



Short communication

High-performance liquid chromatographic analysis of zolmitriptan in human plasma using fluorescence detection

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Received 23 November 2003; received in revised form 20 January 2004; accepted 26 January 2004

Available online 19 March 2004

Abstract

A simple, rapid and sensitive high-performance liquid chromatographic (HPLC) method has been developed to quantify zolmitriptan in plasma using an isocratic system with fluorescence detection. The method included a single-step liquid–liquid extraction with methyl tertiary butyl ether. HPLC separation was carried out by reversed phase chromatography with a mobile phase composed of 0.05% (v/v) triethylamine in water (adjusting to pH 2.75 with 85% phosphoric acid) and acetonitrile (92:8, v/v), pumped at flow rate of 1.5 ml/min. Fluorescence detection was performed at 225 nm (excitation) and 360 nm (emission). The calibration curve for zolmitriptan was linear from 0.2 to 40 ng/ml. The validation method yielded good results regarding linearity, precision, accuracy, specificity and recoveries. The values of the limit of detection (LOD) and limit of quantification (LOQ) were 20 and 40 pg, respectively. The method was sensitive, simple and repeatable enough to be used in pharmacokinetic studies.

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Keywords: Zolmitriptan; High-performance liquid chromatography (HPLC)

1. Introduction

Zolmitriptan (4*S*)-4-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-2-oxazolidinone, is a selective serotonin 5-HT(1B/1D) receptor agonist. Randomised, placebo-controlled, double-blind trials in patients with migraine have shown that zolmitriptan has good efficacy measured using 2 h response and pain-free rates. Oral zolmitriptan (2.5 and 5 mg) has an onset of action within 45 min and efficacy is sustained in most patients who respond at 2 h. Com-

parative clinical studies have shown that zolmitriptan has similar or superior efficacy to sumatriptan in the treatment of migraine [1]. At therapeutic doses, zolmitriptan has good oral bioavailability in healthy volunteers and has dose-proportional pharmacokinetics that is not affected by food to any clinically relevant extent [2]. Due to low dosage and high volumes of distribution, zolmitriptan occurs at low blood levels after therapeutic administration. It was reported that in humans, the plasma concentration of zolmitriptan was very low (less than 10 ng/ml) when zolmitriptan was administered orally in a single dose of 5 mg [3]. There were only two techniques had been reported and validated for zolmitriptan quantification in plasma samples: coulometric detection and liquid

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chromatography/tandem mass spectrometry [4,5]. However, the limit of quantification (LOQ) of coulometric detection was only 2 ng/ml, and it could not have the sufficient sensitivity to properly evaluate the pharmacokinetics of zolmitriptan in human. Vishwanathan et al. reported a liquid chromatography/tandem mass spectrometry method and the limit of quantification was 1 ng/ml. It is to our knowledge, the lowest limit of quantification. However, the LC-MS machine is quite expensive and is not readily available in most clinical research laboratories. In order to provide more guidance to the reasonable use of this drug and study the pharmacokinetic profile of zolmitriptan in Chinese healthy volunteers, a more sensitive and rugged high-performance liquid chromatography (HPLC) with fluorescence detection method has been developed and is now reported in this paper. Based on our method, the pharmacokinetic parameters of zolmitriptan were studied by using blood samples of 20 healthy male volunteers after a single oral dose of 5 mg of zolmitriptan tablet.

2. Experimental

2.1. Reagents and chemicals

Zolmitriptan was obtained from Shanghai Huatuo Pharmaceutical Ltd. (Shanghai, China) and internal standard (IS) rizatriptan benzoate was supplied by Shanghai Institute of Pharmaceutical Industry (Shanghai, China). Chemical structures were presented in Fig. 1. The purity of zolmitriptan and rizatriptan benzoate were all >99.5%. Acetonitrile was purchased from BDH (Dorset, UK), methyl tertiary butyl ether (HPLC/Spectro) was purchased from Tedia Company (Fairfield, OH, USA). Sodium hydroxide, triethylamine, phosphoric acid (analytical reagent grade)

were purchased from Shanghai Chemical Reagent Company (Shanghai, PR China). Double distilled water was purified by Millipore Simplicity(tm) (Millipore, Bedford, MA, USA). The drug-free human heparinized plasma was obtained from Shanghai Blood Center (Shanghai, PR China).

2.2. Preparation of standard solution

A stock standard solution was prepared in methanol and containing 1 mg/ml of zolmitriptan. This solution was further diluted with methanol to prepare the calibration solutions containing 5, 50, 200 ng/ml of zolmitriptan. An internal standard stock solution was prepared in methanol and contained 1 mg/ml of rizatriptan. This solution was further diluted with methanol to prepare the working internal standard solution containing 200 ng/ml of rizatriptan benzoate. All these solutions were stored at 4 °C and no change in stability over a period of 1 month was observed.

