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δvalues of Different Steroids**

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INTRODUCTION

Since isotope ratio measurement became an additional method to draw a "definitive conclusion" [1], some criteria were proposed and implemented in routine working for distinguishing the origin of the naturally occurring steroids. The criteria used in our routine are 1) δ value ratio of the targeted substance to the internal biomarker > 1.15 and/or 2) δ value difference of the targeted substance to the internal biomarker > 4[2]. Recently WADA published the technical document TD2004EAAS in the official website that became effective on Aug. 13, 2004 for the 2004 Athens Olympic Games. Following this technical document the report will be reported as consistent with the administration of a steroid when δ value measured for the metabolite(s) differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen and/or the δ value for the metabolite(s) is below –28 per mil based on the non-derivatised steroid[2].

It was confirmed that the δ value of 1-testosterone was about 19 per mil in our work. This persuaded us to study whether some other steroid preparations had the significantly different δ values from –28 per mil, which may effect the criteria for distinguishing the origin of the naturally occurring steroids detected in human urine, and what could be the reason for the very high δ values. First of all, we searched all available literatures through and found some different raw materials for steroids synthesis. Then we followed the synthetic procedures to analyze the raw materials with GC/MS and GC/C/IRMS step by step. Thirdly, some different steroids from different starting materials were analyzed with GC/MS and GC/C/IRMS. Finally, some urine samples from the excretion study of the steroid with high δ value were analyzed with GC/MS and GC/C/IRMS.

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In our paper different δ values of the steroids from different starting materials for the synthesis are reported and discussion regarding the aspects of routine work are presented. The synthesis procedures mostly followed the literatures[3-6].

EXPERIMENT

Urine Extraction Procedure: The urine sample of 10 ml was applied onto C18 column and eluted with methanol. The methanol phase was evaporated to dryness. To the residue 1 ml of buffer (pH 6.8) and 100 μl of β-glucuronidase from E.coli. were added and vortexed. The solution was incubated at 55°C for 3 hours, and extracted with n-heptane.

Hydrolysis of Steroid Esters: 0.6 mol/L KOH water solution was added to the extract. The solution was heated at 100°C for 0.5 hour then cooled down to the room temperature. The pH value of the hydrolyzed solution was adjusted to 6.8 with 1.2 mol/L HCl water solution then extracted with n-heptane.

Acetylation: The methanol phase was evaporated under gentle nitrogen. The residue was derivatized with 100 μl of pyridine : acetic anhydride (1:1) at 70°C for 0.5 hour. The derivatized sample was evaporated to dryness under nitrogen and resolved in 50 μl of n-hexane for injection into GC/C/IRMS. It is expected that the difference of δvalues introduced by acetylation could be reduced to a very minor range because only the ratio of δvalues of the targeted substance over the PT, the relative value of δper mil, is used for the criteria.

GC/C/IRMS Measurement: The δvalue measurements were carried on Finnigan Delta Plus Instrument (Finnigan, USA) coupled with a Hewlett-Packard (HP) 6890 gas chromatograph. A HP 1 column (30 m x 0.2 mm I.D. x 0.32m film thickness) was used with helium as the carrier gas (1.5 ml/min, room temperature, flow constant mode). The injector was set at 260℃ and the Ox. Reactor at 940℃. Split mode was used with a ratio of 1:5. The oven temperature program was: 180℃(1min) —5℃/min —310℃ (2min). Each sample was extracted twice and each extract injected also twice.

Calibration of GC/C/IRMS: The δ value of the standard carbon dioxide used as a reference gas was calibrated in our National Institute for Standard before being used in our laboratory.

GC/MS: Agilent 6890A/HP5973 was for this experiment. The column used was HP-5 25 m (0.2 mm i.d., 0.33 μm film-thickness) with the head pressure of 100 Kpa. The oven

temperature program was: initial temperature: 80°C (1 min) – 20°C /min → 240 °C – 15 °C /min → 280°C (12 min). The injector temperature was 250°C and transfer line 280°C. The split ratio was 10:1. In EI mode with 70 eV the MS was used in scan mode from 50 to 500 amu. in 0.25 sec.

RESULTS AND DISCUSSION

Identification of the substance: 3β-acetoxyprog-16-en-20-one: The following Fig. 1 is the flowchart for the synthesis of some steroids from tigogenin (hemp). The chromatogram and mass spectrum of 3β-acetoxyprog-16-en-20-one, as a starting materisl, are showed in Fig. 2. From this figure its purity and structure can be identified.

