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Bioavailability Test of Testo^{TM} Tablets (Methyltestosterone, 25 mg) in Male Healthy Volunteers by a Gas-chromatography/Mass Selective Detector

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ABSTRACT – A simple and specific method for determination of methyltestosterone (MT) has been established by a gas chromatography/mass selective detector and applied in plasma of healthy male volunteers received a single oral dose of 50 mg MT (Testo^{TM} tablets, 25 mg) for bioavailability test. This method involves using liquid-liquid extraction of the sample with diethyl ether and derivatization with MSTFA. MT showed good resolution in this condition. The detection limit of quantitation was 5 ng/ml. A good linearity ($r > 0.996$) was obtained at the range of 5-250 ng/ml of MT. Intra-day precision and accuracy were 2.76-12.56% and 0.39-8.01%, and inter-day precision and accuracy were 2.29-17.69% and 0.42-7.99%, respectively. The established method was applied on bioavailability test of MT in human volunteers. The value of $AUC_{0\ to\ last}$ was 264.5 ± 123.9 ng·hr/ml and that of $AUC_{0\ to\ inf}$ was determined to be 275.2 ± 126.5 ng·hr/ml. The values of C_{max} and T_{max} were 95.9 ± 67.1 ng/ml and 1.13 ± 0.79 hr, respectively. The mean elimination half-life ($t_{1/2}$) was 4.4 ± 0.9 hr. This analytical method is suitable and useful for the pharmacokinetics and bioequivalence studies of MT.

Key words – Methyltestosterone, Gas-chromatography/mass selective detector, Bioavailability, Human, Pharmacokinetics

17α-Methyltestosterone (MT; 17β-hydroxy-17α-methyl-4-androstan-3-one) is a synthetic androgen which has been used to treat patients with androgen deficiency and infertility. MT is absorbed from the gastrointestinal tract and from the oral mucosa. It undergoes less extensive first-pass hepatic metabolism than testosterone following oral administration and has much longer half-life than testosterone. Testosterone or dihydrotestosterone binds to an intracellular protein receptor and the hormone-receptor complex acts in the nucleus at specific binding sites on the chromosome. As a result, RNA polymerase activity and the synthesis of specific RNA and protein were increased. The human androgen receptor is a typical member of the superfamily of steroid and thyroid hormone receptors.[1)]

Urinary metabolites of MT have been determined in human, usually by stable isotope dilution analysis technique and its major metabolites are found as glucuronide conjugates.[5)] Also extensive metabolism studies of MT were conducted by Schänzer's group to establish a screening method for anti-doping.[4)] Because most of the quantitation method of MT has used the stable isotope dilution analysis technique and radio-labeled MT such as MT-d_3 and MT-d_6 in the plasma of human,[5,6)] development of a method without using a radio-labeled compound as internal standard may be less expensive and take advantage of removing the requirement relating to the usage of radioactive materials.

Pharmacokinetic parameters of MT in human remain unclear. As a compound with the similar structure to MT, intravenous administration of 7α-methyl-19-nortestosterone (500 μg) to a normal man led to the peak plasma level at 3 minutes after dosing, reaching undetectable levels by 3 hr. The average terminal half-life was about 40 minutes.[3)] Oxymetholone that is structurally very similar to MT was also reported to have 18.8 ± 0.04 ng/ml of C_{max}, 3.5 hr of T_{max} and 7.99 ± 3.56 hr of terminal half-life in healthy volunteers orally taken 50 mg of oxymetholone.[2)]

Although some clinical application and metabolism studies of MT were reported,[4)] the limited data on pharmacokinetics of MT are available. In this respect, determination of plasma MT concentrations and its application to pharmacokinetics in human volunteers are necessary. The objective of this work was to establish a simple and sensitive analytical method of MT for application to pharmacokinetic and bioavailability studies of MT in human volunteers.

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Materials and Methods

Chemicals

MT authentic standard was purchased from Sigma (St. Louis. MI, USA). MT tablets (Testo™, 25 mg) were kindly provided by Samil Pharmaceutical Co. Ltd. (Seoul, Korea). Fluoxymesterone used as internal standard was obtained from Doping Control Center of KIST. Diethyl ether and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), dithiothreitol, and ammonium iodide were obtained from Sigma. The other agents used for MT analysis were of analytical grade.

