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Bioequivalence of Cholicerin Soft Capsule to Gliatilin Soft Capsule (Choline Alphoscerate 400 mg)

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ABSTRACT – The purpose of the present study was to evaluate the bioequivalence of two choline alphoscerate soft capsules, Gliatilin soft capsule (Daewoong Pharmaceuticals Co., Ltd.) and Cholicerin soft capsule (Sam Chun Dang Pharm. Co., Ltd.), according to the guidelines of Korea Food and Drug Administration (KFDA). Serum concentrations of choline after oral administration of choline alphoscerate were determined using a validated LC/MS/MS method. This method showed linear response over the concentration range of 0.5-20 μg/mL with correlation coefficient of 0.9999. The lower limit of quantitation using 100 μL of serum was 0.5 μg/mL which was sensitive enough for pharmacokinetic studies. Thirty six healthy male Korean volunteers received each medicine at the choline alphoscerate dose of 1200 mg in a 2×2 crossover study. There was a one-week washout period between the doses. Blood samples were taken at predetermined time intervals up to 8 hr. AUC_t (the area under the serum concentration-time curve from time 0 to 8 hr) was calculated by the linear trapezoidal rule method. C_{max} (the maximum serum drug concentration) and T_{max} (the 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 Cholicerin/Gliatilin were log0.9998-log1.1172 and log0.9938-1.0944, respectively. These values were within the acceptable bioequivalence intervals of log0.80-log1.25. Thus, the criteria of the KFDA guidelines for the bioequivalence was satisfied, indicating Cholicerin soft capsule and Gliatilin soft capsule are bioequivalent.

Key words – Choline alphoscerate, Gliatilin soft capsule, Cholicerin soft capusule, Bioequivalence, LC/MS/MS

Choline alphosceraete, L-α-glycerylphosphorylcholine, is a precursor of phospholipid containing choline which potential for the treatment of Alzheimer's disease and is used as a nootropic dietary supplement to enhance memory and cognition.[1-4] It is completely absorbed following oral administration and in oral or intramuscular administration it is almost metabolized so that active major metabolite, choline is appeared in plasma. In case of intramuscular injection to healthy adult, it was reported that elimination half life, T_{max} of choline alphoscerate was 1.37±0.26 and 0.5±0.1 hr, respectively and recovery time to base line was 6 to 8 hr.[4]

Choline can be measured in serum using liquid chromatography with tandem mass spectrometry (LC/MS/MS).[5-7] These methods are sufficiently sensitive to allow the determination of the pharmacokinetics of the choline in biological fluids after administration of clinically useful oral doses. The modified LC/MS/MS method presented in this paper was developed for the purpose of providing a rapid, simple and sensitive method for the determination of choline in human serum and validated for the study of choline alphoscerate bioequivalence.

On the other hand, per regulations set by the Korean Food and Drug Administration (KFDA), ANDA filing requires that generic formulation must be bioequivalent with branded formulation before it's approval. Thus, the aim of this study was to evaluate the bioequivalence of two choline alphoscerate soft capsules, which was conducted in accord with KFDA guidelines.[8]

Materials and Methods

Materials and Reagents

Each of the study formulations contained 400 mg of choline alphoscerate. The test formulation (lot no. St2006013; manufactured April 2006) and reference formulation (lot no. 496100; expiration date December 2007) manufactured in

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accordance with the Korean Good Clinical Practice guidelines[9] were supplied as soft capsules by Sam Chun Dang Pharm. Co. Ltd. (Seoul Korea).

Choline was supplied from Sam Chun Dang Pharm. Co. Ltd.. Cimetidine was supplied from Sigma Chemical Co. (St Louis, MO, USA). Methanol (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and the other chemical were of HPLC grade or higher. A Milli Q (Millipore Co., Milford, MA, USA) water purification system was used to obtain the purified water for the LC/MS/MS. The test medication, Cholicerin (400 mg choline alphoscerate soft capsule, Sam Chun Dang Pharm. Co. Ltd., Korea) and the reference medication, Gliatilin (400 mg choline alphoscerate soft capsule, Daewoong Pharmaceuticals Co., Ltd., Korea) were supplied in the form of soft capsule.

