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Pharmacokinetic and Bioequivalence Study of Zolpidem Tartate in Healthy Volunteers

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ABSTRACT − In this study simple and sensitive high performance liquid chromatographic method using a commercially available column, was developed and validated for the determination of zolpidem tartrate in human plasma. The developed method with suitable validation was applied to a bioequivalence study of two different kinds of zolpidem tartrate. Two different formulations containing 10 mg of zolpidem tartate (CAS : 99294-93-6) were compared in 24 healthy male volunteers in order to compare the bioavailability and prove the bioequivalence. The study was performed in an open, single dose randomized, 2-sequence, cross-over design in 24 healthy male volunteers with a one-week washout period. Blood samples for pharmacokinetic profiling were drawn at selected times during 12 h. The mean $AUC_{0\text{-}12h}$, C_{max}, T_{max} and $T_{1/2}$ were 676.6±223.4 ng·h·mL^{-1}, 177.4±34.2 ng·mL^{-1}, and 0.8±0.4 and 3.5±2.1, respectively, for the test formulations, and 640.7±186.6 ng·h·mL^{-1}, 193.0±64.5 ng·mL^{-1}, and 0.9±0.4 and 2.7±0.9, respectively, for the reference formulation. Both primary target parameters $AUC_{0\text{-}12h}$ and C_{max} were log-transformed and tested parametrically by analysis of variance (ANOVA). 90% confidence intervals of $AUC_{0\text{-}12h}$ and C_{max} were in the range of acceptable limits of bioequivalence (80-125%). Based on these results, the two formulations of zolpidem tartate are considered to be bioequivalent.

Key words − Zolpidem tartrate, HPLC, Pharmacokinetics, Bioequivalence, Assay validation

Zolpidem is non-benzodiazepine drug used for the treatment of insomnia as well as brain disorders which selectively binding to gamma-aminobutyric acid (GABA) A receptor as benzodiazepine (Lemmer, 2007) and has a short-term effect and a rapid onset. CYP3A4 in human cytochrome P450 (CYP) in human liver microsomes has rapid effects on zolpidem metabolism, and the metabolism of it is influenced by gender, age, etiology, and the presence of liver disease (Von Moltke et al., 1999, Salvà and Costa, 1995).

Many methods can be used to determine plasma zolpidem levels, i.e., radioimmunoassay (RIA)(De Clerck and Daenens, 1997), high-performance thin-layer chromatography (HPTLC) (El Zeany et al., 2003), gas chromatography (GC) (Stanke et al., 1996), capillary electrophoresis (CE)(Hempel and Blaschke, 1996), high-performance liquid chromatography (HPLC) with ultraviolet (El Zeany et al., 2003) or fluorescence detection systems (Ptácek et al., 2003, Ring and Bostick, 2000), GC-MS (Keller et al., 1999) and LC-MS with ion spray (Giroud et al., 2003), tandem MS(Kintz et al., 2004), and time-of-flight MS (Pelander et al., 2003). However, HPLC and CE have many difficulties and involve complex extractions, GC and HPLC with ultraviolet detections have relatively low sensitivities, and LC-MS methods are usually suitable for determining very small amounts in urine or blood, and as such are used in criminal investigations. Therefore, in the present study, a sensitive and simple HPLC assay using fluorescence detector is developed to determine plasma concentrations of zolpidem tartrate in healthy volunteers.

The main purpose of crossover study is to compare the bioavailability of a generic and a reference zolpidem tartrate formulation under fasting conditions. Pharmacokinetic data are acquired using the non-compartmental method, K-BE Test® 2002 (Lee et al., 1998).

Experimental

Chemicals and reagents

Standard zolpidem tartrate (CAS : 99294-93-6), (purity> 96%) was purchased from Hanmi Pharmaceutical Co., Ltd. (Seoul, Korea), and trazodone hydrochloride (CAS : 19794-93-5) , (purity> 99%), an internal standard (IS) was purchased from Sigma-Aldrich (St. Louis, MO). Zolpidem tartrate 10 mg tablets (Lot No. SX3103B, expiration date: 2007.04.17. obtained from the manufacturer) were used as the reference

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formulation, and Zolpid® 10 mg tablets (Lot No. PZOL01, manufacturing date: 2004. 08.12.) from Hanmi Pharmaceutical Co., Ltd. was used as the generic drug (test formulation), and was randomly sampled from one batch of 100,000 tablets. All other reagents were of analytical grade.

