Bioequivalence Evaluation of Two Brands of Zolpidem Tartrate 10 mg Tablets (Zanilo and Stilnox) in Healthy Male Volunteers

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ABSTRACT – The purpose of the present study was to evaluate the bioequivalence of two zolpidem tartrate tablets, Stilnox tablet (Sanofi-aventis Korea, reference product) and Zanilo tablet (ChoDang Pharm Co., Ltd., Korea, test product), according to the guidelines of Korea Food and Drug Administration (KFDA). After adding an internal standard (cimetropium), 250 μL plasma samples were extracted using 1.3 mL of ethyl acetate. Extracted compounds were analyzed by HPLC with triple-quadrupole mass spectrometry. This method for determination of zolpidem is proved accurate and reproducible with the limit of quantitation of 1 ng/mL in human plasma. Twenty-four healthy male Korean volunteers received each medicine at the zolpidem tartrate dose of 10 mg in a 2×2 crossover study. There was one-week washout period between the doses. Plasma concentrations of zolpidem were monitored for over a period of 8 hr after the administration. AUC_{0-t} (the area under the plasma concentration-time curve) was calculated by the linear trapezoidal rule. C_{max} (maximum plasma drug concentration) and T_{max} (time to reach C_{max}) were compiled from the plasma concentration-time data. Analysis of variance was carried out using logarithmically transformed AUC_{0-t} and C_{max}. No significant sequence effect was found for all of the bioavailability parameters indicating that the crossover design was properly performed. The 90% confidence intervals for the log transformed data were acceptable range of log 0.8 to log 1.25 (e.g., log 0.92-log 1.06 for AUC_{0-t}, log 0.96-log 1.13 for C_{max}). The major parameters, AUC_{0-t} and C_{max}, met the criteria of KFDA for bioequivalence indicating that Zanilo tablet is bioequivalent to Stilnox tablet.

Key words – Zolpidem tartrate, Bioequivalence, Mass spectrometry, Stilnox, Zanilo

Zolpidem tartrate, (N,N-dimethyl-2-[3-methyl-8-(4-methylphenyl)-1,7-diazabicyclo [4,3,0]nona-2,4,6,8-tetraen-9-yl] acetamide), is a nonbenzodiazepine sedative-hypnotic drug that became available in United States in 1993. Although the actions of zolpidem are due to agonist effects on $GABA_A$ receptors and generally resemble those of benzodiazepines, it produces only weak anticonvulsant effects in experimental animals, and its relatively strong sedative actions appear to mask anxiolytic effects in various animal models of anxiety.[1] Unlike the benzodiazepines, zolpidem has little effect on the stages of sleep in normal human subjects. The drug is as effective as benzodiazepines in shortening sleep latency and prolonging total sleep time in patients with insomnia. Zolpidem is absorbed readily from the gastrointestinal tract; first-pass hepatic metabolism results in an oral bioavailability of about 70%, but this value is lower when the drug is ingested with food because of slowed absorption and increased hepatic blood

flow.[1] Zolpidem is converted into inactive metabolites that are mainly eliminated by renal excretion.[2] Its half-life is approximately 2 hr in individuals with normal hepatic blood flow or function.

Several analytical methods have been reported for the quantification of zolpidem in human body fluids and organ samples including capillary electrophoresis,[3] radioimmunoassay,[4] GC,[5] GC/MS,[6] HPLC,[7] and LC-MS/MS.[8] In the present paper, we described a reliable, rapid, sensitive method for quantifying nanograms of zolpidem in plasma using liquid-liquid extraction of zolpidem with ethyl acetate by LC-MS/MS. The chromatographic conditions were optimized and the results of the validation in terms of linearity, accuracy, precision, recovery, detection, limit of quantitation and specificity were provided. The devised method was used in zolpidem tartrate bioequivalence study, which was conducted in accord with KFDA guidelines.[9] Typical bioavailability, including AUC_{0-t} (the area under the plasma concentration-time curve) and C_{max} (the maximum plasma concentration) parameters were compared.