In vitro Disintegration Testing

The *in vitro* disintegration testing, as second equivalence criterion of two choline alphoscerate preparations, was carried out using the disintegration apparatus of KP VIII in water at 5, 10, 15 and 20 min. Disintegration testing should be conducted on 12 individual dosage unit of the test and reference preparations used in the bioequivalence (BE) test. Disintegration is achieved when any residue does not remain in the glass tube. Complete disintegration is also defined as that state in which any residue of the unit, except fragments from insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disks.

Selection of Volunteers

This study in healthy Korean male volunteers was conducted at Chonnam National University Hospital, Gwangju, Korea. The study population consist of thirty six healthy male Korean volunteers with an average age of 24.06 years and an average weight of 68.46 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 existing degree of variation would not be due to an influence of illness or other medications.

Blood Sampling from Volunteers

The study protocol was approved by the Institutional Review Board of the Institute of Bioequivalence and Bridging Study, Chonnam National University. The study was performed in accordance with the revised Declaration of Helsinki[10] and the Good Clinical Practice guidelines.[9] And 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.[8]

All of the volunteers avoided taking other drug for at least one week prior to the study and until its completion. They also refrained from consuming xanthine-containing foods, alcoholic beverage and choline-containing food such as liver of animals, eggs, soybeans, peanut, cocoa, chocolate, oatmeal and cheese for 12 hr prior to each dosing and until the collection of the last blood sample. The study had a single-dose, randomized, 2-treatment, 2-period crossover design. Subjects were hospitalized at 8:00 PM on the day before the study and fasted for 12 hr before and 4 hr after drug administration. At 8:00 AM, a cannula (JELCO^{TM}, 22G, Johnson & Johnson Medical, Pomezia, Italia) was inserted into the median cubital vein and the cannula was flushed with 0.3 mL heparinized normal saline solution for injection (150 units/mL) to prevent clotting. Each subject was randomly assigned to receive a single dose of the reference of test formulation (1200 mg of choline alphoscerate) with 240 mL of spring water at 8:30 AM. Subjects received standardized meals at 4 hr after drug administration. After a washout of 7 days, subjects received the alternative formulation. No drugs, alcohol, xanthine or choline-containing foods or beverage were allowed during the study.

After ~2 mL of blood was discarded, a 8 mL aliquot of blood was drawn from the indwelling cannula into a 8.5 mL Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) before administration (to serve as a control) and at 0.25, 0.5, 0.75, 1, 1.33, 1.67, 2, 3, 4, 6 and 8 hr after administration. After sampling, the cannula was flushed with 0.3 mL of heparinized normal saline solution for injection. On each occasion, the blood sample was centrifuged immediately, and serum sample was frozen at -70°C until the LC/MS/MS analysis.

Subjects were continuously monitored by hospital staff throughout the study period. Vital signs (temperature, blood pressure, and heart rate) were measured before and after each administration of study drug. Adverse events (particularly nausea, vomiting, diarrhea, gastric pain, headache, and fatigue) were collected based on observation and direct questioning, and were recorded for up to 1 week after each treatment period.

LC/MS/MS Analysis of Choline in Serum Samples

An LC/MS/MS method modified from the reported LC/MS/MS methods was developed and validated for choline assay in serum sample.[5-7] A Varian LC/MS/MS system (palo Alto, CA, USA) consisted of a ProStar Dynamix autosampler, a ProStar-410 binary pump, and a 1200L triple quadrupole mass spectrometer equipped with an electrospray ionization source. Varian MS workstation (version 6.5 software) was used for data acquisition and processing.

The chromatographic separation was archived on a Atlantis C18 (2.1×50 mm, 3.5 μm). The mobile phase was prepared by mixing methanol and aqueous ammonium acetate (7.5 M) in the ratio of 48:62 (v/v) at the flow rate of 0.15 mL/min.

The electrospray ionization (ESI) mass spectrometer was operated in the positive ion mode. The electrospray capillary voltage was set to 4500 V and nitrogen was used as a drying gas for solvent evaporation. Multiple reaction monitoring (MRM) of the precursor-product ion transitions from m/z 159.0 to m/z 60.0 for choline and from m/z 253.0 to m/z 104.6 for cimetidine (as internal standard, I.S.) was used for quantitation (Figure 1).

