Journal of Pharmaceutical Investigation
Vol. 41, No. 5, 309-315 (2011)

Bioequivalence Assessment of Acephyll® Capsule to Surfolase® Capsule (Acebrophylline HCl 100 mg) by Liquid Chromatography Tandem Mass Spectrometry

Kyung-Don Nam[1], Ji-Hyung Seo[1,2], Sung-Vin Yim[2] and Kyung-Tae Lee[1,2†]

[1]*College of Pharmacy and*
[2]*College of Medicine, Kyung Hee University, Hoegi-Dong, Dongdaemun-Gu, Seoul, 130-701, Korea*

(Received July 8, 2011 · Revised October 8, 2011 · Accepted October 13, 2011)

ABSTRACT – A sensitive and specific liquid chromatographic method coupled with tandem mass spectrometry (LC-MS/MS) was developed for the analysis of ambroxol (active moiety of acebrophylline). After acetonitrile precipitation of proteins from plasma samples, ambroxol and the domperidone (internal standard, IS) were eluted on a C18 column. The isocratic mobile phase was consisted of 10 mM ammonium acetate and methanol (10 : 90, v/v), with flow rate at 0.2 mL/min. A tandem mass spectrometer, as detector, was used for quantitative analysis in positive mode by a multiple reaction monitoring mode to monitor the *m/z* 379.2→264.0 and the *m/z* 426.2→175.1 transitions for ambroxol and the IS, respectively. Twenty four healthy Korean male subjects received two capsules (100 mg × 2) of either the test or the reference formulation of acebrophylline HCl in a 2 × 2 crossover study, this was followed by a 1week washout period between either formulation. $AUC_{0\text{-}t}$ (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. The 90% confidence intervals for the log transformed data were acceptable range of log 0.8 to log 1.25 (e.g., log 0.8964 - log 0.9910 for $AUC_{0\text{-}t}$ log 0.8690 - log 1.0750 for C_{max}). The major parameters, $AUC_{0\text{-}t}$ and C_{max} met the criteria of Korea Food and Drug Administration for bioequivalence indicating that Acephyll® capsule (test) is bioequivalent to Surfolase® capsule (reference).

Key words – Liquid chromatography/tandem mass spectrometry; Ambroxol; Bioequivalence; Acephyll® capsule, Surfolase® capsule

Acebrophylline, 1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7H-Purine-7-acetic acid with trans-4-[[(2-amino-3,5-dibromophenyl)methyl]amino]cyclohexanol, is an airway mucus regulator with anti-inflammatory action. The drug's approach involves several points of attack in obstructive airway disease. The molecule contains ambroxol, pharmacologically active moiety of acebrophylline, which facilitates various steps in the biosynthesis of pulmonary surfactant, and theophylline-7-acetic acid whose carrier function raises blood levels of ambroxol, thus rapidly and intensely stimulating surfactant production. The resulting reduction in the viscosity and adhesivity of the mucus greatly improves ciliary clearance. On a clinical level, acebrophylline is therapeutically effective in patients with acute or chronic bronchitis, chronic obstructive or asthma-like bronchitis and recurrence of chronic bronchitis (Pozzi, 2007).

Pharmacokinetic properties of ambroxol already have been reported. After administration of two capsules of acebrophylline HCl (100 mg × 2) to 28 healthy Korean subjects, $AUC_{0\text{-}t}$ and C_{max} were 1635.81 ± 405.35 ng·hr/mL and 209.35 ± 50.33 ng/mL, respectively (Cho et al., 2005). And time to reach C_{max} (T_{max}) was 2 hr after oral administraton (Angelo et al., 1992). Terminal elimination half- life ($T_{1/2}$) was approximately 8 hr (Hang et al., 2007). Renal clearance was approximately 53 mL/min; approximately 5 - 6% of a dose was excreted unchanged in the urine (Hyundai Pharm, Co., Ltd., 1998).