Blood sampling for volunteers

Eight healthy human volunteers were selected among volunteers who submitted the agreement to attend to this project 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. The subjects were instructed not to take any medicine for at least 1 week prior to and during the study period. They were accommodated to the lodging facility near the medical center one day before blood collection. They were fasted overnight before administration of the tablets. Lunch and dinner were allowed 4 and 12 h after drug intake, respectively. The physical and biological examinations were carried out before and after completion of the study.

Oral administration of MT tablets to human volunteers

A 21-gauge scalp-vein set was established of the arm vein of each volunteers and 8 ml blood was collected as blank. According to the prescription directed by a doctor, two tablets (total 50 mg MT, Testo™) were orally given to each volunteer with 250 ml of water. Blood was collected into heparin-treated tubes (Vacutainer™, Becton Dickinson, Rutherford, NJ, USA) at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24 and 48 h after the oral administration. The time interval of blood sampling between volunteers was 2 min to consider blood collection time. The blood was centrifuged to obtain plasma. The plasma was stored at -70°C until analyzed.

Gas-chromatography/mass selective detector

The plasma concentration of MT in human volunteers was determined by a gas chromatography/mass selective detector (GC/MSD; Agilent 6980/5973N; Agilent Technologies, Wilminton, DE, USA). The samples were injected to the instrument by an autoliquid sampler (Agilent 7983 Series), being supported with the GC/MSD ChemStation (Kayak PC/G1701DA, Hewlett Packard, USA). Mass selective detector of electron impact mode was used, and ionized energy of the mode was 70 eV. MT was separated by using the column Ultra-1 (50 m length × 0.2 mm inner diameter × 0.33 μm film thickness; Agilent Technologies, USA). Initial temperature of the oven was set to 120°C at which the temperature was increased by a rate of 15°C per min to 300°C of the final temperature and stayed at this temperature for 7 min. Temperatures of inlet, transfer line and detector were all set to 300°C. The flow rate of helium as carrier gas was 0.7 ml/min.

Preparation of the calibration curve of MT

To 1 ml of the MT-free plasma, MT was added to make final concentrations of 0, 5, 10, 25, 50, 100 and 250 ng/ml and fluoxymesterone (10 μg/ml, 20 μl) as an internal standard was added in glass-centrifuged tubes with stopper. The tubes were vortex-mixed and 0.75 ml of 0.5 N sodium hydroxide and 5 ml of diethyl ether were added. The tubes were shaken for 20 min on a shaker (100-150 rpm; 7400 Tubingen, Edmund Buchler, Germany) and centrifuged at 2500 rpm for 10 min (Varifuge 3.0, Heraeus, Germany). The organic layer was transferred to

Table I–*Intra-day and Inter-day Precision (C.V. %) and Accuracy (bias %)*

Concentrations (ng/mL)	Precision (C.V. %)		Accuracy (bias %)	
	Intra-day (n=5)	Inter-day (n=5)	Intra-day (n=5)	inter-day (n=5)
5	12.56	17.69	5.12	7.99
10	2.51	11.50	-8.01	2.12
25	7.97	2.29	-3.29	-4.65
50	5.87	10.32	-1.50	-6.34
100	5.12	8.14	3.07	4.47
250	2.76	4.37	-0.39	0.42

a new tube after freezing the tube in a freezer (-30°C). Ether was evaporated by an evaporator and the tube was placed in a desiccator with potassium pentoxide/potassium hydroxide for at least 1 hr. The residue was derivatized with 50 μl of MSTFA/dithiothreitol/ammonium iodide (100:4:5, v/w/w) in a heating block (60°C, 20 min). After cooling at room temperature, 1 μl of the solution was injected to GC/MSD by an auto liquid sampler.

