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Bioequivalence of Mepiril Tablet to Amaryl Tablet (Glimepiride 2 mg) by Liquid Chromatography/Electrospray Tandem Mass Spectrometry

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ABSTRACT – The purpose of the present study was to evaluate the bioequivalence of two glimepiride tablets, Amaryl tablet (Handok & Aventis Korea, reference drug) and Mepiril tablet (Myungmoon Pharm. Co., Ltd., Korea, test drug), according to the guidelines of Korea Food and Drug Administration (KFDA). After adding an internal standard (glibenclamide) to human plasma, plasma samples were extracted using 1mL of methyl tertiary butyl ether. Compounds extracted were analyzed by reverse-phase HPLC with multiple reaction monitoring (MRM) mode analyte detection. This method for determination glimepiride proved accurate and reproducible, with a limit of quantitation of 2 ng/mL in human plasma. Twenty-four healthy male Korean volunteers received each medicine at the glimepiride dose of 2 mg in a 2 × 2 crossover study. There was a one-week washout period between the doses. Plasma concentrations of glimepiride were monitored by a LC-MS/MS for over a period of 12 hr after the administration. AUC_t (the area under the plasma concentration-time curve from time zero to 12 hr) was calculated by the linear trapezoidal rule method. 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_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 Amaryl/Mepiril were log 0.9583-log 1.1357 and log 1.0570-log 1.2376, respectively. These values were within the acceptable bioequivalence intervals of log 0.80-log 1.25. Taken together, our study demonstrated the bioequivalence of Amaryl and Mepiril with respect to the rate and extent of absorption.

Key words – Glimepiride, Amaryl, Mepiril, LC-MS/MS, Bioequivalence test

Glimepiride,1-[[*p*-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]-phen-yl]sulfonyl]-3-(*trans*-4-methylcyclohexyl) urea, is a "third-generation" sulfonylureas that was first used in everyday clinical practice in 1995. The sulfonylureas are oral antidiabetic agents that can be used in patients with type 2 diabetes because they stimulate the release of insulin from pancreatic beta-cells and have a number of extrapancreatic effects, including increasing the insulin-mediated uptake of glucose in peripheral tissues.[1] Glimepiride is completely absorbed from the GI tract after oral administration.[2] Glimepiride achieved metabolic control with the lowest dose (1-8 mg daily) of all the sulphonylureas. In addition, it maintains a more physiological regulation of insulin secretion than glibenclamide during physical exercise, suggesting that there may be less risk of hypoglycaemia with glimepiride.[3] The pharmacokinetics of glimepiride was dose linear in the dose range 1 to 8 mg, and glimepiride was safe and well tolerated in healthy volunteers.[4]

Several methods have been described in the literature for the determination of glymepiride in biological fluids, including high performance liquid chromatography (HPLC) with UV spectrophotometry[5-6] and LC-MS/MS.[7-8] The HPLC method has low sensitivity and the low efficiency of the sample pretreatment process. The LC-MS/MS method presented in this paper, which was validated in the study of glimepiride bioequivalence to identify pharmaceutical equivalents of the two glimepiride formulations, was developed for the purpose of providing a simple sample preparation procedure and more reproducible results.

Materials and Methods

Materials and reagents

Glimepiride (Figure 1A) and glibenclamide (Figure 1B) were purchased from Sigma-Aldrich Korea. The solvents, i.e., acetonitrile, methyl tertiary butyl ether (MTBE) and formic acid (all first grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q® (Millipore Co., Milford, MA, USA) water purification system was used to obtain the purified water used for the LC-MS/MS analysis. All other chemicals and solvents were of the highest analytical grade

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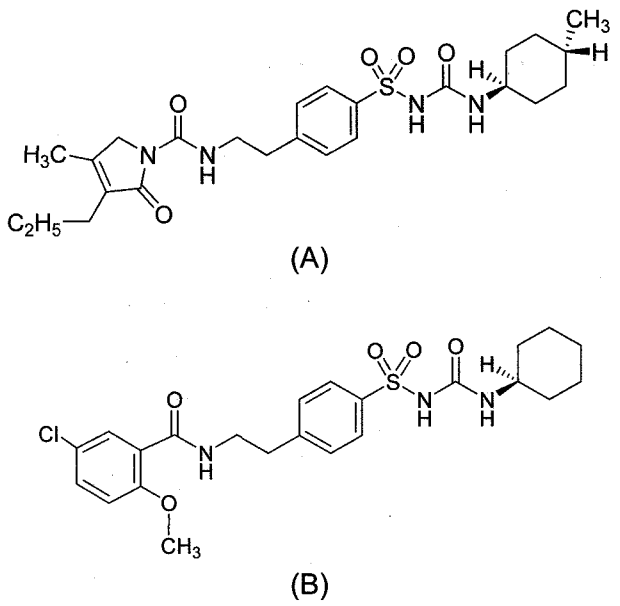

