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Bioequivalence of Boryung Torsemide Tablet to Torem Tablet (Torasemide 10 mg) by High Performance Liquid Chromatography/UV Detector

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ABSTRACT – The purpose of the present study was to evaluate the bioequivalence of two torasemide tablets, Torem tablet (Roche Korea Co., Ltd., Korea, reference drug) and Boryung Torsemide tablet (Boryung Pharmaceutical Co., Ltd., Korea, test drug), according to the guidelines of Korea Food and Drug Administration (KFDA). After adding an internal standard (furosemide) to human serum, serum samples were extracted using 5 mL of ethyl acetate. Compounds were analyzed by reverse-phase HPLC method with UV detection. This method showed linear response over the concentration range of 0.05-5 ug/mL with correlation coefficient of 0.999. The lower limit of quantitation using 0.5 mL of serum was 0.05 ug/mL which was sensitive enough for pharmacokinetic studies. Twenty-eight healthy male Korean volunteers received each medicine at the torasemide dose of 20 mg in a 2 × 2 crossover study. There was a one-week washout period between the doses. Serum concentrations of torasemide were monitored by an HPLC-UV for over a period of 12 hr after the administration. AUC_t (the area under the serum concentration-time curve from time zero to 12 hr) was calculated by the linear trapezoidal rule method. C_{max} (maximum serum drug concentration) and T_{max} (time to reach C_{max}) were compiled from the serum concentration-time data. Analysis of variance was carried out using logarithmically transformed AUC_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 of the AUC_t ratio and the C_{max} ratio for Boryung Torsemide/Torem were log 0.97-log 1.03 and log 0.93-log 1.12, respectively. These values were within the acceptable bioequivalence intervals of log 0.80-log 1.25. Thus, the criteria of the KFDA guidelines for the bioequivalence was satisfied, indicating Boryung Torsemide tablet and Torem tablet are bioequivalent.

Key words – Torasemide, Torem, Boryung Torsemide, HPLC-UV, Bioequivalence test

Torasemide, 1-isopropyl-3-{[4-(3-methyl-phenylamino)pyridine]-3-sulfonyl}urea, is a loop diuretic of the pyridine sulfonylurea class approved for the treatment of oedematous states such as congestive heart failure, liver cirrhosis with ascites, nephrotic syndrome and chronic renal failure.[1] Torasemide acts primarily on the thick segment of the ascending loop of Henle, inhibiting the transport of NaCl out of the tubule into the interstitial tissue by inhibiting the $Na^+/K^+/2Cl^-$ carrier in the luminal membrane.[2]

Torasemide is well absorbed from the gastrointestinal tract after oral administration.[3] The bioavailability of torasemide following single dose of 20 mg was 80 to 90%. The time of peak concentration was reached at 1 hr, the elimination half-life varied from 3 to 4 hr.[4] It is eliminated mainly by hepatic metabolism and excretion. Only 25% of the parent drug is recovered unchanged in the urine.[5]

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Torasemide can be measured in serum using HPLC with UV spectrophotometry. The method is sufficiently sensitive to allow the determination of the pharmacokinetics of the torasemide in biological fluids after administration of all clinically useful intravenous and oral doses.[1,5,6] The HPLC method presented in this paper, which was validated in the study of torasemide bioequivalence to identify pharmaceutical equivalents of the two torasemide formulations, was developed for the purpose of providing a simple sample preparation procedure and more reproducible results.

Materials and Methods

Materials and reagents

Torasemide (Figure 1A) was supplied from Boryung Pharmaceutical Co., Ltd. (Seoul, Korea). Furosemide (Figure 1B) was purchased from Sigma Co. (St. Louis, MO, USA). Methanol, acetonitrile and ethyl acetate (HPLC grade) were purchased from Fischer Scientific (Fair Lawn, NJ, USA) and the

