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Short communication

A fast and sensitive liquid chromatographic–tandem mass spectrometric method for assay of lorazepam and application to pharmacokinetic analysis

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Abstract

A fast and sensitive method of coupled high-performance liquid chromatography–electrospray tandem mass spectrometry for the assay of lorazepam in human plasma was developed. Plasma samples were simply treated with acetonitrile to precipitate and remove proteins and the isolated supernatants were directly injected into the HPLC/MS/MS system. Chromatographic separation was performed on a Zorbax C_{18} (100 × 2.1 mm I.D.) column with a 65:35 (v/v) mixed solution of acetonitrile and 10 mM aqueous formic acid being used as mobile phase. With diazepam as an internal standard, quantification was performed by selected reaction ion monitoring of the transitions of *m/z* 321 → *m/z* 275 for lorazepam and *m/z* 285 → *m/z* 193 for the internal standard. The assay was validated in the concentration range of 0.71–71.3 ng/ml in human plasma. A detection limit of 0.10 ng/ml for lorazepam was achieved, and inter- and intra-run precisions of better than 4.4% (R.S.D.) were observed. The developed method has been successfully applied for pharmacokinetic study of the drug in man.

Keywords: Lorazepam; Electrospray tandem mass spectrometry; Pharmacokinetics

1. Introduction

Lorazepam, one of the 1,4-benzodiazepine derivatives, is widely used or abused as an anti-anxietic and sedative agent [1]. Identification and determination of this drug in biological fluids is of great importance in clinical toxicology and forensic medicine. After oral administration of normal dosage (usually in 2 mg), lorazepam undergoes extensive metabolism to its inactive glucuronide conjugate. The plasma concentration of the unchanged lorazepam is usually at low ng/ml levels. Thus, a sensitive analytical technique is required by both pharmacokinetic study and therapeutic or forensic assay.

Several methods such as high performance liquid chromatography (HPLC) [2–16], gas chromatography (GC) [17–22], micellar electrokinetic capillary chromatography (MECC) [23], immunoassay [24–26], adsorptive-stripping voltammetry [27], gas chromatography/mass spectrometry (GC/MS) [28–32], tandem mass spectrometry (GC/MS/MS) [33] as well as high performance liquid chromatography/mass spectrometry [34,35] have been reported for the determination of lorazepam in bio-samples. Among them, GC and HPLC coupled with various detectors are most widely used. GC/MS [28–32] and GC/MS/MS [33] provided high sensitivity required by the bioanalysis of lorazepam. However, the drug has to be derivatized before GC separation. When HPLC is used for separation of lorazepam with the coexisting species such as its metabolism products, no derivation of the drug is required. Nevertheless, (In these reports [2–15],) either liquid–liquid extraction [2–4,6–12,15] or solid phase extraction [5,13,14] was usually employed to perform matrix removal and analyte pre-concentration prior HPLC separation. The extraction-involved sample pre-treatment

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procedures could enrich the analyte by several folds even 1–2 orders of magnitude, allowing the HPLC or HPLC/MS to be used for determination of lorazepam in low ppb levels. But the procedures were complicated and tedious. An alternative simple sample pre-treatment method involves protein removal by precipitation (with organic solvent) and centrifugation. The isolated supernatants could then be directly introduced into HPLC [16] or HPLC/MS [35] for lorazepam determination. Owing to lack of a pre-concentration step, the direct supernatant introduction technique could not provide the sensitivity high enough for pharmacokinetic study of the drug.

Recently, HPLC–tandem mass spectrometry (HPLC/MS/MS) [36–40] has become one of the major tools for biomedical analysis due to the fact tandem mass spectrometry (MS/MS) can provides not only extraordinarily high sensitivity and selectivity but also low background noise caused by the biological matrices. The purpose of this study is to develop a fast and sensitive liquid chromatographic–tandem mass spectrometric method for determination of lorazepam in plasma samples. This method has been successfully applied to pharmacokinetic studies after low oral administration.

2. Experimental

2.1. Chemicals and material

Test lorazepam was obtained from Zhejiang Wanma Pharmaceutical Company (Hangzhou, China). Reference lorazepam was obtained from Atlantic Laboratories Co. Ltd. (Bangkok, Thailand). Diazepam (internal standard, I.S.) was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and formic acid were of HPLC grade, and other chemicals used were of analytical grade. Distilled water further purified by a milli-Q purification system (Millipore, Bedford, MA, USA) was used throughout the work.

