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Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 707–714
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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High-performance liquid chromatography–atmospheric pressure chemical ionisation-mass spectrometry determination of zaleplon in human plasma

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Received 10 April 2005; received in revised form 18 June 2005; accepted 18 June 2005
Available online 8 August 2005

Abstract

A sensitive and specific liquid chromatography–atmospheric pressure chemical ionisation-mass spectrometry (LC–APCI-MS) method has been developed and validated for the identification and quantification of zaleplon in human plasma using estazolam as an internal standard (IS). After the addition of estazolam and 2.0 M sodium hydroxide solution, plasma samples were extracted with ethyl acetate and then the organic layer was evaporated to dryness. The reconstituted solution of the residue was injected onto a prepacked Shim-pack VP-ODS C_{18} (250 mm × 2.0 mm i.d.) column and chromatographed with a mobile phase comprised of methanol:water (70:30) at a flow-rate of 0.2 ml/min. Detection was performed on a single quadrupole mass spectrometer by selected ion monitoring (SIM) mode via atmospheric pressure chemical ionization (APCI) source. The mean standard curve was linear ($r = 0.9991$) over the concentration range of 0.2–100 ng/ml and had good back-calculated accuracy and precision. The intra-day and inter-day precisions were within 10% relative standard deviation and accuracy ranged from 85% to 115%. The limit of detection was 0.1 ng/ml. The validated LC–APCI-MS method has been used successfully to study zaleplon pharmacokinetic, bioavailability and bioequivalence in 18 adult volunteers.

Keywords: Zaleplon; HPLC–APCI-MS; Human plasma; Pharmacokinetics

1. Introduction

Zaleplon, *N*-3-(3-cyanopyrazolo [1,5-a] pyrimidin-7-yl phenyl)-*N*-ethylacetamide, is a nonbenzodiazepine sedative-hypnotic with a pyrazolopyrimidine structure and selectivity for the gamma-aminobutyric acid (GABA) receptor in the brain. It is currently being developed as an ultra-short-acting sleep inducer with a prompt onset of action. The apparent elimination half-life of zaleplon is approximately 1 h [1,2] and it is effective in reducing latency to sleep without evidence of undesired effects in elderly patients with insomnia [1,3,4]. It has been reported that zaleplon is extensively metabolized, with less than 1% of the dose excreted unchanged in urine, it is primarily metabolized by aldehyde oxidase to form 5-oxo-zaleplon, and secondarily by CYP3A4 to form desethylzaleplon, which is quickly converted by aldehyde oxidase to form 5-oxodesethylzaleplon, these oxidative metabolites are further metabolized by conjugation and subsequently eliminated in urine, all of the metabolites do not contribute to the activity of zaleplon [1,5–7].

Previously, Kawashima et al. [8] developed a HPLC–UV method to study three zaleplon metabolites present in rat and monkey plasma but did not attempt to quantify zaleplon. Similarly, Horstkotter et al. [9] established capillary electrophoresis/laser-induced fluorescence detection and HPLC–ESI-MS methods to quantify metabolites of zaleplon present in human urine but failed to address the quantification of zaleplon present in human urine. Although Giroud et

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doi:10.1016/j.jpba.2005.06.031

al. [10] developed a liquid chromatography-turbo-ion spray mass spectrometry method for determination of zaleplon and zolpidem in whole blood, the LLOQ of 1.0 ng/ml achieved in this method was not sufficient for completing pharmacokinetic analysis of the study samples. Feng et al. [11] established a HPLC–ESI-MS method to assay zaleplon in human plasma by monitoring $[M + Na]^+$, but it was found that the regression curves were nonlinear in our study. In this paper, by comparing the APCI mode with the ESI mode, a more simple, selective and accurate method was first described by using high performance liquid chromatography coupled with atmospheric pressure chemical ionization (APCI) single quadrupole mass spectrometry (MS) for the determination of zaleplon in human plasma.

2. Experimental

2.1. Chemicals and reagents

Zaleplon test orally disintegrating tablets (Batch No: 20030903), zaleplon reference standard (Batch No: 020410) were both supplied and identified by Hainan Pioneer Pharmaceutical Corporation (Hainan, PR China); zaleplon reference dispersible tablets (Batch No: 20040409) were purchased from Sinow Pharmaceutical Corporation (Henan, PR China); estazolam reference standard (99.5% purity) were supplied by Jiangsu Institute for Drug Control (Nanjing, PR China); methanol was chromatographic pure grade and purchased from Merck (Merck Company, Germany). Other chemicals were all of analytical grade and were used as received.

2.2. Instrumentation and operating conditions

2.2.1. Liquid chromatography

Liquid chromatography was performed using a Shimadzu LC-10AD HPLC system consisting of an autosampler (SIL-HTc). The column was a Shim-pack VP-ODS C_{18} (250 mm × 2.0 mm i.d., 5 μm) and was operated at 40 °C. The mobile phase consisted of methanol–water (70:30) was set at a flow rate of 0.2 ml/min.

