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Analytical Data of 1-Testosterone and the Preliminary Results of Excretion Study with 1-Testosterone**

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INTRODUCTION

1-Testosterone, 17β-hydroxy-androst-1-en-3-one, is banned by The International Standard Prohibited List[1]. It has the identical molecular weight and molecular formula as testosterone. The only difference in their chemical structures between testosterone and 1-testosterone is the position of the double bond (Fig. 1). It was reported in the Internet that 1-testosterone shows a

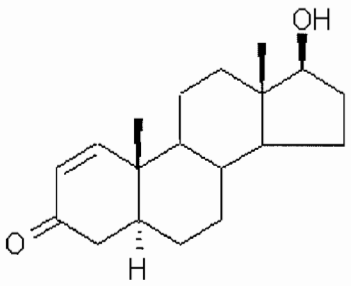

Fig. 1 Chemical structure of 1-testosterone

very strong anabolic effect, even seven times stronger than testosterone[2]. But such information is not scientifically approved. 1-Testosterone is now widely offered as a sport nutrition supplement. To develop the methodology of detecting doping with 1-testosterone, the compound was synthesized and its chemical structure confirmed with different analytical techniques. With this synthesized 1-testosterone, an excretion study was performed. Using the normal procedure for screening IV, some metabolites of 1-testosterone were proposed and confirmed.

Before starting this study ethical committee application, study protocol had been prepared and approved by the local ethic committee in Beijing.

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EXPERIMENTAL

Reagents

All chemical reagents used for analytical purpose were of Analytical Grade.

Instrumentation and Working Condition

GC/MS: Agilent 6890A/HP5973. The column used was a HP-1 17 m (0.2 mm i.d., 0.11 μm film-thickness) with a head pressure of 91 kPa. The oven temperature program was: initial temperature: 180°C (0 min), +3.3°C /min → 231 °C, +30°C /min → 310°C (2 min). The injector and the transfer line were held at 280°C. The split ratio was 10:1. Electron ionisation with 70 eV was used. Scans were acquired from 50 to 500 amu with 0.25 sec/scan.

HRMS: Micromass ZabSpec High Resolution Mass Spectrometer worked with a resolution of 10000. The scan range was from m/z 50 to m/z 600 with a scan rate of 1. With 70 eV the EI mode was used. The temperature of the probe was set to 100°C and the ion source to 200°C.

NMR: Varian Inova 600. $CDCl_3$ was used as the solvent. Pulse Sequence was set in preset mode. Relax delay was 3.000 sec and pulse 27.6 degree. The acquisition time was 1.333 sec.

FTIR: Bio-Rad FTS-65A was used with the resolution of 4 cm^{-1}. The samples were scanned in liquid film.

Excretion Study

The following Tab. 1 contains the information about the two volunteers.

Tab. 1 Information about the volunteers

Age	Gender	Weight (kg)	Dose (mg)
33	F	60	100
52	M	83	120

Prior to oral administration of 1-testosterone in a capsule, two urine samples were collected as the blank urines. After the administration all urine samples were collected for 72 hours and afterwards only the morning urine samples for a further two days. All urine samples were stored at -20°C till analysis. All urine samples were analyzed with our routine procedure IV for the free and conjugated fraction. The steroid profile was studied.

RESULTS AND DISCUSSION

HRMS: The mass spectrum is given in Fig. 2.

