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Labachevska-Gjatovska L., Panovski Nikola

# HPLC METHOD FOR QUANTITATIVE DETERMINATION OF ZOLPIDEM IN HUMAN PLASMA SAMPLES AND ITS APPLICATION FOR BIOEQUIVALENCE STUDIES

Zendelovska D, Simeska S, Kikerkov I, Gjorgjievska K, Jakovski K and Zafirov D

Institute of Preclinical and Clinical Pharmacology and Toxicology, St. Cyril and Methodius University, Medical Faculty, Skopje, Republic of Macedonia

#### Abstract

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A simple and sensitive high-performance liquid chromatographic method was developed to measure plasma concentration of zolpidem. Plasma samples were prepared by adding acetonitrile and the supernatant was injected onto the HPLC system, which used LiChrospher 60 RP select B column. The method utilized mobile phase consisted of 0.025 mol/L  $\rm KH_2PO_4$  and 0.5 % ( $\rm V/V$ ) of triethylamine (pH 6.4) and acetonitrile (50:50,  $\rm V/V$ ) and fluorescence detection (excitation wavelength 244 nm and emission wavelength 388 nm). The method was found to be reproducible with a relative standard deviation less than 5.14 %. The limit of quantification was 2 ng ml $^{-1}$ . The validated method has been applied to clinical pharmacokinetic study.

Keywords: Zolpidem, High-performance liquid chromatography, Bioequivalence study

### НРІС МЕТОД ЗА КВАНТИТАТИВНО ОПРЕДЕЛУВАЊЕ НА ЗОЛПИДЕМ ВО ХУМАНИ ПЛАЗМА ПРИМЕРОЦИ И НЕГОВА АПЛИКАЦИЈА ВО БИОЕКВИВАЛЕНТНИ СТУДИИ

#### Апстракт

Развиена е едноставна и чувствителна метода за високо-ефикасна течна хроматографија за определување на концентрацијата на золпидем во плазма. Примероците од плазма се подготвувани со додавање на ацетонитрил и супернатантот е инјектиран во HPLC систем на кој е инсталирана LiChrospher 60 RP select В колона. Употребена е мобилна фаза составена од раствор на  $KH_2PO_4$  со концентрација од 0.025 mol/L во кој е додаден триетил амин 0.5 % (V/V) (pH 6.4) и ацетонитрил (50:50, V/V) и флуоресцентна детекција (ексцитација на бранова должина од 244 nm и емисија на бранова должина од 388 nm). Методата е репродуцибилна со релативна стандардна девијација помала од 5.14 %. Лимит на квантификација е 2 ng ml $^{-1}$ . Валидираната метода е применета во клиничка фармакокинетска студија

**Клучни зборови**: Золпидем, Високо-ефикасна течна хроматографија, Биоеквивалентна студија

#### Introduction

Zolpidem is an imidazopyridine-derivative sedative and hypnotic. Although zolpidem is structurally unrelated to the benzodiazepines, it shares some of the pharmacologic

properties of benzodiazepines and has been shown to interact with the CNS  $\gamma$ -aminobutyric acid (GABA<sub>A</sub>)-receptor-chloride ionophore complex at benzodiazepine (BZ,) receptors. Zolpidem tartrat, has been in widespread clinical use for many years as a short term treatment for insomnia (1).

In order to monitor plasma levels of zolpidem in patient samples and to carry out pharmacokinetic studies of the drugs, a rapid, sensitive and reproducible method for its determination is required.

There are several methods concerning the determination of zolpidem in biological fluids (2-13). Many authors have performed the determination of zolpidem in fingernails, hair, urine or plasma using liquid chromatography-tandem mass spectrometry (2-4), ultra-high-pressure liquid chromatography tandem mass spectrometry (5, 6) or ultra-performance liquid chromatography tandem mass spectrometry (7, 8) using different sample preparation procedures such as liquid-liquid extraction with diethyl ether, chlorobutane, ethyl acetate, or direct injection of samples. Kratzsch et al. reported a liquid chromatographic mass spectrometric method for determination of zolpidem in plasma samples after liquid-liquid extraction which allows confirmation of the diagnosis of an overdose situation and monitoring of psychiatric patients compliance (9).

