Gas Chromatographic/Mass Spectrometric Characterization of Dromostanolone Metabolites in Human Urine

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The metabolism of dromostanolone (2α-methyl-5α-androstan-17β-ol-3-one) was studied in three adult volunteers after oral dose of 20 mg. Solvent extracts of urine obtained after enzyme hydrolysis were derivatized with MSTFA/TMCS and MSTFA/TMIS. The structures of intact drug and its metabolites were determined by gas chromatography/mass spectrometry (GC/MS) in electron impact (EI) mode. The major metabolite (2α-methyl-5α-androstan-3α-ol-17-one), its 3β-epimer, parent compound, and several hydroxylated metabolites including intact drug were detected by comparing total ion chromatograms of control urine with that of the administered sample. Two epimers of 2α-methyl-5α-androstan-3,17β-diol were detected using selected ion monitoring. The maximum excretion of dromostanolone and 2α-methyl-5α-androstan-3α-ol-17-one was reached in 6.2-15 hr. The half-life of intact dromostanolone was 5.3 hr. About 3.0% of the administered amount was found to be excreted within 95 hr as unchanged form.

Introduction

Dromostanolone (2α-methyl-5α-androstan-17β-ol-3-one) is one of the anabolic steroids that has been widely used in the treatment of anemia[1] and in androgen therapy.[2] Therefore, the International Olympic Committee (IOC) and international sports federations have banned the misuse of anabolic steroids by athletes to improve their performance since 1985. Nevertheless, there were not many reports on the metabolism of dromostanolone in man. In general, the metabolism of dromostanolone in humans involves conversion to 3α-ol-17-one, like the other 17β-ol-3-one analogues such as mesterolone (1α-methyl-5α-androstane-17β-ol-3-one)[3,4] and stanolone (5α-androstane-17β-ol-3-one).[5] Detection of dromostanolone metabolites in human urine using gas chromatography-mass spectrometry (GC-MS) have been reported by Schzer *et al.*[6] and de Boer *et al.*[7] However, minor metabolites such as multi-hydroxylated metabolites which are excreted in rabbit[8] were not monitored even though they are easily detected in man.

In this paper, we describe a simple and rapid extraction method for the detection of dromostanolone metabolites based on the previous report of Schäzer *et al.*[6] We report structural elucidation of metabolites in human urine after oral administration of dromostanolone. We also report the results from an excretion study of unchanged dromostanolone and the urinary excretion pattern.

Experimental

Urine samples

Urine were collected from three healthy subjects of the laboratory staff, at 0, 2.75, 6.25, 15, 23, and 28 hr after oral administration of 20 mg of dromostanolon propionate. Thereafter, the urine samples were collected once a day for three days. The samples were stored at 4 ℃ until analysis.

Chemicals and reagents

Masterid (100 mg of dromostanolone propionate in 2 mL solution) was obtained from Gruenenthal (Stolberg, Germany). β-glucuronidase extracted from *E. coli* was from Boeringer-Mannheim (Mannheim, Germany). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), trimethyliodosilane (TMIS), and dithioerythritol were from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of the guaranteed reagent grade, and diethyl ether was distilled from calcium hydride powder. Phosphate buffer was prepared by mixing 0.2 M potassium hydrogen phosphate and 0.2 M potassium dihydrogen phosphate in a 5 to 1 ratio (v/v) and adjusting the pH to 7.0. Mixtures of MSTFA/TMCS and MSTFA/TMIS were prepared simply in proportion of 100 to 2 and 1000 to 2 (v/v), respectively. Dithioerythritol (0.2%, w/w) was added to the MSTFA/TMIS mixture and the mixture was stored in a dark place in order to prevent iodine isolation from TMIS.

Instruments

Analyses were performed with a Hewlett Packard (Avondale, PA, U.S.A.) 5890A gas chromatograph/5970B mass selective detector for obtaining EI spectra and urine profiles. HP 5988A mass spectrometer was used for chemical ionization (CI) spectra. GC was equipped with HP-1 fused silica capillary (17 m × 0.2 mm i.d.) column cross-linked with dimethylsilicone gum (0.11 μm-thickness). The oven temperature was programmed from 180 ℃ to 224 ℃ at a rate of 4 ℃/min, then to 280 ℃ at a rate of 15 ℃/min, and finally to 300 ℃. The inlet pressure of helium as carrier gas was set to 51.7 kPa at 180 ℃ and the split ratio was 1 : 10.

