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# Development and Validation of a High-Performance Liquid Chromatography–Electrospray Ionization–Mass Spectrometry Assay for the Determination of Zaleplon in Human Plasma

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## Abstract

**In this paper, a sensitive and rapid chromatographic procedure using a selective analytical detection method (electrospray ionization–mass spectrometry in selected-ion monitoring mode) in combination with a simple and efficient sample preparation step is first presented for the determination of zaleplon in human plasma. The separation of the analyte, internal standard, and possible endogenous compounds are accomplished on a phenomenex Luna 5-µm $C_8$(2) column (250- × 4.6-mm i.d.) with methanol–water (75:25, v/v) as the mobile phase. In order to optimize the mass detection of zaleplon, several parameters such as ionization mode, fragmentor voltage, *m/z* ratios of ions monitored, type of organic modifier, and eluent additive in the mobile phase are discussed. An internal standard is selected to guarantee the quantitative accuracy. Each analysis takes less than 6 min. The calibration curve of zaleplon in the range of 0.1–60.0 ng/mL in plasma is linear with a correlation coefficient of > 0.9992, and the detection limit (s/n = 3) is 0.1 ng/mL. The within- and between-day variations (relative standard deviation) in the zaleplon plasma analysis are less than 2.4% (*n* = 15) and 4.7% (*n* = 15), respectively. The application of this method is demonstrated for the analysis of zeleplon plasma samples in a Phase-I human pharmacokinetic study.**

## Introduction

Zaleplon, *N*-[3-(3-cyanopyrazolo[1,5-a]pyrimidin-7-yl)phenyl]-*N*-ethylacetamide, is a novel nonbenzodiazepine hypnotic agent that binds selectively to the benzodiazepine type-1 site on the g-aminobutyric acid subtype-A($GABA_A$)/chloride-ion channel complex (1). The hypnotic effects of zaleplon are similar to those of benzodiazepines and other nonbenzodiazepine hypnotics (2,3), but zaleplon causes less impairment of cognitive and psychomotor function than most older hypnotics (4–6). The recommended zaleplon dose to achieve hypnotic effects is very low (10 mg daily in China), and these doses produced peak plasma concentrations of approximately 16–28 ng/mL (7). As these doses produce very low therapeutic concentrations in plasma, sensitive methods are required in order to determine zaleplon in samples from clinical studies and particularly in bioequivalence single-dose Phase-I studies.

Only one method has been reported for the determination of zaleplon in biological fluids (8). The method involves precipitating protein with acetonitrile, then the supernatant is removed and evaporated to dryness. Residue was resuspended in the mobile phase and analyzed by reversed-phase (RP) high-performance liquid chromatography (HPLC) with fluorescence detection. Using the method to analyze plasma samples in a Phase-I human pharmacokinetic study, we found that the method is not suitable under all conditions. In some cases, endogenous peaks co-eluted with zaleplon and this caused high variations in zaleplon recovery. Also, concentration of aqueous solution by evaporation is time-consuming, especially when several hundreds of samples are analyzed. Another shortcoming of the reported method is that quantitation with external standardization method is sometimes unsatisfactory when fluorescence detection is used because it is easily affected by many factors.

The need for a more productive and sensitive analytical method to assay plasma samples produced during clinical studies required the development of a new analytical method. The use of HPLC coupled with atmospheric pressure electrospray ionization (ESI)–mass spectrometry (MS) is becoming increasingly popular because of its selectivity and sensitivity for the determination of drugs in biological fluids at low concentrations. In this study, a HPLC–ESI–MS method with selected-ion monitoring (SIM) mode was developed for the assay of zaleplon in human plasma samples. Because zaleplon is a lipophilic compound, a one-step clean-up procedure using liquid–liquid extraction prior to HPLC–MS analysis was adopted.

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## Experimental

### Chemical

Zaleplon and triazolam [internal standard, (IS)] were supplied by the department of Pharmaceutical Development (Hua Sheng Pharmaceutical, Chang Zhon, China). Acetonitrile and methanol were HPLC/Spectro grade and purchased from Tedia company (Fairfield, OH). Other chemicals were all of analytical grade. All of the reagents were used as received. Water was purified by a Milli-Q system from Waters Millipore (Vimodrone, Italy).