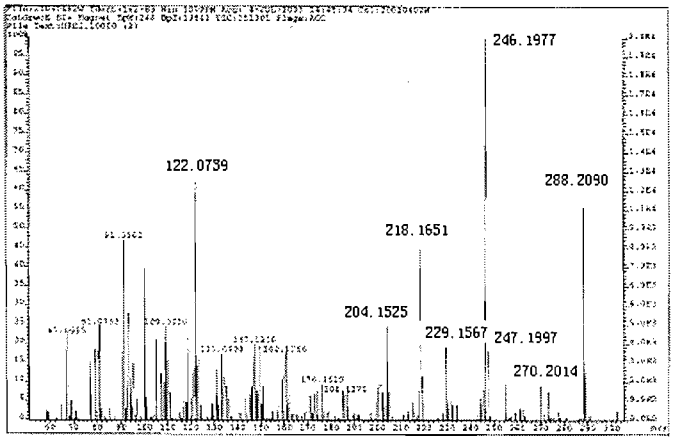

Fig. 2 High resolution mass spectrum of 1-testosterone

The measured exact masses of the fragments are listed in Tab. 2, the fragmentation pattern[3] is proposed in Fig. 3.

Tab. 2 The exact mass and possible elemental composition of the fragments (only the fragment m/z > 100, %Relative Area (RA) >15%, | PPM | < 30 are listed)

m/z	%RA	PPM	Elemental Composition
288.208984	62.1	-0.2	C19 H28 O2
247.199745	18.1	26.1	C17 H27 O1
246.197742	100.0	2.5	C17 H26 O1
229.156693	19.2	11.1	C16 H21 O1
218.165119	44.8	8.9	C15 H22 O1
204.152554	24.6	-5.6	C14 H20 O1
161.132137	19.0	5.5	C12 H17
160.126614	17.5	-8.8	C12 H16
147.120827	19.8	-23.5	C11 H15
122.073887	62.4	-5.9	C8 H10 O1
105.071918	21.0	-14.2	C8 H9
100.052965	39.6	-5.4	C5 H8 O2

NMR: The ^{13}C NMR data of 1-testosterone obtained in our experiment are listed in Tab. 3. For comparison, published ^{13}C NMR data of testosterone[4] are also given in Tab. 3. The ^{1}H NMR spectrum of 1-testosterone is shown in Fig. 4, confirming the number of hydrogen atoms and the major chemical surroundings. The DEPT spectrum of 1-testosterone confirmed the signals only arising from protonated carbons. The H-H-COSY spectrum of 1-testosterone showed the coupled proton pairs. Both HMBC and HMQC spectra of 1-testosterone

confirmed the relationship of single bond between ^{1}H and ^{13}C.

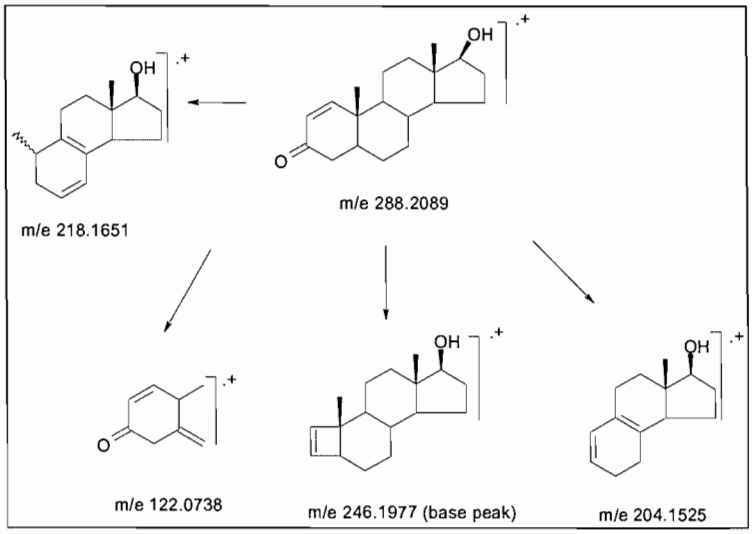

Fig. 3 The proposed fragmentation of 1-testosterone

Tab. 3 ^{13}C-NMR data of 1-testosterone and testosterone

C	Testosterone	1-Testosterone	C	Testosterone	1-Testosterone
1	35.8	158.321	11	21.0	20.843
2	33.8	127.396	12	36.8	36.554
3	197.7	200.130	13	42.9	43.111
4	123.9	39.033	14	50.8	50.120
5	170.1	40.527	15	23.5	23.298
6	32.5	27.484	16	30.4	30.506
7	31.9	30.842	17	81.0	81.836
8	35.8	35.722	18	11.0	11.081
9	54.3	50.695	19	17.0	12.653
10	38.7	44.610	-	-	-