Sample extraction procedure

Preparation of AD-2 column. A Pasteur pipette secured with a glass ball of 3 mm diameter was used as a column. The Serdolit AD-2 slurry (Serva; Heidelberg, Germany), pre-washed sequentially with acetone, methanol and

distilled water, was poured into the column until 25 mm of bed height was reached. The column was washed with 2 mL of distilled water before applying the urine.

Isolation of the steroids from urine. After application of 10 mL of urine in 2 mL portions and the internal standard (500 ng of methyltestosterone) to the Serdolit AD-2 column, the column was washed with the same volume of water and the adsorbed lipophilic fraction containing both the free and conjugated steroids was eluted from the column with three 0.9 mL portions of distilled methanol. The methanolic eluate was evaporated, and the residue was dissolved in 1 mL of the phosphate buffer at pH 7.0 and mixed with 25 μL of β-glucuronidase. The mixture was then maintained at 50 ℃ for 1 hr, followed by alkalization with 100 mg of K_2CO_3 and extraction with 5 mL of diethyl ether. The ethereal phase was evaporated and the residue was dried over P_2O_5/KOH in a vacuum desiccator for at least 30 min.

Derivatization. The dried residue was derivatized[9,10] with 50 μL of MSTFA/TMCS mixture (5 : 1 by volume), at 60 ℃ for 3 min. To confirm the derivatization of carbonyl group, it was performed with 50 μL of MSTFA/TMIS mixture (500 : 1 by volume), at 60 ℃ for 15 min. 1-4 μL volume of each solution was injected on the GC column.

Results and Discussion

Analytical considerations. We have used two kinds of derivatizing agent mixture; the MSTFA/TMIS mixture substitutes both hydroxyl and enolizable carbonyl group while MSTFA/TMCS silylates only hydroxyl group.[9] Generally, the silylation strength of MSTFA/TMCS is so high that all hydroxyl groups are silylated, but for the silylation of carbonyl groups catalysts are required.

The TMS derivatives of the major metabolite, its epimer and the parent dromostanolone were detected by comparing chromatograms of pre- and post-dose urine extracts. Figure 1

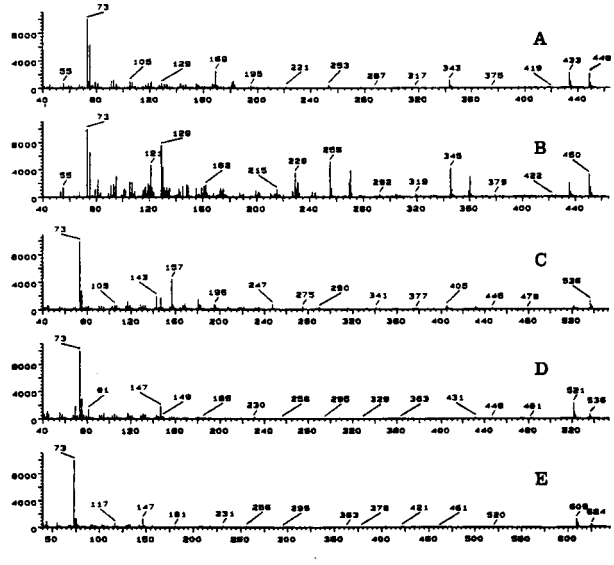

Figure 1. Mass spectra of metabolites of dromostanolone in human urine as TMS ethers. A: 2α-methyl-5α-androstan-3α-ol-17-one; B: 2α-methyl-5α-androstan-3,17β-diol; C, D: monohydroxylated metabolites; E: dihydroxylated metabolite.