The primary stock solution of choline was prepared at 1000 μg/mL in methanol and stored at 4°C. To remove endogenous choline in serum, choline oxidase solution (150 U/mL) was prepared in buffer solution consisted of 10 mM Tris HCl with 2.0 mM ehtylenediaminetetraacetic acid and 134 mM potassium chloride solution. An aliquot of 0.1 mL of this buffer solution and the same amount of 20 mM ammonium acetate (pH 7.8) were added to serum, and shook for 3 hr at 37°C. Choline stock solution was serially diluted with methanol and added to the prepared choline-free serum to obtain final concentration of 0.5, 1, 3, 5, 6, 10 and 20 μg/mL for the preparation of calibration curve. In order to assess the intra- and inter-day coefficient of variation (C.V.) and accuracy for serum samples, the standard samples were analyzed. The lower limit of quantitation (LLOQ) was defined as the lowest concentration at 10 times the signal-to-noise ratio that yielded a precision of <20% (C.V.) and an accuracy between 80% and 120% of the theoretical value. The intra-day precision of the assay was assessed by calculating the %C.V. for the analysis of all calibration samples in 5 replicates, and inter-day precision was determined by analysis of all calibration samples on 5 consecutive days. The precision of the assay was evaluated based on the criterion that the relative S.D. for each concentration level should not exceed ±15%, with the exception of the LLOQ, which should not exceed ±20%. The accuracy of the assay was determined by comparing the measured concentration using the calibration curves with the known concentration. As for precision, the criterion accuracy was that the S.D. for the mean value should not exceed the nominal concentration by more than ±15%, except for the LLOQ, for which the limit was ±20%.

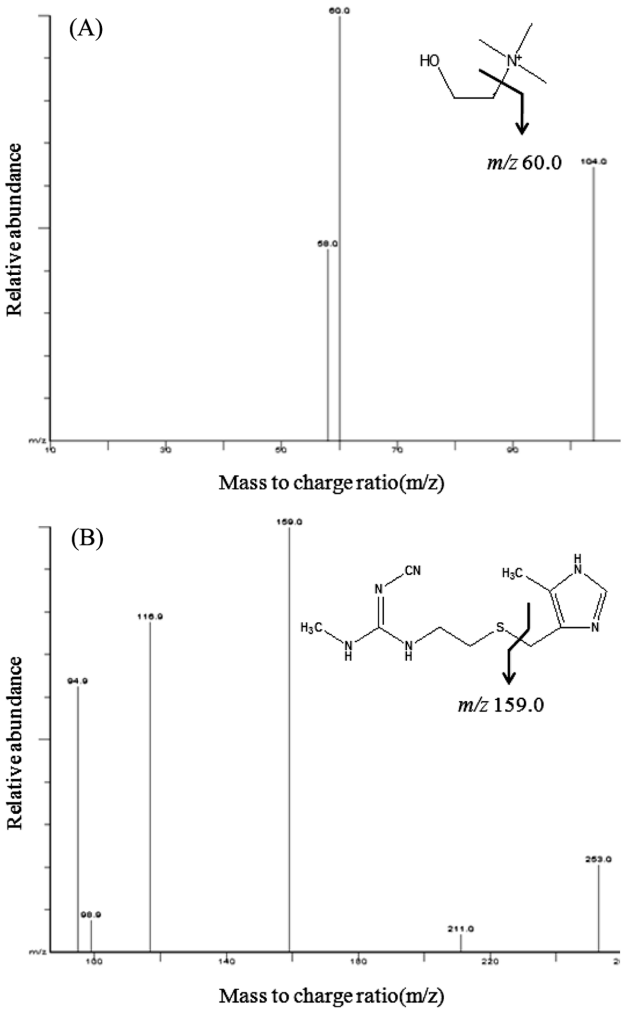

Figure 1—Product ion mass spectrum used in MRM for choline (A) and cimetidine (I.S.) (B) determination.