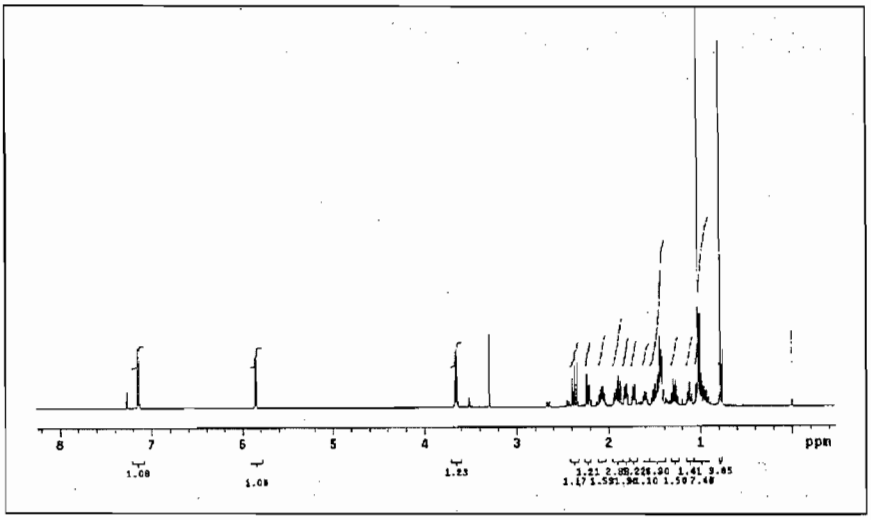

Fig. 4 ^{1}H NMR spectrum of synthesized 1-testosterone

FTIR: Due to the structural similarity of testosterone and 1-testosterone, the FTIR spectra of

these compounds are very similar.

