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Development and validation of a selective and robust LC–MS/MS method for high-throughput quantifying rizatriptan in small plasma samples: Application to a clinical pharmacokinetic study

Yi Chen [a,b], Haijun Miao [c], Mei Lin [d], Guorong Fan [a,b,*], Zhanying Hong [a,b], Huiling Wu [a,b], Yutian Wu [a,b]

[a] *Department of Pharmaceutical Analysis, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China*
[b] *Shanghai Key Laboratory for Pharmaceutical Metabolite Research, No. 325 Guohe Road, Shanghai 200433, PR China*
[c] *Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, PR China*
[d] *Shanghai Food and Drug Administration, No. 288 South Henan Road, Shanghai 200010, PR China*

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Abstract

An analytical method based on liquid chromatography with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry detection (LC–MS/MS) was developed for the determination of a potent 5-$HT_{1B/1D}$ receptor agonist, rizatriptan in human plasma using granisetron as the internal standard. The analyte and internal standard were isolated from 100 μL plasma samples by liquid–liquid extraction (LLE) and chromatographed on a Lichrospher C18 column (4.6 mm × 50 mm, 5 μm) with a mobile phase consisting of acetonitrile–10 mM aqueous ammonium acetate–acetic acid (50:50:0.5, v/v/v) pumped at 1.0 mL/min. The method had a chromatographic total run time of 2 min. A Varian 1200 L electrospray tandem mass spectrometer equipped with an electrospray ionization source was operated in selected reaction monitoring (SRM) mode with the precursor-to-product ion transitions *m/z* 270 → 201 (rizatriptan) and 313.4 → 138 (granisetron) used for quantitation. The assay was validated over the concentration range of 0.05–50 ng/mL and was found to have acceptable accuracy, precision, linearity, and selectivity. The mean extraction recovery from spiked plasma samples was above 98%. The intra-day accuracy of the assay was within 12% of nominal and intra-day precision was better than 13% C.V. Following a 10 mg dose of the compound administered to human subjects, mean concentrations of rizatriptan ranged from 0.2 to 70.6 ng/mL in plasma samples collected up to 24 h after dosing. Inter-day accuracy and precision results for quality control samples run over a 5-day period alongside clinical samples showed mean accuracies of within 12% of nominal and precision better than 9.5% C.V.

Keywords: Rizatriptan; 5-$HT_{1B/1D}$ receptor agonist; LC–MS/MS; Pharmacokinetics

1. Introduction

Rizatriptan {*N*,*N*-dimethyl-2–[5-(1,2,4-triazole-1-ylmethyl) -1H-indol-3-yl]ethylamine} is a selective 5-hydroxytryptamine (5-$HT_{1B/1D}$) receptor agonist, which is efficacious in the treatment of migraine. Oral rizatriptan (5 and 10 mg) has shown significant effects in providing pain relief from associated symptoms, normal functional ability and an improvement in patient life quality. The 10 mg dose of rizatriptan provided faster pain relief than sumatriptan 50 mg, naratriptan 2.5 mg, ergotamine/caffeine 2 mg/200 mg and zolmitriptan 2.5 mg, while displaying similar tolerability, and it is considered as a first-line treatment option in the management of migraine [1–4].

Liquid chromatographic methods including with tandem mass spectrometry detection and fluorescence detection are mainly used for quantification of rizatriptan in biological fluids, either in humans or in animals [5–13]. Since the combination of powerful separation from HPLC and superior selectivity and sensitivity from mass spectrometer made liquid chromatography–tandem mass spectrometric (LC–MS/MS) one of the most useful techniques in bioanalytical chemistry, LC–MS/MS has been used as a normal method to determine rizatriptan in pharmacokinetic studies. Analysis of rizatriptan

* Corresponding author at: Department of Pharmaceutical Analysis, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
E-mail address: Guorfan@yahoo.com.cn (G. Fan).

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in plasma using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry was first reported by McLoughlin et al. [5]. Sumatriptan was simultaneously determined. Using the *n*-diethyl analogues as the I.S.s, the analytes were extracted from 1 mL of plasma by a C_2 Varian Bond-Elut cartridge and the analytical column for rizatriptan and sumatriptan was a Spherisorb CN column (25 cm × 4.6 mm, 5 μm) and a Beckman CN column (25 cm × 4.6 mm, 5 μm) with run time of 6 min and 4 min, respectively. The LLOQ was 0.5 ng/mL for both analytes. Based on this method, Barrish et al. [6] studied the pharmacokinetics and bioavailability of rizatriptan in dogs; Cutler et al. [7] reported the clinical pharmacokinetics of rizatriptan tablets during and between migraine attacks; Vyas et al. [8] investigated the disposition and pharmacokinetics of rizatriptan in humans. Vishwanathan et al. [9] developed a method for determination of rizatriptan, zolmitriptan, naratriptan and sumatriptan using bufotenine as the I.S. The analytes were extracted from 1 mL of serum using Oasis HLB SPE cartridges and the analytical column was an Alltech Solvent Miser Silica column (15 cm × 2.1 mm, 5 μm) with run time of 4.5 min. The LLOQ was 1 ng/mL for all analytes. All of above literature methods required either a long chromatographic run time or large plasma volumes to achieve a satisfactory LLOQ. More recently, a liquid chromatographic–electrospray tandem mass spectrometry was applied to determine rizatriptan in human plasma using zolmitriptan as the I.S. A liquid–liquid extraction (LLE) was used to extract the analyte from 0.5 mL plasma, and a LLOQ of 0.05 ng/mL was achieved. A Zorbax XDB C8 column (150 mm × 4.6 mm, 5 μm) was used for analysis and run time of 5 min was consumed. Moreover, the mean extraction recovery was about 60% [13].