2.2.2. Mass spectrometry

Mass spectrometric detection was performed using a Shimadzu LCMS-2010A quadrupole mass spectrometer with an atmospheric pressure chemical ionization mode. The APCI source was set at positive ionization mode. The $[M + H]^+$ ions at m/z 306.05 and 294.95 were selected as detection ions for zaleplon and estazolam, respectively. The MS operating conditions were optimized as follows: nebulizer gas rate 2.0 l/min, CDL temperature 250 °C, block temperature 200 °C, APCI temperature 350 °C, probe voltage: +4.5 kV. The quantification was performed via peak-area. Data acquisition and processing were accomplished using Shimadzu LCMS solution Software for LCMS-2010A system.

In the comparing study of the ESI and APCI mode, the ESI operating conditions were as follows: nebulizer gas rate 1.5 l/min, CDL temperature 250 °C, block temperature 200 °C, probe voltage: +4.5 kV.

2.3. Preparation of stock solutions

Stock solutions of zaleplon and estazolam (IS) were prepared in HPLC mobile phase (methanol:water = 70:30, v/v) at concentrations of 1.0 mg/ml and were stored at 4 °C.

Working solutions of zaleplon were prepared daily in HPLC mobile phase by appropriate dilution at 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 ng/ml and 1.0, 2.5, 5.0 μg/ml.

The stock solution of estazolam was further diluted with HPLC mobile phase to prepare the working internal standard solution containing 1.0 μg/ml of estazolam.

2.4. Sample preparation and extraction procedure

A 1-ml aliquot of the collected plasma sample from a human volunteer was pipetted into a 10 ml centrifuge tube. The internal standard solution (40 μl × 1 μg/ml), 100 μl of 2.0 M sodium hydroxide solution and 5 ml ethyl acetate were added and then were vortexed for 3 min. After centrifugation of the sample at 1330 × g for 10 min, the organic layer was transferred to another 10 ml centrifuge tube and evaporated to dryness under stream of nitrogen gas at 40 °C. The residue was redissolved in 100 μl mobile phase. An aliquot of 10 μl was injected into the LC–MS system.

2.5. Standard curves

Proper volume of one of the above-mentioned working solutions to produce the standard curve points equivalent to 0.20 (20 μl × 10 ng/ml), 0.50 (20 μl × 25 ng/ml), 1.0 (20 μl × 50 ng/ml), 2.0 (20 μl × 100 ng/ml), 5.0 (20 μl × 250 ng/ml), 10.0 (20 μl × 500 ng/ml), 20.0 (20 μl × 1 μg/ml), 50.0 (20 μl × 2.5 μg/ml) and 100.0 (20 μl × 5 μg/ml) ng/ml of zaleplon was added into 10 ml centrifuge tubes, respectively, and evaporated to dryness under stream of nitrogen gas at 40 °C, and then redissolved in 1 ml blank plasma. Each sample also contained 40.0 ng (40 μl × 1 μg/ml) of the internal standard. The following assay procedures were the same as described above. In each run, a blank plasma sample (no IS) was also analyzed.

2.6. Preparation of quality control samples

Quality control samples were prepared daily by spiking different samples of 1 ml plasma each with proper volume of the corresponding standard solution to produce a final concentration equivalent to low level (0.50 ng/ml), middle level (10.0 ng/ml) and high level (50.0 ng/ml) of zaleplon with 40.0 ng/ml of internal standard each. The following procedures were the same as described above.

2.7. Method validation

The method validation assays were carried out following the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [12].

2.7.1. Assay specificity

Analyses of blank samples of the healthy human blank plasma were obtained from six sources.

In order to evaluate the matrix effect on the ionization of analytes, three different concentration levels of zaleplon (20 μl × 25 ng/ml, 20 μl × 500 ng/ml and 20 μl × 2.5 μg/ml) were added to the dried extracts of 1 ml blank sample, respectively, then were dried and dissolved with 100 μl mobile phase. The same concentration levels of zaleplon were dried directly and dissolved with the same volume of the mobile phase. The matrix effect of internal standard (40 ng/ml) was evaluated using the same method.

2.7.2. Linearity

Standard curves of nine concentrations of zaleplon ranged 0.2–100 ng/ml were extracted and assayed. Blank plasma samples were analyzed to ensure the lack of interferences but not used to construct the calibration function. The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were determined as the concentrations with a signal-to-noise ratio of 5 and 10, respectively.

2.7.3. Precision and accuracy

The precision of the assay was determined from the QC plasma samples by replicate analyses of three concentration levels of zaleplon (0.50, 10.0 and 50.0 ng/ml). Intra-day precision and accuracy was determined by repeated analysis of the QC plasma samples on one day ($n = 5$). Inter-day precision and accuracy was determined by repeated analysis on five consecutive days ($n = 1$ series per day). The concentration of each sample was determined using standard curve prepared and analyzed on the same day.