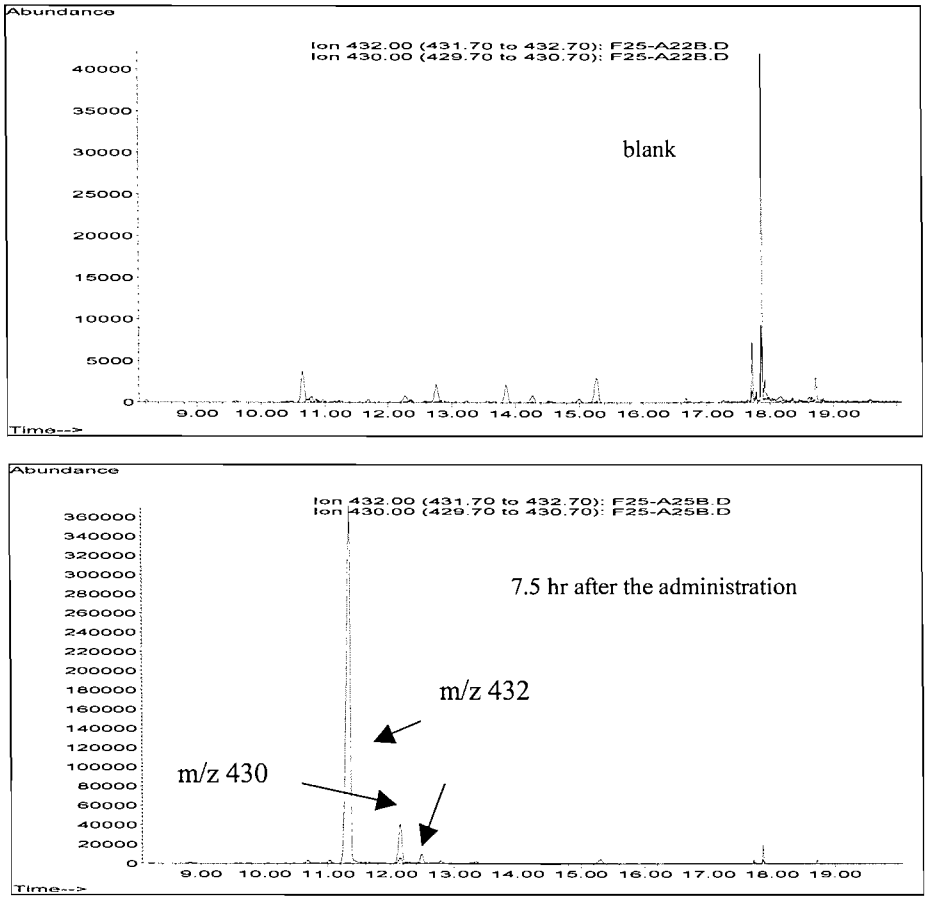

Fig. 5 EIC of urine from the excretion study (conjugated fraction)

Excretion Study: Fig 5 shows the extracted ion chromatogram of a blank urine and the urine from the male volunteer 7.5 hours after administration of 1-testosterone . A peak at a retention time of about 12.5 min is clearly detected in the conjugated fraction of the urine after the administration. By comparison with the mass spectrum and the retention time of the synthesized 1-testosterone (see Fig. 6), this peak in Fig. 5 is identified as 1-testosterone.

The concentration-time curve (Fig. 7) revealed that 1-testosterone could be detected for a long time. About 100 hours after the single dose administration the concentration of 1-testosterone in the urine samples from both volunteers was about at least 5 ng/ml.

Based on the comparison of mass spectra (Fig. 8) and retention times between the synthesized androst-1-ene-3,17-dione and the peak in the urine sample (Fig. 5) with a retention time of 12