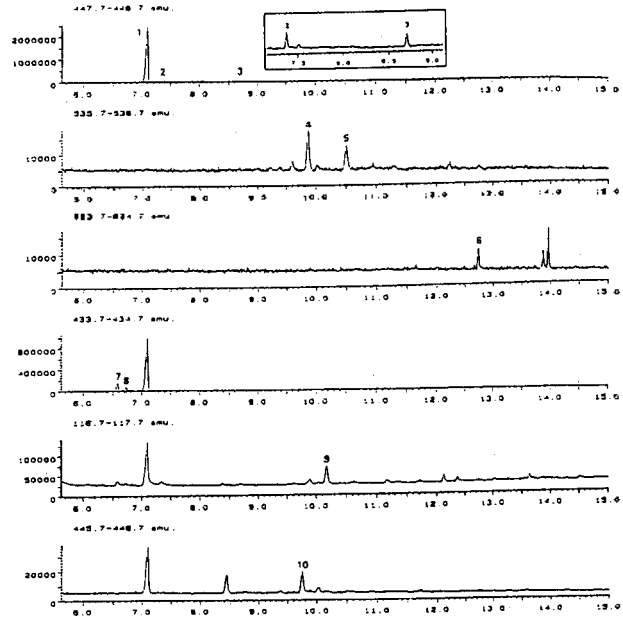

Figure 2. Reconstructed ion fragmentograms obtained by GC/MS analysis in SIM mode of the 6.25 hour post-dose urine sample derivatized with the mixture of MSTFA/TMIS. Peak: 1=2α-methyl-5α-androstan-3α-ol-17-one; 2=2α-methyl-5α-androstan-3β-ol-17-one; 3=dromostanolone; 4,5=mono-hydroxylated metabolites; 6=dihydroxylated metabolite; 7=cis-androsterone; 8=etiocholanolone; 9=pregnandiol; 10=methyltestosterone as internal standard.

shows the mass spectra of trimethylsilyl (TMS) ethers or enol ethers of the metabolites. The molecular weights of the metabolites were confirmed in the chemical ionization mode (methane reagent gas).

The major metabolite in human, 2α-methyl-5α-androstan-3α-ol-17-one and a minor one, 2α-methyl-5α-androstan-3β-ol-17-one, could be easily identified (peaks 1 and 2 in Figure 2); the former was abundant in the chromatograms of the positive samples and the minor metabolite had a slightly longer retention time. The mass spectra of both metabolites were identical (Figure 1A). The stereochemistry at C-3 has not been determined yet, but the major and minor metabolite were presumed to be 3α and 3β, respectively on the basis of their GC retention times and previous reports.[7,11]

The presence of unchanged dromostanolone in urine was confirmed with authentic compound (peak 3 in Figure 2). The 3β-hydroxy isomer of the major metabolite and hydroxylated metabolites were not found in the urine of dromostanolone-administered rabbits.[8] And 3,17-β-diol metabolites were found in the sample derivatized with the mixture of MSTFA/TMCS. In the sample derivatized with MSTFA/TMIS, the bis-TMS derivative of the major metabolite coeluted with that of the diols.

The three unidentified metabolites gave molecular ions at m/z 450, 536, and 624, respectively but their structures have not yet been elucidated.

The molecular ions of the hydroxylated metabolites as TMS derivatives indicate that they have one or two more oxygens than dromostanolone. Each of them seems to have a carbonyl group. They were found only in the sample derivatized with the mixture of MSTFA/TMIS.

Table 1. Excretion profile of dromostanolone and its metabolites after oral administration

Metabolite	M^+	RRT[a]	Response ratio[b] varied with time (hr) post-dose							
			2.75	6.25	15	24	32	48	71	95
3α-OH-2α-Me-5α-androstan-17-one	448		0.73	12	200	118	29	5.7	4.9	2.6
3β-OH-2α-Me-5α-androstan-17-one	448	0.75	0.28	3.1	3.0	1.1	0.34	0.51	0.17	0.13
2α-Me-5α-androstan-3α,17β-diol[c]	450	0.75	0.63	1.3	1.4	0.25	0.09	0.032	0.051	0.042
2α-Me-5α-androstan-3β,17β-diol[c]	450	0.78	0.0032	0.042	0.027	ND	ND	ND	ND	ND
Parent compound	448	0.89	0.27	3.6	5.4	1.4	0.45	0.69	0.21	0.12
Monohydroxylated metabolite	536	1.01	0.052	1.5	1.5	0.42	0.083	0.054	ND	ND
Monohydroxylated metabolite	536	1.08	0.047	0.11	1.5	0.61	0.23	0.26	0.046	0.044
Dihydroxylated metabolite	624	1.31	0.11	0.70	0.63	0.25	0.10	0.022	0.021	0.026

[a]Retention time relative to that of methyltestosterone (9.75 min). [b]Peak area of molecular ion of the metabolite/Peak area of molecular ion of methyltestosterone (M^+=446); Response ratio is the mean value of three subjects. [c]The relative retention times and abundances were calculated indirectly. ND: not detected.