Previous studies have reported different methods for the quantitative detection of ambroxol in human plasma using high performance liquid chromatography with electrochemical detector (HPLC-ECD) (Cho et al., 2005; Yoo et al., 2003). However, these published methods are not ideal for pharmacokinetic work, because they are time-consuming, need complex derivative steps, and require large amount of human plasma. In addition, detection of ambroxol using liquid chromatographic method coupled with tandem mass spectrometry (LC-MS/MS) has not yet reported. Therefore, this study established a novel quantitative method for detecting ambroxol in human plasma using LC-MS/MS. This method is not only sensitive and reliable but also fast and simple compared with other recently reported methods This study was conducted to determine the bioequivalence of two formulations of acebrophylline

†Corresponding Author :
Tel : +82-2-961-0860, E-mail : ktlee@khu.ac.kr
DOI : 10.4333/KPS.2011.41.5.309

HCl 100 mg capsules, reference (Hyundai Pharm Sulfolase® capsule) and test (Yooyoung Pharm Acephyll® capsule) formulation, for the purpose of generic substitution according to the guidelines of Korea Food and Drug Administration (KFDA) (KFDA, 2005).

Materials and Method

Chemicals and reagents

The test formulation, Acephyll® capsule (acebrophylline HCl 100mg, Yooyoung Pharm. Co., Ltd., Kyunggi-Do Korea, lot no. 1002; expiration date, April, 2012) manufactured in accordance with the Korean Good Clinical Practice (KGCP) guidelines (of KFDA) (KFDA, 2007) and the reference formulation, Surfolase® capsule (acebrophylline HCl 100mg, Hyundai Pharm. Co., Ltd., Seoul Korea, lot no. 77022, July, 2010) were supplied in the form of capsules. Ambroxol was supplied from Yooyoung Pharm.co., Ltd (Kyunggi-Do, Korea) and domperidone (internal standard, IS) was purchased Sigma-Aldrich (St. Louis, MO, USA), respectively. The chemical structures of ambroxol (Figure 1A) and IS (Figure 1B) are shown. HPLC-grade acetonitrile and methanol were purchased from J.T. Baker (Pillipsburg, NJ, USA). Ammonium acetate was purchased from Sigma-Aldrich and water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). All other chemicals were of the highest analytical grade.

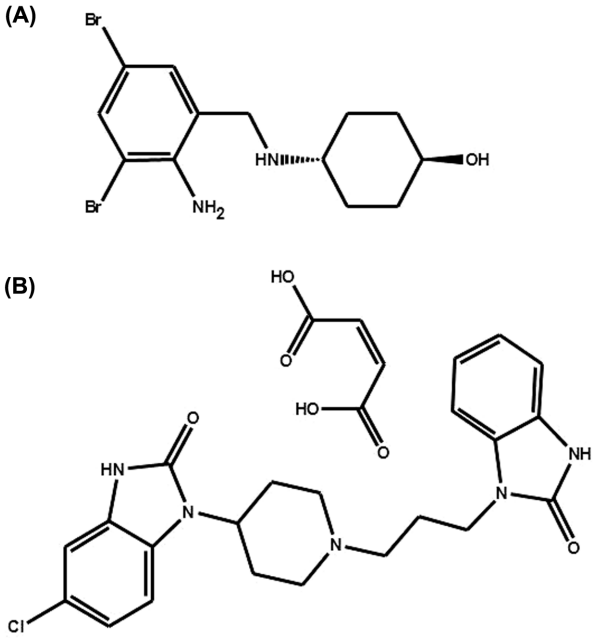

Figure 1. Chemical Structures of (A) ambroxol and (B) domperidone maleate.