On the other hand, many authors have performed the determination of zolpidem in human plasma after liquid-liquid extraction (10-12) or protein precipitation with methanol (13) using high-performance liquid chromatography with fluorescence detection. Some of these methods yielding poor separation of zolpidem from the human plasma endogenous interference's or some methods require an evaporation step and they are time and reagent consuming.

Therefore, in this paper, we report a new, simple, sensitive, and easy to operate HPLC method using fluorescence detection for the determination of zolpidem in human plasma and its application for pharmacokinetic study.

#### Materials and methods

#### Materials

All chemicals and reagents used were of HPLC grade or analytical grade. Zolpidem tartrate was kindly supplied by Alkaloid AD Skopje (Macedonia). Other chemicals of reagent grade and solvents of analytical and HPLC grade were purchased from Merck (Germany).

#### Chromatography

A series of parameters, including composition and pH of mobile phase, column packing, flow rate and detection wavelength, were tested with respect to the location and shape of the peak of zolpidem in the corresponding chromatograms. The final choice of the stationary phase giving satisfying resolution and run time was LiChroCART Lichrospher 60 RP select B (250 x 4 mm) with 5  $\mu$ m particle size, protected with matched guard column (Merck, Germany). The mobile phase consisted of 0.025 mol/L KH<sub>2</sub>PO<sub>4</sub> and 0.5 % (V/V) of triethylamine (pH 6.4) and acetonitrile (50:50, V/V) delivered by a pump ProStar 230, Varian. pH of buffer solution (6.4) was adjusted by adding very small amount of concentrated ortho-phosphoric acid. The flow-rate of the mobile phase was 1.2 ml min<sup>-1</sup>. Chromatographic separation was performed at 25 °C. An fluorescence detector (ProStar 363, Varian) was used for detection (excitation 244 nm and emission 388 nm). The samples

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were introduced in the column using an autosampler ProStar 410 and the injection volume was 20 μl. The chromatographic system was controlled with Star Workstation Version 6.30.

#### Preparation of standards

Stock solution of zolpidem was prepared in methanol at a concentration of 2000  $\mu g$  ml $^{-1}$ . No change in stability over the period of 1 month was observed (storage at 4  $^{\circ}$ C). The working solutions were prepared by diluting appropriate portions of this solution with distilled water separately every day.

#### Blood plasma samples and sample preparation

Human plasma samples were prepared by centrifuging (at 3000 rpm) of heparinized whole blood samples collected from healthy volunteers who later participated in a bioequivalence study of zolpidem. This study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Sts. Cyril and Methodius University, Skopje, Macedonia.

Prepared plasma samples were stored at  $20^{\circ}$ C. Before the analysis blood plasma samples were thawed at 20 °C for about 10 minutes. A 0.5 ml volume of the sample was transferred into a vial and vortexed with 1.0 ml of acetonitrile for 20 s. After centrifugation, (8 min, 13000 rpm), the supernatant was filtered using syringe filter (Minisart RC 4, 0.45  $\mu$ m) and 20  $\mu$ L portion was injected on to HPLC column.

#### Calibration

Typical calibration curves were constructed with seven blank blood plasma samples spiked with appropriate amounts of the standard solutions. The calibration range was 2.0-300.0 ng zolpidem per ml of blood plasma. The standard samples were prepared according to the procedure as unknown samples. The calibration curves were obtained by plotting the peak area of zolpidem versus concentration of zolpidem in ng ml<sup>-1</sup>. The regression equations were calculated by the least-squares method.

#### Clinical study

A total of 28 healthy male volunteers, non-smokers, mean age 32 years, gave their written informed consent to participate in study. The health condition of the volunteers was established on the basis of anamnesis data, physical examination, ECG, biochemical and hematological analyses. This study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, St. Cyril and Methodius University (Skopje, Macedonia). The study was randomized open crossover with a one-week wash-out period. Two weeks before investigation volunteers did not receive any drugs. The subjects were administered a single 10 mg oral dose of zolpidem in the morning after overnight fast.