Clean-up of plasma samples

The plasma sample obtained from human volunteers was thawed at room temperature. To the tube, 1 ml of the plasma samples was added with the internal standard and was prepared as described above. The plasma concentrations of MT in human volunteers were determined, based on the calibration curve from peak area ratios of MT to the internal standard.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the time-plasma concentrations of MT 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 MT. The area under the curve of time-plasma concentrations of MT 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.[9]

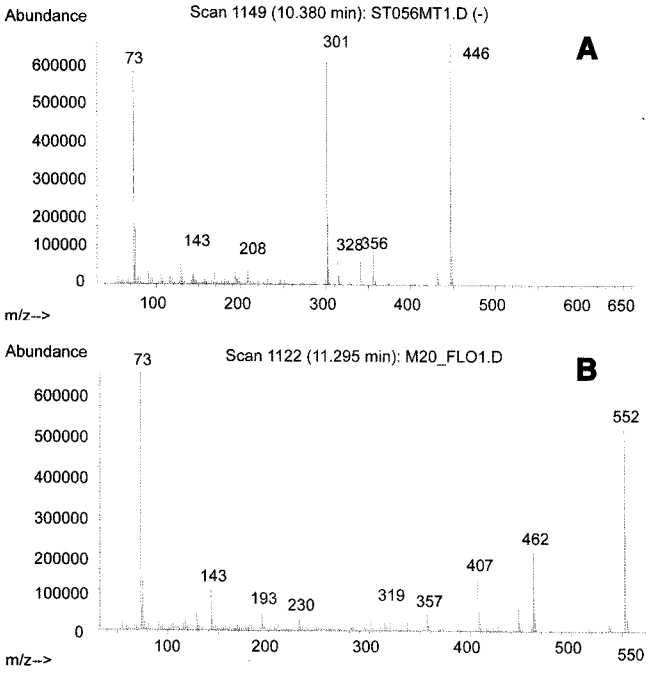

Figure 1–Mass spectra of methyltestosterone (A) and fluoxymesterone (B) obtained by a gas chromatography/mass selective detector. Molecular ions for methyltestosterone and fluoxymesterone were observed as m/z 446 and 552, respectively.

Results and Discussion

GC/MSD mass spectra and ion chromatograms

MT mass spectrum has the characteristic ions of m/z 143, 301, 431(M^+-15), and 446 (M^+) from MT-$(OTMS)_2$. Mass spectrum of fluoxymesterone has the characteristic ions of 143, 407, 462 and 552 (M^+) from fluoxymesterone-$(OTMS)_3$. The characteristic ions of m/z 143 were the common ion that was produced from cleavage of the D-ring in the chemical structure of the MT and fluoxymesterone derivatized with MSTFA. These mass spectra were shown in Figure 1.

The selected ion monitoring mode of GC/MSD was used by using the characteristic ions as mentioned above. The m/z 446 for MT and m/z 552 for fluoxymesterone were selected as a quantitation ion. The ion chromatograms for the quantitation ion were presented in Figure 2. The retention times of MT (m/z 446) and fluoxymesterone (m/z 552) were 11.5 and 12.35 min, respectively. No interfering peaks were observed in these times. With the purpose of confirming MT and fluoxymesterone ion chromatograms, the other characteristic ions are compared at the corresponding retention times.

In order to determine an appropriate internal standard for the plasma MT analysis, retention times and derivatization efficiency of bolasterone, norethandrolone and fluoxymesterone that are structurally similar to MT were compared. As a result, norethandrolone did not show good relative response and the retention time of bolasterone was too long as about 16.5 min, compared to MT and fluoxymesterone (data not shown). Fluoxymesterone showed large mass fragment and good derivatization efficiency to MSTFA, compared to those of the others.

Validation of analytical method of MT in human plasma

The GC/MSD method for determining the MT concentration in the plasma of male healthy volunteers was validated and applied to the bioavailability study of MT. A good linearity ($r > 0.9996$) was obtained in the range of 5-250 ng/ml of MT. The limit of quantitation for MT determination is 5 ng/ml at which both precision and accuracy data were observed to be less than 20%. Because at this concentration the signal to noise ratio is higher than 11, limit of detection is less than 5 ng/ml. Precision data were observed to be less than 12.6 and 17.7%