2.3. Apparatus and chromatographic conditions

A shimadzu HPLC system consisted of a LC-10AT VP pump, RF-10A XL fluorescence detector, CTO-10 AS VP column oven were utilized. Empire HS-2000 software was used for data acquisition. Analyses were performed on an Inertsil ODS-3 reverse column (5 μm particle size, 4.6 mm × 200 mm i.d.) purchased from GL Sciences Inc. (Japan). The mobile phase was composed of 0.05% (v/v) triethylamine in water (adjusting to pH 2.75 with 85% phosphoric acid) and acetonitrile (92:8, v/v). The flow rate was set at 1.5 ml/min, and the total run time was 7 min. The column was maintained at 40 °C. Fluorescence detection was performed at an excitation wavelength of 225 nm and an emission wavelength of 360 nm.

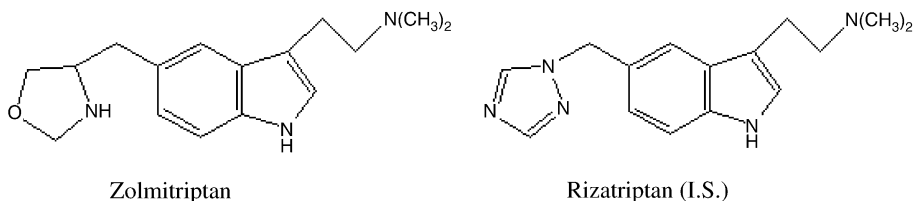


Fig. 1. Chemical structure of zolmitriptan and rizatriptan.

2.4. Sample preparation

To a 1 ml aliquot of plasma in a 15 ml glass tube, 50 μ l of IS solution (200 ng/ml of rizatriptan benzoate solution) and 50 μ l of 1 M sodium hydroxide solution were added. After vortex mixing, 4 ml of methyl tertiary butyl ether was added. The mixture was then shaken for 2 min and centrifuged at $2000 \times g$ for 10 min. The organic phase was decanted into a 10 ml conical glass tube. The organic phase was evaporated to dryness in a water bath at 50 °C under a stream of nitrogen. The residue was reconstituted with 100 μ l mobile phase, vortex mixed briefly. Twenty microliters of this solution was subsequently injected for HPLC analysis.

2.5. Validation

The method was subjected to a validation procedure. Plasma samples were spiked in seven replicates at concentrations of 0.2, 0.5, 2, 5, 10, 20, 40 ng/ml. The samples were assayed using the method described above. The standard calibration curves for zolmitriptan were constructed using the analyte/IS peak-area ratios versus the nominal concentrations of the analytes. Linear least-squares regression analysis with weighting factor of $1/x^2$ was performed to assess the linearity as well as to generate the standard calibration equation: $y = ax + b$, where y is the peak-area ratio, x the concentration, a the slope and b the intercept of the regression line.

The limit of quantification was determined for zolmitriptan, based on the criteria that: (1) the analyte response at LOQ is five times of baseline noise; (2) the analyte response at LOQ can be determined with sufficient precision and accuracy, i.e. precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was determined as the lowest concentration which gives a signal-to-noise ratio of 3 for zolmitriptan.

The specificity of the method was determined by comparing the chromatograms of drug-free human plasma samples with the chromatograms of plasma spiked with zolmitriptan and IS.

The intra- and inter-day precision (relative standard deviation, R.S.D.) and accuracy (relative error, RE) of the assay procedure were determined by the analysis of five samples at lower (0.2 ng/ml), medium (2 ng/ml) and higher (20 ng/ml) QC concentration in the same day and five samples at each QC concen-

trations in five different days, respectively. The absolute recoveries at each concentration were measured by comparing the response of the treated plasma standards (QC) with the response of standards diluted with methanol in the same proportion as the pre-treated standards.

On-instrument stability of zolmitriptan in the mobile phase was verified at three level of concentration (0.2, 2, 20 ng/ml) during 12 h of storage at room temperature, looking for the change of signal height. The long term stability of zolmitriptan in human plasma was verified at three levels of concentration (0.2, 2, 20 ng/ml in plasma) by performing the experiment after 30 days of storage at -20 °C. The freeze-thaw stability was also verified at three levels of concentrations (0.2, 2, 20 ng/ml in plasma) after three freeze-thaw cycles.