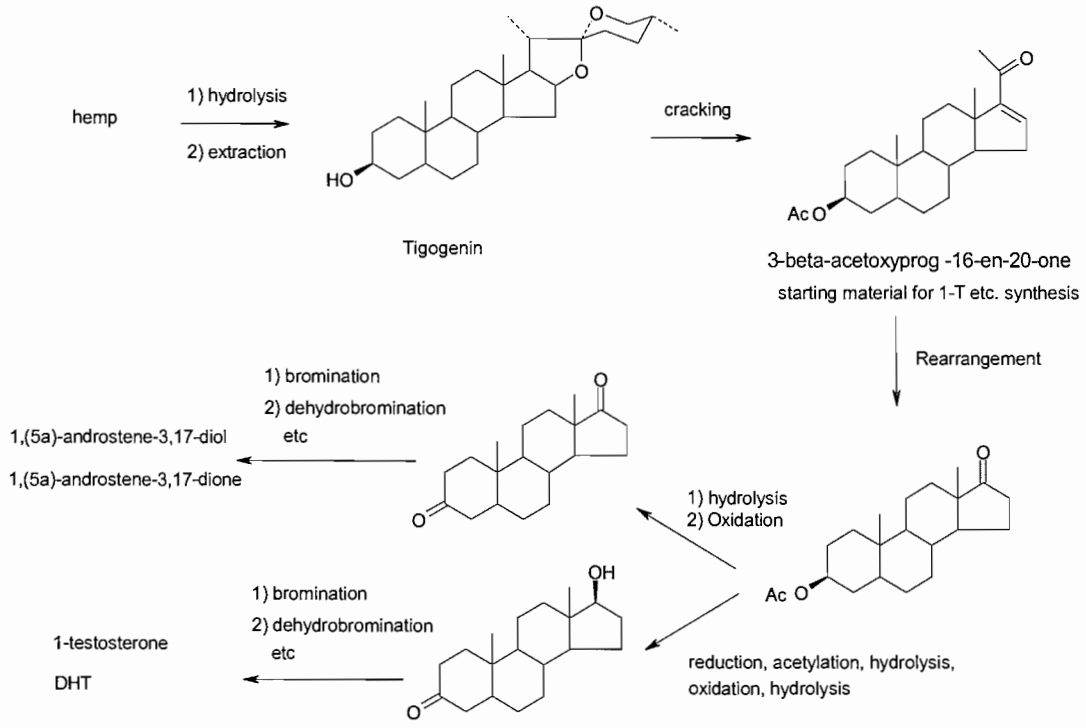

Fig. 1 The flowchart for synthesis of steroids from hemp

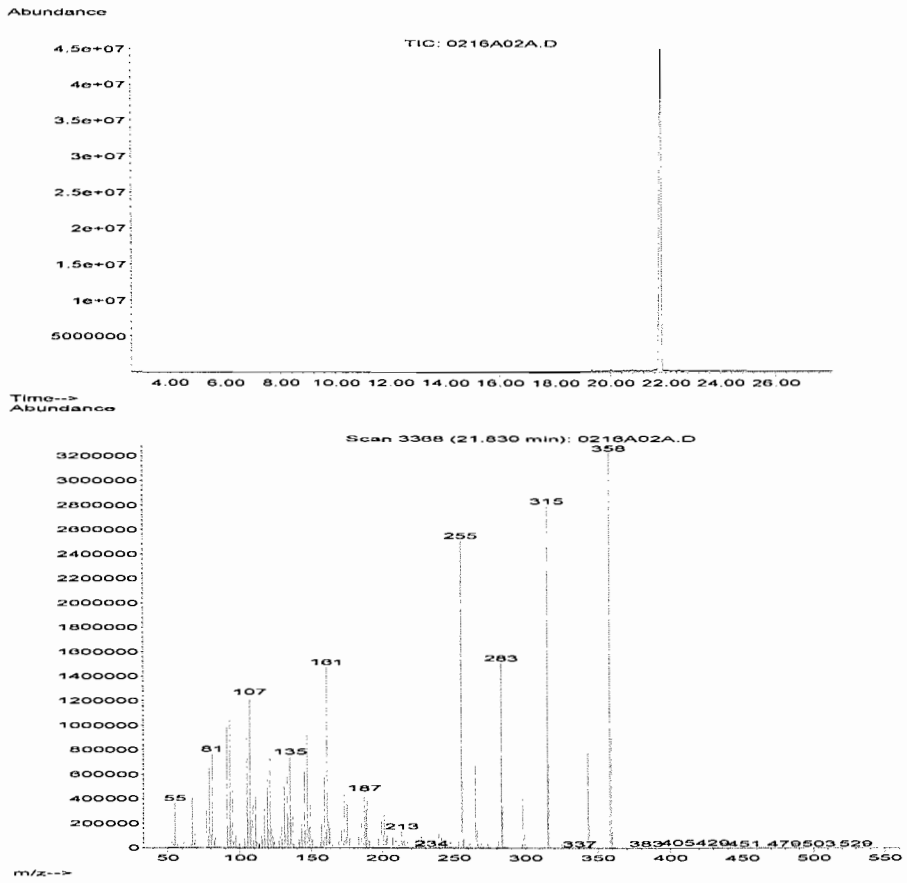

Fig. 2 The chromatogram and mass spectrum of 3β-acetoxyprog-16-en-20-one

5α-Androst-1-ene-3β, 17β-dione and 5α-Androst-1-ene-3β, 17β -diol acetate: As showed in Fig. 1, 5α-Androst-1-ene-3β, 17β-dione was obtained from 3β-acetoxyprog-16-en-20-one. The chromatogram and mass spectrum of 5α-Androst-1-ene-3β, 17β-dione are demonstrated in Fig. 3, which confirmed the structure of the substance. Further more, the result of database searching also confirmed its structure (see Fig. 3 also). The mass spectrum in Fig. 4 confirmed the structure of 5α-Androst-1-ene-3β, 17β -diol acetate.

3β-acetoxyproga-5, 16-dien-20-one: The following Fig. 5 is the flowchart for the synthesis of some steroids from diosgenin (steroidal saponin). The chromatogram and mass spectrum of 3β-acetoxyprog-5, 16-dien-20-one, as a starting materisl, are showed in Fig. 6. From this figure its purity and structure can be identified.

Nutrition supplements with 1-testosterone: In their labels: (1) Molecular Nutrition produced a supplement containing 17β-hydroxyandrost-1-ene-3-one THP ether (Tetra- hydropyranyl), 1-2 caps./time, 2-3 times/day; (2) Vital Pharmaceuticals, Inc. provided also a nutrition containing 17β-hydroxy-5α-androst-1-ene-3-one Cypionate ether (CYP), 1 to 3 cc's per day; (3)

Chemi-Sport Laboratories offered 5-androst-1-ene-3- one-3β-undecanoate (UND), 1-2 softgels 2-3 times daily. All these three kinds of 1-testosterone esters were extracted and analyzed with GC/MS. The identified structures were consistent with that declared in their labels. After hydrolysis, 1-testosterone became free from its esters. All these 1-testosterone from nutrition supplement extracts were confirmed with GC/MS .