2.7.4. Extraction recovery

The extraction recovery of zaleplon was determined by comparing the zaleplon/I.S. peak area ratios (R_1) obtained from extracted plasma samples with those (R_2) from standard solutions at the same concentration. This procedure was repeated for the three different concentrations of 0.50, 10.0 and 50.0 ng/ml.

2.7.5. Stability

Freeze and thaw stability: Three concentration levels of QC plasma samples were stored at the storage temperature (−20 °C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycle were repeated twice, then the samples were tested after three freeze (−20 °C)-thaw (room temperature) cycles.

Short-term temperature stability: Three concentration levels of QC plasma samples were kept at room temperature for a period that exceeded the routine preparation time of samples (around 6 h).

Long-term stability: Three concentration levels of QC plasma samples kept at low temperature (−20 °C) were studied for a period of 3 weeks.

Post-preparative stability: The autosampler stability was conducted reanalyzing extracted QC samples kept under the autosampler conditions (4 °C) for 12 h.

Stock solution stability: The stability of zaleplon and internal standard working solutions were evaluated at room temperature for 6 h.

2.7.6. Standard curve and quality control sample in each batch

A standard curve in each analytical run was used to calculate the concentration of zaleplon in the unknown samples in the run. It was prepared at the same time as the unknown samples in the same batch and analyzed in the middle of the sample set.

The QC samples in four duplicates at three concentrations (0.50, 10.0 and 50.0 ng/ml) were prepared and were analyzed with processed test samples at intervals per batch.

3. Clinical study design

This was an open randomized, balanced, two-period crossover study in 18 Chinese healthy men. Each volunteer received a single oral dose of 10.0 mg zaleplon test tablets or reference tablets in random order in cycle. Blood samples (3 ml) containing zaleplon were collected at the time of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 3.0, 4.0, 6.0 and 8.0 h after oral administration of the medicines. They were put into lithium heparin tubes and immediately centrifuged at 1600 × *g* for 10 min. The plasma samples obtained were frozen at −20 °C in coded polypropylene tubs until analysis.

4. Results and discussion

4.1. Selection of the ionization mode

4.1.1. Comparison of the full scan spectrum of zaleplon in two ionization modes (ESI and APCI)

With the mobile phase of methanol and water, in the ESI full-scan spectrum of zaleplon, the $[M + Na]^+$ ion predominated, and the protonated ion $[M + H]^+$ had very low intensity (see Fig. 1); in the APCI full-scan spectrum of zaleplon, the $[M + H]^+$ ion predominated, and its intensity was at least the 50 times of that of $[M + Na]^+$ ion (see Fig. 2). The phenomenon showed the different mechanisms of ion generation in these two ionization modes.

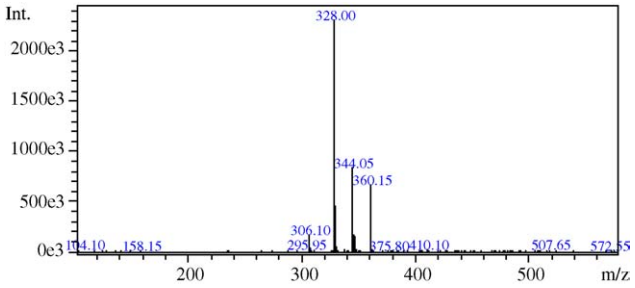

Fig. 1. Positive ion electrospray mass spectrum of zaleplon.

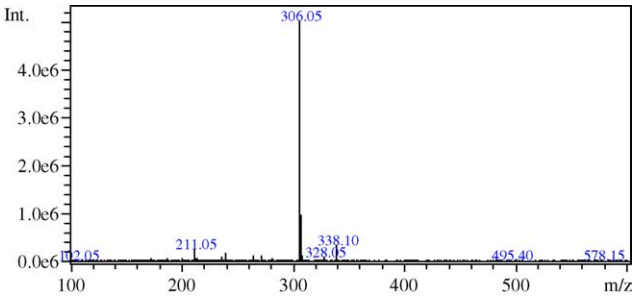

Fig. 2. Positive ion atmospheric pressure chemical ionization mass spectrum of zaleplon.

4.1.2. $[M + H]^+$ ion of zaleplon in ESI

With the mobile phase of methanol and water, the lower limit of quantification of zaleplon was 10 ng/ml in this condition. Because the response of the $[M + H]^+$ ion was usually stable in mass spectrometry, different mobile phases were tried to strengthen the intensity of the $[M + H]^+$ ion. With the mobile phase of methanol and water (different pH), it was found that the response of the $[M + H]^+$ ion was slightly strengthened with the decreasing of the pH. When the pH of the mobile phase was 4.0, the LLOQ of zaleplon was just 1 ng/ml; With the mobile phase of methanol and water (different concentrations of ammonium acetate), it was concluded that the ammonium acetate could restrain the intensity of the $[M + Na]^+$ ion and elevate the response of the $[M + H]^+$ ion, but when the concentration of the ammonium acetate was up to 20 mM, the LLOQ was also just 1 ng/ml. Because zaleplon undergoes extensive presystemic metabolism, its absolute bioavailability was only approximately 30% [5], which meant the low plasma concentration, the two mobile phases mentioned above could not meet the sensitivity of the test.