2.2. Instrumentation and analytical conditions

The HPLC system used was a Surveyor High-performance Liquid Chromatograph (San Jose, CA, USA). Chromatographic separations were performed with a Zorbax Eclipse XDB C_{18} column (100 mm × 2.1 mm i.d., particle size 3.5 μm, Agilent, Wilmington, DE, USA) maintained at 25 °C. The mobile phase was a 65:35 mixed solution of acetonitrile and 10 mM aqueous formic acid at the flow-rate of 0.2 ml/min.

A Finnigan TSQ Quantum discovery triple quadrupole mass spectrometer (San Jose, CA, USA) interfaced to an electrospray ionization (ESI) source was used for mass analysis and detection. The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 3000 V. The heated capillary temperature was set at 300 °C for desolvation. Nitrogen was used as the sheath gas (25 arbitrary units) and auxiliary gas (10 arbitrary units) for nebulization. Quantitation was performed using selected reaction ion monitoring of the transitions of m/z 321 → m/z 275 and m/z 285 → m/z 193 for lorazepam and I.S., respectively. To increase the sensitivity, the flow of collision gas (argon) was set to 1.0 mTorr. The optimized collision energy of 20 V was used for analyte and I.S. to produce the highest intensity of the selected ion peaks.

Data acquisition, peak integration and calculation were interfaced to a computer workstation running Xcalibur 1.3 software (Finnigan). The calibration curve was constructed by plotting peak area ratio (analyte to I.S.) against plasma concentration using a $1/x^2$ weighed linear regression model.

2.3. Standards and sample preparation

2.3.1. Standard solutions

A stock solution of 10.7 μg/ml lorazepam was prepared by dissolving the drug in methanol. Working solutions of lorazepam was obtained by step-wise dilution of the stock solution with methanol. A stock solution of 100 μg/ml diazepam (I.S.) by dissolving the drug in acetonitrile. A working solution of I.S. (40 ng/ml) was prepared by diluting the stock solution with acetontrile. It should be pointed out that acetonitrile served as the solvent for the I.S working solution also acted as the reagent for precipitation of protein in plasma.

2.3.2. Calibration series and quality control samples

Calibration standards containing 0.71, 1.4, 2.8, 7.1, 14.3, 28.6 and 71.3 ng/ml lorazepam, and quality control samples containing 0.71 (low), 14.3 (medium) and 71.3 ng/ml (high) lorazepam were prepared with blank human plasma. To each of 300 μl plasma standards and control samples, 600 μl of I.S. working solution prepared in acetonitrile was added. Then they were vortex mixed for 5 min and centrifuged at 14,000 rpm (4 °C) for 15 min. A 20 μl portion of the supernatant was subjected to HPLC/MS/MS analysis.

2.3.3. Plasma sample preparation for pharmacokinetic study

Twenty healthy male volunteers with a mean age of 24.0 ± 1.7, mean weight of 63.2 ± 6.5 kg, and mean height of 174.2 ± 4.7 cm were selected. The volunteers possessed good health and have not taken any medication for at least 1 month prior to the study. Each volunteer was orally administered with a single 2 mg dose of lorazepam. Blood samples were collected before drug intake and at different time points until 60 h after administration. Plasma was obtained through centrifugation at 2000 rpm for 15 min. Three hundred μl aliquots were transferred into polypropylene tubes and spiked with 600 μl of I.S. working solution prepared in acetonitrile. The samples were vortex mixed for 5 min and centrifuged at 14000 rpm (4 °C) for 15 min. A 20 μl portion of the supernatant was subjected to HPLC/MS/MS analysis.