Plasma samples were obtained before the administration of the drug (0 time) and at 17 time points following a single 10 mg oral dose of zolpidem. Following collection, the samples were stored at -70  $^{\circ}$ C. After thawing, 0.5 ml plasma was analyzed as described before.

#### Results

A series of studies were conducted in our laboratory in order to develop a convenient and easy-to-use method for quantitative analysis of zolpidem in biological fluids. Several HPLC method variables with respect to their effect on the separation of zolpidem from the

matrix were investigated. In our extensive 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 when mobile phase consisted of 0.025 mol/L KH<sub>2</sub>PO<sub>4</sub> and 0.5 % (*V/V*) of triethylamine (pH 6.4) and acetonitrile (50:50, *V/V*). A set of column packings including C8, C18 and RP-select B with different lengths and particle sizes were tested and the RP-select B packing showed best separation. Among several flow-rates tested (0.8-1.5 ml min<sup>-1</sup>) the rate of 1.2 ml min<sup>-1</sup> was the best with respect to location and resolution of the peaks of zolpidem from the interfering peaks. A typical chromatogram of standard solution of zolpidem (20 ng ml<sup>-1</sup>) produced by the developed HPLC method is shown in Fig. 1 (a). Retention time of zolpidem is 4.4 min. Under the chromatographic conditions described, zolpidem peak was well resolved. Endogenous blood plasma components did not give any interfering peaks. Typical chromatograms of blank blood plasma in comparison to spiked samples are shown in Fig. 1 (b, c).

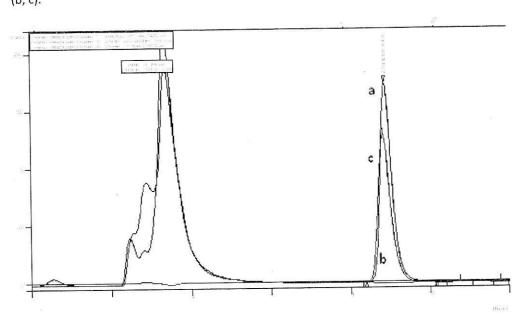


Figure 1 Chromatograms of standard solution of zolpidem (a), blank plasma sample (b) and spiked plasma sample with zolpidem

On the other hand, the method in this report has sufficient sensitivity and reproducibility to permit the pharmacokinetic studies. The developed HPLC method was used for analysis of blood plasma samples from healthy volunteers after oral administration of zolpidem. Typical chromatograms of blood plasma samples of patients before and after administration of 10 mg zolpidem are shown in Fig. 2.

Linearity, precision and accuracy

Calibration standards and spiked quality control samples of zolpidem were prepared by spiking blank human blood plasma with known amounts of zolpidem. Linearity was tested

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## HPLC METHOD FOR QUANTITATIVE DETERMINATION OF....

in nine different days at seven concentration points ranged from 2.0 to 300.0 ng ml $^{-1}$  of zolpidem in human plasma samples. Mean respective regression equation was y = 31382.4 $\cdot \gamma$  + 5343.05. The correlation coefficient was 0.99985.

Intra-day precision was determined by measuring individually prepared six spiked plasma samples at three different concentration levels, first near to LOQ. Also, the inter-day precision was determined by analyzing three different concentrations of zolpidem in two series in 9 different days. Then, the corresponding coefficients of variation were calculated. The intra- and inter-day variations of the method are shown in Table 1.

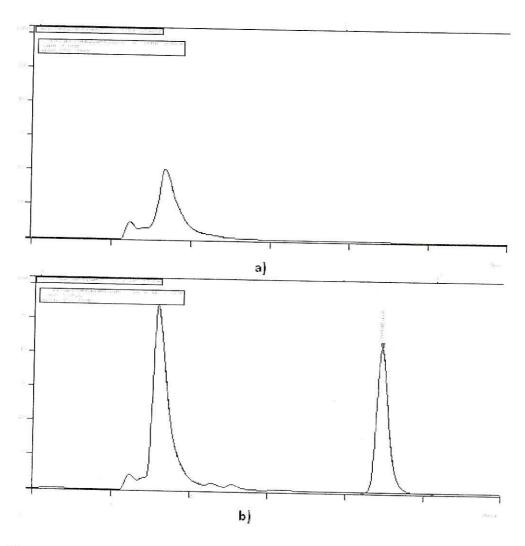


Figure 2 Chromatograms of blood plasma samples from healthy volunteers before (a) and after administration of zolpidem 1 h post-dose (b)