Preparation of calibration standard

To prepare standard stock solutions, appropriate amounts of ambroxol and domperidone were weighed accurately and dissolved separately in acetonitrile and methanol at 1,000 μg/mL, respectively. The stock solution of ambroxol was further diluted with acetonitrile to give a series of standard solution with concentrations of 50, 100, 500, 1,000, 2,000, 5,000 and 10,000 ng/mL. A solution of containing 2 μg/mL of IS was prepared with acetonitrile. Calibration curves were prepared by spiking blank plasma with working solutions to final ambroxol concentrations of 5, 10, 50, 100, 200, 500 and 1000 ng/mL. All the solutions were stored at -20ºC when not in use.

Sample preparation

To 300 μL volume of human plasma were added 50 μL of IS (2 μg/mL) and 700 μL of acetonitrile. The mixture was vortex-mixed for 1 min and centrifuged at 14,000 rpm for 10 min at 4ºC. The supernatant was filtered (150 μL) by a syringe filter (0.22 μm) and then injected (10 μL) into the LC-MS/MS system for analysis.

Chromatographic conditions

The Agilent 1200 series HPLC (Agilent technologies, Santa Clara, CA, USA) is used.

Chromatography was performed on Varian (USA) pursuit XRs C18 column (50 × 2.0 mm I.D., 3 μm) which maintained at 30ºC with a mobile phase consisting of methanol -10 mM ammonium acetate buffer (90:10, v/v) at a flow rate of 0.2 mL/min. The solution filtered using 0.22 μm membrane and degassed with ultra-sonication (NXPC-1505, Kodo Technology Research Co., Ltd) prior to use.

Mass spectrometric conditions

Mass spectrometric detection was performed on API 4000 mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Canada) equipped with Turboionspray ionization source operating in electrospray ionization (ESI) positive ion mode. Optimization of the MS conditions was carried out using a solution of ambroxol and IS, delivered at a constant flow-rate of 10 μL/min. The optimized Turboionspray voltage and temperature were set at 5500 V and 350ºC, respectively. Quantitation was performed using the multiple reaction monitoring (MRM) transition *m/z* 379.2→264.0 for ambroxol, *m/z* 426.2→175.1 for the IS, respectively (Figure 2). The optimized collision energy of 25 V was used for ambroxol and 29 V for the IS. The declustering potential (DP) was set at 104 and 106 for ambroxol and IS, respectively. Data acquisition was performed with Analyst 1.5 software (AB Sciex, Toronto, Canada).