3. Result and discussion

3.1. MS/MS optimization

Quantitation was conducted using selected reaction ion monitoring (SRM) mode. Lorazepam and I.S. gave protonated parent ion $[M+H]^+$ at *m/z* 321 (Fig. 1A) and 285 (Fig. 1B), respectively. The product ion spectra of $[M+H]^+$ showed several fragment ions at *m/z* 229, 275 and 303 for lorazepam (Fig. 2A) and at *m/z* 154, 193 and 222 for I.S. (Fig. 2B). The fragment ions of most significant intensity, which were observed at *m/z* 275 for lorazepam

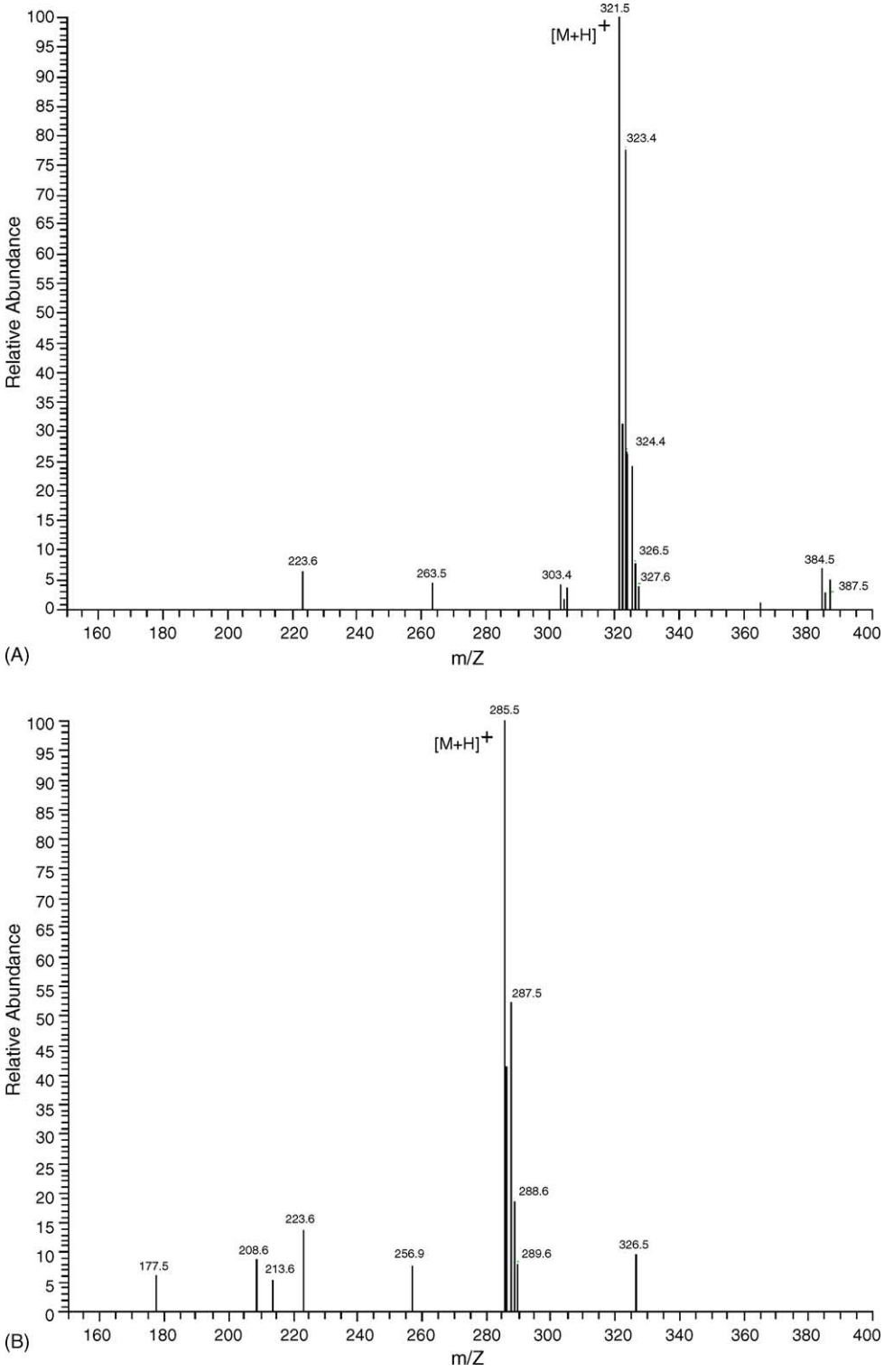

Fig. 1. Mass spectrum of (A) lorazepam and (B) I.S.

and *m*/*z* 193 for I.S., can be attributed to $C_{14}H_6NOCl_2$ and $C_{10}H_8NOCl$, respectively. The mass transitions were chosen for quantitative determination: *m*/*z* 321 → 275 for lorazepam and *m*/*z* 285 → 193 for I.S. Collision-induced dissociation (CID) efficiency for the selected product ions was assessed as a function of collision gas energy. CID conditions were adjusted in order to obtain the maximum intensity of the product ions for the analytes. Collision energy of 20 V