Preparation of standards

Stock standard solutions of zolpidem tartrate (5 μg/mL) and IS (4 μg/mL) were prepared in methanol and kept at 4°C. Standard solutions for spiking (5, 10, 50, 250, 500, 1000, 3000, and 5000 ng/mL) were prepared by diluting the stock solution with methanol. Calibration standards of zolpidem tartrate and IS were prepared by spiking the appropriate amount of stock solutions into human plasma. The calibration curves prepared covered the range 0.5-500.0 ng/mL in plasma.

Instrumentation

The HPLC system (Alliance 2690 series, Waters, USA) was equipped with a degasser, a binary pump, an autosampler, and a fluorescence detector (Alliance 474 series, Waters, USA). A Luna® RP-C_{18} column (4.6 mm×250 mm, 5 μm, Phenomenex, CA) was used. The plasma samples were separated isocratically using an acetonitrile-0.05 M phosphate buffer (pH 6.0) 68:32 (v/v) at a flow-rate of 1 mL/min at room temperature. The eluent was detected at an excitation wavelength of 254 nm and emission wavelength of 400 nm using a fluorescence detector (Alliance 996 series, Waters, USA). HPLC System control and data evaluation were carried out using Millenium 32® (Waters, USA). The gas blowing concentrator used was a dry thermo bath MG-2100® (Eyela, Tokyo).

Extraction procedure

To modify the HPLC-fluorescence-based method for quantifying zolpidem tartrate in human plasma (Ptácek et al., 2003, Ring and Bostick, 2000), the steps used to extract zolpidem tartrate and IS were optimized. Zolpidem tartrate is slightly soluble in water, sparingly soluble in methanol, and freely soluble in chloroform whereas trazodone hydrochloride (IS) is sparingly soluble in water, methanol, and chloroform. Therefore, chloroform was selected for extraction because of higher sensitivity for zolpidem tartrate in human plasma. 50 μL of IS stock solution was added to 500 μL of plasma sample. The plasma was then made alkaline by adding 50 μL of 1.0 M sodium hydroxide. After mixing thoroughly, the mixture was extracted with 4 mL of chloroform for 3 min and then centrifuged (1650 × g, 10 min). The organic layer was then transferred to another clean glass tube and evaporated under a stream of nitrogen at 5°C. 500 μL of the mobile phase (acetonitrile-0.05 M phosphate buffer, pH 6.0, 68:32 (v/v)) was then added to reconstitute this residue, and 50 μL aliquot of the solution was injected into the HPLC system.

Validation of the method

Blank plasma samples for method validation obtained from healthy volunteers were used. Specificity was assessed by extracting samples from six different batches of blank plasma, zero sample spiked with IS only, and then comparing the results obtained with those of plasma samples spiked with IS and zolpidem tartrate in the concentration range 0.5 to 500.0 ng/mL of zolpidem tartrate in calibration curve. The limit of quantification (S/N ratio >10) was defined as the lowest concentration yielding a precision of <20% (relative standard deviation, R.S.D) and an accuracy between 80 and 120% of the theoretical value. Standard samples for linearity were prepared by adding zolpidem tartrate to blank plasma at concentrations of 0.5, 1.0, 5.0, 25.0, 50.0, 100.0, 300.0, 500.0 ng/mL and these were then extracted and assayed as described above. Least-squares linear regression (presented with their correlation coefficients) was used to determine the plasma concentrations from peak area ratios of zolpidem tartrate to IS. Inter and intra-assay relative standard deviations (RSDs) and standard errors of means were used to validate the precision and accuracy of the assay based on assay results for standard samples of zolpidem tartrate and IS in human plasma. Accuracy was determined by comparing calculated concentrations using calibration curves to known concentration. To assess the recovery of zolpidem tartrate and IS extracted from plasma, the peak areas ratios of analytes to IS in the extracted quality control (QC) samples were compared with those obtained from the mobile phase at 15, 100, and 300 ng/mL of zolpidem tartrate with IS in triplicate (Recovery %).

