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Bioequivalence Assessment of Roxithromycin Tablets in Healthy Korean Volunteers

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Abstract – The objective of the study was to evaluate the bioequivalency between the Rozid™ Tablet (Ilhwa Pharm. Co., Ltd.) as a test formulation and the Rulid™ Tablet (Handok Pharm. Co., Ltd) as a reference formulation. Twenty-four healthy male volunteers were administered the formulations by the randomized Latin square crossover design, and the plasma samples were determined by a high performance liquid chromatography (HPLC) with fluorescence detector. AUC_t, C_{max} and T_{max} were obtained from the time-plasma concentration curves, and log-transformed AUC_t and C_{max} and log-untransformed T_{max} values for two formulations were compared by statistical tests and analysis of variation. AUC_t was determined to be 63.30 ± 25.57 μg.hr/ml for the test formulation and 64.02 ± 29.27 mg.hr/ml for the reference formulation. The mean values of C_{max} for the test and reference formulations were 5.07 ± 2.14 and 5.53 ± 2.60 μg/ml, respectively. The AUC_t and C_{max} ratios of the test Rozid™ Tablet to the reference Rulid™ Tablet were -1.12% and -8.32%, respectively, showing that the mean differences were satisfied the acceptance criteria within 20%. The results from analysis of variance for log-transformed AUC_t and C_{max} indicated that sequence effects between groups were not exerted and 90% confidence limits of the mean differences for AUC_t and C_{max} were located in ranges from log 0.80 and log 1.25, satisfying the acceptance criteria of the KFDA bioequivalence. The Rozid™ Tablet as the test formulation was considered to be bioequivalent to the Rulid™ Tablet used as its reference formulation, based on AUC_t and C_{max} values.

Keywords □ Roxithromycin, Bioequivalence, Pharmacokinetics, HPLC, Fluorescence, Human

INTRODUCTION

Roxithromycin [erythromycin, 9-{-O-(2-methoxyethoxy) methyl} oximycin] is relatively new semi-synthetic macrolide antibiotics derived from erythromycin and consists of a 14-membered macrocyclic lactone ring with sugars linked via glycosidic bonds. Macrolides are highly active antibiotics against a wide range of Gram-positive bacteria, but with limited activity against Gram-negative bacteria. Macrolides exert drug effects by interfering with RNA-dependent bacterial protein synthesis, resulting in bacteriostatic effects on pathogens. Based on administration of a single dose of roxithromycin or erythromycin in healthy volunteers, administration of 150 mg roxithromycin showed 3.3-fold C_{max} and 16-fold AUC higher than those of 250 mg erythromycin (Birkett *et al.*, 1990). The half-life for roxithromycin was 12.4 ± 3.9 hr compared with 1.5 ± 0.4 hr for erythromycin (Birkett *et al.*, 1990), indicating that roxithromycin has more potent effects and better compliance to patients than erythromycin.

Various kinds of methods for determining roxithromycin in human plasma or urine and food residues have been reported using HPLC/UV detector (Ptacek and Klima, 1999), HPLC/electrochemical detector (Gonzalez *et al.*, 2005, 2003; Pappa-Louisi *et al.*, 2001; Taninaka *et al.*, 2000; Kees *et al.*, 1998; Fouda and Schneider, 1995), and HPLC/fluorescence detector (Edder *et al.*, 2002; Sastre *et al.*, 1998), as well as LC/MS (Xiao *et al.*, 2005; Hilton and Thomas, 2003; Lai *et al.*, 2000). Most of the methods require electro-chemical detection because of the insensitivity of the macrolides with UV detection. However, a few HPLC/florescence methods were found.

In the current work a sensitive and simple HPLC/fluorescence method is described for the quantitation of roxithromycin

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in human plasma and the bioequivalence study was performed by using the HPLC/fluorescence in healthy human volunteers after oral administration of roxithromycin.

MATERIALS AND METHODS

Chemicals

Roxithromycin tablets as the test product (Rozid™ tablet, a 150 mg tablet, batch No. RZ-03002) and its authentic compound (purity, >98%) were obtained from Ilwha Pharm. Co. (Seoul, Korea). The reference product (Rulid™ tablet, a 150 mg tablet, batch No. L010) was obtained from Handok Pharm. Co. (Seoul, Korea). Erythromycin used as the internal standard was purchased from Sigma (St. Louis, MO, USA). Potassium phosphate dibasic, potassium dihydrogen phosphate, and 9-fluorenylmethyl chloroformate (FMOC) were obtained from Sigma. Hexane, acetonitrile, and methanol were purchased from J. T. Baker (Phillipsburg, NJ, USA). The other agents used for roxithromycin analysis were of analytical grade.