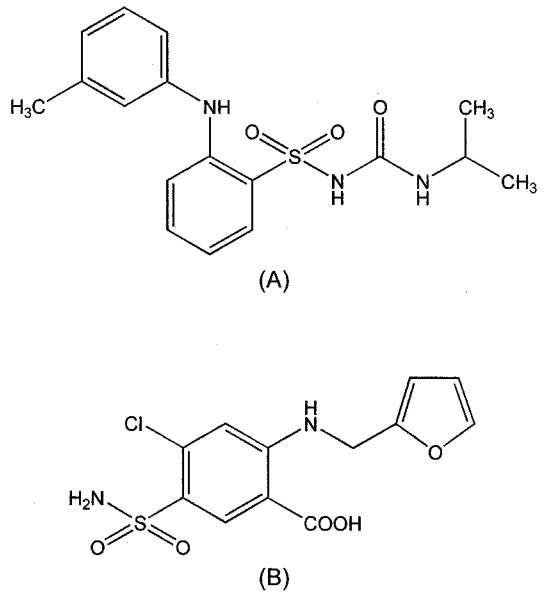

Figure 1–Chemical Structures of (A) torasemide and (B) furosemide.

other chemicals were of HPLC grade or highest quality available. A Milli-Q® (Millipore Co., Milford, MA, USA) water purification system was used to obtain the purified water for the HPLC. The test medication, Boryung Torsemide (10 mg torasemide tablet, Boryung Pharmaceutical Co., Ltd., Korea) and the reference medication, Torem (10 mg torasemide tablet, Roshe Korea Pharm. Co., Ltd., Korea) were supplied in the form of tablets.

Dissolution test

The *in vitro* dissolution tests, as second equivalence criterion of two torasemide preparations, were carried out using the dissolution apparatus II (paddle method) of K.P. VIII at 37 ± 0.5 °C, 50 rpm and 900 mL of the dissolution media, pH 1.2, 4.0, 6.8 buffer solution, water and 0.1 M HCl solution. Drug release testing should be conducted on 12 individual dosage units of the test and the reference preparation used in the BE studies. Samples were removed at 5, 10, 15 and 30 min, filtered and assayed by HPLC coupled with UV detector at 288 nm. Finally, the dissolved torasemide content was expressed as percent of stated amounts.

The acceptance criteria for assessment of equivalence of dissolution profiles between two preparations are as follows. When the average dissolution from reference preparation reaches 85% within 15 min, the average dissolution from test preparation should also reach 85% within 15 min. When the average dissolution from reference preparation reached 85% after more than 15 min, the average dissolution from test preparation should not be deviated by more than 15% from that of the reference preparation at two time points.[7]

Selection of volunteers

The study population consisted of twenty-eight healthy male Korean volunteers with an average age of 22.50 years and an average weight of 68.18 kg. The volunteers were selected after passing a clinical screening procedure including a physical examination and laboratory tests (blood analysis; hemoglobin, hematocrit, RBC, WBC, platelet, differential counting of WBC, total protein, albumin, sGOT, sGPT, alkaline phosphatase, total bilirubin, cholesterol, creatinine, blood urea nitrogen, and glucose fasting and urine analysis; specific gravity, color, pH, sugar, albumin, bilirubin, RBC, WBC, 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.

Prescription for volunteers and Extraction of blood samples from volunteers

All of the volunteers avoided taking other drugs for at least one week 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 a single dose of 20 mg of torasemide in a standard 2 × 2 crossover model in a randomized order. Since half-life of 20 mg torasemide was reported as 2.81 ± 0.26 hr[5] after oral administration, we had an 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.[7] Subjects fasted for at least 10 hr before each drug administration and continued to fast up to 4 hr. At 8:00 a.m., their median cubital vein was cannulated (JELCO™, 22G, Johnson & Johnson Medical, Pomezia, Italia) and 8 mL of blood samples were drawn into Vacutainer® (Becton Dickins and company, Franklin Lakes, NJ, USA). The doses (two torasemide tablets; torasemide 20 mg) were taken at 8:30 a.m. of each dosing day along with 240 mL of water. At 4 hr after the oral administration, standardized meals were given to all of the subjects. Approximately 8 mL of blood samples were collected via the cannula at the following times; predose, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 hr after the

administration. On each occasion, the blood sample was centrifuged immediately, and serum sample was frozen at -70°C until the HPLC analysis.