In this paper, a more rapid and sensitive LC–MS/MS method is described to determine rizatriptan from a small volume of human plasma (100 μL). After a short-duration LLE with mean recovery of above 98% for rizatriptan and 97.5% for the I.S., the samples were submitted to a short-column to achieve high-throughput LC–MS/MS assay. Finally the method reported was successfully applied to a clinical pharmacokinetic study of rizatriptan following single oral dose of 10 mg rizatriptan tablets to 10 healthy Chinese male volunteers.

2. Experimental

2.1. Materials

Tablet formulation of rizatriptan 10 mg (lot 040628) was from Yangtze River Pharmacy Group (Jiangsu, PR China). Rizatriptan benzoate and granisetron hydrochloride (I.S.) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The purities of rizatriptan benzoate and granisetron hydrochloride were >99.5%. Ammonium acetate, acetic acid, sodium hydroxide and ethyl acetate (analytical reagent grade) were purchased from Shanghai Chemical Reagent Company (Shanghai, PR China). Acetonitrile (chromatographic grade) was purchased from Merck (Darmstadt, German). Human control plasma (sodium heparin as an anticoagulant) was obtained from Shanghai Blood Center (Shanghai, PR China). Deionized (18 MΩ/cm) water was generated in-house using a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. Instrumentation

A Varian HPLC–MS/MS system (Palo Alto, CA, USA) consisted of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source. Varian MS workstation version 6.3 software was used for data acquisition and processing.

2.3. Chromatographic conditions

The chromatographic separation was performed on a Lichrospher C18 column (4.6 mm × 50 mm I.D., 5 μm particle size, Hanbon Science & Technology Co. Ltd., Jiangsu, PR China) thermostated at 30 °C. The mobile phase consisted of acetonitrile–10 mM aqueous ammonium acetate–acetic acid (50:50:0.5, v/v/v) and was pumped at a flow rate of 1.0 mL/min. Before use, the mobile phase was filtered through a 0.45 μm nylon membrane filter. The injection volume was 20 μL and the analysis time was 2 min per sample.

2.4. Mass spectrometer conditions

The HPLC eluant was split 1:5 to flow 200 μL into the mass spectrometer. The ESI-MS spectrometer was operated in the positive ion mode. The electrospray capillary potential was set to 55 V. Nitrogen was used as a drying gas for solvent evaporation. The API housing and drying gas temperatures were kept at 50 °C and 380 °C. Protonated analyte molecules were subjected to collision induced dissociation using argon as the collision gas to yield product ions for each analyte. The collision energy was 10 eV for rizatriptan and 16 eV for the I.S. The scan time was 1 s and the detector multiplier voltage was set to 1500 V. Selected reaction monitoring of the precursor-product ion transitions *m/z* 270 → 201 for analyte and 313.4 → 138 for I.S. was used for quantitation. Product ion mass spectra for analyte and I.S. are shown in Fig. 1.

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

Primary stock solutions of rizatriptan were prepared by dissolving the accurately weighed rizatriptan benzoate in methanol to yield a final concentration of 1 mg/mL (calculated as free base). The solutions were sonicated for 5 min to ensure complete dissolution of the solid. Following sonication, the solutions were allowed to equilibrate to room temperature after which they were diluted to volume with methanol. Working standards of rizatriptan were prepared from individual aliquots of 1 mg/mL stock solution at 0.001–1.0 μg/mL using acetonitrile–water (40:60, v/v) as the diluent. The stock standard solution of I.S. was prepared by dissolving appropriate amounts of granisetron hydrochloride in methanol to give a final base concentration of 1 mg/mL. A 200 ng/mL internal standard working solution