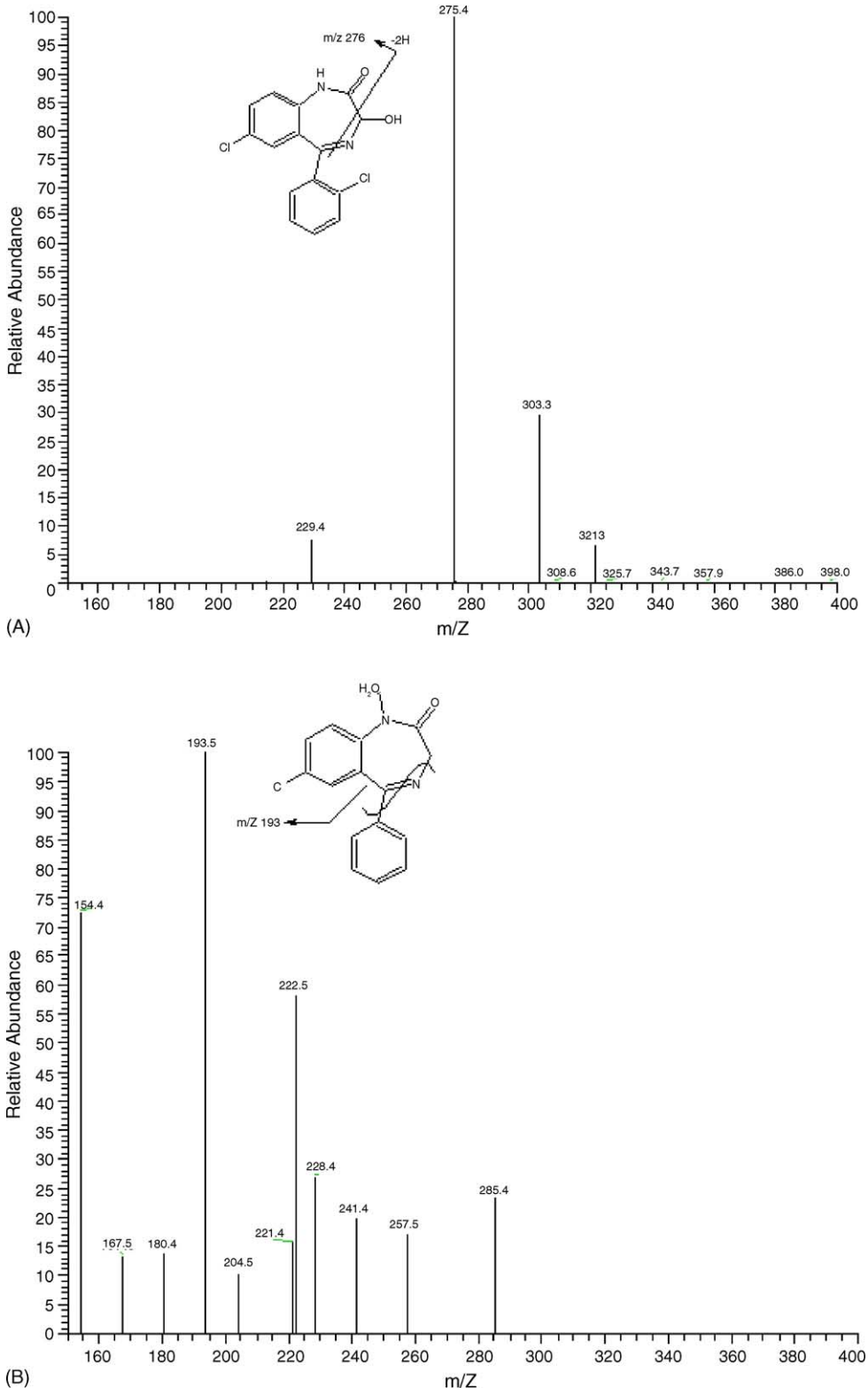

Fig. 2. The product ion spectra of $[M+H]^+$ of (A) lorazepam and (B) I.S.