Figure 1–Chemical Structures of (A) glimepiride and (B) glibenclamide.

available. The test medication, Mepiril (2 mg glimepiride tablet, Myungmoon Pharm. Co., Ltd., Korea) and the reference medication, Amaryl (2 mg glimepiride tablet, Handok & Aventis Korea) were supplied in the form of tablets.

Selection of volunteers

The study population consisted of twenty-four healthy male Korean volunteers with an average age of 22.04 years and an average weight of 67.13 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, creatinine, total bilirubin, cholesterol, total protein, albumin, alkaline phosphatase, glucose fasting, sGOT, and sGPT, 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.

Prescribe for volunteers and extract blood samples from volunteers

All of the volunteers avoided using other drugs for at least one week prior to the study and until after its completion. They also refrained from consuming xanthine-containing foods, alcoholic beverages and other beverages for 48 hr prior to each dosing and until the collection of the last blood sample. Each volunteer received an oral dose of 2 mg of glimepiride in a standard 2 × 2 cross-over model in a randomized order. Half-life of glimepiride dose 2 mg was reported 1.3 ± 0.4 hr,[4] and then we had a 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 (Kyung Hee Medical Center, Seoul, Korea) at 10:00 p.m. on the eve of the study and fasted overnight (10 hr) 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. of 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, 3, 4, 6, 8, 10 and 12 hr after the administration. On each occasion, the blood sample was centrifuged immediately, and this sample was frozen at -70°C until the LC-MS/MS analysis.

Analysis of glimepiride in blood sample

The chromatographic system consisted of an Agilent 1100 HPLC system. The separation was achieved on a Capcell Pak MG II C18 (50 × 2.0 mm i.d., 3 μm) reversed-phase column from Shiseido (Tokyo, Japan) at a column temperature of 35°C. The mobile phase prepared by mixing 5 mmol/L ammonium formate solution (pH 5.8 with formic acid) : acetonitrile in the ratio of 46 : 54 (v/v). The flow rate was 280 μL/min for the toal running time of 3 min. The autosampler was controlled at 4°C.

The HPLC system was coupled to an API 2000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada) equipped with a Turbo Ion Spray source. Electrospray ionization (ESI) was performed in the positive mode and the optimum conditions for nebulizing gas (GS1) of nitrogen, turbo spray gas (GS2) and curtain gas (CUR) were set to 40, 50 and 20, respectively. The source temperature for GS2 was set at 320°C. The ion spray (IS) voltage is 5500 V. Unit resolution was set for both Q1 and Q3 mass detection. The collision energy (CE) was set at 37 and 49 V for glimepiride and glibenclamide, respectively, with the collision gas of 7 arbitrary units. For the measurements of analyte, multiple reaction monitoring (MRM) mode was carried out with a

dwell time 150 ms for each transition. The analytical data were processed by Analyst software (version 1.4).

The primary stock solutions of glimepiride were prepared up at 1000 μg/mL in acetonitrile and stored at −70°C. The internal standard stock solution was in acetonitrile producing a concentration of 2 μg/mL. Glimepiride stock solution was serially diluted with acetonitrile and added at drug free plasma to obtain concentration of 2, 5, 10, 20, 100, 200 and 400 ng/mL. These standard solutions were employed for the preparation of calibration graphs. In order to assess the intra-day coefficient of variation (CV) and accuracy for plasma samples, samples of glimepiride and glibenclamide were spiked into human plasma at final concentrations of 2, 5, 10, 20, 100, 200 and 400 ng/mL. Limit of detection (LOD) was determined from signal to noise ratio (S/N)=3 and lower limit of quantitation (LLOQ) 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 μL) was pipetted into an eppendorf tube and glibenclamide (I.S.) solution (20 μL, 2 μg/mL) was added. After vortexing briefly, 1 mL of MTBE 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 Speed-Vac (Holbrook instruments INC., USA). The residue was reconstituted into 50 μL of 50% acetonitrile, vortexed again for 10 min and then centrifuged for 20 min at 15000 rpm. The resulting clear supernatant from each sample was transferred to an autosampler vial and a 5 μL aliquot was injected into the LC-MS/MS system and the peak area and retention time were recorded.