### Apparatus

An HP Model 1100 series liquid chromatography (LC) system equipped with a binary pump was connected to an autosampler (Hewlett-Packard, Palo Alto, CA). Chromatographic separation was carried out at room temperature using a Phenomenex Luna $C_8$(2) analytical column (250- × 4.6-mm i.d., packed with 5-µm $C_8$ silica RP particle) supplied by DIKMA (Bejing, China). The mobile phase consisted of methanol–water (75:25, v/v). Baseline separation was achieved by isocratic solvent elution at a flow rate of 1 mL/min.

Detection was performed on an HP1100 single-quadruple MS (Hewlett-Packard) equipped with an ESI source. The ionization mode was ESI, positive, and SIM. The detected ions were zaleplon [*m/z* 328.1, $(M+Na)^+$] and triazolam [*m/z* 365.0, $(M+Na)^+$]. The interface variables were the following: temperature (350°C), drying gas [$N_2$ (10.0 L/min)], nebulizer pressure [40 p.s.i. ($N_2$)], capillary voltage (4000 V), fragmentor voltage (90 V), and dwell time (500 ms).

The detector was interfaced to an HP computer. All data were acquired and processed by Agilent Chemstation Rev. A.08.03 software (Agilent, Palo Alto, CA) with sample control and MacQuan, respectively.

Optimization of the interface variable (such as gas flow and fragment voltage) was done during direct infusion of 10-µg/mL separate solutions of the target analyte dissolved in mobile phase.

### Sample Preparation

Twenty healthy volunteers received a single oral dose of zaleplon (10 mg). Venous blood (3.0 mL) was collected into sodium-heparinized Venoject sampling tubes. Plasma fractions were separated immediately prior to dosing at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4, 6, and 8 h after drug administration. The tubes were immediately centrifuged at 4°C at 3000 g for 15 min. The plasma was then removed and stored at –20°C until analysis was conducted.

To 1 mL of plasma samples in a centrifuge tube, 20 ng triazolam (IS) in the mobile phase was spiked. After adding 0.1 mL of NaOH (2 mol/L) and 5.0 mL of ethyl acetate, the mixture was vortexed for 3 min and centrifuged at 3500 RPM for 10 min. The supernatant was transferred to a 10-mL tube and was then evaporated to dryness in a water bath at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 0.2 mL of HPLC mobile phase. An aliquot of the solution of 35 µL was injected onto the LC–MS system.

### Calibration standards and quality control samples

Stock solutions of zaleplon and triazolam, with a concentration of 1 mg/mL, were prepared separately by dissolving 10 mg of each compound in the HPLC mobile phase.

Quality control samples were prepared by spiking control plasma samples (1 mL) with zaleplon at three concentration levels (low, medium, and high) on the day of blood sampling. The quality control samples were stored together with the unknown samples at temperatures below –20°C. Six of the quality control samples at each concentration level were analyzed in each sample sequence.

Calibration samples were prepared by spiking control plasma samples (1 mL) with zaleplon at 0.1, 0.5, 1.0 , 5.0, 10.0, 20.0, 40.0, 60.0 ng/mL and triazolam (IS) at 20 ng/mL on the day of sample preparation. To each batch of sample, a calibration curve covering the whole analytical working range was run in duplicate with the unknown samples. The ratio (Ai/As) of the peak areas of the analyte (Ai) and IS (As) in each fraction was calculated. Calibration covers were constructed by plotting the peak area ratio versus standard concentration of zaleplon, and the plot was then subjected to linear regression analysis.

### Validation

The method was validated by analysis of human plasma quality control samples prepared at three concentrations spanning the calibration range. Quality control samples were analyzed on six different days. On the first day, the number of samples of quality control was 15 (5 at each concentration level). On the following five days, the number of samples of quality control was three per day. Precision and accuracy were determined. Precision of a method was expressed as the percentage of the relative standard deviation (RSD) of replicate measurements. Accuracy was represented by percentage difference from theoretical value. To be acceptable, the measures should be lower than 15% at all concentrations.

The selectivity of the analytical procedure was determined by individual analysis of blank samples. The retention times of possible endogenous compounds in the matrix were compared with those of zaleplon and triazolam.

The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. The limit of quantitation (LOQ) was defined as the lowest drug concentration that can be determined with accuracy and precision. In this work, LOD of the assay method was determined by analysis of the peak baseline noise in ten blank samples, resulting in a peak area of three times the noise in ten blank samples.

Over one month, the stability of zaleplon was studied in both frozen plasma (–20°C) and processed samples left at room temperature (20 ± 3°C) over 24 h.