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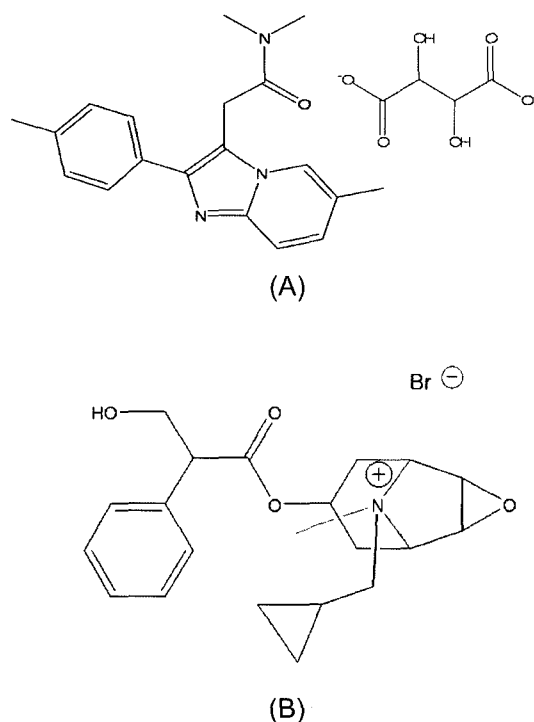Tel : 02)961-0860, E-mail : ktlee@khu.ac.kr

(A)

Br ⊖

(B)

Figure 1–Chemical Structures of (A) zolpidem tartrate and (B) cimetropium bromide.

Materials and Methods

Materials and reagents

Zolpidem tartrate (Figure 1A) and cimetropium bromide (Figure 1B) were provided from ChoDang Pharm. Co., Ltd. (Seoul, Korea) and Alkaloids Private Ltd. (Andhra Pradesh, India), respectively. The solvents, i.e., acetonitrile, ethyl acetate, methanol, and formic acid (all HPLC grades) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q® (Millipore Co., Ltd., Milford, MA, USA) water purification system was used to obtain the purified water. All other chemicals and solvents were of the highest analytical grade available. The test product, Zanilo (10 mg zolpidem tartrate, ChoDang Pharm. Co., Ltd., Korea) and the reference product, Stilnox (10 mg zolpidem tartrate, Sanofi-aventis Korea) were supplied in the form of tablets.

Calibration standards

The primary stock solution of zolpidem was prepared at 1000 μg/mL in methanol as free forms. The internal standard (IS) stock solution was producing a concentration of 100 μg/mL in methanol. This stock solution was diluted appropriately with methanol to obtain working solutions for calibration at 10, 50, 100, 500, 1000, 5000 and 10,000 ng mL^{-1} of zolpidem. Aliquots of 25 μL of working solutions were added to 225 μL

of drug free plasma to obtain zolpidem concentrations of 1, 5, 10, 50, 100, 500, and 1000 ng/mL These working solutions were employed for the preparation of calibration samples and were stored at -70°C.

Selection of volunteers

The study population consisted of twenty-four healthy male volunteers with an average age of 22.4±2.3 years and an average weight of 69.2±8.4 kg. The volunteers were selected after passing a clinical screening procedure including a physical examination and laboratory tests (blood analysis; hemoglobin, hematocrit, WBC, platelet, differential counting of WBC, blood urea nitrogen, total bilirubin, cholesterol, total protein, albumin, alkaline phosphatase, glucose fasting, sGOT, and sGPT, urine analysis; specific gravity, color, pH, bilirubin, RBC and cast). The volunteers were excluded if there was any possibility of their being sensitive to this type of medication, had a history of any illness of the hepatic, renal or cardiovascular systems, or a history of excessive alcohol intake or other medications. This was done to ensure that the existing degree of variation would not be due to an influence of illness or other medications.

Administration for volunteers and sample collection from volunteers

All of the volunteers avoided using other drugs for at least 10 days prior to the study and until its completion. They also refrained from consuming xanthine-containing foods, alcoholic beverages and other beverages for 12 hr prior to each dosing and until the collection of the last blood sample. Each volunteer received an oral dose of 10 mg of zolpidem tartrate in a standard 2×2 cross-over model in a randomized order. We had one week washout period between the doses. All of the participants signed a written consent form after they had been informed of the nature and details of the study in accordance with the Korean Guidelines for Bioequivalence Test.[9] The subjects were hospitalized (Seoul Adventist Hospital, Korea) at 10:00 p.m. on the eve of the study and fasted overnight and 4 hr after each drug administration. At 7:00 a.m., their median cubital vein was cannulated and 7 mL blood samples were drawn into heparinized tubes. The doses were taken at 8:00 a.m. on each dosing day along with 240 mL of water. At 4 hr after the oral administration, all of the subjects were given standardized meals. The subjects were not allowed to remain in the supine position or to sleep until 8 hr after the oral administration. Approximately 7 mL blood samples were collected via the cannula at the following times; predose, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 8 hr after the administration. On each occasion, the blood sample was centrifuged immediately

(3000 rpm, 10 min), and this sample was frozen at -70 until the LC-MS/MS analysis.