After thawing at room temperature and vortexing briefly, an aliquot of each sample (0.1 mL) was pipetted into clean test tube and cimetidine solution (50 μL, 100 ng/mL) and 0.8 mL of methanol were added. After vortexing for 10 sec, the mixture was centrifuged at 10,000 g for 5 min. An aliquot of 0.1 mL of upper layer was pipetted into a clean test tube and 20 mM ammonium acetate (pH 7.8) buffer solution were added, then 10 μL of the solution was injected into the LC/MS/MS system, and the peak area and retention time of selected ions were recorded.

Statistical Analysis of Pharmacokinetic Parameters

Each volunteer received an oral dose of 1200 mg of choline

alphoscerate in a standard 2×2 crossover method in a randomized order. Pharmacokinetic parameters such as AUC_t, C_{max} and T_{max} were calculated from total serum concentration-time curves of choline, because choline alphoscerate is metabolized to endogenous choline which has pharmacological effect. C_{max} and T_{max} were recorded as actual measurement values and AUC_t was calculated by trapezoidal formular in 0-8 hr. Their ratios (test/reference) using log-transformed data, together with their means and 90.0% confidence intervals, were analyzed with the analysis of variance (ANOVA) that performed with the Equivtest[11] and K-BE Test program®[12] at a significant level of 0.05. The bioequivalence of two choline alphoscerate soft capsules were estimated by AUC_t and C_{max}. T_{max} used as a reference value.

Results and Discussion

Thirty six healthy Korean male volunteers (mean [S.D.] age, 24.06 [1.80] years; height, 174.60 [4.60] cm; weight, 68.46 [9.93] kg) were enrolled in and completed the study. Both choline alphoscerate formulations were well tolerated; no clinically relevant or drug-related adverse effects were observed.

In vitro Disintegration Test

Accordance of KP VIII, disintegration testing was done to test drug and reference drugs. In reference drugs, disintegration occurred at 10 min to all of 12 capsules. In test drugs, disintegration occurs at 10 min and 15 min to 9 capsules and the other 3 capsules, respectively. So two formulations has no dif-