## Results and Discussion

HPLC–MS has grown into one of the most powerful analytical techniques currently available. Among its advantages, HPLC–MS technology has provided a high level of sensitivity and selectivity. This made it possible to analyze nonvolatile, low-concentration compounds in biological samples.

At the start of this investigation, we attempted to use the pub-

lished method (8) to analyze zaleplon in plasma with fluorescence detection. However, the results obtained in our laboratory showed that the retention time for zaleplon could not be perfectly reproduced. In addition, there were some endogenous interferences that could not be resolved from zaleplon. Because the quantitative method for chromatography was an external standardization method, the precision was not very good, especially when the concentration was low (0.5 ~ 1.0 ng/mL). The methodology described in this paper provides a simple, fast, and effective method for the determination of zaleplon in human plasma for routine assays in a large sample series (i.e., hundreds of samples analyzed in sequential series) for pharmacokinetic purposes.

### MS conditions and optimization

The selectivity and sensitivity in the MS depends on the compound (i.e., how easily it is ionized and the amount and nature of organic solvent used in the mobile phase).

Because zaleplon is a weak base, positive ESI is the best option for the molecule to obtain a high sensitivity. Figures 1 and 2 show the mass spectra of zaleplon and triazolam recorded under standard operating conditions (see "Experimental"). A strong signal corresponding to ions with *m/z* 328.1 is produced by the quasimolecule ion $(M+Na)^+$ of zaleplon. To triazolam (IS), the signal (*m/z* 365.9) resulted from the $(M+Na)^+$ ion. These two ions were selected for monitoring in the MS. To increase the sensitivity of detection further, different values of fragment voltage were examined. Using a "fragment ionization action" sequence of 50, 70, 90, 120, and 150 V fragment voltages, the test solutions containing zaleplon at 10 ng/mL were introduced separately into the MS. The strongest response was produced by 90 V.

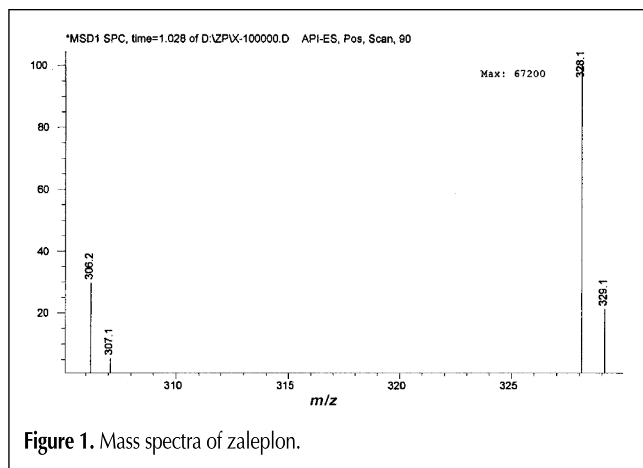


**Figure 1.** Mass spectra of zaleplon.

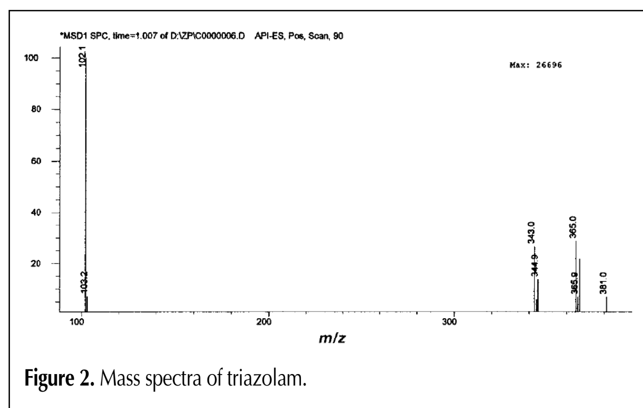


**Figure 2.** Mass spectra of triazolam.

The level of sensitivity of HPLC–MS is also related to the percentage of organic solvent content in the mobile phase. Usually when the organic concentration increases (relative to water), the signal-to-noise ratio of compounds increases. In our work, methanol was selected as the organic modifier because in a positive mode, methanol generally gave stronger signals than acetonitrile (9).

Other MS parameters (gas temperature, pressure, and flows) were adjusted to get a maximum signal for the zaleplon sodium adduct $(M+Na)^+$.