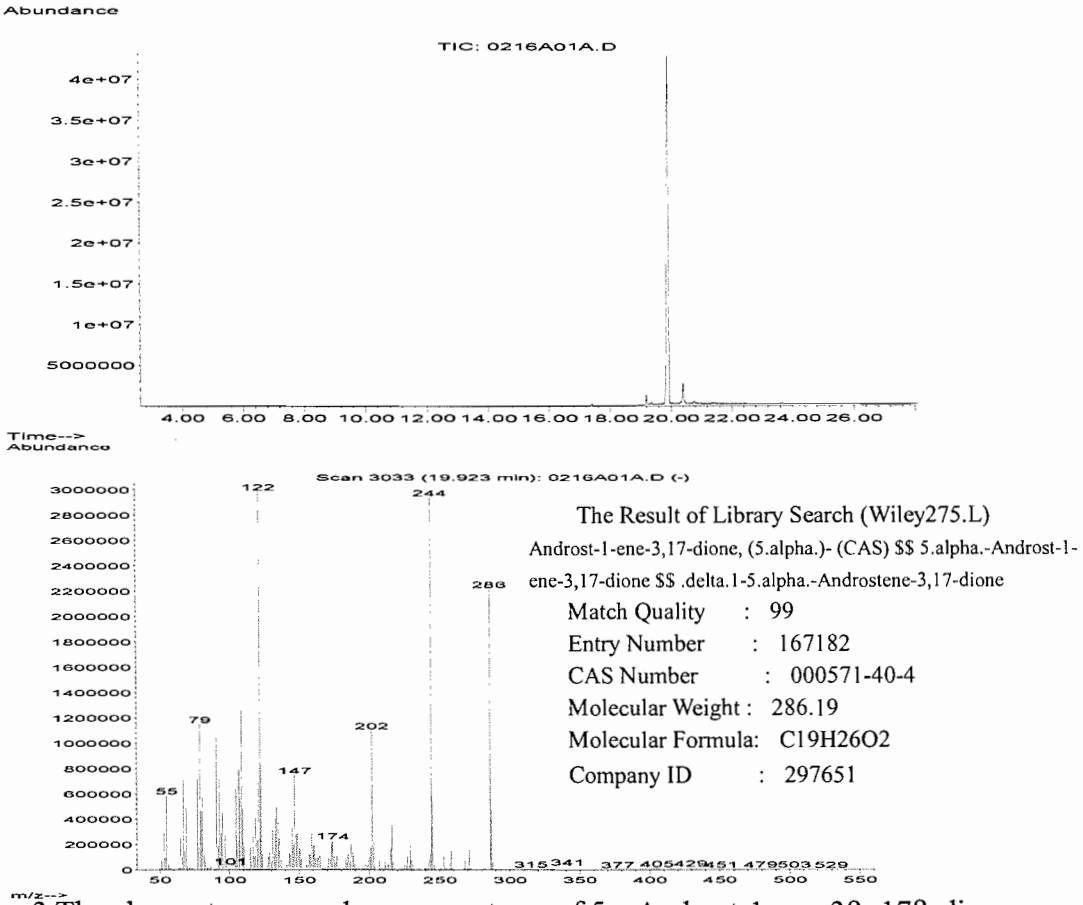

Fig. 3 The chromatogram and mass spectrum of 5α-Androst-1-ene-3β, 17β-dione

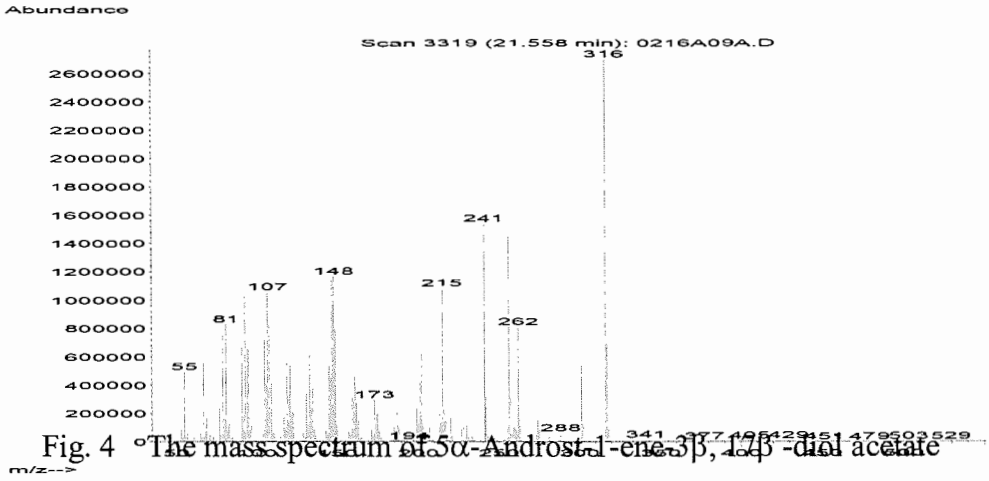

Fig. 4 The mass spectrum of 5α-Androst-1-ene-3β, 17β -diol acetate

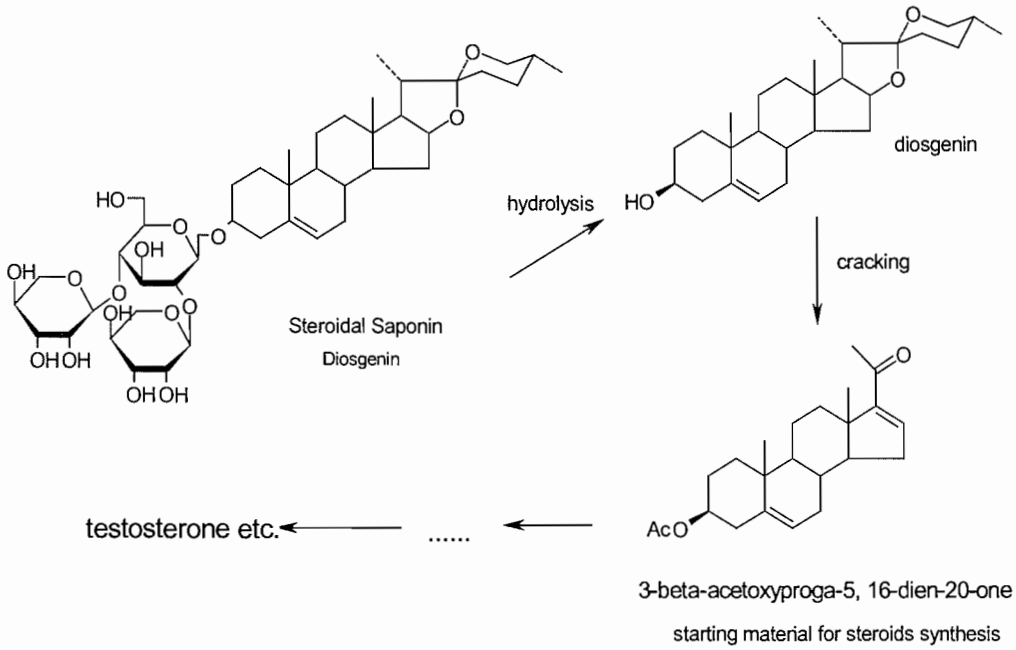

Fig. 5 The flowchart for synthesis of steroids from Saponin

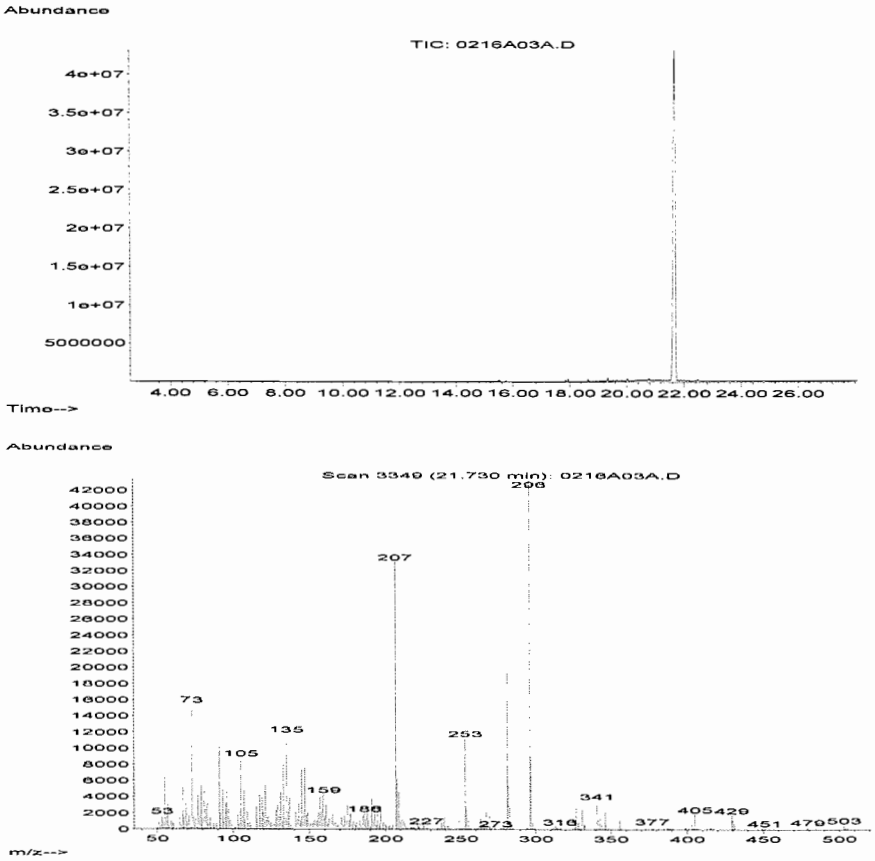

Fig. 6 The chromatogram and mass spectrum of 3β-acetoxyprog-5, 16-dien-20-one

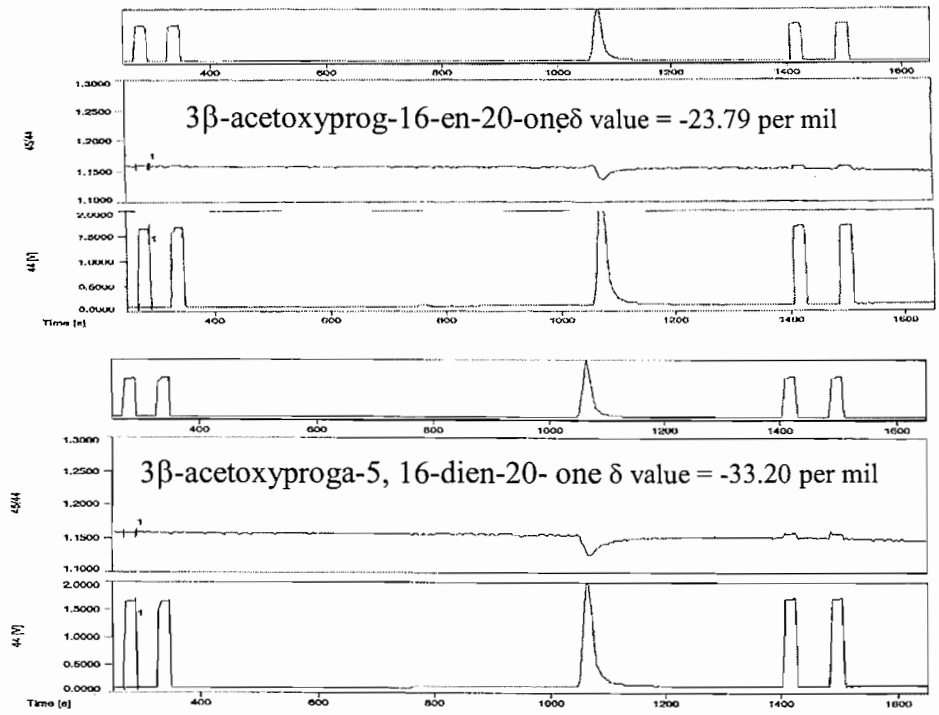

Fig. 7 GC/C/IRMS spectra and δvalues of the starting materials

Isotope measurements

Starting materials: The following Fig. 7 shows the GC/C/IRMS results for these two starting materials, 3β-acetoxyprog-16-en-20-one and 3β -acetoxyproga-5, 16-dien-20- one for steroid synthesis with different δvalues, which are listed in their GC/C/IRMS figures respectively. It is reasonable that some steroids (for example 1-testosterone and testosterone) must have different δvalues because they are from different starting materials that are obtained from different botanic resources containing different δvalues.