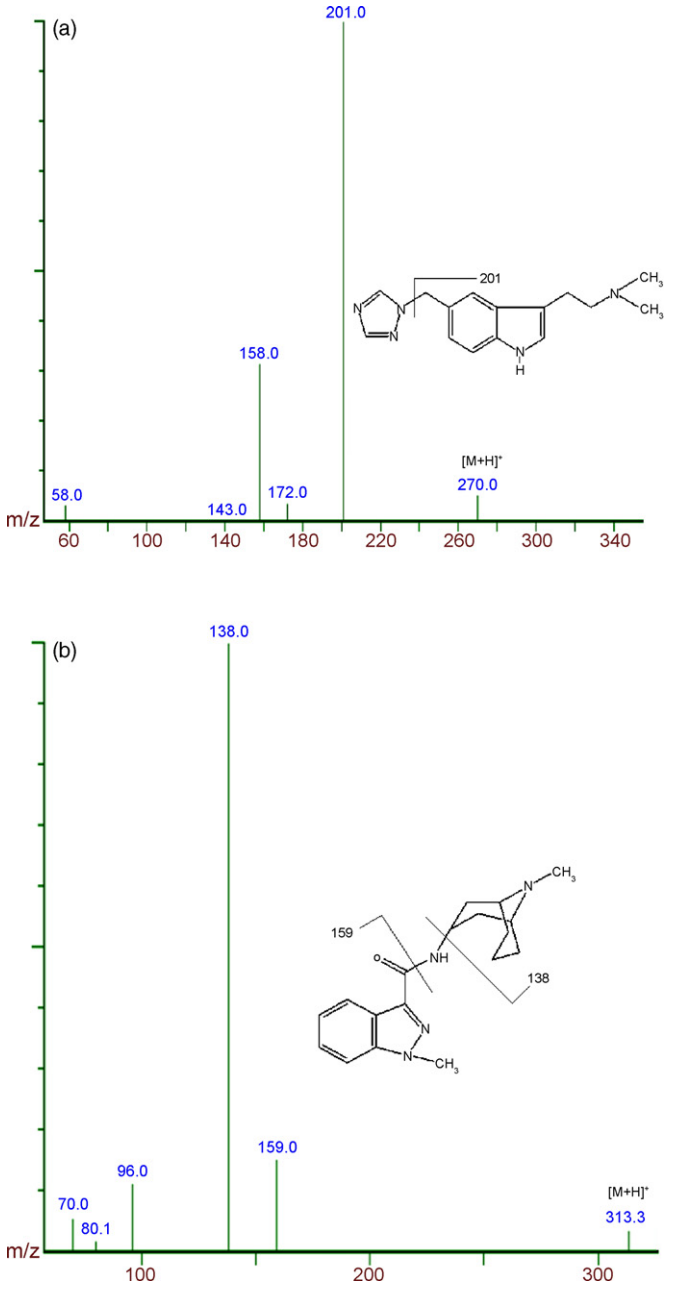

Fig. 1. Chemical structures and product ion spectra of $[M+H]^+$ of rizatriptan (a) and granisetron (b).

was obtained by diluting the stock solution of granisetron with acetonitrile–water (40:60, v/v). All the solutions were stored at 4 °C and brought to room temperature before use. Plasma standards (0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 ng/mL) were prepared by spiking 5 μL of each working standard (0.001, 0.002, 0.004, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4, 1.0 μg/mL) into 100 μL of human control plasma. These standards were used to construct calibration curves for the quantitation of rizatriptan at plasma concentrations ranging from 0.05 to 50 ng/mL. QC stock solutions (400 μg/mL) were prepared from a separate weighing of rizatriptan. QC samples of 0.2, 2 and 20 ng/ml were prepared by spiking 5 μL of diluted solution of QC stock solutions (0.004, 0.04, 0.4 μg/mL) into 100 μL of human control plasma. The QC samples and calibration standards were pipetted into 200 μL non-sterile eppendorf tubes and stored under the same conditions as the clinical samples (i.e. −20 °C) for the purpose of evaluating intra- and inter-day accuracy and precision. Freshly prepared calibration standards on the 8th day were used to evaluate the sample stability. Samples found to contain rizatriptan at concentrations above 50 ng/mL were diluted appropriately with control plasma and re-assayed.

2.6. Extraction procedure

Plasma samples were removed from −20 °C storage and immersed in a heated (37 °C) water bath to thaw. After vortexing and centrifugation (6207 × *g* for 5 min) of the sample tubes, a 100 μL aliquot of plasma was transferred to a 1.5 mL eppendorf tube. A 5 μL aliquot of acetonitrile–water (40:60, v/v) was added to the blanks, quality controls, and subject samples to compensate for the volume of diluent added during spiking of the calibration standards. Next, 5 μL of the working internal standard solution and 50 μL of 0.1 M NaOH were added. The mixed samples were then extracted with 1 mL ethyl acetate by vortex for 1 min. After centrifugation at 2124 × *g* for 5 min, 0.8 mL of the upper organic layer was transferred to another tube and was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 50 μL of acetonitrile–water (40:60, v/v). A 20 μL aliquot of the solution was injected into the LC–MS/MS system for analysis.

2.7. Clinical pharmacokinetic study

The developed LC–MS/MS procedure was used to investigate the plasma profiles of rizatriptan after a single oral dose of 10 mg rizatriptan tablets. A clinical study on healthy Chinese male volunteers, aged from 20 to 35 years, non smokers and drinkers were conducted. The mean weight and height of these subjects were 60.2 kg and 173 cm, ranging from 55 to 65 kg and from 168 to 178 cm, respectively. On the basis of medical history, clinical examination and laboratory investigation (hematology, blood biochemistry, and urine analysis), all volunteers were judged to be in good health. The volunteers were instructed to abstain from taking any medication including over-the counter (OTC) drugs for at least 2 weeks prior to and during the study period and avoid any alcohol or xanthine containing food and beverages 36 h prior to, or during the course of the study. The study was approved by a local ethics committee. The subjects received a single dose of 10 mg of rizatriptan under fasting conditions. Thirteen blood samples for volunteers were withdrawn at different time until 24 h after the administration of the medication.

3. Results and discussion

3.1. Method development

Granisetron, considered as a reasonable choice of I.S. in this assay, is an anti-vomit drug specially used for anticancer drug treatment and very unlikely to be co-administered with rizatrip-

tan. Moreover, granisetron is the same weak organic base as rizatriptan and gave good chromatographic behavior after optimization of the mobile phase. The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and I.S., as well as a short run time. Modifiers such as ammonium acetate and acetic acid alone or in combination in different concentrations were added. It was found that a mixture of acetonitrile–10 mM aqueous ammonium acetate–acetic acid (50:50:0.5, v/v/v) could achieve this purpose and was finally adopted as the mobile phase. The percentage of acetic acid was optimized to maintain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer. After careful comparison of many columns, a Lichrospher C18 column (4.6 mm × 50 mm, 5 μm) was finally used with a flow rate of 1.0 mL/min to produce good peak shapes and permit a run time of 2 min.