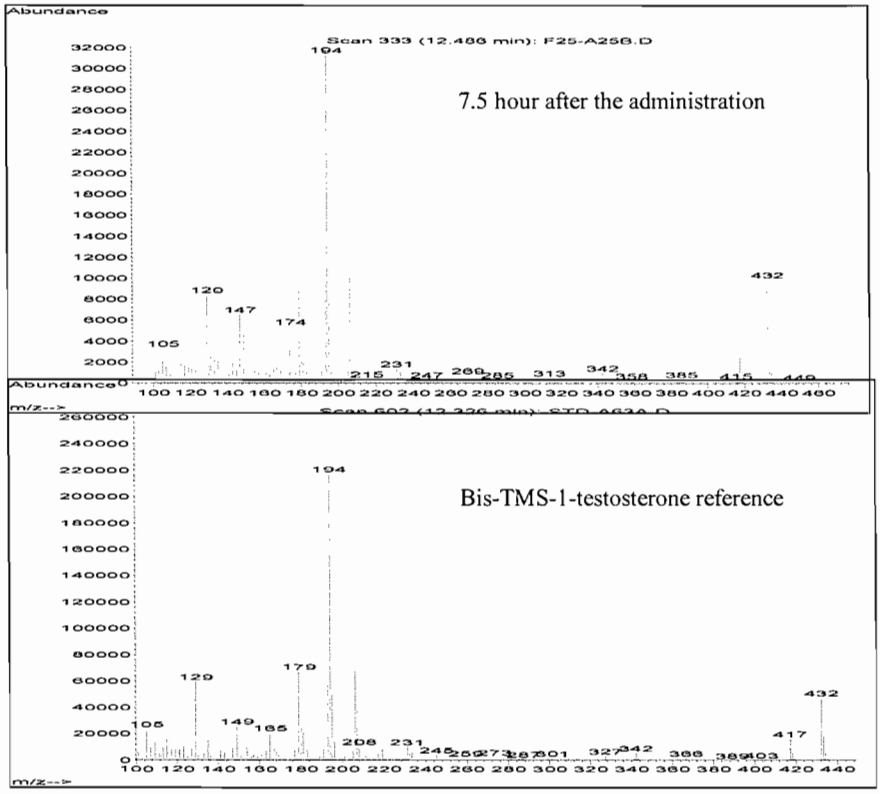

Fig. 6 Mass spectra of bis TMS-1-testosterone from urine and reference

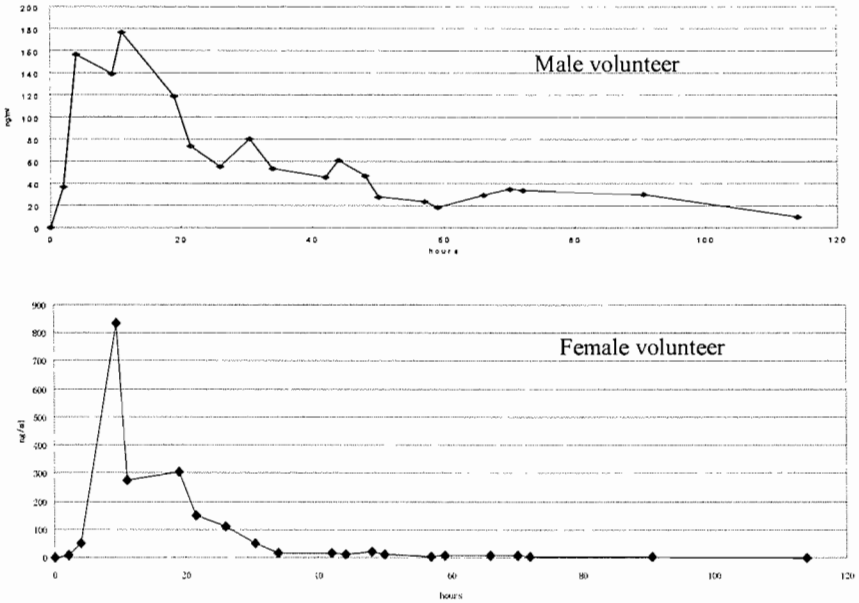

Fig. 7 Concentration-time curves of 1-testosterone in two volunteers.

min (m/z = 430) it was confirmed that androst-1-ene-3,17-dione is a metabolite of 1-testosterone.. When the excretion study was going on, the synthesis and identification of androst-1-ene-3,17-dione was not completely finished, the time curve, which is shown in Fig. 9, could only be expressed as the ratio of peak area of the peak to the internal standard (17α-