Sample preparation for LC-MS/MS injection

20 μL of IS working solution (cimetropium 100 μg/mL) was added to 250 μL plasma sample. The samples were vortexed briefly for 30 sec and 1.3 mL of ethyl acetate was added. This mixture was shaken for 10 min and centrifuged at 10,000 rpm for 10 min. The organic layer was separated and evaporated to dryness under N_2 at 40. The final residue was reconstituted into 100 μL of mobile phase, vortexed again for 10 min and then centrifuged at 10,000 rpm for 10 min. The resulting clear supernatant from each sample was transferred to 96-well plates using an automatic pipette with a disposable plastic tip. Finally, a 10 μL aliquot was injected into the LC-MS/MS system.

LC-MS/MS analysis and validation of method

All plasma samples were analyzed for zolpidem concentration according to a sensitive, selective and accurate high-performance liquid chromatography with triple quadrupole mass spectrometry method, which was developed and validated.

The chromatographic system consisted of Waters 2795 separation module HPLC system and Waters Micromass Quattro Premire triple quadrupole mass spectrometer The separation was achieved on a hydrosphere C_{18} (50 × 2.0 mm i.d., S-3 μm, 12 nm YMC, Kyoto, Japan) reversed-phase column at 40°C. The mobile phase consisted of 90% acetonitrile and 10% Mili-Q water containing 10 mM ammonium formate (pH 3.09 with formic acid) was filtered and degassed. The mobile phase was eluted at a flow rate of 0.2 mL/min. The autosampler was controlled at 4°C. The peak area was measured, and the peak area ratio of the zolpidem to that of IS and the concentration was calculated. All samples from each single volunteer were measured on the same day in order to avoid interassay variation. Micromass Masslynx 4.0 and Quanlynx were used for data management.

Optimum tandem MS parameters including resolution, ion energy, entrance potential, exit potential and collision energy were set to maximize product ions. The obtained optimum instrumental parameters are summarized in Table I. Two channels of positive ion multiple reaction monitoring mode were used to detect zolpidem and IS. The most abundant product ions were at m/z 235.4 from the parent m/z 308.4 ion of zolpidem and at m/z 210.3 from the m/z 358.4 of the IS (Figure 2).

The standard solutions over the range of 1-1000 ng/mL using 250 μL plasma samples were employed for the preparation of calibration curves. In order to assess the intra-day coefficient of variation (CV) and accuracy of the method, five

Table I–*LC-MS/MS Analysis Condition of Zanilo and Stilnox*

Parameters	Value	
Source temperature (°C)	150	
Desolvation temperature (°C)	300	
Desolvation gas flow (L/hr)	600	
Cone gas flow (L/hr)	60	
Collision cell gas pressure (mbar)	2.90×10^{-3}	
Capillary voltage (kV)	3	
Extractor (V)	3	
RF lens (V)	0	
Low mass 1 resolution	14	
High mass 1 resolution	14	
Ion energy 1 (V)	1	
Low mass 2 resolution	14	
High mass 2 resolution	14	
Ion energy 2 (V)	1	
Entrance (V)	2	
Exit (V)	2	
Multiplier	650	
Dwell time (s)	0.5	
	Analyte	**IS**
Molecule ion (m/z)	308.4	358.4
Product ion (m/z)	235.4	210.3
Cone energy (V)	45	30
Collision energy (V)	30	25

calibration curves were obtained in one day. The precision and accuracy for inter-day assay were also assessed at the same concentration and repeated for five different days.