HPLC analysis of torasemide in serum sample

An HPLC method modified from the reported HPLC methods[5] was developed and validated for torasemide assay in serum samples at Institute of Bioequivalence and Bridging Study, Chonnam National University. The chromatographic system consisted of Shimadzu LC 10 AD/*vp* system. The separation was achieved on a Shim-Pack ODS (5 um particle size, 150 × 4.6 mm I.D.) reversed-phase column from Shimadzu (Kyoto, Japan) at a room temperature. The detection wavelength was UV-290 nm. The mobile phase was prepared by mixing 5 mM ammonium acetate buffer containing 0.1% acetic acid and acetonitrile in the ratio of 75:25 (v/v). The flow rate was 1.0 mL/min for the total running time of 13 min.

The primary stock solution of torasemide was prepared at 1000 ug/mL in methanol and stored at 4°C. The internal standard stock solution was prepared in methanol producing a concentration of 40 ug/mL. Torasemide stock solution was serially diluted with methanol and added to drug free serum to obtain final concentrations of 0.05, 0.1, 0.5, 1, 2 and 5 ug/mL. These standard solutions were employed for the preparation of calibration curve. In order to assess the intra-day coefficient of variation (C.V.) and accuracy for serum samples, samples of torasemide and furosemide were spiked into human serum at final concentrations of 0.05, 0.1, 0.5 and 2 ug/mL. Lower limit of quantitation (LLOQ) was determined from S/N = 10. The precision and accuracy for inter-day assay were assessed at the same concentration and repeated for five different days.

After thawing at room temperature, an aliquot of each sample (500 uL) was pipetted into a clean test tube and furosemide (I.S.) solution (50 uL, 40 ug/mL) was added. After vortexing briefly, 5 mL of ethyl acetate was added to each sample. The mixture was shaken and centrifuged at 3000 rpm for 10 min. The organic layer was separated and evaporated to dryness at 40°C in CVE-200D (EYELA, Tokyo Rikakikai Co., LTD., Japan). The residue was reconstituted with 500 uL of mobile phase and then 50 uL of the solution was injected into the HPLC system and the peak area and retention time were recorded.

Statistical analysis of pharmacokinetic parameters

Each volunteer received an oral dose of 20 mg of torasemide in a standard 2 × 2 crossover model in a randomized order. Pharmacokinetic parameters such as AUC_t, C_{max} and T_{max} were calculated from serum concentration-time curve. C_{max} and T_{max} were recorded as actual measurement values and AUC_t was calculated by trapezoidal formula in 0-12 hr. Their ratios (test/reference) using log-transformed data, together with their means and 90.0% confidence intervals, were analyzed with two-way analysis of variance (ANOVA) that performed with the K-BE Test program at a significant level of 0.05.[7] The bioequivalence of two torasemide tablets were estimated by AUC_t and C_{max}. T_{max} was used as a reference value.

Results and Discussion

Dissolution test

Both preparations released more than 85% of torasemide within 15 min in pH 1.2, 4.0, 6.8 buffer solution, water and 0.1 M HCl solution. The release profiles of two torasemide preparations were very similar at all dissolution media (Table I). The dissolution profiles of two torasemide preparations in 0.1 M HCl solution are shown in Figure 2.

HPLC analysis of torasemide in serum sample

The method was validated according to FDA guidance and international guidelines.[8] In this HPLC method, torasemide and furosemide (I.S.) were well separated from the biological background under the described chromatographic conditions at retention times of 7.9 and 9.5 min, respectively (Figure 3B). The peaks were of good shape, completely resolved one. No interference with constituents from serum was observed (Figure 3A).

Table I–*Dissolution Data of Two Torasemide Preparations in Five Dissolution Media (n=12)*

Dissolution media	pH 1.2[a]		pH 4.0[a]		pH 6.8[a]		Water[a]		0.1 M HCl[a]	
Preparations	Ref.	Test	Ref.	Test	Ref.	Test	Ref.	Test	Ref.	Test
Mean	93.5	95.3	95.2	98.2	94.1	95.8	93.5	97.7	96.7	96.7
S.D.	2.23	0.65	2.08	2.10	0.64	1.39	3.84	2.50	0.67	0.70
C.V.	2.39	0.68	2.18	2.14	0.68	1.45	4.11	2.56	0.69	0.72

[a]Percent of dissolved torasemide content within 15 min.