Statistical analysis of pharmacokinetic parameters

Each volunteer received an oral dose of 2 mg of glimepiride in a standard 2 × 2 cross-over model in a randomized order. Pharmacokinetic parameters such as AUC_t, C_{max} and T_{max}, were calculated from blood concentration-time curve. C_{max} and T_{max} were recorded actual measurement value 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 analysis two-way analysis of variance (ANOVA) that per-

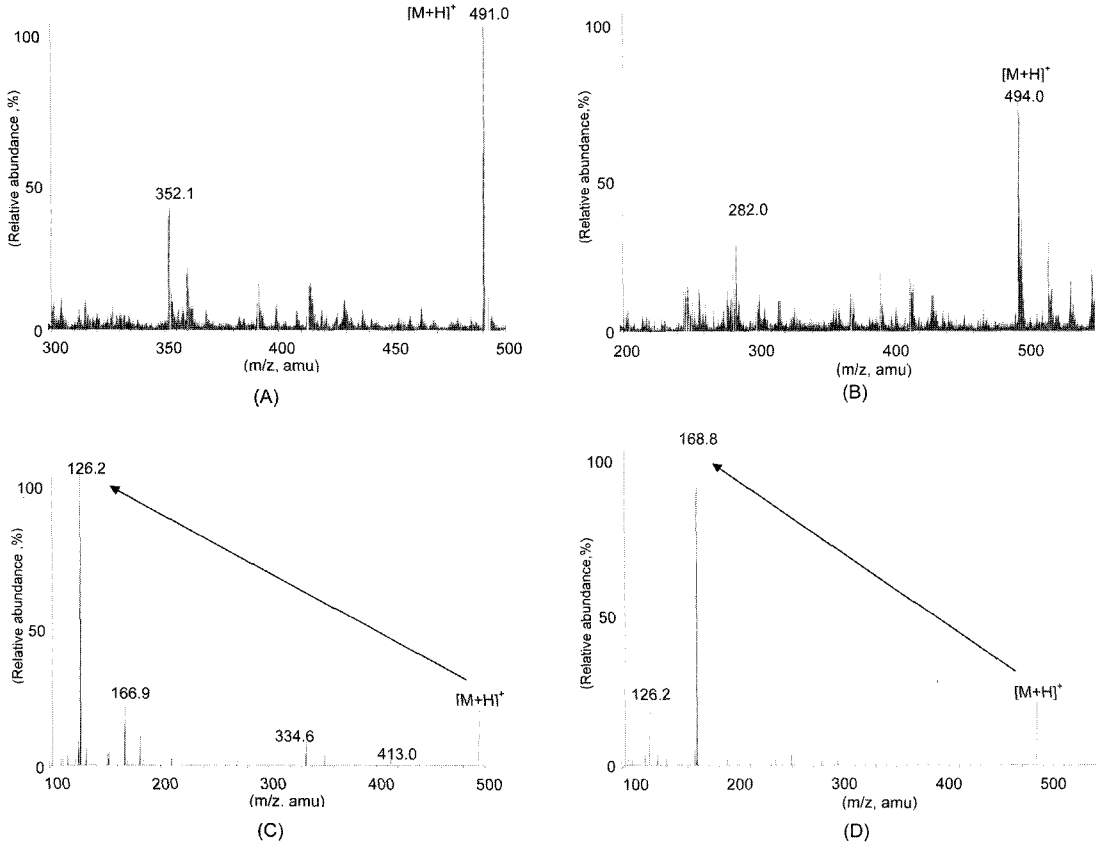

Figure 2–Full-scan mass spectra (A) glimepiride, (B) glibenclamide (I.S.) and product ion spectra of $[M+H]^+$ ions of (C) glimepiride, (D) glibenclamide (I.S.).

formed with the K-BE Test program at a significant level of 0.05.[9] The bioequivalence of two glimepiride tablets estimated by AUC_t and C_{max}. T_{max} was used with reference value.