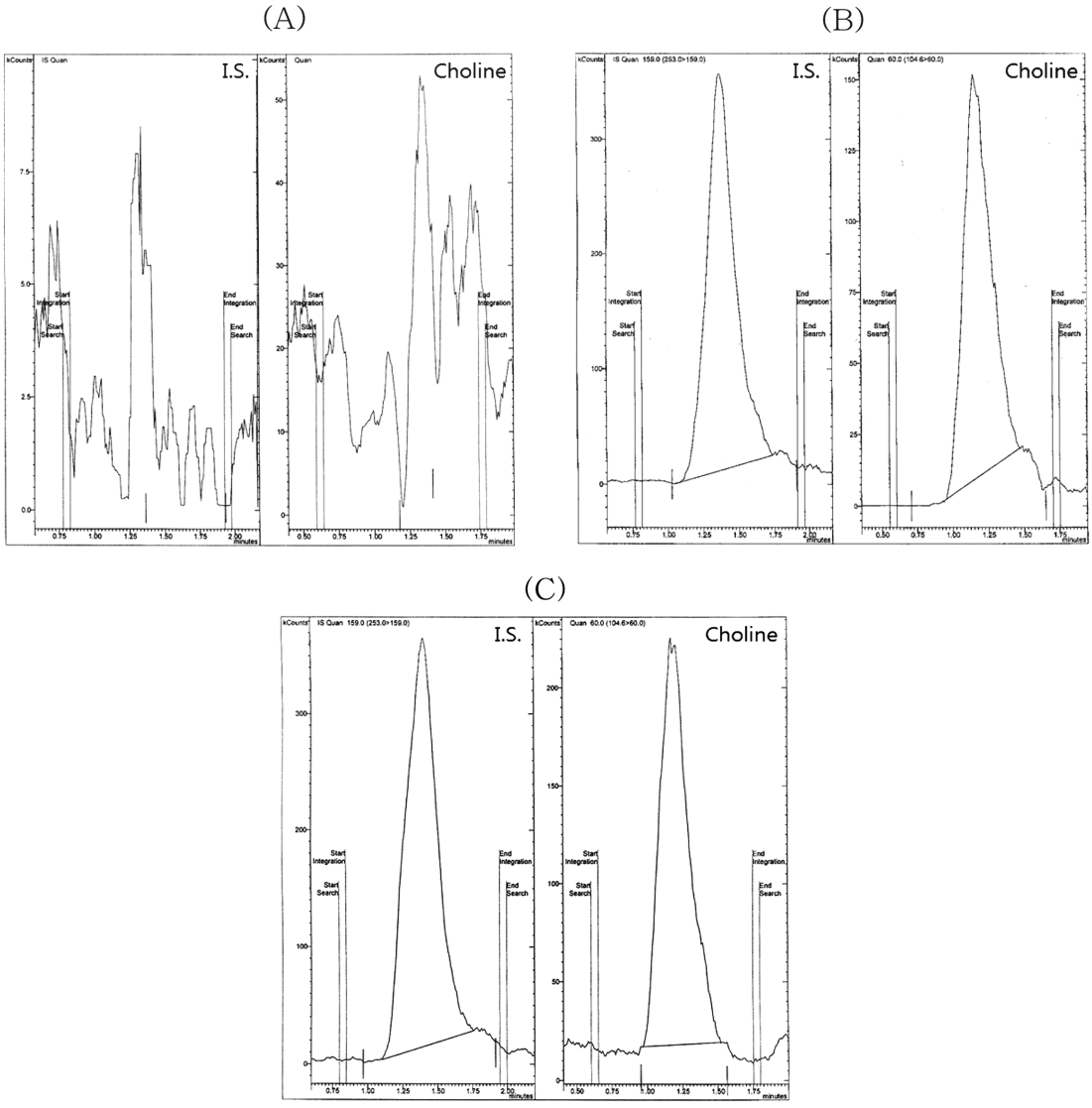

Figure 2—Chromatograms of (A) blank human serum, (B) blank human serum spiked with choline (10 μg/mL) and internal standard (I.S., cimetidine 100 ng/mL) and (C) serum sample (13.67 μg/mL) at 1 hr after oral administration of 1200 mg Cholicerin soft capsule.

ference in disintegration testing because time difference between two formulations was within 5 min and also all the disintegration times were within 15 min.

LC/MS/MS Analysis of Choline in Serum Samples

The method was validated according to the FDA guidance.[13] In this LC/MS/MS method, choline and cimetidine were well separated from the biological background under the described chromatographic condition, respectively (Figure 2). This peaks were of good shape, completely resolved one. No interference with constituents from serum was observed.

The calibration curve was linear in the whole range tested (0.5-20 μg/mL) and described by following equation: Y=0.39113·X+0.009998 (X=choline concentration (μg/mL), Y=ratio of peak areas) with a correlation coefficient of 0.9999. In the range of 0.5-20 μg/mL, the accuracy percentage was ranged from 96.5 to 118.7%. The intra-day precision percentage was ranged from 3.5 to 14.2%, the inter-day precision from 3.9 to 14.6%. These results indicate that the present method has a satisfactory accuracy and precision (Table I).

Pharmacokinetic Analysis

The developed method was successfully used for a bioequivalence test in which serum concentrations of choline in thirty six healthy male volunteers were determined up to 8 hr after the oral administration of 1200 mg choline alphoscerate. Figure 3 shows the mean serum concentration-time curves of choline following oral administration of Cholicerin and Gliatilin soft capsules, and descriptive statistics of the derived pharmacokinetic parameters such as AUC_t, C_{max}, and T_{max} for two preparations are summarized in Table II.