The stabilities of zolpidem tartrate and IS in human plasma were assessed by storing standard solutions containing 15 or 300 ng/mL of zolpidem tartrate and IS under ambient conditions for 6 or 12 h. Freeze-thaw stability testing was also assessed using three freeze-thaw cycles versus non-freeze thawed standard solutions; specifically, freezing was performed at -70°C for 24 h and thawing was conducted at room temperature. Long-term stability was examined at -70°C over 1 month. Whereas stability tests of stock standard (zolpidem tartrate, 15 and 300 ng/mL) and IS (trazodone hydrochloride, 400 ng/mL) solutions were assessed using only such three tests as short-term temperature, auto sampler, and long-term stabilities.

Application of the method

The protocol of this bioequivalence study was approved by

the Korean Food and Drug Administration (KFDA) and by the institutional review board (IRB) for human studies at Chungnam National University.

The study was performed in an open, single dose randomized, 2-sequence, cross-over design in 24 healthy male volunteers with a A total of twenty-four healthy male volunteers with a one-week washout period.

Aged 20 to 30 years and weighing from 60 to 80 kg were advised not to take any kind of medications or foods containing xanthin within the 2 weeks period prior to the study or during the study. A single dose (10 mg) of one tablet of the reference (Lot No. SX3103B) or test formulation (Lot No. PZOL01) was given with 240 ml of water to subjects in fasting state in a randomized manner. During the 12 hours period after drug administration, no strenuous physical or mental activity was permitted, and food and drinks were controlled. Approximately 10 ml blood samples were drawn into Vacutainer™ tubes (BD biosciences, NJ, USA) containing heparin from a forearm vein using an indwelling catheter or by direct vein puncture before dosing (0 h) and at 0.3, 0.6, 1.0, 1.3, 1.6, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0 and 12.0 h of post-dosing. Blood samples were centrifuged within 1h at 3000 rpm for 10 min, and then plasma samples were transferred into coded test tubes, and stored at -70°C until required for analysis. The washout period between the two treatments was 1 week, which is more than 5 times greater than the elimination half-life (2 ~ 4 h) of zolpidem tartrate.

Pharmacokinetic parameters including $AUC_{0\rightarrow12h}$ (the area under the plasma concentration-versus-time curve from time 0 to 12 h), $AUC_{0\rightarrow\infty}$, C_{max} (peak plasma concentration) and T_{max} (time to C_{max}) were calculated using K-BE TEST® 2002, which was performed according to the standard non-compartmental method and analysis of variance (ANOVA, $\alpha = 0.05$) for experiment of a crossover design (Lee et al., 1998). Parametric 90% confidence intervals of mean log-transformed AUC and C_{max} values between the two formulations were computed using mean residual errors obtained using ANOVA.

Results and Discussion

Validations of the method

Specificity and selectivity

A plasma blank (free of analyte and IS), a zero sample (plasma blank spiked with IS only), and plasma sample (0.5-500.0 ng/mL) were used to check for interference due to endogenous materials. Typical chromatogram of plasma spiked with zolpidem tartrate 500.0 ng/mL (the highest) and IS is shown in Figure 1. The retention times of zolpidem tartrate (around 8.5 min) and of IS (around 7.0 min) were less than 10 min. For all plasma samples, the analyte and the IS regions were found to be free of interference.