Results and Discussion

Analysis of glimepiride in blood sample

In order to optimize ESI conditions for glimepiride and glibenclamide (I.S.), first quadrupole full-scans (Q1 scan) of glimepiride and glibenclamide were carried out in positive ion detection mode. The mass spectra of glimepiride and glibenclamide revealed base peaks at *m/z* 491.0 and *m/z* 494.0, respectively, and protonated molecular ions $[M+H]^+$ (Figure 2A and 2B). Major fragment ions of glimepiride and glibenclamide were observed at *m/z* 126.2 (Figure 2C) and *m/z* 168.8 (Figure 2D), respectively. Full-scan mass spectra and product ion mass were collected during direct infusion experiment, and the collision activated dissociation (CAD) of each protonated $[M+H]^+$ was conducted at different collision energies to optimize the output signal. The product ions of *m/z* 126.2 and *m/z* 168.8 provided high sensitivity for quantification in multiple reaction monitoring (MRM) mode. Instrumental parameters are summarized in Table I.

Table I–*LC-MS/MS Instruments Parameters of Glimepiride and Glibenclamide*

Parameters	Glimepiride	Glibenclamide
Curtain gas (CUR) (arbitrary unit)	20	20
Nebulizing gas (GS1) (arbitrary unit)	40	40
Turbo spray gas (GS2) (arbitrary unit)	50	50
Protonated molecule (*m/z*)	491.0	494.0
Product ion (*m/z*)	126.2	168.8
Dwell time (ms)	150	150
Declustering potential (V)	16	11
Focusing potential (V)	370	370
Entrance potential (V)	6.5	8
Collision cell entrance potential (V)	24	24
Collision energy (V)	37	49
Collision cell exit potential (V)	2	2

Gimepiride and glibenclamide (I.S.) were well separated from the biological background under the described chromatographic conditions at retention times of 2.3 and 1.9 min, respectively (Figure 3B). The peaks were of good shape, completely resolved one. No interference with constituents from plasma matrix was observed (Figure 3A). The mobile phase used guaranteed good repeatability of retention times.

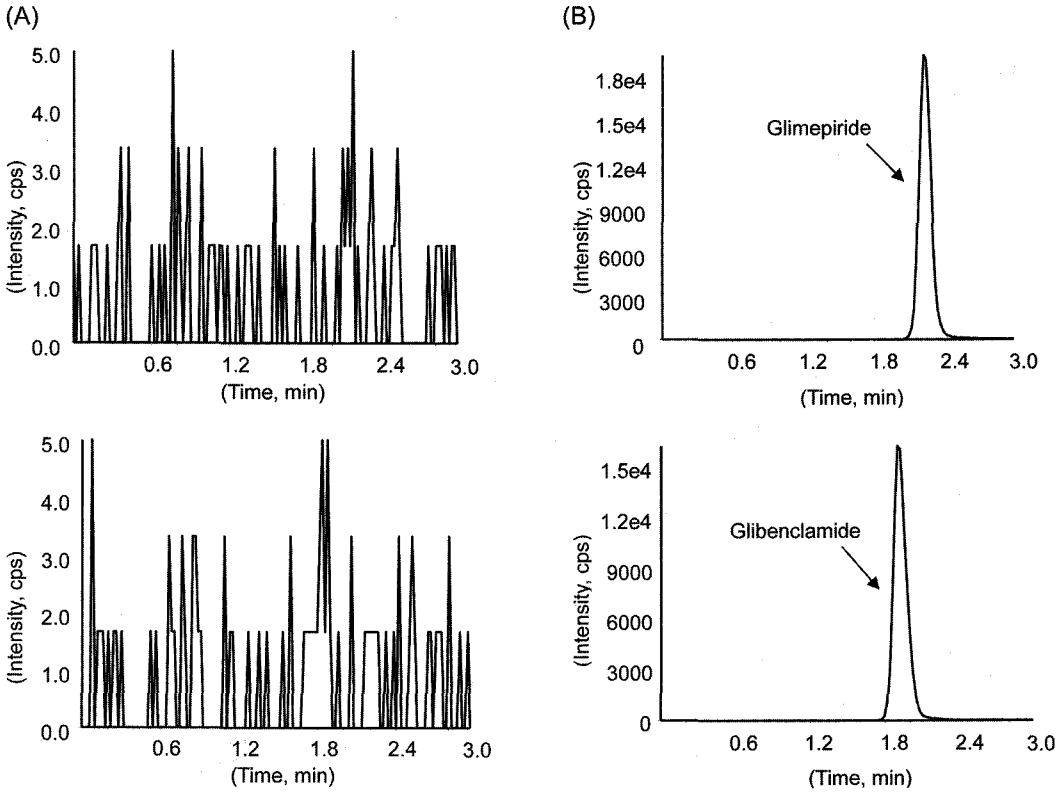

Figure 3–Multiple reaction monitoring chromatogram of (A) blank human plasma and (B) plasma spiked with glimepiride (200 ng/mL) and internal standard.