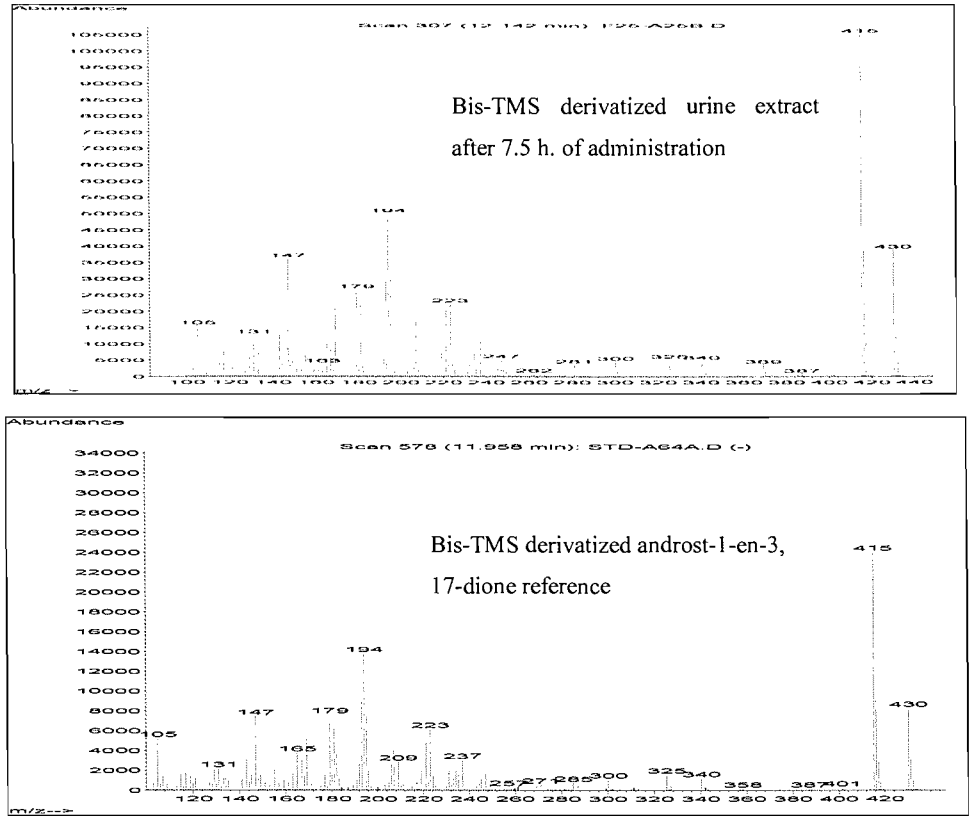

Fig. 8 Mass spectra of androst-1-en-3, 17-dione from urine and reference

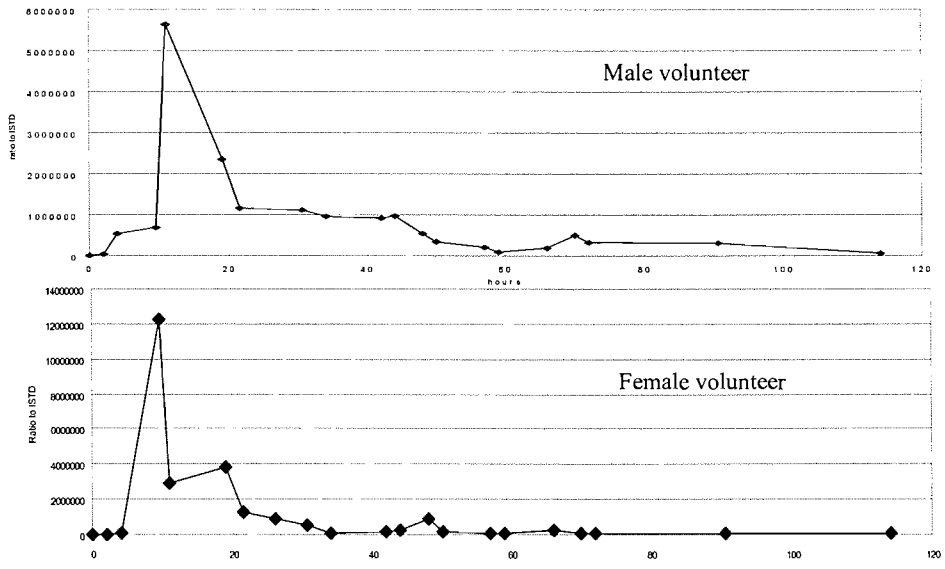

Fig. 9 Time curve of androst-1-ene-3,17-dione of the excretion study

methyltestosterone). In the urine samples from both volunteers, androst-1-ene-3,17-dione could be detected till, at least, about 100 hours after the administration. Most (about 90%) of thr androst-1-ene- 3,17-dione is in the free fraction.