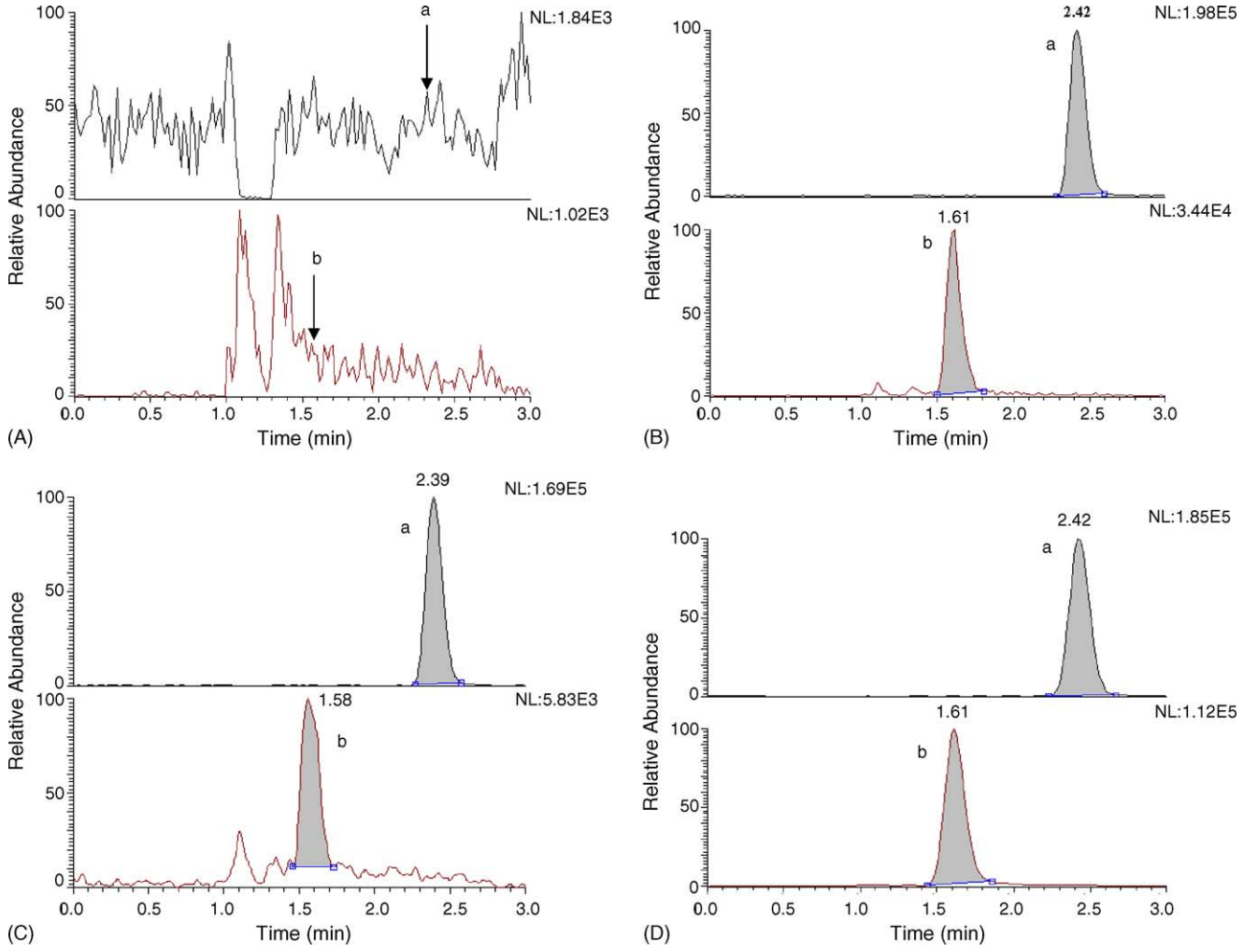

Fig. 3. Chromatograms of (A) plasma blank, (B) plasma spiked with lorazepam (7.13 ng/ml) and I.S. (26.7 ng/ml), (C) plasma spiked with lorazepam at the limit of quantitation (0.71 ng/ml) and I.S. (26.7 ng/ml) and (D) plasma sample from a volunteer 2.0 h after administration of 2 mg lorazepam. Peak: **a**, I.S.; **b**, lorazepam.

was chosen to obtain the highest intensity of the product ions.