4.1.3. $[M + Na]^+$ ion of zaleplon in ESI

Working solutions of zaleplon were prepared in HPLC mobile phase by appropriate dilution at 10, 50, 100, 200, 500, 1000 ng/ml.

To evaluate the formation of sodium adduct, $[M + Na]^+$, respective to available Na^+ in the mobile phase, mobile phases containing different concentrations of sodium salt (10, 50, 100 and 200 nM) were evaluated. It was found that the deviation of the response of $[M + Na]^+$ at high concentration of zaleplon was exist, which might result from the lack of sodium ions during the ionization process for high concentration samples. Table 1 shows the result of zaleplon working solution HPLC–ESI-MS determination in different mobile phases mentioned above.

4.1.4. Comparison of $[M + H]^+$ ion of zaleplon in APCI and $[M + Na]^+$ ion of zaleplon in ESI

Comparison of the standard curves: Proper volume of one of the above-mentioned working solutions to produce the standard curve points equivalent to 0.50, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/ml of zaleplon was added into 10 ml centrifuge tubes, respectively, and evaporated to dryness under stream of nitrogen gas at 40 °C, and then redissolved in 1 ml blank plasma. Each sample also contained

Table 1
The results of zaleplon working solution HPLC–ESI-MS determination in different mobile phases ($n = 5$)

Concentration of NaAc (nmol/l)	Concentration of zaleplon (ng/ml)					
	1000	500	200	100	50	10
10						
Back-calculated concentration	653.38	431.44	224.20	116.30	51.40	9.69
Accuracy (%)	65.34	86.29	112.10	116.30	102.79	96.86
r	0.9787					
50						
Back-calculated concentration	702.36	427.32	209.07	113.61	57.50	9.61
Accuracy (%)	70.24	85.46	104.54	113.61	115.01	96.11
r	0.9814					
100						
Back-calculated concentration	752.07	440.63	210.34	111.87	56.02	9.66
Accuracy (%)	75.21	88.13	105.17	111.87	112.03	96.56
r	0.9870					
200						
Back-calculated concentration	784.28	467.68	213.41	107.90	54.28	9.74
Accuracy (%)	78.43	93.54	106.70	107.90	108.56	97.36
r	0.9916					

Table 2
The comparing study of the standard curves by two different ionization mode ($n = 5$)

Concentration of zaleplon in plasma (ng/ml)	0.5	1	2	10	20	50	100
ESI							
R.S.D. (%)	17.79	11.13	13.38	10.13	9.22	16.75	18.65
Back-calculated concentration	0.4492	1.1481	2.2517	10.0076	19.7125	45.1823	86.6062
Accuracy (%)	89.84	114.81	112.58	100.08	98.56	90.36	86.61
r				0.9914			
APCI							
R.S.D. (%)	5.61	6.08	2.38	5.41	3.24	2.57	1.62
Back-calculated concentration	0.5116	0.9694	1.9293	9.5217	20.2732	52.8784	100.2005
Accuracy (%)	102.32	96.94	96.47	95.22	101.37	105.76	100.20
r				0.9990			

300.0 ng (30 μl × 10 μg/ml) of the internal standard. The following assay procedures were the same as described above in Section 2.4. An aliquot of 10 μl of each extracted sample was injected into the LC–MS system, respectively, in the conditions of ESI/SIM/$[M + Na]^+$(both for zaleplon and IS) with the mobile phase of methanol:water (100 nM sodium acetate) = 70:30 and APCI/SIM/$[M + H]^+$ (both for zaleplon and IS) with the methanol:water = 70:30 as mobile phase. At last it was found (see Table 2) that the linearity and repeatability of the $[M + H]^+$ response in APCI mode was obviously better than that of the $[M + Na]^+$ response in ESI mode.

Comparison of precision and accuracy tests: Using the analytical methods described in Section 2.7.3, an aliquot of 10 μl of each extracted sample was injected into the LC–MS system, respectively, in the two conditions mentioned above. It was found (see Table 3) that the method using APCI mode had the better precision, accuracy and stability.

With the comparison of these two ionization modes, the method using high performance liquid chromatography coupled with atmospheric pressure chemical ionization single quadrupole mass spectrometry (MS) for the determination of zaleplon in human plasma was at last adopted.