Endogenous steroid profile: Based on the chemical structure of 1-testosterone, it looks reasonable that, when the double bound of Δ1 is reduced, it may be converted into 5α-dihydrotestosterone (DiHT) without some obvious effect on the T/epiT ratio. In fact, the T/epiT ratios of all the urine samples from the male volunteer were quite stable with a mean value of 0.222 with the standard deviation of 0.08 (n=22), while T/epiT ratios of the urine samples from the female volunteer were slightly increased after the administration (see Fig. 10). The concentration of DiHT after the administration did not obviously change but the ratio of 5α- to 5β-

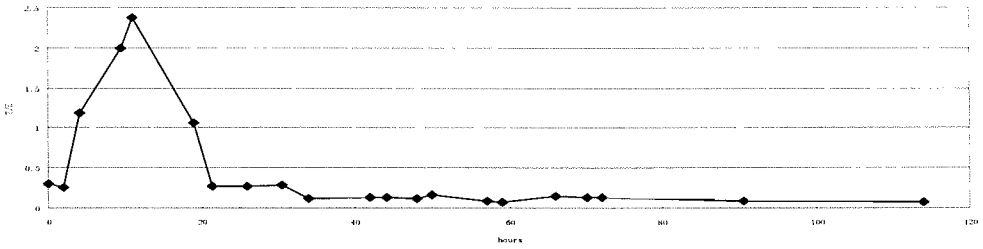

Fig. 10 The change of T/epiT ratio in the urine from the female volunteer

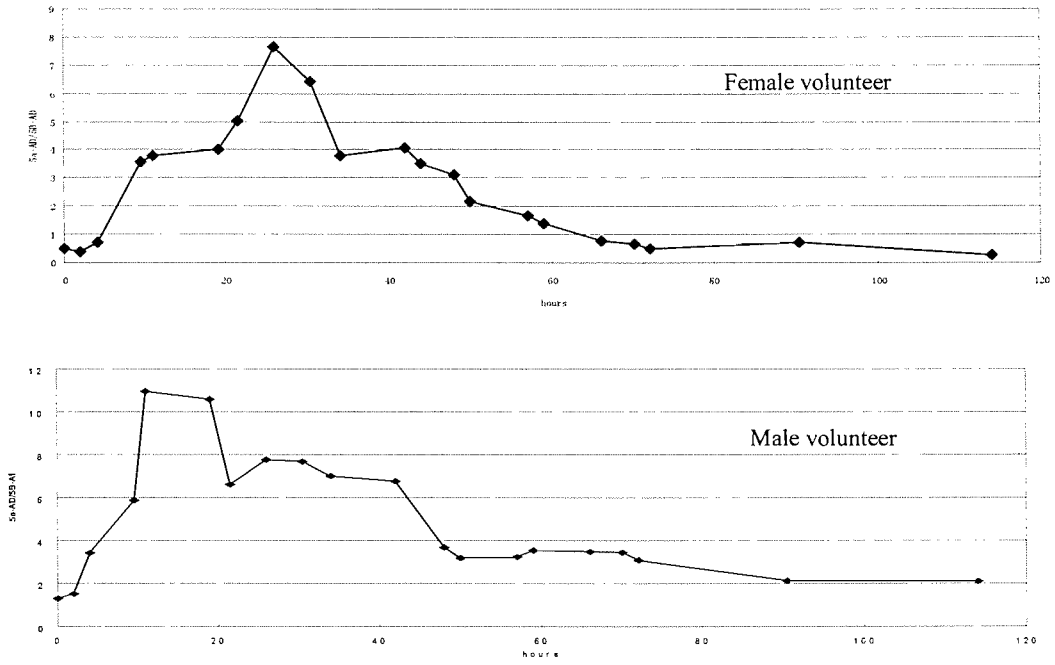

Fig. 11: Change of 5α- to 5β-androstanediol ratios in the urines from the volunteers

androstane-3α,17β-diol, as a marker for detecting doping with DiHT, was greatly increased after the administration, exceeding the criteria for an adverse result[5 - 7]. The maximum value of this 5α- to 5β-androstane-3α,17β-diol ratio from the male volunteer was 12 and from the female was 8. The values of this ratio in both urine samples exceeded the threshold value for

many hours (Fig. 11). From Fig. 7, 9, 11 and 12, it appears that the time curve for 5α- to 5β-androstanediol ratio reached its maximum later than in the other two cases and the type of this curve was much wider. Concentrations of 5β-androstanediol were not high, the maximum concentrations were about 140 ng/ml for the female and 50 ng/ml for the male volunteer, respectively. Maximum values of the ratio for androsterone to etiocholanolone were 9 for the female and 25 for the male volunteer /Fig. 12). The maximum concentrations of androsterone were 7000 ng/ml for the female and 3500 ng/ml for the male volunteer.