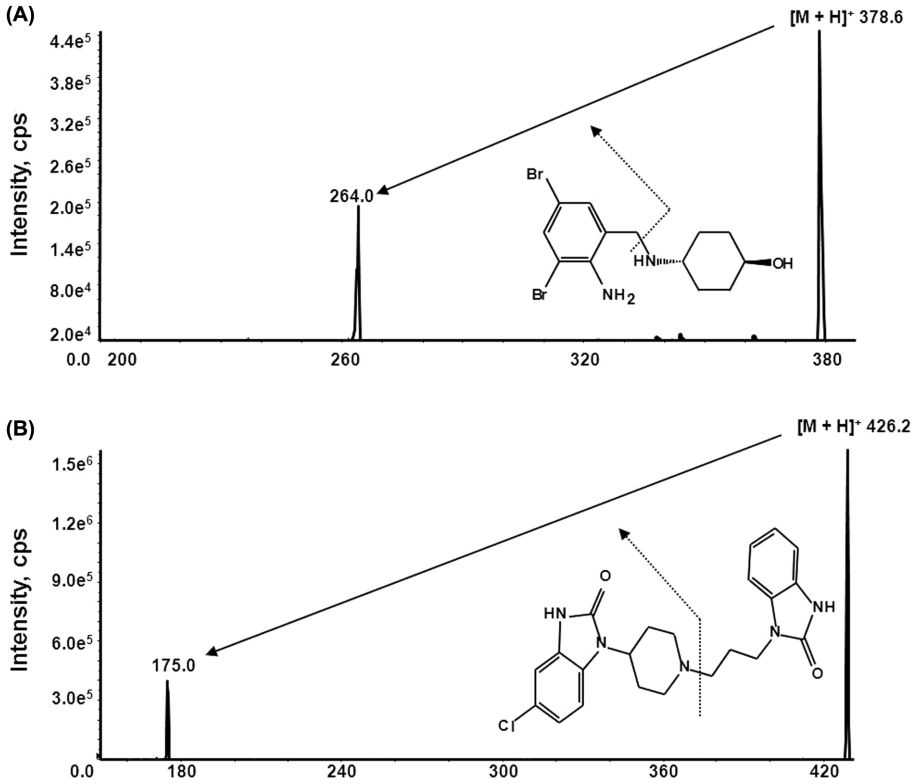

Figure 2. Product ion spectra of (A) ambroxol and (B) domperidone (IS).

Method validation

The method was validated for specificity, accuracy, precision, sensitivity and linearity according to the KFDA (KFDA, 2003) guideline for validation of bioanalytical methods. The specificity of the method was measured by analysis of six blank plasma samples of different origin for interference at the retention times of the analyte and IS. Specificity was assessed by comparing the chromatograms obtained from the sample spiked with a concentration of ambroxol at lower limit of quantification and IS with or without those obtained from blank samples.

In order to assess the intra- and inter-day precision and accuracy were performed on the same day and on four consecutive days. Precision is expressed as coefficient of variance (C.V.), at each level. The accuracy of the assay was defined as a percentage of the measured concentration over the theoretical concentration. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value, except for LLOQ, which was set at 20%. The LLOQ was defined as the concentration of ambroxol at which the signal to noise ratio (S/N) was greater than 10.

The linearity of the calibration curves, ranging from 5 to 1000 ng/mL, was validated with five different calibration curves. The calibration curves were constructed using the weighted regression method ($1/x^2$) of peak area ratios of ambroxol to IS (y) versus actual concentrations (x).

Selection of subjects

The method described in this paper was applied to a bioequivalence study of two oral formulations of acebrophylline HCl (test formulation: Acephyll® capsule; reference formulation: Surfolase® capsule). The study population consisted of twenty-four healthy male subjects with an average age of 23.81 ± 1.92 years and an average weight of 68.6 ± 5.9 kg. Subjects were enrolled in this study after performing a medical history assessment, a physical examination and standard laboratory (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) testing. Subjects were excluded if they had possible sensitivity to acebrophylline, had a history of hepatic, renal, respiratory, endocrine, or cardiovascular illness; or had ingested alcohol or medications, including over the counter drugs, within 4 weeks before the study. This was done to ensure that existing degree of variation would not be due to an influence of illness or other medications. Written informed

consent was obtained from all subjects after the nature and purpose of the study had been explained, in accordance with the KFDA (KFDA, 2005) guidelines for bioequivalence test.

Brood sampling from subjects

The study was performed in accordance with the revised Declaration of Helsinki and the Good Clinical Practice guidelines (KFDA) (KFDA, 2007). All of the subjects 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 (KFDA, 2005). After an overnight fast (12 hr), each subject was given an oral dose (two acebrophylline HCl capsules,100 mg × 2) with 240 mL of water. The subjects were hospitalized (Kyung Hee Medical Center, Seoul, Korea) at 5:00 P.M. on the eve of the study and fasted overnight until 4 hr after each drug administration.