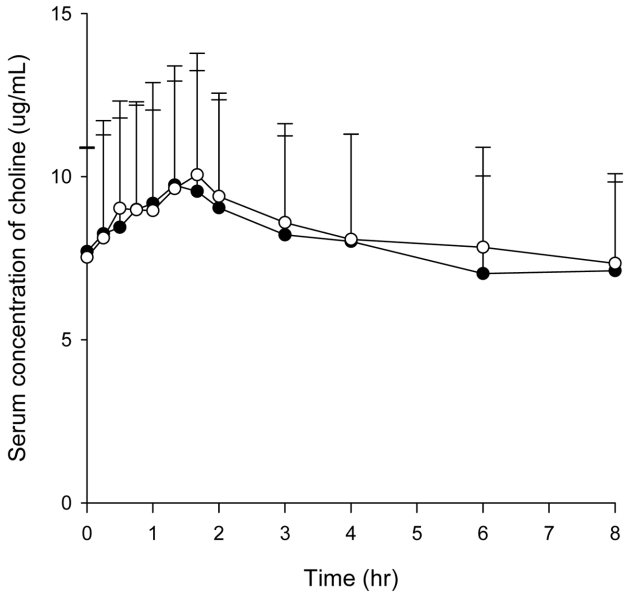

Figure 3–Mean serum concentration-time curves of choline following oral administration of Gliatilin (●) and Cholicerin (○) at the dose of choline alphoscerate 1200 mg (±S.D., n=36).

Table I–*Reproducibility for the LC-MS/MS analysis of choline in human serum*

Concentration (g/mL)	Precision C.V.(%)		Accuracy (%, n=5)
	Intra-day C.V.(%) (n=5)	Inter-day C.V.(%) (n=5)	
0.5	3.5	6.4	118.7
1	11.1	14.6	112.3
3	14.2	12.1	97.9
4	10.9	7.9	107.0
6	5.2	11.8	101.5
10	10.9	9.4	96.5
20	6.3	3.9	98.8

C.V.(Coefficient of Variation)=100×S.D./mean.

AUC_t, C_{max} and T_{max} for choline were 63.92±22.87 μg·hr/mL (Gliatilin), 66.63±23.40 μg·hr/mL (Cholicerin) and 11.15 ±3.88 μg/mL (Gliatilin), 11.34±3.34 μg/mL (Cholicerin) and 1.49±0.84 hr (Gliatilin) and 1.48±0.94 hr (Cholicerin), respectively (Table II). The differences of the means of the test to reference medication for AUC_t and C_{max} were 4.24% and 1.70%, respectively, which are generally accepted if the differences of mean values for AUC_t and C_{max} lie within ±20% (Table III).

Bioequivalence Test of Choline Alphoscerate Products

No significant sequence, subject, formulation or period effects were detected for any pharmacokinetic parameters. The parametric 90% confidence intervals for the AUC_t and C_{max} (Table III) were in the range of log0.9998-log1.1172 and log 0.9938-log(1.0944), respectively, which were satisfied with the accepted bioequivalence criteria of log0.80-log1.25. 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 Gliatilin (reference drug) and Cholicerin (test drug).

Conclusion

This validated modified LC/MS/MS method was sensitive, reproducible and accurate for the determination of choline concentration in human serum samples collected for bioequivalnce studies. Using this method, the bioequivalence of two different choline alphoscerate soft capsule formulations was examined

Table II–*Bioavailability parameters in normal and logarithmic scales for each volunteer obtained after oral administration of gliatilin and cholicerin soft capsule at the choline alphoscerate dose of 1200 mg*