Statistical analysis of pharmacokinetic parameters

Non-compartmental analysis was performed to estimate pharmacokinetic (PK) parameters of zolpidem. C_{max} and T_{max} were obtained directly from the data without interpolation. AUC_{0-t} was calculated using the linear and logarithmic trapezoidal rule. The terminal first order elimination constant (K_e) was determined by a least squares fit of the terminal plasma concentrations. The constant K_e was used to extrapolate $AUC_{t-\infty}$ (area under the plasma concentration versus time curve from time of last quantifiable concentration to infinite). $AUC_{0-\infty}$ (area under the plasma concentration versus time curve from time zero-pre-dose-extrapolated to infinite time) is obtained from AUC_{0-t} plus $AUC_{t-\infty}$.[10]

Primary PK parameters required in assessing bioequivalence were evaluated by PK software WINNONLIN® standard version 3.1 software[11] and K-BE test.[12] Bioequivalence between the products was determined by calculating 90% confidence interval for the ratio of AUC_{0-t} and C_{max} values for the test and reference
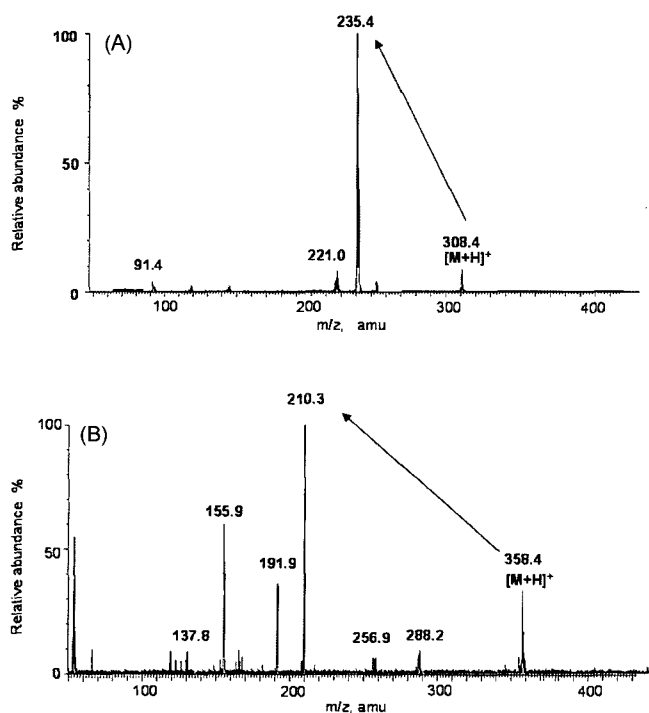
Figure 2–Product ion spectra of $[M+H]^+$ ions of (A) zolpidem and (B) cimetropium (IS).

products, using logarithmic transformed data. Analysis of variance (ANOVA) was used to assess group and period effects.

Results and Discussion

Our chromatographic condition gave a short chromatographic run time (2 min) with satisfactory separation of zolpidem and IS. Figure 3 shows typical sample chromatograms.
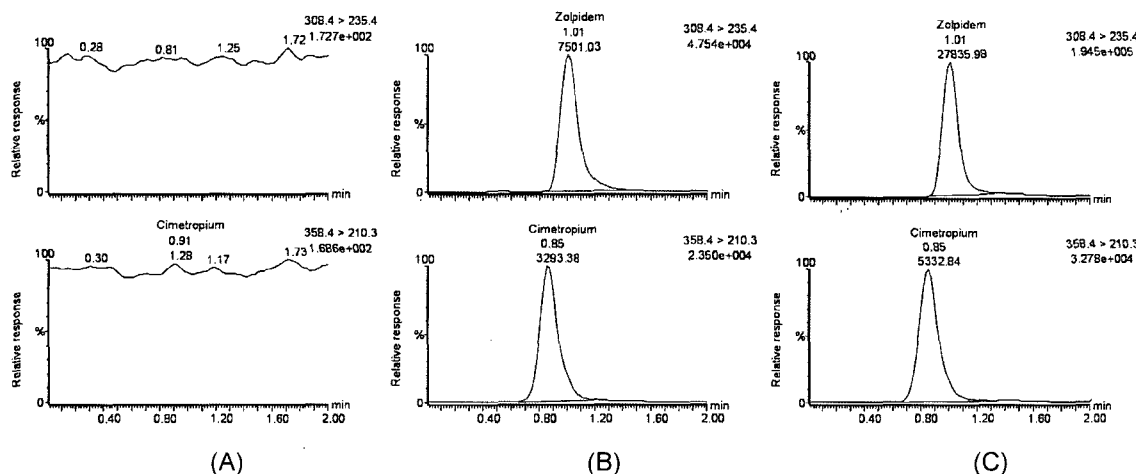
No interference with constituents from drug-free human plasma was observed. Zolpidem and IS were separated from the biological background at 1.01 and 0.85 min, respectively. The total analysis time for each run was 2 min.