Table 3
The inter-day and intra-day precision, accuracy of the method with the determination of zaleplon by two different ionization mode ($n = 5$)

Concentration of zaleplon in plasma (ng/ml)	0.5	10	50
ESI			
Intra-day			
R.S.D. (%)	15.44	9.51	12.06
Detected concentration	0.4678	9.8921	46.7215
Accuracy (%)	93.56	98.92	93.44
Inter-day			
R.S.D. (%)	17.09	9.42	14.04
Detected concentration	0.4505	9.7934	46.9350
Accuracy (%)	90.10	97.93	93.87
APCI			
Intra-day			
R.S.D. (%)	8.53	2.81	2.23
Detected concentration	0.5065	10.1514	52.0507
Accuracy (%)	101.30	101.51	104.10
Inter-day			
R.S.D. (%)	9.23	3.57	2.44
Detected concentration	0.5094	9.7551	51.9763
Accuracy (%)	101.88	97.55	103.95

4.2. Selection of mass spectrometric conditions

Different rates of the nebulizer gas were tested and the rate of 2.0 l/min was adopted at last for the highest intensity of the analyte response in this condition. The other MS parameters were adopted as the recommended value of the instrument.

4.3. Selection of IS

It is necessary to use an IS to get high accuracy when a mass spectrometer is used as the HPLC detector. Estazolam, diazepam and flurazepam were investigated to find the more suitable one. Estazolam was adopted in the end because of its similarity of retention and ionization with the analyte and the less endogenous interferences at estazolam $[M + H]^+$, m/z 294.95. The structure of zaleplon and estazolam are shown in Fig. 3.

4.4. Sample preparation

Zaleplon undergoes extensive presystemic metabolism, so its absolute bioavailability is approximately 30% [5], the concentration of zaleplon in plasma is also very low. In order to elevate the detector's sensitivity of zaleplon, liquid–liquid extraction [10] was necessary and important because this technique can not only purify but also concentrate the sample. Ethyl acetate was finally adopted as extraction solution

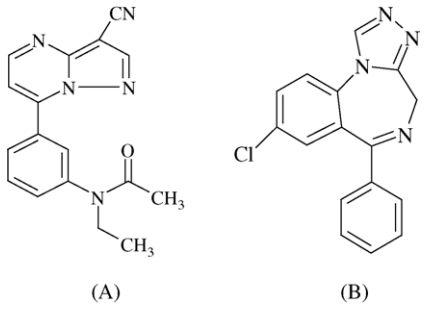

Fig. 3. Chemical structure of zaleplon (A) and estazolam (B).

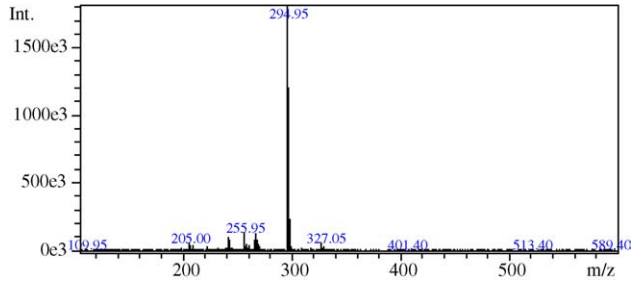

Fig. 4. Positive ion atmospheric pressure chemical ionization mass spectrum of estazolam.

because of its high extraction efficiency. Different concentration levels of sodium hydroxide (0.1, 1 and 2 M) were tested, finally we used sodium hydroxide (0.1 ml × 2.0 M) because it can not only reduce the amount of acidic, endogenous interferences but also accelerate the drugs' dissociation from the plasma and enhance the extraction stability.

4.5. Separation

In positive mode, atmospheric pressure chemical ionization mass spectra of zaleplon and IS are shown in Figs. 2 and 4, respectively. The major ions observed were $[M+H]^+$, $m/z = 306.05$; $[M+Na]^+$, $m/z = 328.05$; $[M+CH_3OH_2]^+$, $m/z = 338.10$ for zaleplon and $[M+H]^+$, $m/z = 294.95$; $[M+CH_3OH_2]^+$, $m/z = 327.00$ for estazolam. The ions of $[M+H]^+$, $m/z = 306.05$ for zaleplon and $[M+H]^+$, $m/z = 294.95$ for estazolam were selected for the SIM (+) due to their high stability and intensity.

The SIM (+) chromatograms extracted from supplemented plasma are depicted in Fig. 5. As shown, the retention times