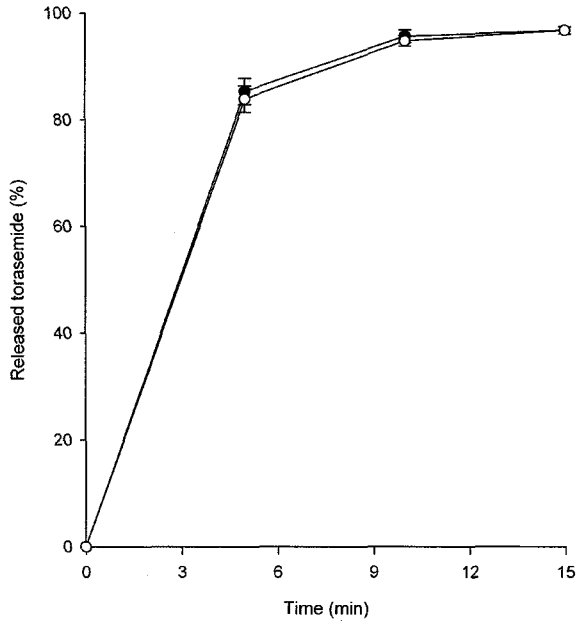

Figure 2–Dissolution profiles of torasemide from Torem (●, reference) and Boryung Torsemide tablets (○, test) in 0.1 M HCl solution. Data are presented as mean±S.D. (n=12).

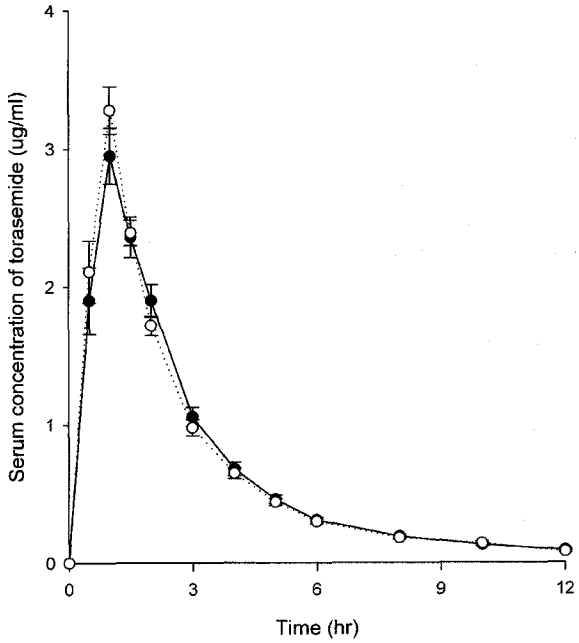

Figure 4–Mean (±S.E., n=28) serum concentration-time curves of torasemide following oral administration of Boryung Torsemide (○) and Torem (●) tablets at the dose of 20 mg torasemide.

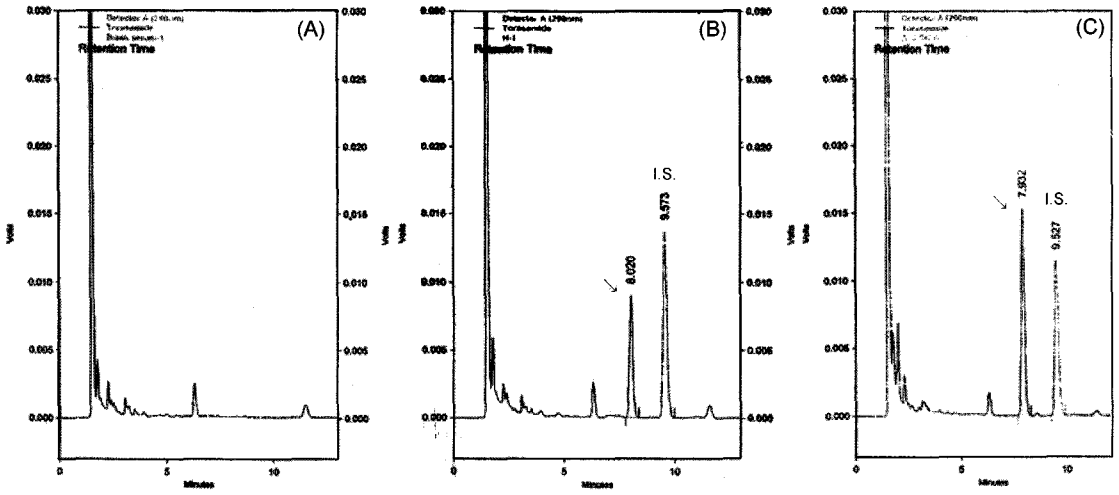

Figure 3–Chromatograms of (A) blank human serum, (B) blank human serum spiked with torasemide (1 ug/mL) and internal standard (I.S., furosemide 40 ug/mL) and (C) serum sample (1.97 ug/mL) at 2 hr after oral administration of 10 mg torasemide two tablets. ↘=torasemide peak.