In order to produce a spectroscopically clean sample and avoid the introduction of non-volatile materials onto the column and MS system, LLE was used for the sample preparation in this work. Clean samples are essential for minimizing ion suppression and matrix effect in LC–MS/MS analyses. Since rizatriptan and granisetron are weak organic bases, plasma samples were alkalified with 0.1 M NaOH prior to extraction in order to ensure liberation of the analyte molecules in an effort to more effectively retain the compound on the sorbent. Experience has shown that the addition of 50–100 μL of 0.1 M NaOH per 100 μL of plasma is often effective at improving the extraction recovery of analytes. In order to develop a single step liquid–liquid extraction procedure with sufficient recovery, several commonly used extraction solvents, such as chloroform, ethyl acetate, diethyl ether, dichloromethane alone or in combination in different proportions were investigated. As a different result with prior works reported by Chen et al. [10] and Guo et al. [13], the absolute recovery of rizatriptan after single extraction from plasma using relatively large volume of ethyl acetate (1 mL ethyl acetate versus 100 μL plasma) was excellent (above 98% for rizatriptan and 97.5% for the I.S.). In this work, a simple organic solvent ethyl acetate was used as an extraction solvent under basic pH. As a result, a clean chromatogram for a blank plasma sample and high recovery for the analytes from plasma was obtained.

3.2. Method validation

The validation was performed with 44 runs for each batch. The runs were composed of duplicate runs for each QC sample at high, medium and low concentration, 10 runs for calibration curve, blood samples of two people (14 points including blank for each person). The blood samples ran with the order from 0 to 24 h, with randomized standards and QCs inserted. The validation experiments and results obtained are described below.

3.2.1. Selectivity

Assay selectivity was evaluated by analyzing six separate lots of drug free human control plasma. Based on the analysis of drug free plasma from control plasma and blank samples included in each validation run, endogenous or chemical components did not interfere with the drug and internal standards over the concentration range described herein (Fig. 2a and b). Analysis of study pre-dose samples did not show any significant interference. Rizatriptan has very little metabolism [8]. In humans the principal metabolite of rizatriptan is the indole-3-acetic acid derivative generated via oxidative deamination, which is catalyzed by monoamine oxidase A and the metabolites were detected in human urine. Both renal and nonrenal routes of elimination of rizatriptan are important, suggesting that neither renal dysfunction nor hepatic impairment would alter the plasma concentration profile of rizatriptan to a clinically notable degree. The metabolites were found to co-elute with rizatriptan and a SRM-enhanced product ion experiment was performed to screen the presence of the metabolites in study samples. Various study samples were re-analyzed randomly and no metabolite signal was detected in any of them. For the assay, potential metabolite cross-talk, in the quantification of rizatriptan was not found to be critical.

In addition, the HPLC–MS/MS system was evaluated for the presence of "cross-talk" between the channels used for monitoring rizatriptan and granisetron. Fig. 2c and d clearly show the absence of any MS/MS response from the analyte into internal standard channel and vice versa.

The extent of matrix suppression or enhancement of ionization was assessed by comparing the peak areas of rizatriptan across the QC concentration range and granisetron for samples spiked after extraction from five different lots of human plasma (i.e. lots originating from five unique donors) with the analogous peak areas obtained by injecting neat standards directly. Similar with literature [13], a mean ionization enhancement of 36% ($C.V._{\text{lot to lot}} = 2.4\%$), 23% ($C.V._{\text{lot to lot}} = 1.7\%$), 29% ($C.V._{\text{lot to lot}} = 1.9\%$) for rizatriptan (0.2, 2 and 20 ng/ml) and 27% ($C.V._{\text{lot to lot}} = 2.1\%$) for granisetron (10 ng/mL) was observed. The similar ionization enhancement did not affect the ratios of rizatriptan/granisetron that were used for constructing the standard curves.

3.2.2. Sensitivity and linearity

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 15% of nominal and precision not exceeding 15% C.V., was 0.05 ng/mL. The reproducibility of LLOQ was determined by examining five LLOQ samples independent from standard curve with 10.5% accuracy and 13.1% C.V. Calibration curves were constructed by plotting the peak area ratios (rizatriptan/granisetron) of plasma standards versus nominal concentration. Linear least-squares regression analysis with weighting factor of $1/x^2$ was performed to assess better linearity than $1/x$. Linear calibration curves were obtained over the range 0.05–50 ng/mL of rizatriptan in plasma. Unknown sample concentrations exceeding 50 ng/mL were diluted appropriately with control plasma and re-assayed. The correlation coefficient (r^2) for the mean standard curve of five different lots of plasma was 0.9995. The typical equation for the calibration curve was $y = 0.05725x - 0.001856$. The correlation coefficients were above 0.999 for each run. The difference between the nominal standard concentration and the back-calculated con-