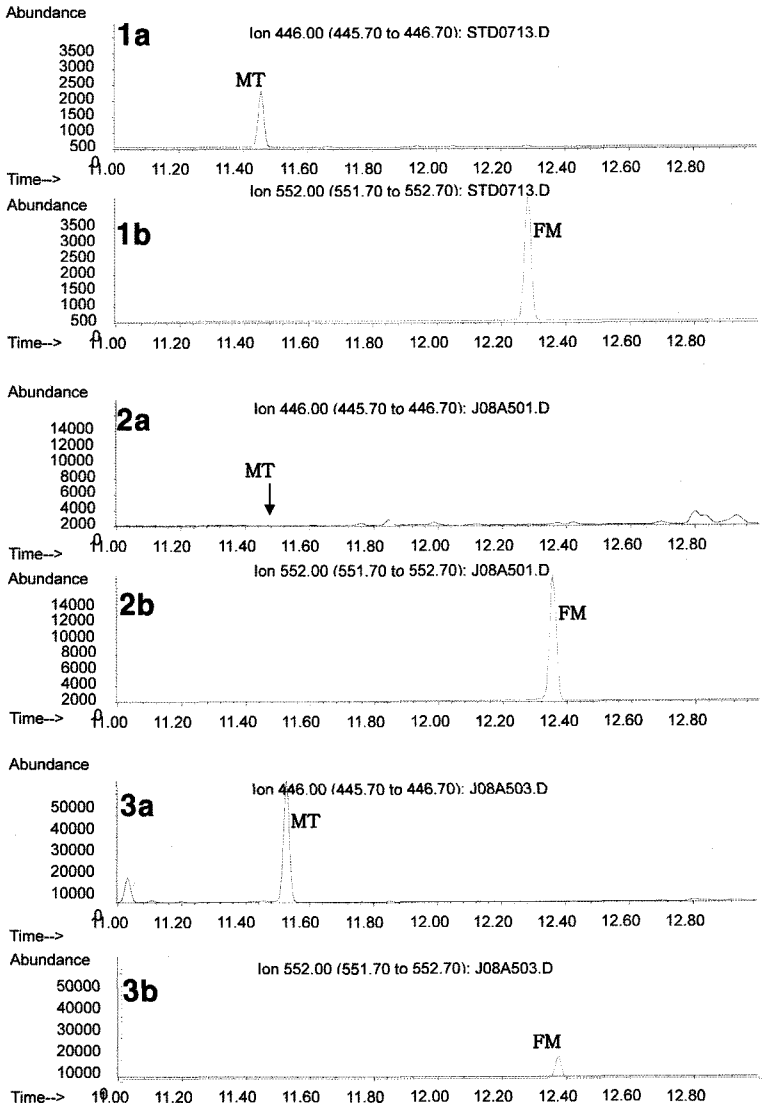

Figure 2–Ion chromatograms of authentic standards (1a, 1b), plasma blank spiked with internal standard (2a, 2b) and the plasma sample obtained from a human volunteer administered 50 mg methyltestosterone (3a, 3b). MT, methyltestosterone; FM, fluoxymesterone (internal standard).

for intra-day and inter-day, respectively. Accuracy for intra- and inter-day was less than 8.0%.

The plasma concentration of MT was below the limit of quantitation 24 and 48 hr after oral administration of 50 mg MT in 7 of 8 human volunteers. In only one volunteer, plasma concentrations of MT were 6.98 and 5.07 ng/ml at 24 and 48 hr, respectively.

Determination of bioavailability parameters

The developed method was applied on the pharmacokinetic study of MT after oral administration of MT (50 mg) to 8 healthy human volunteers. The principle pharmacokinetic parameters of AUC, C_{max}, T_{max}, K_e and $t_{1/2}$ were determined from the plasma concentration-time curves (Figure 3; Table II). $AUC_{0\ to\ last}$ was 264.5 ± 123.9 9 ng·hr/ml and $AUC_{0\ to\ inf}$ was 275.2 ± 126.5 ng·hr/ml. C_{max} and T_{max} were 95.9 ± 67.1 ng/ml and 1.13 ± 0.79 hr, respectively. The mean elimination half-life ($t_{1/2}$) was 4.4 ± 0.9 hr. Based on these data, the time of blood sampling at the early phase needs to be adjusted by inserting one more point of the blood sampling within 30 min after administration (Figure 3).