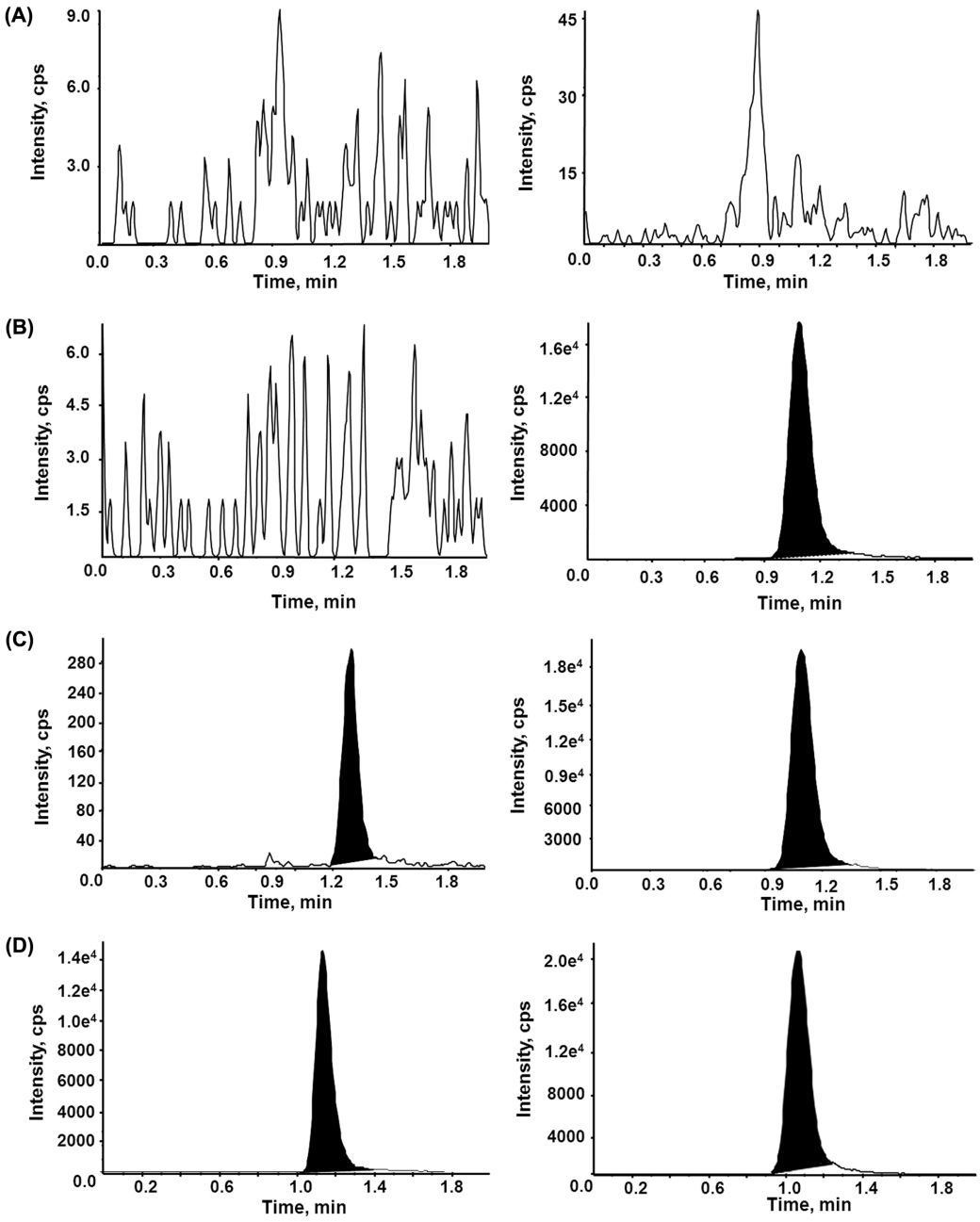

Figure 3. Multiple reaction monitoring chromatograms of (A) blank human plasma, (B) zero blank human plasma, (C) plasma spiked with ambroxol (5 ng/mL, LLOQ) and IS, and (D) plasma from a subject 1 hr after the oral administration of two Acephyll® capsules (acebrophylline HCl 200 mg).

About 7.0 mL of blood samples were collected from each subject using a cannula inserted into the median cubital vein into heparinized tubes before (0 hr) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12 and 24 hr after dosing. Plasma separated by centrifugation at 3,000 rpm for 10 min and kept frozen at -70°C until analysis. Subjects were continuously monitored by hospital staff throughout the study period. No drugs, alcohol, smoking, xanthine-containing foods or beverage were allowed during the study.

