peak area to that of IS and x represents the plasma concentration.

The LLOQ for the determination of tropisetron in plasma was 0.100 ng/mL. The precision and accuracy at LLOQ were 3.1% and −0.7%, respectively. This method was sensitive enough to ensure the determination of tropisetron concentrations in elimination-phase with desired accuracy and precision for actual subject samples after a single oral administration of 5 mg tropisetron hydrochloride (4.44 mg base).

3.4.3. Precision and accuracy

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days using a one-way analysis of variance (ANOVA) [16]. Intra-day precision was 6.0% or less and the inter-day precision was 4.2% or less at each QC level (0.200, 8.00 and 80.0 ng/mL).

The accuracy of the method was determined by calculating RE and the results ranged from −4.2% to 0.6% at each QC level. Assay performance data are presented in Table 1. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.4.4. Extraction recovery

Mean extraction recoveries of tropisetron were 74.5 ± 1.6%, 72.3 ± 1.9% and 77.3 ± 1.5% ($n = 6$) at concentrations of 0.200, 8.00 and 80.0 ng/mL, respectively. The extraction recovery of the IS was 74.1 ± 2.0% ($n = 6$).

3.4.5. Stability

The stability tests of the analyte were designed to cover anticipated conditions for the preservation of the clinical samples. The stability results showed that tropisetron spiked into human plasma was stable for 2 h at room temperature, for 30 days at −20 °C, and during three freeze–thaw cycles. Stability of tropisetron extracts in the sample solvent on autosampler was also observed over a 24 h period. The results of stability experiments are shown in Table 2.

3.5. Application of the method to a pharmacokinetic study in healthy volunteers

The validated analytical method was applied to the assay of tropisetron in human plasma after a single oral administration of 5 mg tropisetron hydrochloride (4.44 mg base) to healthy male volunteers. The plasma samples were processed based on the proposed extraction protocol for the quantification of tropisetron. Mean plasma concentration versus time profile is presented in Fig. 3. A high inter-individual variability in plasma concentrations was observed in this study. As a result, individualization of tropisetron therapy should be considered under clinical circumstances to ensure drug efficiency.

Table 1
Precision and accuracy data for the analysis of tropisetron in human plasma (in prestudy validation, 3 days, 6 replicates per day).

Concentration (ng/mL)	RSD (%)		RE (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.200	4.7	0.9	−4.2	0.2
8.00	6.0	4.2	0.6	0.1
80.0	3.3	2.7	−2.7	−0.5

Table 2
Summary of stability of tropisetron under various storage conditions ($n = 3$).

Condition	Concentration (ng/mL)		RSD (%)	RE (%)
	Added	Found		
Ambient, 2 h	0.200	0.207 ± 0.006	2.8	3.3
	8.00	8.87 ± 0.44	5.0	10.8
	80.0	85.6 ± 0.9	1.0	7.0
−20 °C, 30 days	0.200	0.199 ± 0.011	5.3	−0.5
	8.00	8.03 ± 5.88	5.9	0.4
	80.0	79.3 ± 3.5	4.4	−0.8
3 freeze thaw	0.200	0.213 ± 0.006	2.7	6.7
	8.00	8.76 ± 0.36	4.1	9.5
	80.0	85.4 ± 8.0	9.3	6.7
Autosampler	0.200	0.208 ± 0.004	1.7	4.0
Ambient, 24 h	8.00	7.46 ± 0.22	3.0	−6.7
	80.0	80.7 ± 3.6	4.5	0.9

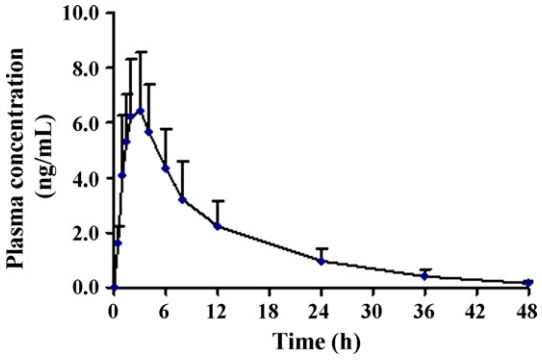

Fig. 3. Mean plasma concentration of tropisetron after a single oral dose of 5 mg tropisetron hydrochloride (4.44 mg base) to 20 healthy volunteers.

4. Conclusions

An LC–MS/MS method for the determination of tropisetron in human plasma was developed and validated over the concentration range of 0.100–100 ng/mL. The LLOQ at 0.100 ng/mL ensures the estimation of tropisetron with desired accuracy and precision for elimination-phase concentration in actual subject samples of bioequivalence or bioavailability studies. This method has been applied to analyze more than 2000 clinical samples with precision, accuracy, and high throughput, and it has been proved suitable for routine measurement of clinical plasma samples.

Acknowledgment

This work was supported by the National Basic Research Program of China (No. 2009CB930300). We thank Dr. Kate Yu at Waters Corporation (Milford, USA) for helpful discussions.

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