2.6. Clinical application

This method developed was used to investigate the plasma profile of zolmitriptan after single 5 mg oral dose of zolmitriptan. A clinical study on 20 Chinese healthy male volunteers (age from 18 to 24 years old) was conducted under fasting conditions. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. The protocol was approved by the Base for Drug Clinical Trial of SDA of Shanghai No. 2 Medical University, Shanghai, China. Following written informed consent, each volunteer received the zolmitriptan tablet with 250 ml of water. No food was allowed until 4 h after dose administration. Water intake was allowed after 2 h and low-fat standard meals were provided at 4 h and 10 h post dose. Blood samples were drawn into tubes through an indwelling cannula before (0 h) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 11 and 14 h after oral administration. The blood samples were centrifuged at $1643 \times g$ for 15 min, plasma was separated and kept frozen at -18 °C in coded polypropylene tubs until analysis.

3. Results

3.1. Separation and specificity

Fig. 2 shows the representative chromatograms of a blank plasma, plasma samples spiked with

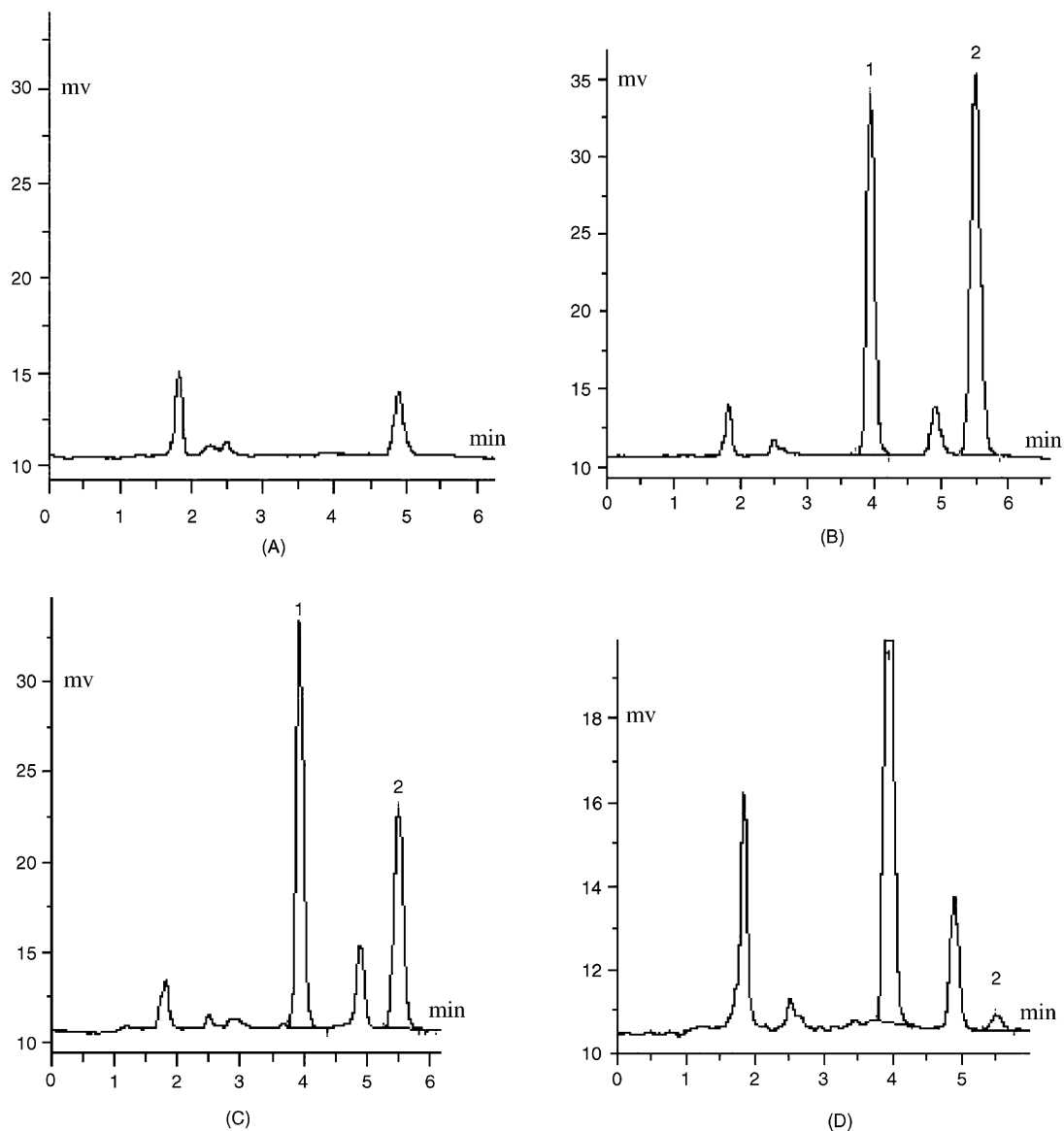


Fig. 2. Chromatograms of: (A) a blank plasma; (B) plasma sample spiked with 10 ng/ml zolmitriptan and IS; (C) plasma sample from a healthy subject following a 5 mg oral dose of zolmitriptan, the plasma concentration was determined to be 5.32 ng/ml for zolmitriptan; (D) spiked plasma sample at LOQ (0.2 ng/ml).

zolmitriptan at 10 ng/ml and at LOQ (0.2 ng/ml), and plasma sample obtained from a healthy subject following an oral 5 mg dose of zolmitriptan. The analytes were well separated using the present chromatographic conditions. The retention times were 5.6 min for zolmitriptan and 3.9 min for IS. No interfering peaks from the endogenous plasma components

were observed at the retention time of zolmitriptan or IS.

3.2. Calibration and linearity

Good linearity was observed over the concentration range of 0.2–40 ng/ml plasma ($r = 0.9979$ – 0.9996).

Table 1
Inter-day precision in the slope and intercept of standard curves ($r = 0.9979$ – 0.9996)

Days	Slope	Intercept	Correlation
1	0.06736	0.000779	0.9996
2	0.06993	−0.000326	0.9984
3	0.06919	0.000905	0.9995
4	0.06682	0.000668	0.9987
5	0.06740	0.001705	0.9979
Mean ± S.D.	0.06814 ± 0.001343	0.00075 ± 0.000725	0.9988 ± 0.00074