The limit of detection (LOD) arbitrarily set at a signal to noise ratio (S/N)=3 and the lower limit of quantitation (LLOQ) at an S/N=10. The precision and accuracy of LLOQ was required to be within 20% according to FDA guidelines. Under the validation conditions described, the lower limit of quantification was 1 ng/mL for zolpidem (S/N ratio>10). The relationship between the concentration and peak area ratio was found to be linear within the range 1-1000 ng/mL for zolpidem (y=0.0442791x+0.00652196, r=0.9999). The intra-day accuracy of the method for zolpidem ranged from 81.6% to 105.2% while precision ranged from 2.5% to 5.7%. The inter-day accuracy of the method for zolpidem ranged from 100.5% to 119.7% while precision ranged from 2.2% to 17.9% (Table II). The mean recoveries of the 10, 100 and 1000 ng/mL levels were 96.4, 92.1 and 104.1%, respectively. The proposed method used in this study was found to be reliable, accurate, sensitive and rapid for detecting plasma levels of zolpidem.

After validation, about 600 plasma samples were processed

Table II–*Precision and Accuracy for the Determination of Zolpidem in Human Plasma (n = 5)*

Concentration (ng/mL)	Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
1	2.9	17.9	81.6	119.7
5	5.7	3.6	105.2	101.2
50	4.8	5.1	98.7	103.0
500	2.5	2.2	101.0	100.5

(A)   (B)   (C)

Figure 3–Multiple reaction monitoring chromatogram of (A) blank human plasma, (B) plasma spiked with zolpidem (10 ng/mL) and IS (100 µg/mL, 20 µL) and (C) plasma (116.6 ng/mL) from a volunteer 1 h after the oral administration of Zanilo (zolpidem tartrate 10 mg).

with LC-MS/MS. The mean concentration-time profile of zolpidem for the two products is shown in Figure 4. The figure indicates that the mean plasma concentration profiles of the two
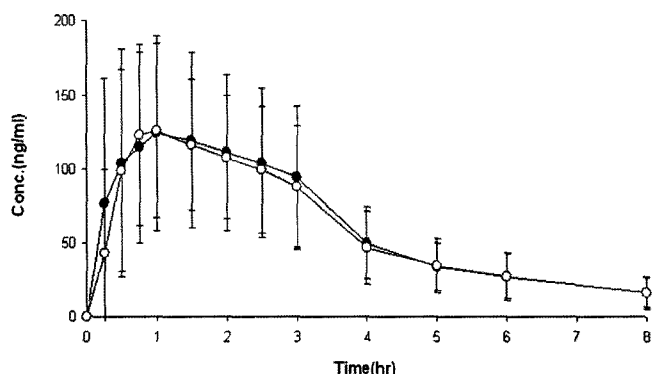

Figure 4–Mean (±S.D., n=24) plasma concentration-time curves of zolpidem following oral adminstration of Zanilo (○) and Stilnox (●) tablet at the dose of 10 mg of zolpidem tartrate.

zolpidem tartrate products were closely similar or overlapped. Peak concentrations of 123.8 ng/mL (test) and 125.3 ng/mL (reference) for zolpidem were attained at 1 hr equally after drug administration and then declined broadly up to end time point (8 hr).

Table III shows the average values of pharmacokinetic parameters after administration of two brands of zolpidem tartrate. The extent of absorption is a key characteristic of drug formulation, and therefore AUC is an important parameter for bioequivalence study.[13] In addition, other two parameters, such as C_{max} and T_{max}, are also important features and could affect the therapeutic behavior of a drug[14] and hence were also considered in the study. The sampling schedule should be planned to provide a reliable estimate of the extent of absorption. This is generally achieved if AUC_{0-t} is at least 80% of $AUC_{0-\infty}$. The AUC_{0-t} value of this test estimated was more than 80% of AUC_{0-} value. In our study, AUC_{0-t} and C_{max} for

Table III–*Bioavailability Parameters in Normal and Logarithmic Scales for Each Subject Obtained after Oral Administration of Stilnox and Zanilo Tablets at the Zolpidem tartrate Dose of 10 mg*