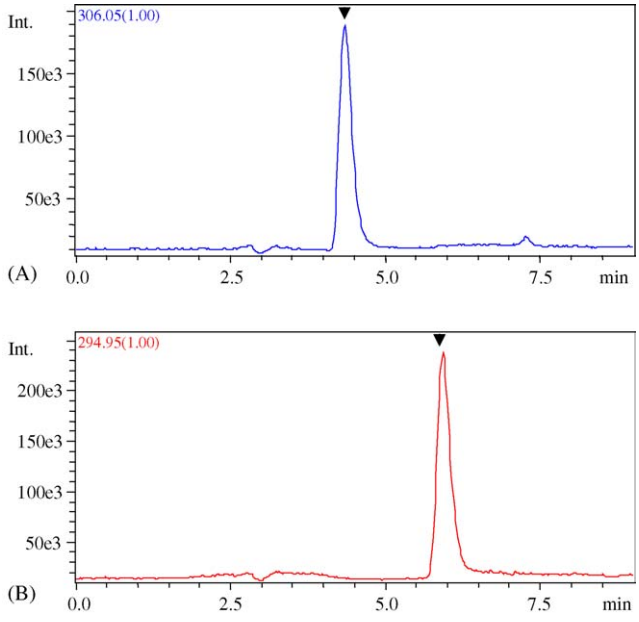

Fig. 5. The SIM (+) chromatograms extracted from supplemented plasma. Peaks were assigned with (▼).The retention times of zaleplon and the IS were 4.3 min (A) and 5.9 min (B), respectively.

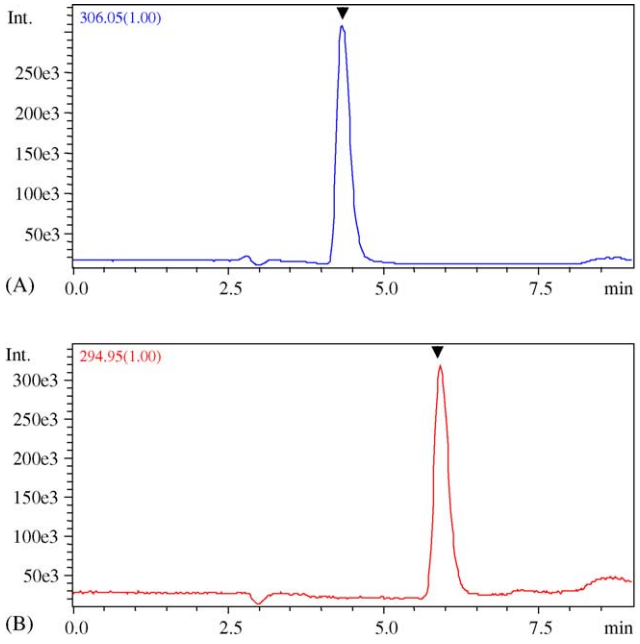

Fig. 6. The SIM (+) chromatograms for plasma sample of a healthy volunteer. Peaks were assigned with (▼). The retention times of zaleplon and the IS were 4.3 min (A) and 5.9 min (B), respectively.

of zaleplon and the IS were 4.3 and 5.9 min, respectively. They were well separated, which avoided the interference of ionization between them.

The total HPLC–MS analysis time was 8 min per sample. A representative chromatogram of a plasma sample obtained at 1.25 h from a subject who received a single oral dose (10 mg) is shown in Fig. 6.

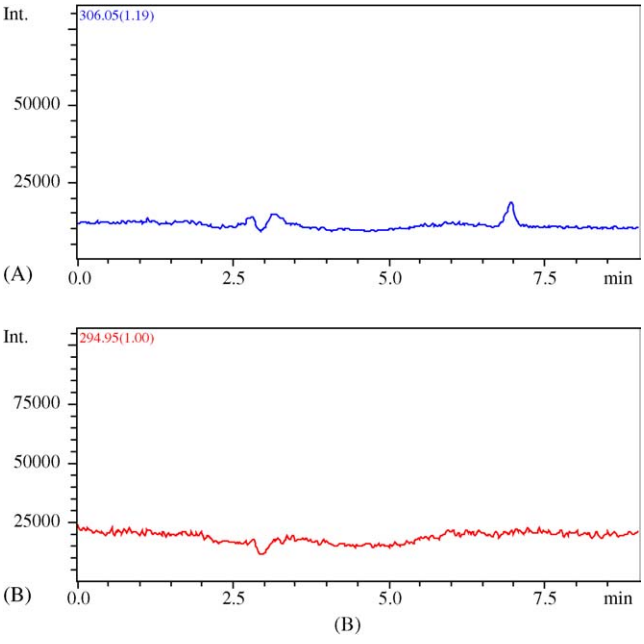

Fig. 7. The SIM (+) chromatograms extracted from a blank plasma.

Table 4
Results of five representative standard curves for zaleplon LC–MS determination

Added concentration (ng/ml)	0.2	0.5	1.0	2.0	5.0	10.0	20.0	50.0	100.0
Back-calculated concentration	0.2022	0.5401	0.8929	1.9256	5.0403	10.0731	20.4287	53.1986	104.6092
	0.2025	0.5293	1.0088	2.0397	4.6418	9.6569	20.7347	52.1745	102.7076
	0.2145	0.5541	0.9577	2.0753	4.5716	9.1851	20.2235	53.3458	102.0856
	0.1695	0.4813	0.9829	1.9053	4.6930	10.0838	21.2185	51.1642	100.5668
	0.2142	0.4561	0.8900	1.8223	4.7059	9.7818	20.3530	52.3022	103.5710
Mean	0.2006	0.5122	0.9464	1.9536	4.7305	9.7561	20.5917	52.4371	102.7080
R.S.D. (%)	8.10	7.72	5.49	5.21	3.81	3.77	1.93	1.68	1.49
Accuracy (%)	100.30	102.43	94.64	97.68	94.61	97.56	102.96	104.87	102.71