R.S.D. (%)	1.97	97.13	0.07

The R.S.D. ($n = 5$) of the slope calculated with calibration curve data was 1.97%, showing good repeatability (Table 1).

3.3. Recovery

For plasma concentrations at 0.2, 2, 20 ng/ml, the mean recovery of zolmitriptan were 83.02 ± 2.78 , 84.38 ± 3.52 and $83.69 \pm 2.32\%$, respectively, and the mean recovery of IS was $88.35 \pm 2.03\%$.

3.4. Precision and accuracy

The precision and accuracy for measurement of zolmitriptan was summarized in Table 2. The R.S.D. of zolmitriptan ranged from 1.75 to 4.54% for intra-day and 2.49 to 5.74% for inter-day, respectively. The RE of zolmitriptan ranged from −1.39 to 4.08% for intra-day and −0.60 to 8.02% for inter-day, respectively.

3.5. Sensitivity

The lower limit of quantification was 0.2 ng/ml for zolmitriptan. The limit of detection was 0.1 ng/ml for zolmitriptan.

3.6. Stability

The results obtained after three freeze-thaw cycles demonstrated that 97.46–105.6% of the initial content of zolmitriptan were recovered and that the analytes were stable under these conditions. Zolmitriptan in re-constitution solution was found to be stable for approximately 12 h since the found concentrations of analytes were within 99.17–102.7% of the initial concentrations. The residual percentages of zolmitriptan stored in plasma at -20°C for 30 day ranged from 97.5 to 102.8%, indicating no stability problems occurred.

3.7. Application to clinical study

The present HPLC method of zolmitriptan was for the first time employed to determine the pharmacokinetic parameters of zolmitriptan in volunteers' plasma sample. After a single oral dose of 5 mg zolmitriptan tablet in 20 healthy volunteers, concentration versus time profiles were constructed for up to 14 h for zolmitriptan determination. Fig. 3. shows the mean concentration–time curves of zolmitriptan in 20 subjects following a 5 mg oral dose of zolmitriptan under fasting condition. The maximum zolmitriptan plasma

Table 2
Intra- and inter-day precision and accuracy of zolmitriptan spiked in human plasma ($n = 5$)

	Actual concentration (ng/ml)	Detected concentration (mean ± S.D.) (ng/ml)	Precision (R.S.D., %)	Accuracy (error, %)
Intra-day	0.2	0.1972 ± 0.0090	4.56	−1.39
	2	2.0817 ± 0.0673	3.23	4.09
	20	19.78 ± 0.35	1.77	−1.10
Inter-day	0.2	0.1988 ± 0.0114	5.73	−0.60
	2	2.1604 ± 0.0686	3.18	8.02
	20	19.88 ± 0.49	2.46	−0.60