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Table 1 Intra-and inter-day precision data

Nominal concentration (ng ml <sup>-1</sup> )	Intra-day		Inter-day	
	Mean (n=6) observed concentratio n (ng ml <sup>-1</sup> )	Relative standard deviation (%)	Mean (n=18) observed concentration (ng ml <sup>-1</sup> )	Relative standard deviation (%)
Zolpidem				
10.0	9.81	1.80	9.92	5.14
70.0	70.24	1.37	70.52	3.04
250.0	252.13	2.50	256.73	2.15

Intra- and inter-day accuracy was determined by measuring human plasma quality control samples at low, middle and high concentration levels of zolpidem. An indication of accuracy was based on the calculation of the relative error of the mean observed concentration as compared to the nominal concentration. Accuracy data are presented in Table 2.

Table 2 Intra-and inter-day accuracy data

Nominal concentration (ng ml <sup>-1</sup> )	Intra-day		Inter-day	
	Mean (n=6) observed concentration (ng ml <sup>-1</sup> )	Relative error (%)	Mean (n=18) observed concentration (ng ml <sup>-1</sup> )	Relative error (%)
Zolpidem				
10.0	9.81	-1.87	9.92	-0.78
70.0	70.24	0.34	70.52	0.74
250.0	252.13	0.85	256.73	2.69

#### Limit of quantification

The limit of quantification (LOQ) was defined as the lowest concentration of zolpidem on the standard curve which can be measured with acceptable accuracy and precision. The LOQ was determined using the lowest calibration standard in five series and it was found to be 2.0 ng ml<sup>-1</sup> for zolpidem for human plasma samples.

#### Extraction efficiency

The efficiency of extraction procedure was determined at 10, 70 and 250 ng ml<sup>-1</sup> zolpidem. The mean values for extraction efficiency (six replicates for each concentration) were ranged from 98.64% to 101.86% which shows that the separation of zolpidem from plasma samples using the proposed method is satisfactory.

#### Stability of zolpidem in human plasma samples

Stability of zolpidem in human plasma was investigated using spiked samples at two different concentration levels prepared in triplicate. Spiked samples were analysed after

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#### HPLC METHOD FOR QUANTITATIVE DETERMINATION OF ....

different storage conditions: immediately, after staying at room temperature for 4 and 24 hours, after one and two freeze/thaw cycles and after 1 month stored at -70 °C. Also, stability of zolpidem plasma extracts was investigated after staying in autosampler for 24 hours.

#### Ruggedness

Ruggedness was performed by injecting the standard solution (n=3) of zolpidem at concentration of 20 ng ml $^{-1}$  on new HPLC column of the same type. Relative error was calculated by comparing the mean peak area for zolpidem to those obtained by changing pH value of the mobile phase from 6.4 to 6.7 and from 6.4 to 6.1 or to those obtained by changing the buffer concentration from 0.025 mol l $^{-1}$  to 0.05 mol l $^{-1}$  and from 0.025 mol l $^{-1}$  to 0.01 mol l $^{-1}$ .

#### **Application**

The described HPLC method has been successfully applied in a bioequivalence study of zolpidem formulations on 28 healthy volunteers. Fig. 3 presents the mean plasma zolpidem concentration-time profiles after oral administration of 10 mg test zolpidem formulation or 10 mg zolpidem (a reference formulation of zolpidem - Stilnox). As can be seen from Fig. 3, the character of both curves is practically the same. Following oral administration of zolpidem and Stilnox tablets, maximum plasma concentration of 155.19 and 157.59 were achieved after 0.81 h and 0.85 h, respectively.

The corresponding pharmacokinetic parameters (mean±SD) obtained by 28 independed analyses and the relative bioavailability of the generic zolpidem tablet are presented in Table 3.