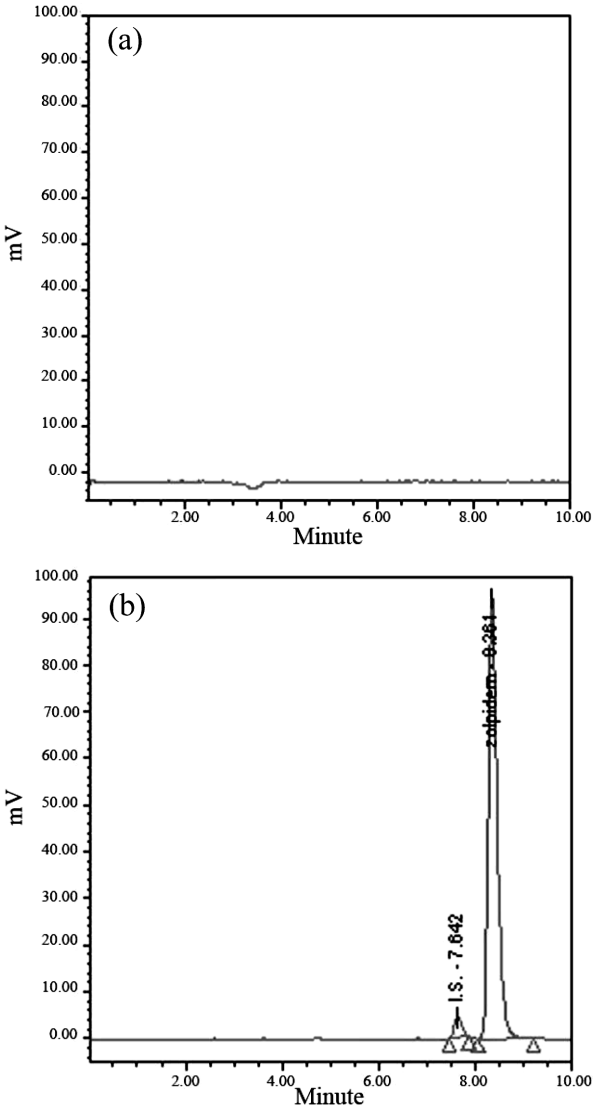

Figure 1. Representative chromatogram of (a) blank plasma, (b) plsma spiked with zolpidem tartrate (the highest QC, 500 ng/mL) and trazodone (IS, 400 ng/mL).

Sensitivity and linearity

The lower detection limit (LOD), defined at an S/N > 3, was 0.05 ng/ml and the lower limit of quantification (LOQ) of zolpidem tartrate (defined at an S/N >10) was 0.5 ng/mL with intra and inter-day precisions of 4.876% and 5.300%, respectively and an accuracy of 92.375%. The linearity of response/concentration curve was established in human plasma over the concentration range 0.5 - 500.0 ng/mL, and correlation coefficients of 0.998 were obtained.

Precision, accuracy and recovery

Intra-day precision (RSD, %) ranged from 1.985 to 5.707% and inter-day precision from 3.233 to 6.424%. Both intra-day and inter-day precisions were <15%. Accuracies of all investigated concentrations were within 85 - 115%. Results are presented in Table I. The recoveries % (mean±SD) of zolpidem tartrate /IS at different concentration of zolpidem tartrate (15, 100, and 300 ng/mL) were 99.530±2.999%, 98.307±6.453% and 102.680±7.086%, respectively and were thus almost 100% for zolpidem tartrate at all concentrations.

Stability studies

Freeze-thaw, short-term, and auto-sampler stabilities showed no substantial effect on results, and samples were stable (≥ 91.5%) for at least 1 month of storage at -70ºC. Results are presented in Table II. Moreover, samples of stock solutions of zolpidem tartrate (15, 300 ng/mL) and IS (400 ng/mL) were stable (difference ≤7.1%) over 1 month of storage. Results are presented in Table III.

Therefore, the described assay method showed the required precision, accuracy, linearity, stability, and specificity. And, the method was applied to the bioequivalence study of the two formulations of zolpidem tartrate in 24 healthy male volunteers after a single oral administration (10 mg).

Application of the method

Mean plasma concentrations of zolpidem tartrate obtained after orally administering the formulations to 24 healthy volunteers are shown in Figure 2, and the pharmacokinetic parameters of the two formulations are summarized in Table IV. $AUC_{0\rightarrow12h}$, C_{max} and T_{max} values of the reference and test formulations were 640.7±186.6 ng·h·mL^{-1} and 676.6±223.4 ng·h·mL^{-1}, 193.0±64.5 ng·mL^{-1} and 177.4±34.2 ng·mL^{-1}, and 0.9±0.4 h and 0.8±0.4 h, respectively.

Statistical analysis of the pharmacokinetic study of reference and test formulation were carried on ($\alpha = 0.05$). No significant effects of groups/sequences or formulations were observed. Also 90% confidence intervals of log values of $AUC_{0\rightarrow12h}$ and C_{max} were log 0.8473-log 1.2343 and log 0.8298-log 1.0827, respectively, which satisfied the standard range of the bioequivalence test, log 0.8-log 1.25. The time to reach maximum serum concentrations (T_{max}) of reference formulation and test formulation was between 0.5-1.3 h and between 0.4-1.2 h, respectively. Therefore, we conclude that the two for-

Table I. *Precision and accuracy of the developed method for zolpidem in human plasma*

Concentration (ng/mL)	Precision (RSD %)		Accuracy (%, n=11)
	Intra-day (n=6)	Inter-day (n=6)	
0.5	4.876	5.300	92.375
1	1.985	5.584	100.787
5	2.715	6.424	85.757
25	5.707	5.223	94.840
50	3.074	5.553	105.388
100	4.711	3.599	99.734
300	2.826	3.233	99.658
500	3.223	3.248	100.014