Pharmacokinetics and statistical analysis

Pharmacokinetics parameters were calculated from plasma levels applying a non-compartmental statistic using BA Calc 2007® (Lee et al., 2000). Blood samples were drawn up to a period of three to five times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration time curve ratio ($AUC_{0-t}/AUC_{0-\infty}$) higher than 80%. The C_{max} and T_{max} values were determined by visual inspection of the plasma ambroxol concentration-time curve (AUC_{0-t}) by the trapezoidal method in 0-24 hr. Their ratios (test/reference) using log-transformed data, together with their means and 90% confidence intervals, were analyzed with analysis of variance (ANOVA) that performed with the K-BE Test program® at a significant level of 0.05. Results are indicated as mean ± standard deviation throughout the paper (Lee et al., 1998).

Results and Discussion

Specificity

Figure 3 shows the typical chromatograms of a double blank (without ambroxol and IS), a zero blank (without ambroxol, with IS), a blank plasma spiked with ambroxol (5 ng/mL) and IS, and a plasma sample collected at 1 hr after oral administration of 200 mg acebrophylline HCl. The peak retention time for ambroxol and IS were 1.30 and 1.09 min, respectively. There was no significant interference from endogenous substances in human plasma at the retention time of the ambroxol or IS.

Linearity

Calibration curves were prepared over the ambroxol concentration range of 5 - 1000 ng/mL by linear regression using a $1/x^2$ weighting factor. Correlation coefficients (r) were greater than 0.9967 (n = 5) for all curves and the within- and between-run C.V.s of the response factors for the concentrations assayed were <15%. The calibration curves showed good linearity within the range 5 - 1000 ng/mL and the signal to noise ratio (S/N ratio) of LLOQ (5 ng/mL) was greater than 10.

Precision and accuracy

Intra- and inter-day precision and accuracy of the method used for ambroxol analysis are presented in Table I. Intra-day precisions were between 3.56 and 8.35%, and inter-day precisions between 3.61 and 13.74%. Intra- and inter-day accuracies ranged from 87.08 to 97.31% and from 94.08 to 103.82%, respectively. All results were within the ranges of precision (%) and accuracy (%) specified by the KFDA (KFDA, 2005) for bioanalytical applications.

Pharmacokinetic analysis

Figure 4 shows time-concentration of ambroxol in plasma profile after oral administration of the different acebrophylline HCl formulations. Plasma profiles of the ambroxol concentration versus time after the oral administration of a single dose of both formulations exhibited closely similar patterns. All calculated pharmacokinetic parameter values were shown in Table II. In our study, AUC_{0-t} and C_{max} for ambroxol were 1565.92 ± 426.48 ng·hr/mL (reference drug) and 1491.50 ± 469.50 ng·hr/mL (test drug), 215.40 ± 69.25 ng/mL (reference drug) and 214.08 ± 92.98 ng/mL (test drug), respectively. In addition, T_{max} for ambroxol was 1.77 ± 0.82 hr (reference drug) and 1.92 ± 0.89 hr (test drug), respectively.

Table I. *Precision and accuracy for the determination of ambroxol in human plasma (n=5)*

Concentration (ng/mL)	Precision (C.V. %)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
5	5.35	13.74	87.08	103.82
50	8.35	5.63	97.31	97.93
200	4.05	3.61	92.99	96.29
1000	3.56	4.09	95.91	94.08