The calibration curve was obtained by analyzing six samples. The curve was linear in whole range tested (0.05-5 ug/mL) and described by following equation: $Y = 0.564X + 0.00312$ (X = torasemide concentration (ug/mL), Y = ratio of peak areas) with a correlation coefficient of 0.999. In the range of 0.05-5 ug/mL, the intra-day accuracy ranged from 94.72 to 109.28% while the intra-day precision ranged from 8.10 to 10.95%. The inter-day accuracy ranged from 97.71 to 109.15 % while the intra-day precision ranged from 5.22 to 12.22%. These results indicated that the present method has a satisfactory accuracy and precision.

Pharmacokinetic analysis

The developed method was successfully used for a bioequivalent test in which serum concentrations of torasemide in twenty-eight healthy male volunteers were determined up to 12 hr after the oral administration of 20 mg torasemide. Figure 4 shows mean serum concentration-time curves of torasemide

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

Concentration (ug/mL)	Precision C.V. (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.05	8.91	12.22	94.72	97.83
0.1	9.10	11.76	109.28	109.15
0.5	10.95	5.37	99.80	99.41
2	8.10	5.22	100.01	97.71

following oral administration of Torem and Boyung Torsemide tablets, and descriptive statistics of the derived pharmacokinetic parameters such as AUC_t, C_{max} and T_{max} for two preparations are summarized in Table III.

AUC_t, C_{max} and T_{max} for torasemide were 8.43 ± 1.73 ug·hr/mL (Torem) and 8.42 ± 1.60 ug·hr/mL (Boryung Torsemide), 3.47 ± 0.66 ug/mL (Torem) and 3.52 ± 0.68 ug/mL (Boryung Torsemide), and 1.11 ± 0.44 hr (Torem) and 0.96 ± 0.36 hr (Boryung Torsemide), respectively (Table III). The differences of means of the test medication to reference medication for AUC_t, C_{max} and T_{max} were -0.12, 1.44 and -11.29%, which are generally accepted if the differences of mean values for AUC_t, C_{max} and T_{max} lie within ±20%.

Bioequivalence test of torasemide products

No significant sequence effect was found for all of the bioavailability parameters indicating that the crossover design was

Table III–*Bioavailability Parameters in Normal and Logarithmic Scales for Each Subject Obtained after Oral Administration of Torem and Boryung Torsemide Tablets at the Torasemide Dose of 20 mg*