	Stilnox Tablet					Zanilo Tablet				
	AUC_{0-t} (ng·hr/mL)	Ln AUC_{0-t}	C_{max} (ng/mL)	Ln C_{max}	T_{max} (hr)	AUC_{0-t} (ng·hr/mL)	Ln AUC_{0-t}	C_{max} (ng/mL)	Ln C_{max}	T_{max} (hr)
A1	427	6.06	135	4.90	0.50	452	6.11	135	4.90	1.50
A2	441	6.09	203	5.31	1.00	555	6.32	173	5.15	0.75
A3	696	6.54	287	5.66	0.25	642	6.46	228	5.43	0.75
A4	387	5.96	128	4.85	2.50	402	6.00	179	5.19	0.50
A5	699	6.55	183	5.21	2.50	604	6.40	192	5.26	2.50
A6	725	6.59	212	5.35	0.50	758	6.63	228	5.43	1.00
A7	949	6.86	281	5.64	1.00	741	6.61	268	5.59	0.50
A8	665	6.50	172	5.15	3.00	626	6.44	172	5.15	3.00
A9	414	6.03	141	4.95	0.75	406	6.01	138	4.93	0.75
A10	455	6.12	166	5.11	0.75	348	5.85	113	4.73	1.00
A11	439	6.08	148	5.00	2.00	346	5.85	232	5.44	1.00
A12	531	6.27	145	4.98	3.00	483	6.18	152	5.03	2.50
B1	164	5.10	79	4.37	0.25	205	5.32	107	4.67	0.50
B2	373	5.92	125	4.83	0.75	386	5.96	136	4.91	0.75
B3	368	5.91	137	4.92	0.75	439	6.08	161	5.08	0.75
B4	453	6.12	146	4.98	3.00	545	6.30	203	5.31	0.50
B5	620	6.43	149	5.01	3.00	450	6.11	107	4.67	2.50
B6	557	6.32	202	5.31	1.00	642	6.46	191	5.25	0.50
B7	217	5.38	84	4.42	0.75	216	5.37	77	4.34	0.75
B8	221	5.40	85	4.44	3.00	297	5.69	107	4.67	0.50
B9	374	5.93	143	4.96	1.00	380	5.94	153	5.03	0.75
B10	660	6.49	202	5.31	0.50	403	6.00	180	5.19	0.25
B11	704	6.56	221	5.40	0.25	624	6.44	169	5.13	0.25
B12	187	5.23	70	4.24	1.50	276	5.62	116	4.75	0.75
Mean	488.61	6.10	160.15	5.01	1.40	467.76	6.09	163.15	5.05	1.02
(S.D.)	197.22	0.46	56.79	0.37	1.04	155.66	0.36	47.55	0.30	0.78

Table IV–*Statistical Results of Bioequivalence Evaluation between Two Zolpidem Tartrate Tablets*

	Parameters		
	AUC_{0-t}	$AUC_{0-\infty}$	C_{max}
Difference (%)	-4.27	-4.49	1.88
F value[a]	0.06	0.98	0.64
Test/Ref estimate	0.99	0.98	1.04
90% Confidence interval	0.92-1.06	0.90-1.07	0.96-1.13

[a] $\alpha = 0.05$, $F(1,22) = 4.260$

zolpidem were 488.61 ± 197.22 ng·hr/mL (Stilnox) and 467.76 ± 155.66 ng·hr/mL (Zanilo), and 160.15 ± 56.79 ng/mL (Stilnox) and 163.15 ± 47.55 ng/mL (Zanilo), respectively (Table III). No significant sequence effect was found for all of the bioequivalence parameters indicating that the cross-over design was properly performed. Statistical significances of the parameters (Table IV) are given for the test and reference products separately for each period and as combined estimates. The differences of the test product/the reference product for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were -4.27%, -4.49% and 1.88%, respectively. No significant period effect in AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} was detected in this study. 90% confidence intervals also demonstrated that the ratios of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} of the two products lie within the KFDA acceptable range of log 0.8-log 1.25.[15] The results of this study suggest equivalent of the two brands of zolpidem.

Conclusions

It was shown that this method is suitable for the analysis of zolpidem in human plasma samples for bioequivalence studies. Using this method, the bioequivalence of two different 10 mg of zolpidem tartrate tablet products was examined in twenty-four healthy male volunteers. The statistical analysis results based on comparisons of the two pivotal parameters (AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}) demonstrated the bioequivalence of these two tablet products of zolpidem tartrate.

Acknowledgements

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