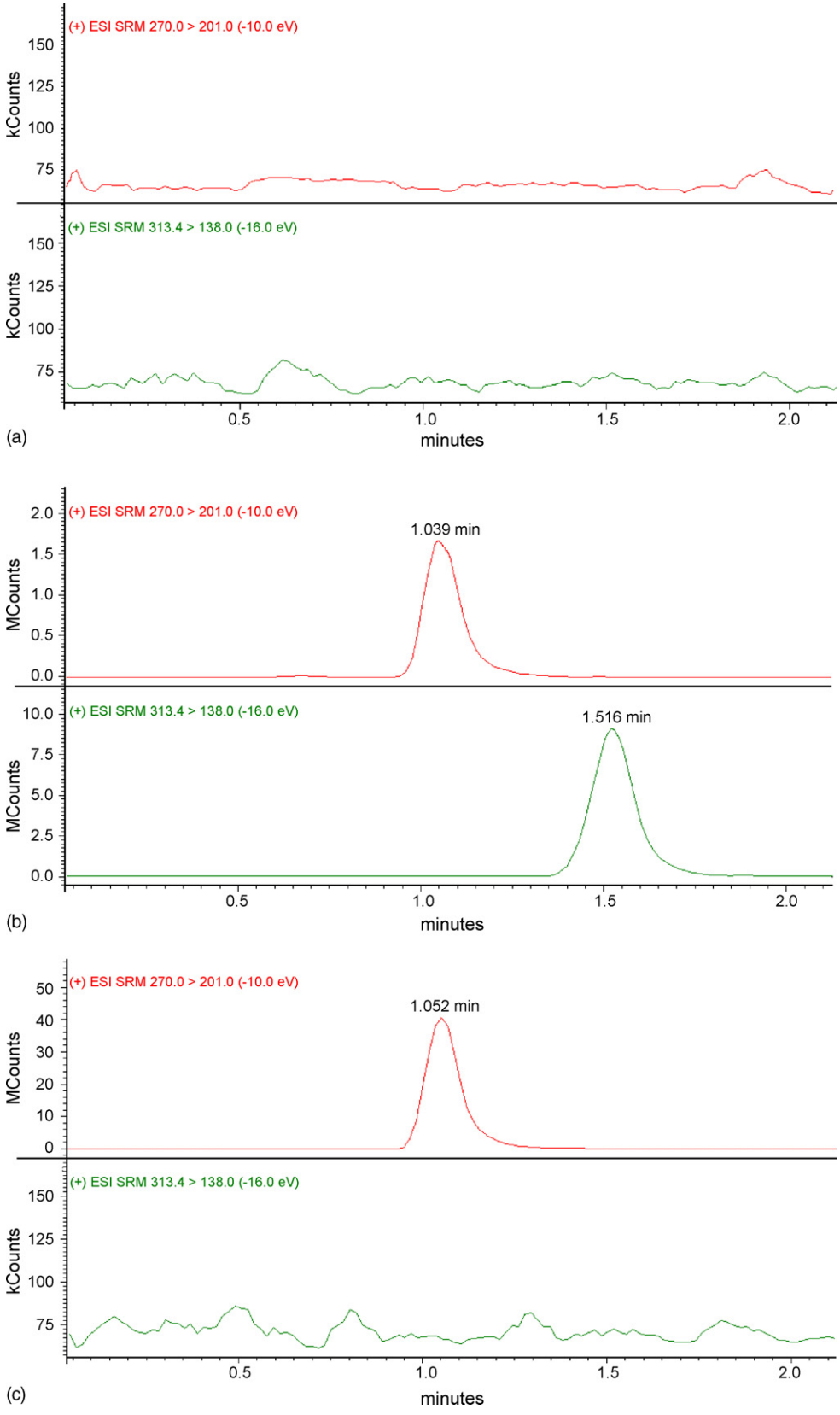

Fig. 2. Representative chromatograms: (a) control plasma double blank; (b) 2 ng/mL QC sample; (c) blank plasma spiked with 50 ng/mL of rizatriptan; (d) blank plasma spiked with 10 ng/mL of the I.S.

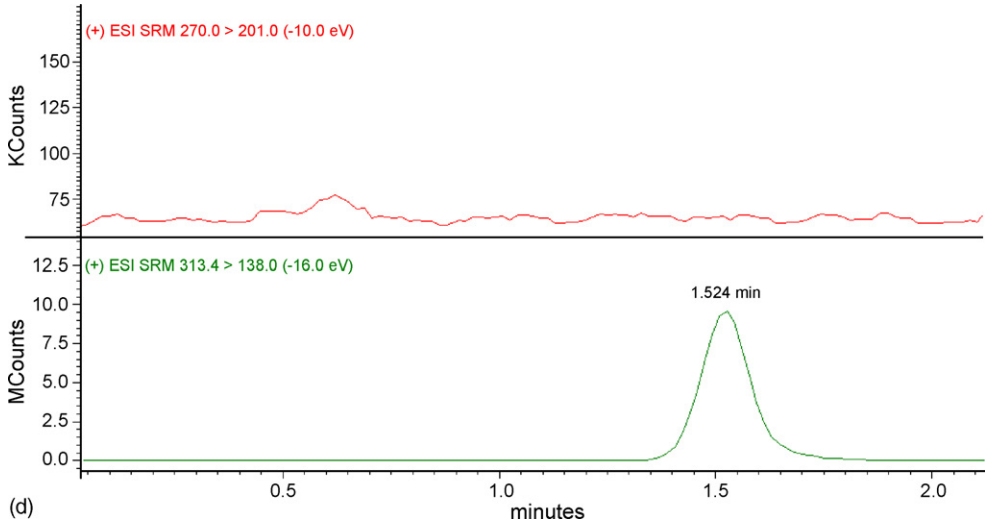

Fig. 2. (*Continued*).

Table 1
Intra-day accuracy and precision for the determination of rizatriptan in five unique lots of human plasma

Nominal concentration (ng/mL)	Back-calculated concentration (ng/mL)						Accuracy[a] (%)
	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Mean[b]	
0.05	0.0641	0.0619	0.0541	0.0470	0.0516	0.0557 (12.8)	111.5
0.1	0.119	0.117	0.098	0.096	0.111	0.108 (9.9)	108.2
0.2	0.235	0.197	0.247	0.224	0.233	0.227 (8.3)	113.6
0.5	0.468	0.499	0.565	0.560	0.541	0.527 (7.9)	105.3
1	1.04	1.03	1.01	1.11	1.16	1.07 (5.9)	107.0
2	1.78	1.87	2.03	1.87	2.08	1.93 (6.5)	96.3
5	4.86	4.89	4.70	4.69	4.36	4.70 (4.5)	94.0
10	9.16	9.57	9.05	9.01	8.61	9.08 (3.8)	90.8
20	19.69	18.65	19.60	20.54	19.72	19.64 (3.4)	98.2
50	51.46	50.82	51.20	50.22	53.55	51.45 (2.5)	102.9

[a] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] $n = 5$; numbers in parentheses are coefficients of variation (%C.V.).

centration from the weighted linear regression line was varied from −9.2% to 13.6% for each point on the standard curve (C.V. varied from 2.5% to 12.8%) indicating that the linear regression analysis applied ($1/x^2$) provided an adequate fit of the data (Table 1).