The calibration curves were obtained by analyzing seven samples. The curve was linear in whole range tested (2-400 ng/mL) and described by following equation: Y=0.0115X−0.00304 (X=glimepiride concentration (ng/mL), Y=ratio of peak areas) with a correlation coefficient of 0.9999. The intra-day accuracy of the method for glimepiride ranged from 98.1% to 102.8% while the intra-day precision ranged from 0.51% to 9.99%. The inter-day accuracy of the method for glimepiride ranged from 99.0% to 104.0% while the intra-day precision ranged from 1.16% to 7.15% (Table II).

Change of glimepiride concentration in human plsma

The developed method was successfully used for a pharmacokinetic study in which plasma concentrations of glimepiride in twenty-four healthy male volunteers were determined up to 12 hr after the oral administration of 2 mg of glimepiride dose. Figure 4 shows plasma concentration-time curves of glimepiride following oral administration of Amaryl and Mepiril.

Pharmacokinetic studies of glimepiride have been performed.[4,10] Cho *et al.*,[10] assayed plasma glimepiride concentrations using a HPLC-UV method, and reported the following pharmacokinetic parameters: AUC_t, 1019.06-1058.16 ng · hr/mL; C_{max}, 253.54-276.44 ng/mL; and T_{max}, 3.13-3.15 hr after a single oral dose of 2 mg glimepiride tablet. Following the oral administration of 1, 2, 4 or 8 mg single oral dose of glimepiride, Malerczyk *et al.*,[4] assayed plasma glimepiride concentrations using HPLC after pre-column derivatization, and reported a dose-dependent increase for C_{max} with a correlation coefficient of r=0.90. In our study, AUC_t, C_{max} and T_{max} for glimepiride were 523.43 ± 204.80 ng · hr/mL (Amaryl) and 534.06 ± 171.22 ng · hr/mL (Mepiril), 130.05 ± 54.63 ng/mL (Amaryl) and 145.55 ± 47.06 ng/mL (Mepiril), and 2.38 ± 1.16 hr (Amaryl) and 2.09 ± 0.68 hr (Mepiril), respectively (Table III). The difference of the test medication/mean of the reference medication for AUC_t, C_{max} and T_{max} were 2.035%, 11.918% and -12.184%. However, AUC_t and C_{max} of glimepiride reported by Cho *et al.*,[10] was almost quantitative at least 50% even though we used a same amount administration. It was demonstrated that they used a different analytical system.

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

Concentration (ng/mL)	Precision(%)		Accuracy(%)	
	Intra-day	Inter-day	Intra-day	Inter-day
2	2.78	3.78	98.1	99.0
5	4.63	1.16	101.8	100.1
10	9.99	4.38	102.4	101.7
20	3.56	1.50	99.8	99.0
100	8.58	2.14	102.8	101.3
200	5.17	3.96	101.2	102.3
400	0.51	7.15	99.5	104.0

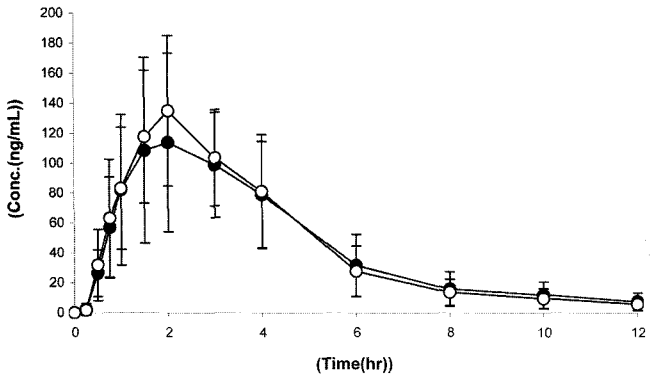

Figure 4–Mean (±S.D., n=24) plasma concentration-time curves of glimepiride following oral adminstration of Mepiril (○) and Amaryl (●) tablet at the dose of 2 mg of glimepiride.

Bioequivalence test of glimepiride products

No significant sequence effect was found for all of the bioavailability parameters indicating that the cross-over design was 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 0.9583-log 1.1357 and log 1.0570-log 1.2376, respectively, which were within the commonly accepted bioequivalence range of log 0.80-log 1.25.[11] 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 Amaryl (reference drug) and Mepiril (test drug).