Blood sampling from volunteers

After approval of pre-planed proposal by Korea Food and Drug Administration (KFDA), male volunteers who submitted the agreement to attend to this project were medically examined and 24 volunteers were selected by a medical doctor in Bestian Medical Center (Seoul, Korea), based on clinical examination including seropathological (hemoglobin, hematocrit, WBC, platelet), serochemical (blood urea nitrogen, creatinine, total protein, albumin, SGOT, SGPT, total bilirubin, cholesterol, glucose fasting, alkaline phosphatase), and urological (specific gravity, color, pH, sugar, albumin, bilirubin, RBC, WBC) data. An exclusion criterion for selecting volunteers includes taking frequently any medicine such as hypertensive agent and vitamines or nutrient aids. They were accommodated at the lodging facility near the Medical Center one day before blood collection. They were fasted for at least 12 hr before administration of tablets. Lunch and dinner were allowed, respectively, 4 and 12 hr after drug intake. Physical and biological examinations were carried out before and after completion of the study.

Oral administration of roxithromycin tablets to human volunteers

A 21-gauge scalp-vein set on arm vein was established on the arm vein of each volunteer and 8 ml of the blood were collected for blank. According to the prescription directed by a doctor, two tablets (a 150 mg tablet, 300 mg roxithromycin) were orally taken by each volunteer of the designated group at random design (12 volunteers a group) with 240 ml of water. One group received test tablets, and the other reference tablets. These two groups were taken the formulation by the randomized Latin square crossover design after a 1-week washing-out period. Blood was collected into heparin-treated tubes (Vacutainer®, Becton Dikinson, Rutherford, NJ, USA) at 0, 0.33, 0.67, 1, 1.5, 2, 4, 8, 12, 24, 48 and 72 hr after the oral administration. The time interval of blood sampling between volunteers was 2 min to consider blood collection time. Blood was centrifuged to obtain plasma. The plasma was stored at -70°C until analyzed.

HPLC analysis of roxithromycin

Roxithromycin analysis was performed by HPLC with a fluorescence detector (Waters 600S controller/Waters 474, Waters, Milford, MA, USA). A Waters 717 autoliquid sampler was used and HPLC ChemStation was supported by Millenium™ 3.2 software. The detector was set at 255 nm for excitation wavelength and 315 nm for emission wavelength. Symmetry C_{18} (3.9 × 150 mm, 5 mm, Waters, Milford, MA, USA) column was used. The mobile phase for roxithromycin analysis consists of acetonitrile and potassium phosphate buffer (50 mM, pH 7.5), with which the running by gradient program was made in 65% acetonitrile at 0 min for 1 min, 82% acetonitrile at 11 min until 24 min, and 65% acetonitrile at 26 min to 30 min. The flow rate of the mobile phase was set to 1 ml/min.

Preparation of the calibration curve of roxithromycin in human plasma

The stock solution of roxithromycin was prepared by dissolving it in methanol as 1000 μg/ml, from which the working solutions of 1, 10 and 100 μg/ml were prepared by serial dilution. To 1 ml of the roxithromycin-free blank plasma, 0, 0.1, 0.5, 1, 5, 10 and 20 mg of roxithromycin were added, respectively. Erythromycin (500 μg/ml, 20 μl) was added to the tube and the tube was vortex-mixed for 10 sec. 100 μl sodium hydroxide (1N) was added to adjust pH value to alkaline condition, and 5 ml of hexane/isoamyl alcohol (98:2, v/v%) was added. The tube was agitated for 20 min on a shaker (100-150 rpm; SM-25, Edmund Buhler, Germany). After centrifugation (Hm-150IV, Hanil Industrial Co., Seoul, Korea) at 2500 rpm for 10 min, the organic layer was transferred to a new tube after freezing the tube in a freezer (-70°C; Ecoline RE112, Lauda, Germany), and evaporated by a nitrogen evaporator (Tur-

voVap, Zymark, Hopkinton, MA, USA) at 40°C. To the residue, 150 ml of FMOC-Cl and 50 μl of potassium phosphate buffer were added and heated at 40°C for 40 min. After cooling at room temperature, the solution was filtered by filtering tubes (Ultrafree-MC centrifugal filter units, 0.22 μm; Millipore, Canton, MA, USA) at 2000 rpm for 5 min. Ten μl of the filtrate was applied to the instrument. Calibration curves were made by plotting the concentrations of roxithromycin added at x-axis and peak area ratios of the roxithromycin to the internal standard at y-axis. Intra- and inter-day precisions and accuracies were obtained from the five repeated experiment, respectively.