Some of the minor metabolites found in rabbits urine, such as 2α-methyl-5β-androstane-3,15(or 16),17-triol and 2α-hydroxymethyl-5α-androstane-3,16,17-triol, were not found in human urine, even in the ion fragmentograms of m/z 538 and 629 obtained in SIM mode.

Excretion profiles of dromostanolone and its metabolites. Excretion patterns of dromostanolone and its metabolites were studied using GC/MS in SIM mode. The selected ion, response ratio and relative retention time (RRT) of each compound are shown in Table 1. Excretion profiles were evaluated by excretion rate which were calculated by dividing response ratio (each compound area/internal standard area) by time interval.

Excretion rates of several metabolites and unchanged dromostanolone were reached at maximum rate within 6.25 hr after dosing. But only two metabolites, 2α-methyl-5α-androstan-3α-ol-17-one and one of the monohydroxylated metabolites (RRT=1.08) were excreted slowly. Most of the metabolites could be detected until 4 days after administration.

The amounts of the unchanged dromostanolone excreted in urine after oral administration of dromostanolone (20 mg) are shown in Table 1. We found that 87.9% of total excreted amount was eliminated within 24 hr, and 91.6% within 32 hr. Also, in the excretion pattern of 2α-methyl-5α-androstane-3α-ol-17-one as a major metabolite, Table 1 shows the variations of the peak height ratios, which reflect the concentrations of the metabolite, with time after oral administration. In the same way, we found that the 88.7% of total excreted amount was obtained within 24 hr, and 96.5% within 32 hr.

The urinary excretion half-life of unchanged dromostanolone was approximately 5.31 hr and the maximum excretion of dromostanolone (unchanged metabolite) and 2α-methyl-5α-androstan-3α-ol-17-one after oral administration was reached within 6.25-15 hr.

Conclusion

The analytical method showed good specificity and sensitivity for the quantitation of unchanged dromostanolone and the peak area ratio of some of the metabolites in human urine using GC/MS. All metabolites were found in the conjugated fraction of the specimen after hydrolysis using an enzyme solution of β-glucuronidase. Some of the metabolites such as two triols and 3,17-diones detected in rabbit urine were not found in human urine.

The excreted amount of dromostanolone and the excretion patterns of its metabolites were determined. The excretion pattern was similar in all subjects. The half-life of intact dromostanolone was 5.31 hr. About 3.0% of the administered amount was found to be excreted within 95 hr as unchanged form.

References

1. Choi, S. K.; Chung, T. J.; Morrison, R. S.; Myers, C.; Greenberg, M. S. *J. Clin. Nutr.* **1974**, *27*, 901.
2. Seay, D. G.; Bradshaw, J. D.; Nicol, N. T. *Cancer Chemotherapy Reports* **1972**, *56*, 89.
3. Gehard, E.; Gibian, H.; Kolb, K. H. *Arzneimittel-Forsch.* **1966**, *16*, 458.
4. Huck, H.; Egg, D. *Wien. Klin. Wochenschr.* **1972**, *7*, 114.
5. Cartoni, G. P.; Giarruso, A.; Ciardi, M.; Rosati, C. *J. High Resol. Chromato. Commun.* **1985**, *8*, 539.
6. Schänzer, W.; Donike, M. *Anal. Chim. Acta.* **1993**, *275*, 23.
7. De Boer, D.; De Jong, E. G.; Maes, R. A. A.; van Rossum, J. M. *J. Steroid Biochem. Molec. Biol.* **1992**, *42*, 411.
8. Templeton, J.; Kim, R. S. *Steroids.* **1977**, *29*, 371.
9. Donike, M.; Zimmerman, J. *J. Chromatogr.* **1980**, *202*, 483.
10. Masse, R.; Ayotte, C.; Dugal, R. *J. Chromatogr.* **1989**, *489*, 23.
11. Houghton, E.; Ginn, A.; Teale, P.; Minoo, C.; Copsey, J. *J. Chromatogr.* **1989**, *479*, 73.