3.2.3. Accuracy and precision

The intra-day accuracy and precision of the assay were determined by analyzing replicate ($n = 5$) standard curves. To more fully characterize the ruggedness of the assay, the standard curves were prepared in five different lots of plasma (i.e. lots originating from five unique donors). The results of this analysis are provided in Table 1.

Duplicate QC samples at each concentration were analyzed daily along with standards and clinical samples. Inter-day accuracy and precision data for QC samples analyzed over a 5-day period are provided in Table 2.

3.2.4. Extraction recovery

To investigate extraction recovery, a set of samples ($n = 5$ at each concentration in unique lots of plasma) was prepared by spiking rizatriptan into plasma at 0.2, 2, and 20 ng/mL. Each of the samples was also spiked with granisetron at the working concentration of 10 ng/mL. The samples were subsequently processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction

Table 2
Inter-day QC accuracy and precision

Day	Assayed concentration (ng/mL)[a]		
	Low QC	Mid QC	High QC
1	0.23	1.9	19.2
2	0.24	1.9	19.6
3	0.19	1.9	20.6
4	0.24	2.1	21.1
5	0.22	1.8	21.5
Mean	0.22	1.9	20.4
Accuracy[b] (%)	112.0	96.1	102.0
C.V. (%)	9.3	6.5	4.8

[a] Data presented are the mean of duplicate QC samples at each concentration.
[b] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 3
Extraction recovery[a]

Nominal concentration (ng/mL)	Peak area[b] (e^6) (A)	Peak area[c] (e^6) (B)	Extraction recovery[d] (%) (A/B)
0.2	1.07	1.11	96.6
2	11.63	12.07	98.7
20	114.30	114.52	99.8
10 (I.S.)	90.16	92.47	97.5

[a] $n = 5$.
[b] Standards spiked before extraction.
[c] Standards spiked after extraction.
[d] Extraction recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma pre-extraction (A) to the mean peak area of the analytes spiked into plasma post-extraction (B).

with the same concentrations of rizatriptan and granisetron that actually existed in the pre-extraction spiked samples (i.e. 80% of the concentrations of rizatriptan and granisetron in the pre-extraction spiked samples). Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to the raw peak areas of the samples spiked after extraction. The results are indicated in Table 3. Mean extraction recoveries of rizatriptan at concentrations 0.2, 2, 20 ng/mL were 96.6%, 98.7% and 99.8%, respectively, and the extraction recovery of the I.S. was 97.5%.

3.2.5. Working standards and plasma samples stability

The stability of the working standard solutions of rizatriptan and internal standard working solution of granisetron were determined when stored at 4 °C for 7 days. The stability of rizatriptan in human plasma were determined from QC plasma samples of 0.2, 2 and 20 ng/ml stored at −20 °C for seven days. The difference between the compared sets of results was 10% or less, indicating that rizatriptan was stable for a period of seven days in both working solutions and plasma samples.

3.2.6. Benchtop stability

The stability of rizatriptan in plasma exposed to ambient laboratory conditions prior to processing was investigated. Quality control samples ($n = 3$ at three concentration levels) were removed from −20 °C storage, thawed by immersion in a 37 °C water bath, and allowed to sit on a laboratory bench top for 4 h prior to processing. A set of QC samples ($n = 3$ per concentration level) processed immediately after thawing served as a control group. The results, which are presented in Table 4, demonstrate the stability of rizatriptan under these conditions.

3.2.7. Freeze–thaw stability

The stability of rizatriptan in human plasma over multiple freeze–thaw cycles was assessed. QC samples were subjected to three freeze–thaw cycles and analyzed along with a set of control QCs subjected to a single freeze–thaw cycle. Each freeze–thaw cycle consisted of storage at −20 °C for a minimum of 12 h followed by thawing by immersion in a 37 °C water bath. As shown in Table 5, rizatriptan was found to be stable through three cycles of freezing and thawing.

3.2.8. Processed sample stability/re-injection accuracy and precision

The stability of rizatriptan in processed samples was investigated as follows. A set of samples consisting of a standard curve and QCs ($n = 5$ per concentration) was processed and analyzed. After this analysis, the samples were allowed to remain on the autosampler exposed to ambient laboratory conditions for five days, after which the samples were re-injected. Assayed QC concentrations were determined based on the initial injection of the standard curve. The results, which are presented in Table 6, demonstrate the stability of rizatriptan in processed samples. Sample re-injection accuracy and precision was assessed in the same experiment. Assayed concentrations of the re-injected QC samples were determined using the re-injected standard curve and compared with the assayed concentrations determined from the original injection of the samples. As indicated in Table 6, no meaningful difference in accuracy or precision was found among these data sets.

3.2.9. Sample dilution

To demonstrate the ability to dilute and analyze samples containing rizatriptan at concentrations above the assay upper limit of quantitation, a set of plasma samples was prepared containing rizatriptan at a concentration of 200 ng/mL and placed in a −20 °C freezer overnight prior to analysis. After thawing by immersion in a 37 °C water bath, a 20 μL aliquot was withdrawn for analysis ($n = 5$), diluted with 80 μL of control human plasma, and processed as described in Section 2.6. The results of this experiment are shown in Table 7.