Clean-up of human plasma samples

The frozen plasma samples were thawed at room temperature, vortex-mixed, and 1 ml of the sample was added to the centrifuged glass tubes with stopper. Erythromycin (500 μg/ml, 20 μl) was added to human plasma. The rest of the clean-up procedure was the same as described above. The roxithromycin plasma concentrations in human volunteers were determined based on the calibration curve from peak area ratios of roxithromycin to the internal standard.

Pharmacokinetic parameters

Pharmacokinetic parameters were determined from the time-plasma concentrations of roxithromycin by non-compartmental analysis by using WinNonlin software (Scientific Consulting Inc., Cary, NC, USA). The highest concentration (C_{max}) and the time to reach the highest concentration (T_{max}) were read directly from the time-plasma concentration curves of roxithromycin. The area under the curve of time-plasma concentrations of roxithromycin until the last sampling time ($AUC_{0\ to\ last}$) was determined by the equation of $AUC_{0\ to\ inf} = AUC_{0\ to\ last} + C_{last}/\beta$, where β, is the slope of the terminal phase of the time-log plasma concentration curve and C_{last} is the concentration at the last sampling time (Shargel and Yu, 1993).

Statistics

Data are presented as mean ± standard deviation. K-BE test® software (Seoul National University, Seoul, Korea) was used for data handling of bioavailability difference, and 90% confidence limit (δ%) for log-transformed C_{max} and AUC_t [15,17]. In addition, ANOVA test was performed by the general linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC, USA) to determine F-values and probability. The statistical analysis was performed for C_{max} and AUC_t after log-transformation: the 90% confidence intervals and two one-sided t-tests of Schuirmann were estimated and if 90% confidence limits of log-transformed mean value of C_{max} and AUC_t ranged from log 0.80 to log 1.25 at α=0.05, two products are concluded to be bioequivalent (Schuirmann, 1987; KFDA, 2002).

RESULTS AND DISCUSSION

Validation of analytical method of roxithromycin in human plasma

The plasma concentrations of roxithromycin in healthy volunteers were determined and validated by HPLC with a fluorescence detector. The HPLC chromatograms obtained from either its authentic standard or plasma samples were showed in Fig. 1. Retention times of roxithromycin and the internal standard were about 14.2 and 9.3 min, respectively, and no interfering peaks were observed at these times, showing good separation between peaks. Total run time for determining one sample was within 30 min. Precision and accuracy data were presented in Table I. The lower limit of quantitation for roxithromycin in human plasma was decided to be 0.1 μg/ml, at which the within- and between day precision and accuracy were less than 20% (5.59~8.75% precision; -0.25~7.62% accuracy) and the signal to noise ratios for roxithromycin peaks

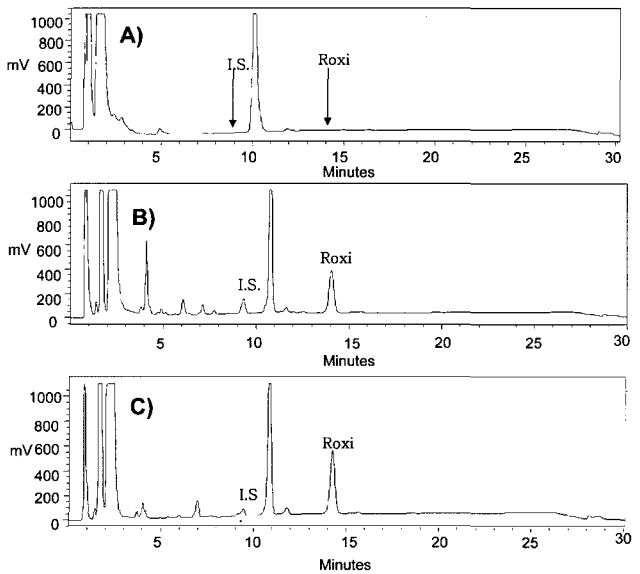

Fig. 1. Chromatograms obtained by HPLC with a fluorescence detector from human plasma blank with the internal standard (A), authentic standard of roxithromycin (B), and the plasma sample obtained at 1.5 hr after oral administration of 300 mg roxithromycin to a volunteer (C). Erythromycin was used as the internal standard (I.S.). Roxithromycin (Roxi) was eluted at 14.2 min and erythromycin at 9.3 min.