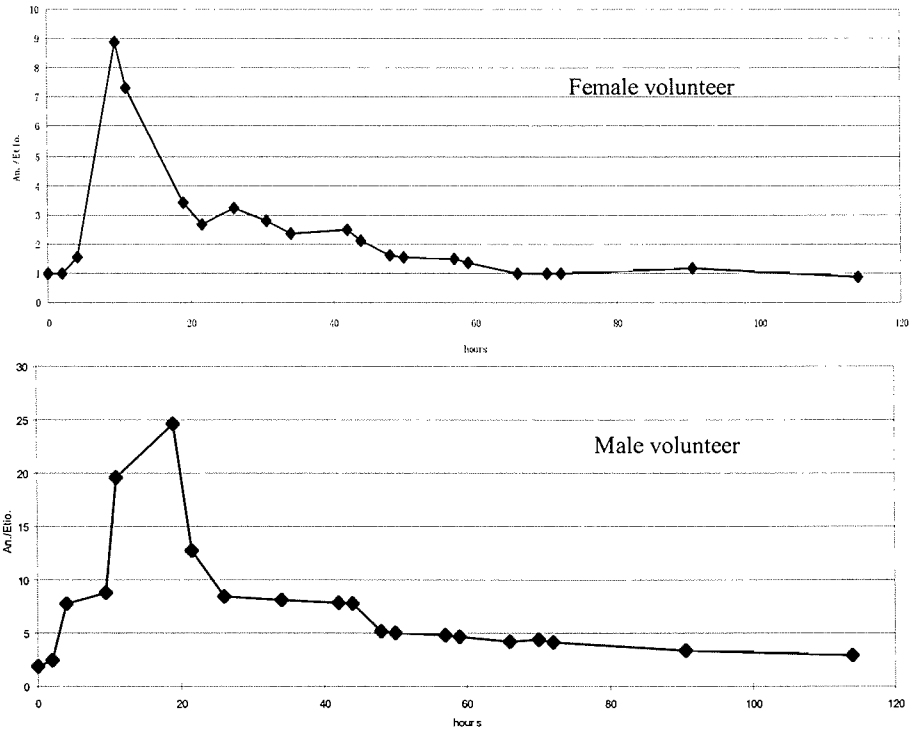

Fig. 12 The change of androsterone to etiocholanolone ratios in the urine from the volunteers

Unconfirmed metabolite: In urine samples both from the female and male volunteer a peak with the retention time of about 11.3 min was found. The mass spectrum showed the same molecular ion as 1-testosterone. The substance was tentatively assigned as 1-epitestosterone. Further work is in progress.

SUMMARY

The identity of synthesized 1-testosterone was confirmed with different analytical methods. An excretion study after oral administration was performed in two volunteers. The presence of some exogenous metabolites of 1-testosterone, including 1-testosterone, androst-1-ene-3,17-dione and another unidentified metabolite may be used as evidence for doping with 1-testosterone.The abnormal steroid profile (e.g. T/epiT ratio, 5α-/5β-androstanediol ratio, androsterone/etiocholanolone ratio) can be proof of extra evidence of doping with 1-testosterone. Due to the great variation in concentrations of endogenous steroids, androsterone, 5a-androstanediol etc. are not good markers for the administration of 1-testosterone. . After a single oral dose, some metabolites of 1-testosterone can be detected at least for about 100 hours.

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