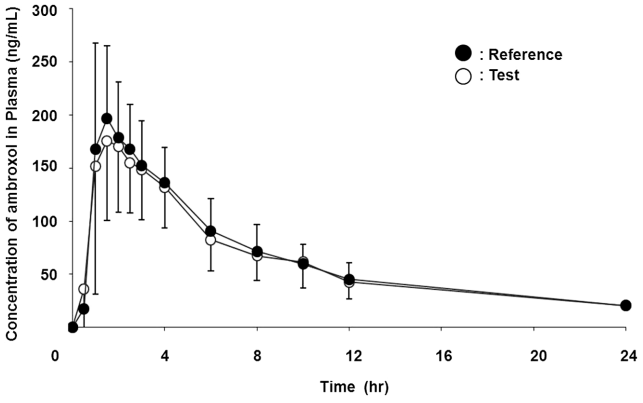

Figure 4. Mean (±S.D., n=24) plasma concentration-time curves of ambroxol following oral administration of Surfolase® capsule (●) and Acephyll® capsule (○) at the dose of 200 mg of acebrophylline HCl.

Table II. *Bioavailability parameters in normal and logarithmic scales for each subject obtained after oral administration of Surforase® capsule and Acephyll® capsule at the acebrophylline HCl dose of 200 mg.*

Subjects	Sulforase® capsule (reference)					Acephyll® capsule (test)				
	AUC_{0-t} (nghr/mL)	Log AUC_{0-t}	Cmax (ng/mL)	Log Cmax	Tmax (hr)	AUC_{0-t} (nghr/mL)	Log AUC_{0-t}	Cmax (ng/mL)	Log Cmax	Tmax (hr)
A1	2024.85	3.31	328.3	2.52	1.5	1751.78	3.24	315.3	2.50	1.0
A2	2708.80	3.43	399.6	2.60	1.0	2155.35	3.33	257.8	2.41	3.0
A3	1104.42	3.04	126.5	2.10	3.0	832.69	2.92	95.11	1.98	4.0
A4	2034.03	3.31	199.9	2.30	3.0	1480.09	3.17	173.8	2.24	1.0
A5	1360.18	3.13	184.6	2.27	1.5	1252.72	3.10	182.9	2.26	1.0
A6	1490.65	3.17	190.2	2.28	1.0	1646.12	3.22	193.2	2.26	4.0
A7	915.85	2.96	152.9	2.18	1.5	841.74	2.93	155.4	2.19	2.0
A8	1369.65	3.14	161.4	2.21	1.0	1140.35	3.06	187.2	2.27	1.5
A9	1111.67	3.05	173.6	2.24	2.0	1093.26	3.04	190.2	2.28	1.0
A10	1276.76	3.11	170.9	2.23	1.5	943.53	2.97	171.1	2.23	1.5
A11	2013.03	3.30	277.8	2.44	1.5	1699.20	3.23	203	2.31	2.0
A12	1168.90	3.07	215.2	2.33	1.5	1242.36	3.09	249.8	2.40	1.5
B1	1463.62	3.17	184.9	2.27	1.5	1485.32	3.17	227.5	2.36	2.0
B2	1598.68	3.20	264.8	2.42	1.0	1709.49	3.23	270.3	2.43	1.0
B3	1711.43	3.23	146.9	2.17	4.0	1680.82	3.23	173.6	2.24	2.0
B4	1188.41	3.07	140.7	2.15	2.5	1182.27	3.07	126	2.10	3.0
B5	1839.49	3.26	317.6	2.50	1.0	1483.48	3.17	168.1	2.23	2.5
B6	2154.10	3.33	192.1	2.28	2.5	2233.78	3.35	253.1	2.40	1.5
B7	1376.93	3.14	200.1	2.30	1.0	1585.68	3.20	190.7	2.28	2.0
B8	1682.29	3.23	213.4	2.33	2.0	1531.39	3.19	263.4	2.42	1.0
B9	1502.17	3.18	227.1	2.36	1.5	1375.14	3.14	142.9	2.16.	2.0
B10	1992.25	3.30	328.3	2.52	1.0	2885.76	3.46	558.4	2.75	1.0
B11	1099.73	3.04	186.7	2.27	1.5	1007.16	3.00	105.7	2.02	2.5
B12	1394.28	3.14	186	2.27	3.0	1556.63	3.19	293.3	2.47	2.0
Mean	1565.92	3.18	215.40	2.31	1.77	1491.50	3.15	214.08	2.30	1.92
(S.D.)	426.48	0.11	69.25	0.13	0.82	469.50	0.13	92.98	0.16	0.89