### LC

In the preliminary studies, the separation experiments of zaleplon in plasma sample were done with a Model 10A HPLC system equipped with a fluorescence detector (Shimadzu, Kyoto, Japan). The chromatographic conditions for analysis were according to what was reported (8). Results showed that retention time of zalepon was much longer than the reported one. Besides, there were some endogenous compounds coeluting when detected with the fluorescence detector. By replacing the organic modifier with methanol and varying the percentage of organic modifier in the mobile phase, we found that the use of the SIM detection mode of the MS detector would result in excellent separation only if the percentage of methanol was larger than 60%; low LOD (nearly 0.05 ng/mL of zaleplon in plasma) could be detected.

Because the retention and chromatographic performance of drugs were strongly modified by the mobile phase additives used in ESI–MS, acetic acid and ammonium acetate were investigated further for the separation of zaleplon, triazolam, and the endogenous interferences. However, no outstanding improvements were observed.

When considering the analysis of a series related to 600–700 samples from clinical trials, speed and simplicity are important factors that need to be thought of. Methanol–water (75:25, v/v) was adopted as the mobile phase in the end because it could wash nearly all the compounds out of the chromatographic column within 6 min.

### Sample preparation

The design of a sample preparation method is heavily dependent on the context in which it is intended to be used. Because the concentration of zaleplon in plasma is very low (especially after 8 h of oral administration), sensitivity is a critical issue to determination. Although the usage of an MS detector can satisfy the demand of sensitivity to certain extent, the use of liquid–liquid extraction is necessary because the technique can not only purify but also concentrate the outcome extracts if a suitable procedure is adopted. Ethyl acetate, cyclohexane, dichloromethane, a different ratio of ethyl acetate, and cyclohexane were used to find the best results of extraction. Ethyl acetate was selected in the end. To reduce the amount of acidic, endogenous interferences in the outcome extracts, 0.1 mL NaOH (1.0 mol/L) was added to the mixture of aqueous organic phase to neutralize the acids.

### Selection of IS

It is necessary to use an IS to get high accuracy when a mass detector is used as the HPLC detector. Triazolam, dixipan, and nitrazepam were investigated to find the more suitable one. Triazolam was adopted in the end because of its similarity of retention and ionization with the analyte and the less endogenous interferences at *m/z* 365.9.

### Specificity

Comparison of the LC–MS chromatograms of blank and validation-control samples obtained during the validation experiment (including those of control human plasma from individual volunteers) showed the method has good specificity for zaleplon. The analytes have good chromatographic peak shape, and no significant interferences were observed from endogenous material at the retention time of zaleplon and triazolam. A representative chromatogram of a plasma sample obtained at 1 h from a subject who received a single oral dose (10 mg) is shown in Figure 3.

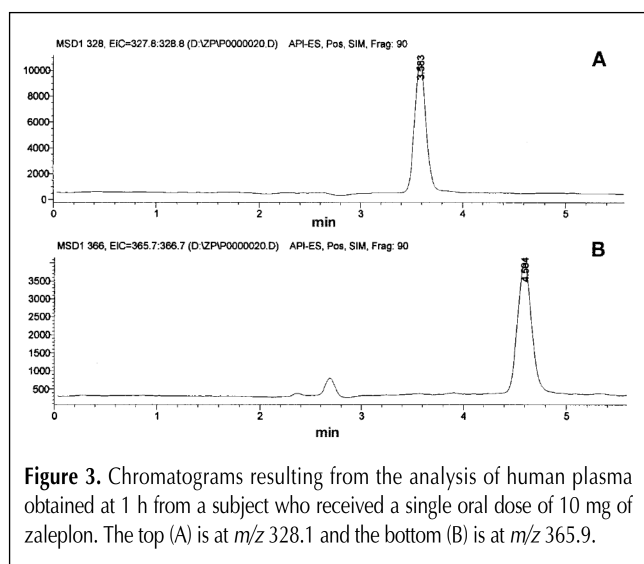


**Figure 3.** Chromatograms resulting from the analysis of human plasma obtained at 1 h from a subject who received a single oral dose of 10 mg of zaleplon. The top (A) is at *m/z* 328.1 and the bottom (B) is at *m/z* 365.9.

### Assay performance

Assay performance of the present method was assessed by the following criteria: linearity, accuracy, precision, stability, recovery, LOD, and LOQ.

The assays exhibited linearity between the response (Ai/As) and corresponding concentration of zaleplon (Ci) over the range of 0.1–60 ng/mL in the sample. The results are presented in Table I.

Accuracy values were within acceptable limits (Table II). The results for within- and between-day precision for control samples are presented in Table III and the value ranged from 1.9% to 4.7%, respectively.