Subjects	Torem Tablet					Boryung Torsemide Tablet				
	AUC_t (ug·hr/mL)	Ln AUC_t	C_{max} (ug/mL)	Ln C_{max}	T_{max} (hr)	AUC_t (ug·hr/mL)	Ln AUC_t	C_{max} (ug/mL)	Ln C_{max}	T_{max} (hr)
A1	10.85	2.38	4.53	1.51	1.00	11.67	2.46	4.64	1.53	1.00
A2	7.91	2.07	3.86	1.35	0.50	9.80	2.28	3.26	1.18	1.00
A3	7.61	2.03	3.41	1.23	1.00	9.12	2.21	4.67	1.54	1.00
A4	6.20	1.83	3.68	1.30	0.50	6.27	1.84	2.61	0.96	0.50
A5	7.95	2.07	3.91	1.36	1.00	8.19	2.10	3.52	1.26	0.50
A6	9.05	2.20	2.97	1.09	1.50	9.32	2.23	3.58	1.28	1.00
A7	7.41	2.00	3.28	1.19	1.00	8.31	2.12	2.27	0.82	2.00
A8	9.07	2.21	3.37	1.21	1.00	8.93	2.19	3.62	1.29	1.00
A9	5.00	1.61	1.32	0.28	2.00	5.84	1.76	3.13	1.14	0.50
A10	9.76	2.28	3.58	1.28	1.50	9.25	2.22	3.66	1.30	1.00
A11	6.98	1.94	3.90	1.36	2.00	6.78	1.91	3.05	1.12	1.00
A12	8.46	2.13	3.37	1.21	1.00	9.52	2.25	4.24	1.44	1.00
A13	7.14	1.97	2.96	1.09	1.00	7.00	1.95	1.97	0.68	2.00
A14	6.49	1.87	3.49	1.25	1.50	6.20	1.82	3.64	1.29	1.00
B1	9.48	2.25	3.17	1.15	1.50	8.62	2.15	3.43	1.23	1.00
B2	13.53	2.60	4.96	1.60	1.00	11.31	2.43	4.51	1.51	1.00
B3	7.75	2.05	3.72	1.31	1.00	6.70	1.90	3.32	1.20	1.00
B4	7.14	1.97	3.58	1.28	1.00	7.82	2.06	4.22	1.44	0.50
B5	8.06	2.09	3.19	1.16	0.50	8.06	2.09	3.48	1.25	0.50
B6	9.53	2.25	2.78	1.02	1.00	10.41	2.34	3.73	1.32	1.00
B7	11.73	2.46	2.89	1.06	2.00	11.63	2.45	4.72	1.55	0.50
B8	8.45	2.13	3.64	1.29	1.00	8.77	2.17	2.57	0.94	1.00
B9	8.16	2.10	3.40	1.22	1.00	7.34	1.99	3.22	1.17	1.00
B10	10.19	2.32	3.75	1.32	1.00	9.09	2.21	3.17	1.15	1.00
B11	7.78	2.05	3.36	1.21	1.00	6.99	1.94	3.62	1.29	1.00
B12	7.99	2.08	3.85	1.35	0.50	7.36	2.00	3.71	1.31	1.00
B13	7.82	2.06	2.73	1.00	1.50	8.14	2.10	3.55	1.27	1.00
B14	8.60	2.15	4.38	1.48	0.50	7.29	1.99	3.55	1.27	1.00
Mean	8.43	2.11	3.47	1.22	1.11	8.42	2.11	3.52	1.24	0.96
(S.D.)	1.73	0.20	0.66	0.23	0.44	1.60	0.19	0.68	0.21	0.36

Table IV–*Bioavailability Parameters for Each Volunteer Obtained after Oral Administration of Boryung Torsemide and Torem Tablets at the Torasemide Dose of 20 mg*

	Parameters*		
	AUC_t	C_{max}	T_{max}
Difference (%)	-0.12	1.44	-11.29
F_G[a)]	1.5508	1.0366	2.0392
Test/Ref point estimate	0.0006	0.0193	-0.1429
Confidence interval (δ)[b)]	$\log 0.97 \leq \delta \leq \log 1.03$	$\log 0.93 \leq \delta \leq \log 1.12$	$-28.71 \leq \delta \leq 6.13$

*The AUC_t and C_{max} values were calculated on the basis of ln-transformed data, and the T_{max} values on the basis of untransformed data.
[a)]α=0.05, F(1,26)=4.230, [b)]α=0.05

properly performed. Geometric means of the parameters (Table IV) are given for the test and reference formulations separately for each period and as combined estimates. The parametric 90% confidence intervals for AUC_t and C_{max} were log 0.97-log 1.03 and log 0.93-log 1.12, respectively, which were within the commonly accepted bioequivalence range of log 0.80-log 1.25.[9)] Geometric means of the parameters such as AUC_t and C_{max} of the test drug were similar to those of the reference drug, which proved that there was no significant difference between the bioavailability of Torem (reference drug) and Boryung Torsemide (test drug).

Conclusions

This HPLC method was suitable for the analysis of torasemide in human serum samples collected for bioequivalence studies. Using this method, the bioequivalence of two different torasemide tablet formulations was examined at a dose of 20 mg in 28 healthy normal male volunteers. It can be concluded that Boryung Torsemide is bioequivalent to Torem on the basis of the pharmacokinetic and statistical analysis results obtained in this study, and that the two formulations may be prescribed interchangeably in medical practice.

Acknowledgements

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