3.3. Application of the assay

The assay has successfully been utilized to analyze samples obtained from subjects administered oral doses of rizatriptan. Representative chromatograms of a control plasma double blank, 0.05 ng/mL plasma standard, are shown in Fig. 3a and b.

A representative chromatogram from a post-dose sample is provided in Fig. 3c. Following the administration of 10 mg of the compound, plasma concentrations of rizatriptan ranged from 0.2 to 70.6 ng/mL. Mean plasma concentration–time curves are shown in Fig. 4. The pharmacokinetic parameters calculated are summarized in Table 8. Vyas et al. [8], reported a mean AUC (area under the curve) of rizatriptan of 59.8 ng h/mL in healthy volunteers obtained with a dose of 10 mg rizatriptan in US male volunteers. The mean AUC observed in Chen's study [12], is 79.86 ng h/mL, about 25% higher in Chinese male volunteers than in US male volunteers. Guo et al. [13], reported a higher mean AUC of rizatriptan of 95.06 ng h/mL in 10 healthy Chinese volunteers (5 males and 5 females) obtained with a oral dose of 10 mg rizatriptan (administration condition of fasted/non-fasted unprovided). In our study, the mean AUC observed is 151.20 ng h/mL, about 150% higher than Vyas's study. The maximum rizatriptan concentration (C_{max}) and the time of the maximum plasma concentration (T_{max}) were determined by the inspection of the individual drug plasma concentration–time profiles. C_{max} is 40.34 ng/mL in our report, which is higher than Vyas's study (19.8 ng/mL), Chen's report (20.45 ng/mL), and

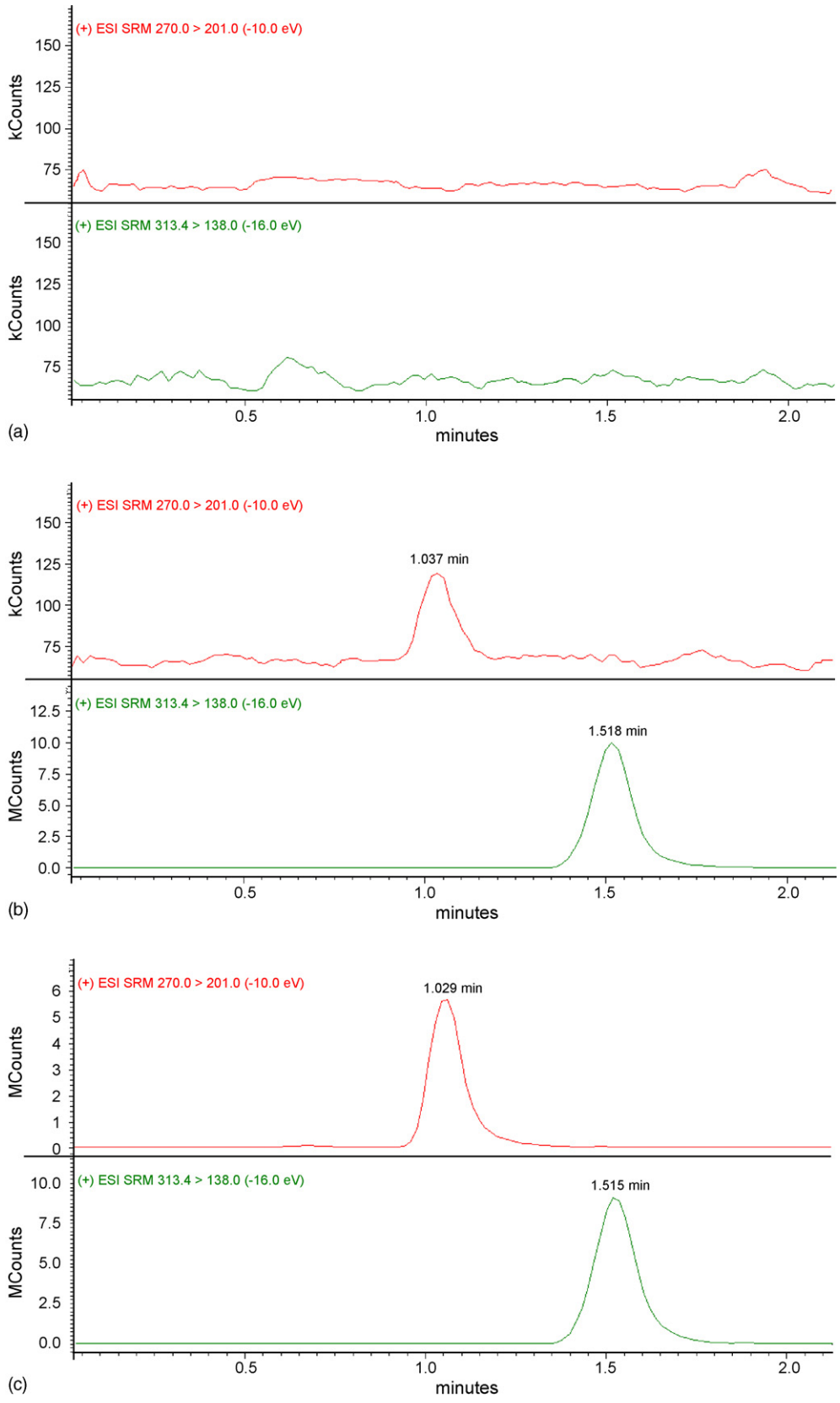

Fig. 3. Representative chromatograms: (a) control plasma double blank; (b) 0.05 ng/mL plasma standard; (c) plasma sample collected from a subject 3 h after receiving a 10 mg oral dose of rizatriptan. The assayed concentration of rizatriptan in this sample was 16.9 ng/mL.