Shinohara *et al.* (1985) has determined plasma concentrations of MT and MT-d_3 after oral administration of each 10 mg mixture of MT and MT-d_3 solution to a healthy subject.[6] Plasma concentrations were determined to 8 hr after administration even if they do not show any pharmacokinetic data.[6]

Table II–*The Pharmacokinetic Parameters of Methyltestosterone in 8 Healthy Male Human Volunteers after a Single Oral Dose of 50 mg Methyltestosterone (Testo™ tablet, 25 mg)*

Subjects	Parameters					
	AUC (ng·hr/ml)		C_{max} (ng/ml)	T_{max} (hr)	K_e (1/hr)	$t_{1/2}$ (hr)
	$AUC_{0\ to\ inf}$	$AUC_{0\ to\ last}$				
A1	337.19	330.49	125.91	1.0	0.185	3.74
A2	139.24	138.16	57.58	1.0	0.146	4.75
A3	438.85	438.13	245.81	0.5	0.216	3.20
A4	160.24	152.73	43.15	1.0	0.134	5.16
A5	251.39	248.22	106.77	1.0	0.202	3.43
A6	272.30	264.61	54.71	1.0	0.160	4.32
A7	151.44	140.39	53.92	0.5	0.149	4.65
A8	405.93	364.04	79.41	3.0	0.119	5.84
Mean	275.2	264.5	95.9	1.13	0.16	4.4
S.D.	126.5	123.9	67.1	0.79	0.03	0.9

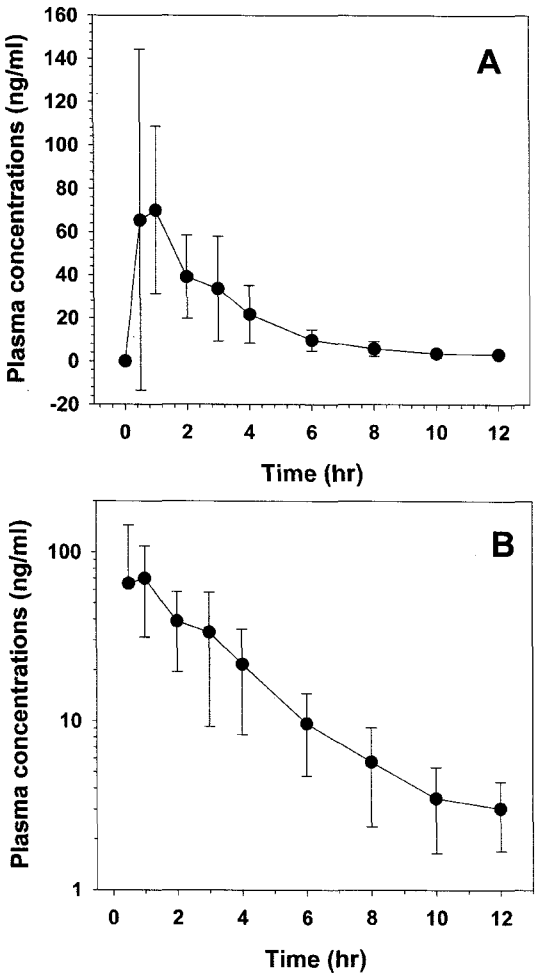

Figure 3–Plasma methyltestosterone concentration-time curves showing in either regular (A) or semilogalithmic (B) scales after a single oral administration of 50 mg methyltestosterone in 8 healthy male volunteers. Methlytestosterone concentrations in the human plasma were determined until 12 hrs. From this curve time intervals to be collected the blood within 30 min after administration of methyltestosterone should be adjusted to obtain unambiguous C_{max}.

In another study, urinary excretion rate was determined in normal adult male volunteers who received a single oral dose of 500 mg MT. The urinary excretion rate for the main metabolites was obtained over 72 hr.[5] Most of the data were focused on the metabolism of MT in urine, and there are no papers reporting MT pharmacokinetics in human volunteers except for pharmacokinetic study of MT in rainbow trout[7] and drug interaction with cyclosporine.[8] Therefore, this work presents practically the principle pharmacokinetic parameters of MT in normal male volunteers. The established method may be useful and applicable to the pharmacokinetic and bioequivalence studies of MT.

Conclusion

A simple and specific method for determining MT has been established and validated by a gas chromatography/mass selective detector and the method was applied to the analysis of MT in the plasma of eight normal male volunteers orally taken a single dose of 50 mg MT (Testo™ tablet, 25 mg). C_{max} and T_{max} were 95.9 ± 67.1 ng/ml and 1.13 ± 0.79 hr, respectively. The mean elimination half-life ($t_{1/2}$) was 4.4 ± 0.9 hr. This method is considered to be suitable and useful for the pharmacokinetics and bioequivalence studies of MT.

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