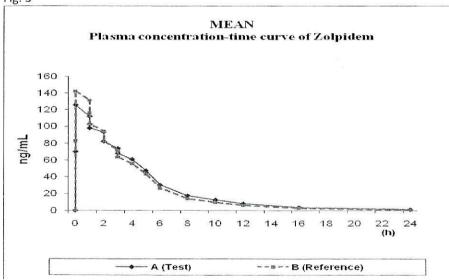


Figure 2 Plasma concentration profiles of zolpidem following administration of two dosage forms of zolpidem (dose 10 mg)

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d 250 ng ml<sup>-1</sup> zolpidem. th concentration) were tolpidem from plasma

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Table 3 Mean pharmacokinetic parameters of zolpidem after a single 10 mg oral dose

Pharmacokinetic parameter	Zolpidem tablet	Stilnox tablet	
C <sub>max</sub> (ng ml <sup>-1</sup> )	155.19±49.82	157.59±53.97	
t <sub>max</sub> (h)	0.81±0.48	0.85±0.45	
t <sub>1/2</sub> (h)	2.92±1.52	2.74±1.21	
AUC <sub>0-t</sub> (ng h ml <sup>-1</sup> )	556.25±326.63	524.71±283.70	
$AUC_{0-\infty}$ (ng h ml <sup>-1</sup> )	574.77±340.41	537.93±290.39	
$F_r$	1.067±0.29	=	

C<sub>max</sub> - mean plasma zolpidem concentration

t<sub>max</sub> - time to achieve the peak concentration

 $t_{1/2}$  – elimination half-life

 $AUC_{0-t}$  – area under the plasma concentration-time curve from 0 to t

 $AUC_{0-\infty}$  - area under the plasma concentration-time curve from 0 to infinity

Fr - relative bioavailability defined as AUCzolpidem/AUCStilnox

#### Discussion

From the results from method validation we can concluded that for intra-day precision of the method the RSDs ranged from 1.37 to 2.50 % and for inter-day precision, RSDs ranged from 2.15 to 5.14 %. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs. Also, relative errors at all three concentrations studied for zolpidem (quality control samples) are less than 2.69 % and it is obvious that the method is remarkably accurate which ensures obtaining of reliable results. The results from the stability investigation show that zolpidem added to human plasma samples is stable in the different storage conditions. At the end of the method validation process, stability investigation data show that the relative errors were ranged from 0.14 to 5.51 % which means that this HPLC method for determination of zolpidem in spiked human plasma samples is rugged.

The aim of this study was to develop and validate rapid, sensitive, precise and accurate method which can be applied for analysis of plasma samples obtained from healthy volunteers after single oral dose of 10 mg zolpidem. The statistical analysis of pharmacokinetic parameters: C<sub>max</sub>, t<sub>max</sub>, t<sub>1/2</sub>, AUC<sub>0-t</sub> and AUC<sub>0-∞</sub> with the two-way analysis of variance, single observation (ANOVA) shows that there are no differences between the compared drugs. The relative bioavailability of the investigated formulation, i.e., the zolpidem tablet was 106.7 % of that obtained for the proprietary product, Stilnox. Based on these results, it may be concluded that 10 mg zolpidem tablets are bioequivalent to 10 mg Stilnox tablets.

#### Conclusion

The developed HPLC method employing sample precipitation affords a rapid, sensitive, accurate and precise analyses of as low as 2.0 ng ml<sup>-1</sup> zolpidem in human plasma. The typical assay time is about 6 min. No internal standard is required and this simplifies the method. This method is advantageous compared to the methods for the determination of zolpidem in human plasma because of its simplicity, short time of analysis, efficient clean up of the complex biological matrix and high recovery of zolpidem. The validation data demonstrate method. Also

#### References

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demonstrate good precision and accuracy, which proves the reliability of the proposed method. Also, the method can be used in bioequivalence and pharmacokinetic studies.

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single 10 mg oral dose

tablet -53.97 45 21 -283.70 -290.39

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or intra-day precision of y precision, RSDs ranged egree of precision and between different runs. Dipidem (quality control is remarkably accurate ability investigation show erent storage conditions. Lion data show that the at this HPLC method for ged.

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fords a rapid, sensitive, in human plasma. The d and this simplifies the for the determination of analysis, efficient clean em. The validation data