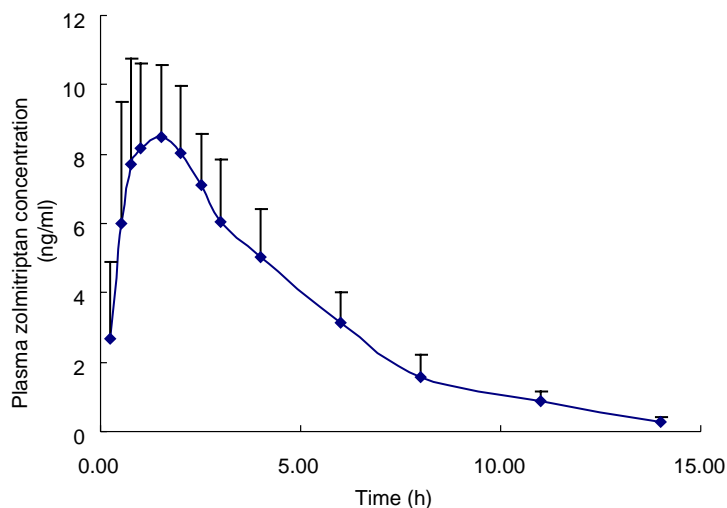


Fig. 3. Mean concentration–time profiles of zolmitriptan in 20 healthy subjects following a 5 mg oral dose of zolmitriptan.

concentration was 8.64 ± 3.05 ng/ml, T_{\max} was 1.83 ± 0.42 h, and $t_{1/2}$ in the terminal elimination phase was 2.48 ± 0.32 h. The parameter values are in good agreement with those reported previously [6].

4. Discussion

For the determination of zolmitriptan by HPLC, fluorescence detection was used because this compound was found to have fluorescence. This detection was more advantageous than UV detection in its stability of chromatographic baseline and selectivity for the compounds of interest in body fluids. Selection of the most adequate wavelengths for fluorescence detection was based on the scan of excitation and emission of zolmitriptan peak.

In our preliminary experiments, a series of aqueous mobile phases containing buffer solutions with different pH values in combination with different modifiers including acetonitrile, methanol and triethylamine with different volume fractions were tested. The results were most satisfactory with respect to location and resolution of the peaks of zolmitriptan and internal standard from the interfering peaks when mobile phase consisted of 0.05% (v/v) triethylamine in water with pH 2.75 and acetonitrile in volume fractions 92:8. A set of column packing including C₁₈, C₈ and Inertsil ODS-3 were tested and the Inertsil ODS-3

packing showed the best peak shape for zolmitriptan and IS.

Both two previous methods described a solid phase extraction using Oasis HLB to extract the zolmitriptan from plasma; the extraction recovery was 82%. In order to develop a single step liquid–liquid extraction procedure with sufficient recovery, we investigated a large range of extraction solvents. The absolute recovery of zolmitriptan after single extraction from plasma using chloroform, ethyl acetate, diethyl ether, dichloromethane were all <70%; however, when the methyl tertiary butyl ether was used, the absolute recovery was quite high (>80% for both zolmitriptan and IS). Alkalinization of the plasma with 1 M sodium hydroxide solution was found to be appropriate for the extraction of zolmitriptan and IS.

Rizatriptan was found to be very suitable to be used as the IS, since their molecular structures are very similar and they both exhibit almost same excitation and emission spectra. Also the selection of rizatriptan was based on its extraction behavior and it did not interfere in the analysis of zolmitriptan.

Clement et al. developed a coulometric detection method for the determination of zolmitriptan in human plasma with a limit of quantitation of 2 ng/ml [4]. It appeared that the HPLC method did not have the sufficient sensitivity to properly evaluate the pharmacokinetics of zolmitriptan in human. Also the internal standard was not available in most

laboratories. Vishwanathan et al. [5] developed a liquid chromatography/tandem mass spectrometry method with solid-liquid extraction for the determination of zolmitriptan in human serum. The method established could quantify zolmitriptan with a quantification of limit of 1 ng/ml by using 1.0 ml serum. In this paper, we described a very simple method for the determination of zolmitriptan in human serum, and this method could detect zolmitriptan at concentration of 0.1 ng/ml, which was as low as liquid chromatography/tandem mass spectrometry method [5].

5. Conclusion

The method described in this report is able to determine low levels of zolmitriptan in human plasma. The detection limit of this method for zolmitriptan is 20 pg, which is enough to detect terminal phase concentrations of zolmitriptan after oral administration of 5 mg

dose of zolmitriptan to healthy volunteers. In addition, this method has a short chromatographic run (<7 min), so the method is more suitable for high-through quantitative analysis such as human pharmacokinetic studies.

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