Conclusions

It was shown that this method is suitable for the analysis of glimepiride in human plasma samples collected for bioequivalence studies in humans. Using this method, the bioequivalence of two different 2 mg glimepiride tablet formulations, in 24 healthy normal male volunteers was examined by monitoring. The statistical analysis results based on comparisons of the two pivotal parameters (AUC_t and C_{max}) point to the bioequivalence of these two tablet formulations of glimepiride, leading to the conclusion that they may be prescribed interchangeably.

Table III–*Bioavailability Parameters in Normal and Logarithmic Scales for Each Subject Obtained after Oral Administration of Amaryl and Mepiril Tablets at the Glimepiride Dose of 2 mg*

Subjects	Amaryl Tablet					Mepiril Tablet				
	AUC_t (ng · hr/mL)	Ln AUC_t	C_{max} (ng/mL)	Ln C_{max}	T_{max} (hr)	AUC_t (ng · hr/mL)	Ln AUC_t	C_{max} (ng/mL)	Ln C_{max}	T_{max} (hr)
A1	439.80	2.64	149.00	2.20	1.50	580.96	2.76	113.00	2.10	2.00
A2	486.52	2.69	138.00	2.10	1.50	661.24	2.82	195.00	2.30	1.50
A3	629.78	2.80	98.20	2.00	6.00	505.83	2.70	159.00	2.20	2.00
A4	455.27	2.66	106.00	2.00	4.00	679.59	2.83	295.00	2.50	2.00
A5	596.29	2.78	148.00	2.20	1.50	301.00	2.48	101.00	2.00	1.50
A6	558.04	2.75	141.00	2.10	1.50	622.88	2.79	147.00	2.20	1.50
A7	772.17	2.89	165.00	2.20	2.00	222.27	2.35	61.60	1.80	2.00
A8	615.50	2.79	121.00	2.10	4.00	344.39	2.54	95.20	2.00	2.00
A9	269.54	2.43	69.60	1.80	1.50	279.75	2.45	82.40	1.90	3.00
A10	234.38	2.37	73.60	1.90	1.50	684.91	2.84	168.00	2.20	2.00
A11	332.00	2.52	88.90	1.90	1.50	554.28	2.74	142.00	2.20	2.00
A12	511.61	2.71	134.00	2.10	3.00	463.62	2.67	174.00	2.20	2.00
B1	811.57	2.91	165.00	2.20	4.00	437.55	2.64	166.00	2.20	0.75
B2	634.39	2.80	154.00	2.20	2.00	547.10	2.74	159.00	2.20	2.00
B3	519.13	2.72	122.00	2.10	2.00	796.94	2.90	161.00	2.20	4.00
B4	1079.47	3.03	324.00	2.50	2.00	526.80	2.72	127.00	2.10	3.00
B5	285.46	2.46	86.40	1.90	1.50	686.65	2.84	182.00	2.30	2.00
B6	593.52	2.77	119.00	2.10	2.00	549.06	2.74	127.00	2.10	1.50
B7	208.20	2.32	71.20	1.90	3.00	833.63	2.92	177.00	2.20	2.00
B8	336.73	2.53	80.00	1.90	3.00	525.91	2.72	149.00	2.20	1.50
B9	357.49	2.55	70.20	1.80	3.00	377.72	2.58	120.00	2.10	2.00
B10	683.18	2.83	194.00	2.30	1.50	358.43	2.55	98.00	2.00	3.00
B11	675.48	2.83	163.00	2.20	1.50	834.78	2.92	172.00	2.20	2.00
B12	476.84	2.68	140.00	2.10	2.00	442.52	2.65	122.00	2.10	3.00
Mean	523.43	2.68	130.05	2.08	2.38	534.06	2.69	145.55	2.15	2.09
(S.D.)	204.80	0.18	54.63	0.17	1.16	171.22	0.15	47.06	0.14	0.68

Table IV–*Bioavilability Parameters for Each Volunteer Obtained After Oral Administration of Mepiril and Amaryl Tablets at the Glimepiride Dose of 2 mg*

	Parameters		
	AUC_t	C_{max}	T_{max}
Difference(%)	2.035	11.918	-12.184
F_G[a)]	0.119	0.005	0.407
Test/Ref point estimate	1.043	1.143	-11.843
Confidence interval(δ)[b)]	0.9583 – 1.1357	1.0570 – 1.2376	-29.9484 – 2.2642

#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(1,22)=4.260, [b)]α=0.05

Acknowledgements

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