Table 5
The inter-day and intra-day precision, accuracy of the method for determination of zaleplon ($n = 5$)

Added concentration (ng/ml)	Intra-day			Inter-day		
	Detected concentration (mean ± S.D., ng/ml)	Mean accuracy (%)	R.S.D. (%)	Detected concentration (mean ± S.D., ng/ml)	Mean accuracy (%)	R.S.D. (%)
0.5	0.5045 ± 0.0357	100.90	7.08	0.5129 ± 0.0412	102.59	8.03
10.0	9.9367 ± 0.2269	99.37	2.28	9.6586 ± 0.3650	96.59	3.78
50.0	52.0191 ± 0.6760	104.04	1.30	51.8859 ± 0.8728	103.77	1.68

4.6. Method validation

4.6.1. Assay specificity

No interferences of the analytes were observed. Fig. 7 shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of zaleplon or internal standard (estazolam). Compared the peak area resolved in blank sample with that resolved in mobile phase, all the ratios of these two values are between 85% and 115%, which means no ion suppression for zaleplon and estazolam in this method.

4.6.2. Linearity and LLOQ

The quantification of zaleplon in plasma samples was carried out by determining the slope (b), intercept (a) and regression coefficient (r) of the standard curves of the peak area ratio of zaleplon/estazolam versus zaleplon concentration. Using linear regression analysis, the data confirmed linear relationships over the selected concentration range. Standard curves were constructed on 5 different days. The mean standard curve was typically described by the least-square equation: $R = 0.0394 \times C + 0.0010$ ($r = 0.9991$), where R corresponds to the peak area ratio of zaleplon to the I.S. and C refers to the concentration of zaleplon added to plasma over a concentration range of 0.2–100 ng/ml. Results of five representative standard curves for zaleplon LC–MS determination are given in Table 4.

Table 6
Recoveries of zaleplon from plasma ($n = 5$)

Added (ng/ml)	Recovery (mean ± S.D. %)	R.S.D. (%)
0.5	92.94 ± 7.02	7.55
10.0	90.81 ± 3.62	3.99
50.0	95.01 ± 1.61	1.69

The lower limit of quantification for zaleplon was proved to be 0.20 ng/ml and the lower limit of detection was 0.10 ng/ml. Fig. 8 shows the chromatogram of an extracted sample that contained 0.20 ng/ml (LLOQ) of zaleplon.

4.6.3. Precision and accuracy

Data for intra-day and inter-day precision and accuracy of the method for zaleplon are presented in Table 5. The accuracy deviation values are within 15% of the actual values. The precision determined at each concentration level doesnot

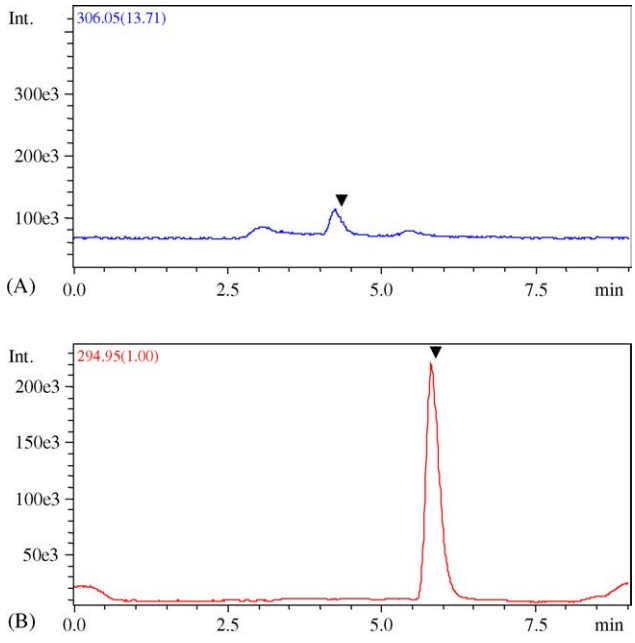

Fig. 8. The SIM (+) chromatograms of LLOQ (0.2 ng/ml). Peaks were assigned with (▼). The retention times of zaleplon and the IS were 4.3 min (A) and 5.9 min (B), respectively.