Zaleplon is stable in plasma samples stored at –20°C for at least one month. The stability of zaleplon in processed samples left at room temperature (20 ± 3°C) over 24 h was also studied from our laboratory. Quality control was set up for the drug at concentrations of 1, 15, and 25 ng/mL, respectively. Zaleplon and the IS were also stable under these conditions.

The extraction recovery of zaleplon was determination by comparison of the peak area ratio after extraction with that obtained by the addition of triazolam to the extracted plasma, which contained the same concentration of zaleplon. The registered recovery was higher than 96%.

As defined by the lower and upper validation control concentrations possessing acceptable accuracy and precision (± 15% of the nominal value), the LOQ of the method for zaleplon was 0.1 ng/mL. Under the experimental conditions used, the LOD was

**Table I. Regression Parameters for the Calibration Curves of Zaleplon in Human Plasma**

| Batch | Slope (A) | Intercept (B) | $R^2$ |
|---|---|---|---|
| 1 | 0.1815 | 0.0601 | 0.9990 |
| 2 | 0.1817 | 0.0537 | 0.9992 |
| 3 | 0.1815 | 0.0276 | 0.9988 |
| 4 | 0.1816 | 0.0110 | 0.9984 |
| 5 | 0.1829 | 0.0386 | 0.9994 |

**Table II. Accuracy of the Method for Determining Zaleplon Concentrations***

| Concentration added (ng/mL) | Concentration found (mean ± SD, ng/mL) | Accuracy (%) |
|---|---|---|
| 1 | 1.13 ± 0.03 | 13.0 |
| 15 | 14.37 ± 0.35 | 4.2 |
| 30 | 30.70 ± 0.72 | 2.3 |

* Expressed as relative error (%).

**Table III. Between- and Within-Day Variability of the HPLC Method for Determining Zaleplon Concentrations**

| Concentration added (ng/mL) | Between-day variability (*n* = 5) Concentration found (mean ± SD, ng/mL) | Between-day RSD (%) | Within-day variability (*n* = 15) Concentration found (mean ± SD, ng/mL) | Within-day RSD (%) |
|---|---|---|---|---|
| 1 | 1.13 ± 0.03 | 1.9 | 1.04 | 4.7 |
| 15 | 14.37 ± 0.35 | 2.4 | 14.70 | 3.6 |
| 30 | 30.70 ± 0.72 | 2.4 | 30.42 | 3.6 |

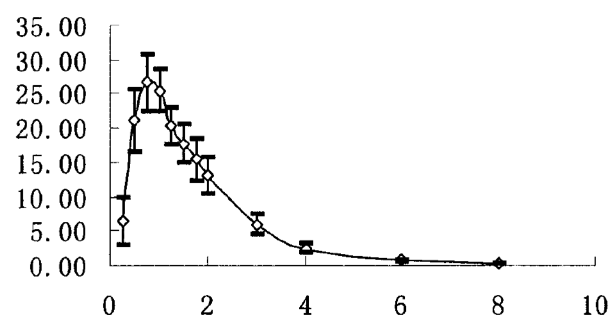


**Figure 4.** Time courses of mean concentrations of zaleplon in plasma following oral administration zaleplon dose of 10 mg to 20 healthy male volunteers.

0.05 ng/mL (s/n = 3). These values are lower than those described by Beer et al. (0.5 ng/mL) in biologic media (8).

### Applicability in pharmacokinetic studies

The applicability of this method has been demonstrated by the determination of zaleplon in plasma samples from 20 subjects that received oral doses of zaleplon in a bioavailability study. The sensitivity of the assay was such that zaleplon concentrations in plasma could be quantitated over a period of 12 h after a single oral dose of 10 mg of zaleplon. Figure 4 shows the mean plasma concentration-time profile of zalepolon following a single oral dose of 10 mg of zaleplon. The mean maximum of 28.47 ng/mL appeared at 0.8 h after the administration and the mean half-life was 1.1 h.

## Conclusion

A highly sensitive and specific method for the determination of zaleplon in clinical plasma samples by LC–ESI–MS has been established. The method was validated according to internationally accepted criteria. Compared with the reported method (8), the present method has solved previous problems of low specificity and reproductivity. Because the method only requires a simple liquid–liquid extraction procedure and short run time, large sample batches (more than 120 samples) can be processed daily. The experiments have demonstrated that HPLC–MS, together with the extraction procedure, is an effective alternative to fluorescence chromatographic methods that are traditionally employed for the determination of drugs in biomedical and clinical research.

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