3.2. HPLC separation and sample preparation

HPLC conditions were optimized with respect to sensitivity, speed and peak shape. It was found that acetontrile was more favorable for chromatographic separation of lorazepam from matrix than methanol. It was also founded that addition of formic acid to the mobile phase could improve sensitivity by promoting the ionization of analytes. The composition of the mobile phase was optimized by varying percentages of organic solvents while keeping the flow rate at 0.2 ml/min. It was found that a mobile phase composed of 65% (v/v) of acetonitrile and 35% (v/v) of 10 mM aqueous formic acid produced the highest sensitivity, lowest background noise, and most stable MS signal. Under the optimized HPLC/MS/MS conditions, excellent separation was achieved for lorazepam and I.S., and the retention times were 1.61 and 2.42 min, respectively (Fig. 3B). A total separation time of 3.5 min made it possible to analyze a large number of samples in a relatively short period of time.

Since the sensitivity and selectivity afforded by tandem mass spectrometry was high enough for pharmacokinetics study of lorazepam, either liquid–liquid extraction or solid phase extraction was excluded in the sample pre-treatment procedure. Therefore, direct sample injection model was adopted after the simple removal of the plasma protein by precipitation with acetonitrile that was used as the solvent for I.S. solution.

3.3. Analytical performance

3.3.1. Specificity

The specificity of the method was tested by screen analysis of 6 blank plasma samples. It was found no endogenous

Table 1
Intra- and inter-day precision and accuracy for lorazepam from human plasma by the present method ($n = 5$ days, 6 replicates per day)

Amount added (ng/ml)	Intra-day			Inter-day		
	Amount detected (ng/ml)	Precision (%)	Accuracy (%)	Amount detected (ng/ml)	Precision (%)	Accuracy (%)
0.71	0.69 ± 0.02	2.9	97.2	0.68 ± 0.03	4.4	95.8
14.3	14.8 ± 0.2	1.5	103	15.1 ± 0.6	4.0	106
71.3	75.0 ± 0.9	1.2	105	76.0 ± 1.4	1.8	106

Table 2
Stability of lorazepam in human plasma ($n = 6$)

Lorazepam (ng/ml)		0.71	14.3	71.3
Freeze-thaw stability (relative error %)	0 cycle	−3.1	2.0	0.3
	3 cycles	−4.0	−1.8	−0.5
Storage stability (<−20 °C) (relative error %)	0 day	−3.3	1.7	−0.5
	10 days	−3.9	−3.1	0.6
	20 days	5.8	1.4	0.2
Processed plasma samples at room temperature (relative error %)	0 h	−3.6	1.8	0.3
	10 h	−4.1	1.1	2.1
	27 h	−5.2	−2.2	1.8

plasma components were observed at the retention times corresponding to the analyte and I.S. Typical chromatograms of a plasma blank, plasma spiked with analyte at the limit of quantitation (0.71 ng/ml), plasma spiked with analyte at 7.13 ng/ml, and plasma sample from a volunteer 2.0 h after administration of 2 mg lorazepam, are respectively illustrated in Fig. 3.

3.3.2. Linearity, LOD, and LOQ

The standard curves showed good linearity over the concentration range of 0.71–71.3 ng/ml for lorazepam in human plasma. A linear regression equation of $y = 0.0400x - 0.0005$ was observed with a correlation coefficient of 0.9999. Under the optimized conditions, the limit of detection ($S/N = 3$) and the limit of quantification ($S/N = 10$) were observed to be 0.10 and 0.71 ng/ml, respectively.

3.3.3. Accuracy, precision and recovery

The precision and accuracy of the method was evaluated by analyzing quality control samples (QCs), prepared at high, medium and low concentrations (71.3, 14.3, and 0.71 ng/ml). The intra-assay precision and accuracy was assessed by measuring QCs in replicates ($n = 6$) on a single day. Inter-assay precision and accuracy was determined from the results of three different QCs of five days. The results (intra- and inter-day precision and accuracy from the QC samples) are listed in Table 1. The intra- and inter-run precisions (expressed by RSD) for the high, medium and low concentrations were better than 2.9% and 4.4%, respectively. The intra- and inter-run accuracy was found to be in the range 95.8–106%. Recovery tests were performed by comparing the peak areas obtained from injection of the spiked plasma to those obtained by injection of the analyte dissolved in the mobile phase. The recoveries of lorazepam were 98.1 ± 4.2%, 102 ± 3.1% and 97.1 ± 1.8% at concentrations of 0.71, 7.13 and 71.3 ng/ml, respectively.