Table 7
Data showing stability of zaleplon in human plasma at different QC levels ($n = 5$)

	0.5 ng/ml Accuracy (mean ± S.D. %)	10.0 ng/ml Accuracy (mean ± S.D. %)	50.0 ng/ml Accuracy (mean ± S.D. %)
Short-term stability	90.23 ± 7.50	96.01 ± 3.85	98.49 ± 3.38
Freeze and thaw stability	91.14 ± 6.42	95.27 ± 4.47	93.31 ± 3.96
Long-term stability	88.69 ± 6.77	93.56 ± 3.52	93.72 ± 3.59
Post-preparative stability	95.46 ± 7.86	98.64 ± 2.76	97.57 ± 2.48

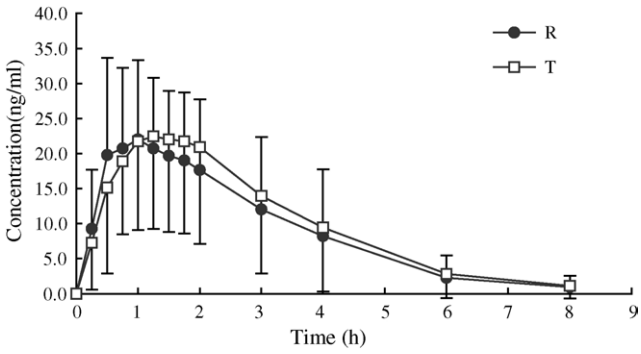

Fig. 9. Mean drug plasma concentration-time curve of zaleplon in 18 volunteers after oral administration (R: reference tablets; T: test tablets).

exceed 15% of the relative standard deviation (R.S.D.). The results revealed good precision and accuracy.

4.6.4. Extraction recovery

The extraction recovery determined for zaleplon was shown to be consistent, precise and reproducible. Data are shown below in Table 6.

4.6.5. Stability

Table 7 summarizes the freeze and thaw stability, short-term stability, long-term stability and post-preparative stability data of zaleplon. All the results showed the stability behavior during these tests and there were no stability-related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies.

The stability of working solutions was tested at room temperature for 6 h. Based on the results obtained, these working solutions were stable within 6 h.

4.7. Results of pharmacokinetic study

The procedure developed was sensitive enough to assure the quantitative analysis of zaleplon in plasma with acceptable accuracy during a period of 8 h after a single oral administration. The mean plasma concentration–time profiles of 18 volunteers are represented in Fig. 9. Pharmacokinetic parameters of the test orally disintegrating tablets and reference dispersible tablets are listed in Table 8. The test tablet was found to be bioequivalent to the reference one.

Table 8
Pharmacokinetic parameters of zaleplon in 18 men after oral administration

Parameters	Test	Reference
C_{max} (ng ml^{-1})	27.66 ± 14.29	26.09 ± 12.23
T_{max} (h)	1.4 ± 0.5	1.3 ± 0.9
$T_{1/2}$(Kel) (h)	1.26 ± 0.24	1.23 ± 0.38
$MRT_{0\to\infty}$ (h)	2.60 ± 0.53	2.42 ± 0.65
$Auc_{0\to 8}$ (ng h ml^{-1})	80.23 ± 55.25	73.65 ± 46.30
$Auc_{0\to\infty}$ (ng h ml^{-1})	82.85 ± 59.59	76.19 ± 50.84

5. Conclusion

A sensitive, selective, accurate and precise HPLC method with selected ion monitoring by single quadrupole mass spectrometer with APCI interface was developed and validated for determination of zaleplon in human plasma. The reported method offers several advantages, such as a rapid and simple extraction scheme, improved detection stability and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from the pharmacokinetic, bioavailability or bioequivalent study of zaleplon.

References

[1] W.E. Heydorn, Expert Opin. Investig. Drugs 9 (2000) 841–858.
[2] M.G. Terzano, M. Rossi, V. Palomba, A. Smerieri, L. Parrino, Drug Saf. 26 (2003) 261–282.
[3] S. Ancoli-Israel, J.K. Walsh, R.M. Mangano, M. Fujimori, Prim. Care. Companion J. Clin. Psychiatry 1 (1999) 114–120.
[4] D.R. Drover, Clin. Pharmacokinet. 43 (2004) 227–238.
[5] A.S. Rosen, P. Fournie, M. Darwish, P. Danjou, S.M. Troy, Biopharm. Drug Dispos. 20 (1999) 171–175.
[6] B.G. Lake, S.E. Ball, J. Kao, A.B. Renwick, R.J. Price, J.A. Scatian, Xenobiotica 32 (2002) 835–847.
[7] A.B. Renwick, H. Mistry, S.E. Ball, D.G. Walters, J. Kao, B.G. Lake, Xenobiotica 28 (1998) 337–348.
[8] K. Kawashima, K. Hosoi, T. Naruke, T. Shiba, M. Kitamura, T. Watabe, Drug Metab. Dispos. 27 (1999) 422–428.
[9] C. Horstkotter, D. Schepmann, G. Blaschke, J. Chromatogr. A 1014 (2003) 71–81.
[10] C. Giroud, M. Augsburger, A. Menetrey, P. Margin, J. Chromatogr. B 789 (2003) 131–138.
[11] F. Feng, J. Jiang, H. Dai, J. Wu, J. Chromatogr. Sci. 41 (2003) 17–21.
[12] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), May 2001.