Table I. Precision and Accuracy in Within-a-day and Between-days for the Determination of Roxithromycin in the Human Plasma

Concentrations (μg/ml) of roxithromycin added	Precision (CV%)*		Accuracy (Bias%)*	
	Within a day	Between days	Within a day	Between days
0.1	8.75	5.59	-0.25	-7.62
0.5	3.10	8.77	-8.21	-11.53
1.0	0.62	6.46	7.11	8.29
5.0	4.84	6.63	-1.91	-2.22
10.0	3.51	6.00	1.69	1.97
20.0	2.30	3.86	-0.06	-0.36

*Validations for precision and accuracy were made from 5 repeated experiments.

were larger than 10. Within-day and between-day precisions were determined to be less than 8.75 and 8.77 %, respectively, and within-day and between-day accuracies were less than -8.21 and -11.53 %, respectively. The linearity of roxithromycin calibration curve was good (r^2=0.9998) with the equation of y=1.4938x + 0.049 at concentrations ranging from 0.1 to 20 μg/ml. This data suggest that the method was suitable to determine the plasma concentrations of roxithromycin and applicable to the pharmacokinetics and bioequivalence studies.

Pharmacokinetics of roxithromycin in healthy volunteers

From the time-plasma concentrations of roxithromycin in healthy human after oral administration of two different products (Fig. 2), principal pharmacokinetic parameters were determined. The parameters for individual subjects are seen in Table II. The mean values of C_{max} for the test and reference products were 5.07 ± 2.14 and 5.53 ± 2.60 μg/ml, respectively. T_{max} was 2.42 ± 2.01 for the test product and 1.79 ± 1.36 hr for the reference product. AUC_t was determined to be 63.30 ± 25.57 μg.hr/ml for the test product and 64.02 ± 29.27 μg.hr/ml for the reference product. As shown in Table III, the AUC_t ratios of the test Rulid™ Tablet to the reference Rozid™ Tablet were -1.12%, and C_{max} ratios of the test Rulid™ Tablet to the reference Rozid™ Tablet were -8.32%. T_{max} ratio was only used as a reference value. These values for AUC_t and C_{max} indicate that the difference of the mean values satisfy the criteria that it should be located within 20%.

The statistical consideration of parameters for bioequivalence evaluation

The analysis of variance for AUC_t, C_{max} and T_{max} obtained from human volunteers was conducted and the results were presented in Table III. F values determined for log-transformed AUC_t and C_{max} and log-untransformed T_{max} at the level of α=0.05 were compared to the values listed in F table, and they

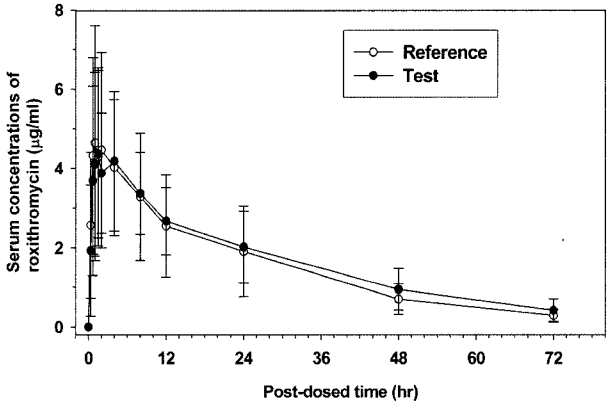

Fig. 2. The time-plasma concentration curves of roxithromycin in human volunteers after oral administration of two different products of roxithromycin to healthy volunteers over 72 hr at a dose of 300 mg. Mean (± SD) values of plasma roxithromycin concentrations of 24 volunteers for test or reference formulations were represented.

were resulted in being less than F (1,22)=4.301, indicating that group or sequence in the Latin-square crossover experiment were appropriately designed. The mean differences of log-transformed AUC_t and C_{max} at 90% confidence limit were determined to be log 0.96~log 1.22 and log 0.83~log 1.08, in which these two parameters satisfied the condition of the bioequivalence criteria that should be ranged from log 0.80 to log 1.25. The mean difference of T_{max} at 90% confidence limit was beyond from the ranges required as of log 0.80 ~log 1.68 (Table III). However, this value should be used as the reference value because roxithromycin is not used with the purpose of inducing a rapid effect or of emergency cases. Our data showed that most of these parameters satisfy the acceptance criteria of bioequivalence for two products.