3.3.4. Stability

Stability studies were conducted in blank plasma spiked with 0.71, 14.3, and 71.3 ng/ml lorazepam, respectively, which was stored at −20 °C. The samples were analyzed on the day of samples preparation and then every 10 days for 20 days. Table 2 summarizes stability of lorazepam in human plasma. The results demonstrate that the compound is stable in human plasma through at least three cycles of freeze (−20 °C)–thaw (room temperature) and for at least 20 days under −20 °C freeze conditions. Tests also revealed that the protein-removed samples were stable at room temperature for at least 27 h.

3.3.5. Pharmacokinetic study

The developed HPLC/MS/MS method was applied to evaluate the pharmacokinetic parameters of the drug. A typical plasma concentration–time profile is shown in Fig. 4.

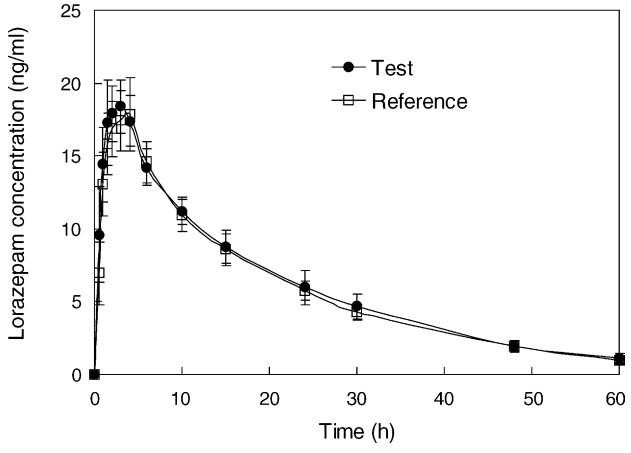

Fig. 4. Mean plasma concentration–time curve of lorazepam in 20 volunteers after a single oral dose of 2 mg lorazepam.

Table 3
Pharmacokinetic parameters (means ± S.D.) after oral administration of 2 mg lorazepam to 20 healthy individuals

Parameter[a]	Test	Reference
t_{max} (h)	2.8 ± 0.8	2.9 ± 1.0
C_{max} (ng/ml)	20.2 ± 4.6	19.6 ± 5.0
$AUC_{0-60\,h}$ (ng h/ml)	370.2 ± 88.3	357.6 ± 70.9
$AUC_{0-\infty}$ (ng h/ml)	396.4 ± 103.6	379.5 ± 81.0
$t_{1/2}$ (h)	14.4 ± 3.7	14.3 ± 2.8

[a] Parameter: t_{max}: time of peak concentration; C_{max}: peak plasma concentration; $AUC_{0-60\,h}$: area under the plasma concentration–time curve (0–60 h); $AUC_{0-\infty}$: area under the plasma concentration–time curve extrapolated to infinity; $t_{1/2}$: terminal elimination half life.

The calculated pharmacokinetic parameters obtained by using Bio-Equivalence computing software (Magnasoft Corp., Shanghai) are summarized in Table 3. The bioavailability of lorazepam is determined using the $AUC_{0-60\,h}$ value from test versus that from reference to the last collected data point (60 h) and found to be 103% ($n = 20$). The pharmacokinetic data is in accordance with the parameters observed by Greenblatt and Shader [41] (1×2 mg; t_{max}: 2 h, C_{max}: 16.9 ng/ml and $t_{1/2}$: 12 h). Fig. 4 and Table 3 indicate that the proposed method has been successfully applied to pharmacokinetic studies to determine the concentration of lorazepam in human plasma.

4. Conclusion

It has been demonstrated that lorazepam presented in plasma samples at low ppb levels can be reliably assayed with coupled HPLC/MS/MS system by using a direct sample injection technique after the plasma protein was simply removed by acetonitrile precipitation. The quantification of lorazepam has been validated in the concentration range from 0.71 to 71.3 ng/ml, which demonstrated a highly reliable precision and accuracy; no signal interferences from endogenous compounds have been observed. Further quality controls revealed adequate analyte stability under all conditions applied. More than 150 samples could be assayed daily, including sample preparation, data acquisition and processing. The present method was used successfully to evaluate the pharmacokinetic study of lorazepam in human after an oral administration.

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