Table 4
Benchtop stability of rizatriptan in human plasma

Nominal concentration (ng/mL)	$t = 0$ h (control) mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	$t = 4$ h mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	Difference from control (%)
0.2	0.22 (6.3)	107.7	0.21 (7.1)	106.9	0.7
2	1.9 (3.7)	97.3	1.9 (3.5)	95.8	1.5
20	20.7 (2.4)	103.5	20.8 (2.9)	104.2	0.7

[a] $n = 3$; numbers in parentheses are coefficients of variation (%C.V.).
[b] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 5
Freeze–thaw stability of rizatriptan in human plasma

Nominal concentration (ng/mL)	1 F/T cycle (control) mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	3 F/T cycles mean assayed concentration[a] (ng/mL)	Accuracy[b] (%)	Difference from control (%)
0.2	0.22 (6.7)	107.6	0.22 (7.9)	108.1	0.5
2	1.9 (3.8)	97.4	1.9 (4.1)	96.5	0.9
20	20.8 (2.5)	104.1	20.7 (3.0)	103.6	0.5

[a] $n = 3$; numbers in parentheses are coefficients of variation (%C.V.).
[b] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 6
Processed sample stability and re-injection accuracy and precision

Nominal concentration (ng/mL)	Initial injection[a]		Re-injection[b]	
	Mean assayed concentration[c] (ng/mL)	Accuracy[d] (%)	Mean assayed concentration[c] (ng/mL)	Accuracy[d] (%)
0.2	0.21 (6.3)	106.5	0.22 (7.1)	111.3
2	1.9 (3.7)	96.2	1.9 (5.3)	93.7
20	20.9 (2.2)	104.3	21.2 (2.7)	105.9

[a] Initial injection of QCs calculated from initial injection of standard curve.
[b] Re-injected QCs calculated from re-injected standard curve.
[c] $n = 5$; numbers in parentheses are coefficients of variation (%C.V.).
[d] Accuracy is expressed as [(mean observed concentration)/(nominal concentration)] × 100.

Table 7
Sample dilution accuracy and precision

Assayed concentration (ng/mL)	Reported concentration (ng/mL)
38.2	191.0
38.9	194.5
38.7	193.5
39.2	196.0
37.6	188.0
Mean	192.6
C.V.	1.6
Accuracy (%)	96.3

Nominal concentration: 200 ng/mL; dilution factor: 5.

Table 8
Pharmacokinetic parameters for 10 volunteers after administration of a single dose of rizatriptan

Parameter	Value
C_{max} (ng/mL)	40.34 ± 13.30
T_{max} (h)	1.0 ± 0.2
$t_{1/2}$ (h)	6.52 ± 1.46
MRT (h)	5.65 ± 1.16
$AUC_{0-24\,h}$ (ng h/mL)	144.88 ± 23.21
$AUC_{0-\infty}$ (ng h/mL)	151.20 ± 25.07

Guo's article (32.59 ng/mL). These discrepancies of AUC and C_{max} from different studies seemed to be linked to the interracial variability and the between-study difference (for example, in the study population, the study conduct, the study formulation and inter-individual variability). Therefore, further study should be focused on the disposition and pharmacokinetics of rizatriptan in interracial groups and demographic data should be paid much attention to. T_{max} (1.0 h) among our pharmacokinetic parameters, is a little shorter than other's report (1.4, 1.33, and 1.18 h), which can be regarded as clinically insignificant. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{e1}$. The elim-

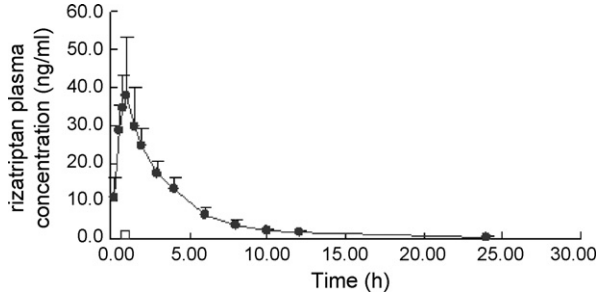

Fig. 4. Mean plasma concentration–time curve in 10 adult, healthy Chinese subjects when administered oral doses of 10 mg rizatriptan (linear plot).

ination rate constant (k_{e1}) was obtained by regression analysis of the semi-logarithmic plasma drug concentration–time curve and used to extrapolate the area from the last plasma drug concentration to infinity. In our study, $t_{1/2}$ is 6.52 h, longer than other's report (2.2, 2.23, and 2.80 h). However, the parameter of ours is obtained from the time points of 0–24 h, the same as Guo's method (0–24 h), and different from Vyas's and Chen's (0–12 h or less). According to the formulation of $t_{1/2}$ and plasma drug concentration–time curves, the data of ours is creditable, comparing with Vyas's and Chen's.

The present method proved to be suitable for determining the plasma concentrations of rizatriptan for up to 24 h following a 10 mg dose in a small sample volume (100 μL). So, a sufficient number of samples can be obtained in human or other small animals, without any impairment to its physiological state.

4. Conclusion

An HPLC–MS/MS assay has been developed and validated for the determination of rizatriptan in human plasma. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability, using a small sample volume (100 μL). The simplicity of the method, and using rapid liquid–liquid extraction and sample turnover rate of 2.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of rizatriptan.

The method was successfully applied to a pharmacokinetic study of rizatriptan in human, following single oral dose of 10 mg rizatriptan tablets to 10 healthy Chinese male volunteers.

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