Taken together, the Rulid™ Tablet as a test formulation was considered to be bioequivalent to the Rozid™ Tablet used as its reference formulation, based on pharmacokinetic data of roxithromycin obtained from the time-plasma concentrations.

Table II. Bioavailability Parameters in Normal and Logarithmic Scales Obtained after Oral Administration of the Rulid Tablet™ or Rozid Tablet™ containing 150 mg Roxithromycin (as Total 300 mg) to Human Healthy Volunteers

Subjects	Rulid Tablet™ (Reference)					Rozid Tablet™ (Test)				
	AUC_t (μg.hr/ml)	Ln AUC_t	C_{max} (μg/ml)	Ln C_{max}	T_{max}	AUC_t (μg.hr/ml)	Ln AUC_t	C_{max} (μg/ml)	Ln C_{max}	T_{max}
A1	101.51	2.01	8.35	0.92	1.00	109.89	2.04	5.18	0.71	4.00
A2	107.92	2.03	7.54	0.88	1.50	130.11	2.11	2.92	0.47	6.00
A3	125.22	2.10	12.89	1.11	1.00	59.71	1.78	4.67	0.67	1.50
A4	39.30	1.59	3.28	0.52	0.67	47.31	1.68	4.25	0.63	1.50
A5	43.39	1.64	5.01	0.70	2.00	64.94	1.81	2.68	0.43	6.00
A6	79.90	1.90	6.18	0.79	1.50	75.57	1.88	3.20	0.51	1.50
A7	36.20	1.56	3.29	0.52	4.00	112.13	2.05	5.08	0.71	6.00
A8	58.23	1.77	3.66	0.56	0.67	50.83	1.71	8.11	0.91	4.00
A9	43.03	1.63	4.45	0.65	1.50	55.31	1.74	4.43	0.65	0.67
A10	39.73	1.60	4.62	0.67	0.33	69.24	1.84	3.25	0.51	1.50
A11	28.03	1.45	1.69	0.23	6.00	47.00	1.67	5.26	0.72	0.67
A12	34.03	1.53	2.73	0.44	0.67	50.01	1.70	7.49	0.88	4.00
B1	90.88	1.96	7.74	0.89	1.50	75.65	1.88	6.47	0.81	0.67
B2	121.69	2.09	9.61	0.98	1.50	115.90	2.06	11.77	1.07	2.00
B3	102.96	2.01	7.40	0.87	1.50	107.68	2.03	6.80	0.83	2.00
B4	53.77	1.73	3.70	0.57	1.50	40.68	1.61	3.08	0.49	4.00
B5	52.55	1.72	8.67	0.94	0.67	44.75	1.65	3.86	0.59	0.67
B6	66.68	1.82	4.78	0.68	4.00	65.95	1.82	5.29	0.72	1.00
B7	56.44	1.75	5.00	0.70	1.50	79.25	1.90	7.66	0.88	1.00
B8	49.07	1.69	3.90	0.59	4.00	36.69	1.60	3.52	0.55	0.67
B9	71.17	1.85	5.23	0.72	1.50	60.48	1.78	5.72	0.76	0.33
B10	51.11	1.71	3.50	0.54	2.00	46.46	1.67	4.07	0.61	0.33
B11	39.52	1.60	3.62	0.56	1.50	35.88	1.56	3.36	0.53	2.00
B12	44.15	1.65	5.76	0.76	1.00	45.60	1.63	3.52	0.55	6.00
Mean	64.02	1.77	5.53	0.70	1.79	63.30	1.80	5.07	0.67	2.42
(SD)	29.27	0.19	2.60	0.20	1.36	25.57	0.17	2.14	0.16	2.01

Table III. Statistics for Pharmacokinetics and Bioequivalence Parameters of Roxithromycin Formulations

	Parameters		
	AUC_t	C_{max}	T_{max}
Difference (%)	-1.12	-8.32	35.79
Test/Reference point estimate	1.08	0.943	1.157
F_G [a)]	0.001	0.775	0.933
90% Confidence limit [b)]	$0.9598 \leq \delta \leq 1.2166$	$0.8270 \leq \delta \leq 1.0755$	$0.7990 \leq \delta \leq 1.6772$

Log-transformed values of AUC_t and C_{max} were used for statistics of bioequivalence evaluation. The statistic results of T_{max} value were obtained without the log-transformation. [a)] $\alpha=0.05$, $F(1,22)=4.301$. [b)] $\alpha=0.05$.

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