Table III. *Statistical results of bioequivalence evaluation between two acebrophylline HCl capsules*

	Parameters	
	AUC_{0-t}	C_{max}
Difference (%)	-4.75	-0.61
Test/Ref estimate	0.942	0.966
Confidence internal	Log $0.8964 \leq \delta \leq$ Log 0.9910	Log $0.8690 \leq \delta \leq$ Log 1.0750

Bioequivalence analysis

No significant sequence, subject, formulation or period effects were detected for any pharmacokinetic parameters. The point estimates for the mean ratio of the test to reference formulation for the AUC_{0-t} and C_{max} were 0.942, 0.966, respectively (Table III). The 90% confidence intervals for the ratios of AUC_{0-t} and C_{max} were log 0.8964 - log 0.9910 and log 0.8690 - log 1.0750, respectively (Table III), which were entirely within the regulatory acceptance limits for bioequivalence (80 - 125%). This proved that there was no significant

difference between the bioavailability of reference and test formulations.

Conclusion

It was shown that this developed and validated analytical method using LC-MS/MS is suitable for the analysis of ambroxol in human plasma samples for bioequivalence studies. Using this method, the bioequivalence of two different acebrophylline HCl capsule (100 mg) formulations was examined in twenty-four healthy male subjects. The statistical analysis results based on comparisons of the two pivotal parameters (AUC_{0-t} and C_{max}) demonstrated the bioequivalence of these two capsule formulations of acebrophylline HCl.

References

Angelo, L.D., 1992. Plasma levels and pharmacokinetic parameters of ambroxol and theophylline-7-acetic acid in adults after single and repeated acebrophylline doses, Italian J. chest Diseases, 85-89.

Cho, H.Y., Park, E.J., Kang, H.H., Kim, S.M., Park, C.H., Oh, I.J., Lim, D.K., Lee M.H., Lee, Y.B., 2005. Bioequivalence of Burophil Capsule to Surfolase Capsule, J. Kor. Pharm. Sci., 35(3), 179-185.

Hang, T.J. et al., 2007. simultaneous determination and pharmacokinetic study of roxithromycin and ambroxol hydrochloride in human plasma by LC-MS/MS, Clinical Chimica Acta. 382, 20-24.

Hyundai Pharm, Co., Ltd., 1998. Sulfolase®capsule Drug Information, 7-8.

Korea FDA, 2003 Guideline for the Validation of Bioanalytical Method 2003. 05.

Korea FDA, 2005 Guideline for Bioequivalence Test 2005-31.

Korea FDA, 2007 Guideline for Korean Good Clinical Practice 2007-34.

Lee, Y.J., Choi, J.H., Song, S.H., Seo, C.H., Kim, D.S., Park, I.S., Choi, K.H., Na, H.K., Chung, S.J, Lee, M.H. and Shim, C.K., 1998. Development of K-BEtest, a computer program for the analysis of bioequivalence J. Kor. Pharm. Sci. 28, 223-229.

Lee, Y.J. Kim, Y.G., Lee, M.G., Chung, S.J., Lee, M.H., Shim, C.K., 2000. Analysis of bioequivalence study using log-transformed model Yakhakhoeji. 44, 308-314

Pozzi, E., 2007. Acebrophylline: an airway mucoregulator and anti-infalmmatory agent, Monaldi. Arch. Chest. Dis., 67(2), 106-15.

Yoo, J.Y., Joung, S.K., Choe, M.H., Han, S.B., Lee, K.R., Lee, H.J., 2003. Bioequivalence of ambrect™ Tablet J. Kor. Pharm. Sci., 35(3), 215-221.