Table II. *Summary of freeze-thaw, and short-term, auto sampler, and the long-term stabilities of zolpidem tartrate*

Stability (n=3)	Zolpidem tartrate in plasma (ng/mL)					
	15 ng/mL			300 ng/mL		
	Mean (ng/mL)	RSD (%)	Accuracy (%)	Mean (ng/mL)	RSD (%)	Accuracy (%)
Standard	14.914	0.218	99.429	293.928	4.097	97.976
Freeze/thaw stability	16.617	1.314	110.783	295.587	4.262	98.529
Short term stability	14.938	0.380	99.590	288.853	5.107	96.284
Post-preparative stability	16.188	2.528	107.923	273.551	0.073	91.184
Long term stability	17.035	5.206	113.567	286.197	2.209	95.399

Table III. *Stock solution stabilities of zolpidem tartrate and trazodone (IS)*

Samples (n=3)	Standard (0 h)		6 h			1 month		
	Mean peak area	RSD (%)	Mean peak area	RSD (%)	Difference (%)	Mean peak area	RSD (%)	Difference (%)
15 ng/mL	146024	0.820	150929	1.436	3.359	156358	7.077	7.077
300 ng/mL	10530084	0.089	10493305	0.017	-0.349	10498559	-0.299	-0.299
400 ng/mL (IS)	845810	0.689	850151	1.100	0.513	874885	3.438	3.438

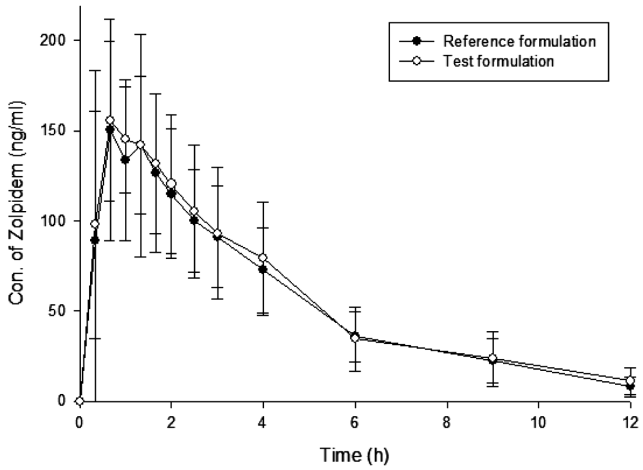

Figure 2. Mean plasma concentration-time profiles of zolpidem tartrate in 24 human volunteers after a single oral administration of the reference (closed circle) or test (open circle) formulations. Each point represents a mean±S.D.

Table IV. *Pharmacokinetic parameters of zolpidem tartrate (10 mg) in 24 human volunteers*

	Parameters (Mean±S.D.)			
	$AUC_{0\rightarrow 12h}$ ($ng{\cdot}mL^{-1}{\cdot}h$)	C_{max} ($ng{\cdot}mL^{-1}$)	T_{max} (h)	$t_{1/2}$ (h)
Reference	640.7±186.6	193.0±64.5	0.9±0.4	2.7±0.9
Generic	676.6±223.4	177.4±34.2	0.8±0.4	3.5±2.1

*$AUC_{0\rightarrow 12h}$: the area under the concentration versus time curve ($ng{\cdot}h{\cdot}mL^{-1}$); C_{max}: peak plasma concentration ($ng{\cdot}mL^{-1}$); T_{max}: time at C_{max} (h); $t_{1/2}$: elimination half-life (h)

mulations of zolpidem tartrate 10 mg tablets are bioequivalent in terms of their extents and rates of absorption.

Conclusion

A simple and sensitive method for the quantitation of zolpidem tartrate in human plasma described in the present paper is suitable for bioequivalence study, which needs to process hundreds of samples in a limited time. This proposed method involves a single and simple liquid-liquid extraction procedure and successfully improved sample preparation, run time of analysis and LOQ utilizing a fluorescence detector. The modified and validated assay satisfies the precision, accuracy, linearity, stability, and specificity requirements for bioequivalence studies. The statistical analysis performed on the pharmacokinetic results indicates that the generic formulation of zolpidem tartrate is bioequivalent to the reference formulation in terms of the rate and extent of absorption.

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