Subjects	Gliatilin Soft Capsule					Cholicerin Soft Capsule				
	AUC_t (μg·hr/mL)	Ln AUC_t	C_{max} (μg/mL)	Ln C_{max}	T_{max} (hr)	AUC_t (μg·hr/mL)	Ln AUC_t	C_{max} (μg/mL)	Ln C_{max}	T_{max} (hr)
A1	36.39	3.59	6.74	1.91	1.67	49.95	3.91	7.13	1.96	2.00
A2	30.54	3.42	5.75	1.75	1.33	44.33	3.79	7.11	1.96	0.75
A3	38.24	3.64	6.82	1.92	1.33	50.97	3.93	9.93	2.30	1.33
A4	72.96	4.29	11.43	2.44	4.00	94.90	4.55	15.98	2.77	1.67
A5	59.12	4.08	12.68	2.54	1.67	96.13	4.57	13.62	2.61	2.00
A6	26.81	3.29	6.26	1.83	2.00	58.41	4.07	9.40	2.24	6.00
A7	74.84	4.32	15.82	2.76	1.33	118.01	4.77	16.86	2.82	0.75
A8	81.28	4.40	14.46	2.67	1.33	112.52	4.72	17.15	2.84	1.67
A9	44.98	3.81	6.87	1.93	1.33	54.94	4.01	9.54	2.26	2.00
A10	88.19	4.48	15.47	2.74	1.00	122.70	4.81	18.37	2.91	1.67
A11	49.21	3.90	9.41	2.24	0.25	69.42	4.24	13.03	2.57	0.25
A12	49.02	3.89	7.76	2.05	0.25	60.11	4.10	9.33	2.23	1.67
A13	62.52	4.14	13.62	2.61	0.50	80.16	4.38	12.90	2.56	1.00
A14	77.13	4.35	11.58	2.45	4.00	98.43	4.59	16.90	2.83	1.67
A15	34.81	3.55	5.67	1.74	2.00	44.28	3.79	8.74	2.17	1.67
A16	50.69	3.93	9.28	2.23	1.67	68.30	4.22	12.37	2.52	1.67
A17	24.98	3.22	4.84	1.58	1.67	50.91	3.93	9.58	2.26	2.00
A18	108.19	4.68	17.65	2.87	1.00	76.91	4.34	15.69	2.75	1.67
B1	78.57	4.36	15.48	2.74	1.33	64.36	4.16	11.63	2.45	1.33
B2	83.86	4.43	16.59	2.81	0.25	63.36	4.15	13.15	2.58	0.50
B3	73.35	4.30	11.59	2.45	2.00	44.22	3.79	9.69	2.27	1.67
B4	78.12	4.36	12.16	2.50	3.00	57.50	4.05	9.43	2.24	0.25
B5	99.28	4.60	14.53	2.68	1.00	68.70	4.23	11.17	2.41	1.67
B6	68.41	4.23	9.75	2.28	0.75	51.84	3.95	9.11	2.21	0.75
B7	73.89	4.30	13.09	2.57	1.33	66.71	4.20	10.81	2.38	1.67
B8	66.00	4.19	13.33	2.59	1.67	51.45	3.94	10.50	2.35	1.00
B9	76.33	4.34	13.67	2.62	1.00	74.75	4.31	12.87	2.55	1.00
B10	30.02	3.40	4.70	1.55	1.67	28.96	3.37	6.02	1.80	1.67
B11	38.75	3.66	6.08	1.81	1.67	33.36	3.51	5.14	1.64	2.00
B12	85.97	4.45	15.25	2.72	1.33	65.41	4.18	10.84	2.38	1.33
B13	106.56	4.67	16.53	2.81	1.67	80.01	4.38	12.37	2.52	2.00
B14	50.11	3.91	9.19	2.22	0.75	40.33	3.70	8.17	2.10	0.50
B15	75.13	4.32	13.65	2.61	2.00	93.92	4.54	14.97	2.71	1.00
B16	81.83	4.40	13.08	2.57	0.75	61.92	4.13	11.25	2.42	1.67
B17	80.56	4.39	13.43	2.60	1.67	65.21	4.18	11.42	2.44	1.33
B18	44.45	3.79	7.06	1.95	1.33	35.15	3.56	6.16	1.82	0.50
Mean	63.92	4.09	11.15	2.34	1.49	66.63	4.14	11.34	2.38	1.48
(S.D.)	22.87	0.40	3.88	0.39	0.84	23.40	0.35	3.34	0.31	0.94

Table III–*Statistical results of bioequivalence evaluation between two choline alphoscerate*[#]

	Parameters		
	AUC_t	C_{max}	T_{max}
Difference	4.24%	1.70%	-0.67%
F_G[a)]	0.032	0.017	2.423
Test/Reference point estimate	1.057	1.043	-0.006
Confidence interval(δ)[b)]	$\log 0.9998 \le \delta \le \log 1.1172$	$\log 0.9938 \le \delta \le \log 1.0944$	$-21.54\% \le \delta \le 20.72\%$

[#]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, 34)=4.13, [b)]α=0.05.

at dose of 1200 mg in 36 healthy normal male volunteers. No significant differences in AUC_t or C_{max} were found between the test and reference formulations and the calculated 90% confidence intervals for the ratios of mean AUC_t and C_{max} were within the regulatory acceptance range for bioequivalence (80%-125%).

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