δ values of 1-testosterone: Three 1-testosterone ester standards, 1-testosterone hydrolyzed from nutrition supplements, 1-testosterone standard and 1-testosterone ethylcarbonate were analyzed and confirmed by GC/MS and their δvalues were obtained with GC/C/IRMS. The results of are listed in Tab. 1.

Tab. 1 δvalues of 1-testosterone and preparations

Substance	δ value in per mil (without acetylation)
1-T from 1-T CYP in NS	-19.06
1-T from 1-T THP in NS	-18.67
1-T from 1-T UND in NS	-21.04
1-T Standard	-20.54
1-T CYP Standard	-21.08
1-T THP Standard	-15.85
1-T UND Standard	-23.26
1-T –ethylcarbonate Stand.	-19.50

NS: nutrition supplement

δ values of some endogenous steroids after administration with 1-testosterone: A male volunteer, 53 years old, 83 kg and 169 cm high, administered 120 mg of 1-testosterone orally. The urine samples were collected before and after the administration and analyzed with both GC/MS and GC/C/IRMS. The δ values of some endogenous steroids are listed in Tab. 2. For comparison, some δ values of our routine urine samples with adverse findings are also listed in Tab. 2. It is clearly confirmed that the δ values of some endogenous steroids, such as the metabolites of testosterone, were strongly affected by the steroids administered, which contained different δ values due to the different starting materials for synthesis.

Some other steroids from tigogenin: The following Tab. 3 shows the δ values of other steroids from tigogenin. All of these data demonstrated the δ values much higher than "normal" δ values for steroid preparations.

CONCLUSION

From the data presented in this paper the following points may be concluded:

1) From different starting materials steroids may have very different δvalues, some of which are not significantly different from that of naturally produced steroids.

2) The administration of some precursors or preparations of endogenous steroid could not be

excluded only based on the δvalues. It should be very careful and needs further evidence to make the conclusion for confirming no exogenous origin.

3) This result reported here does not mean any peradventure for concluding the administration of some precursors or some preparations of endogenous steroid with the criteria (Ratio > 1.15 and/or Difference > 4).

4) The wording, "The results of such analyses will be reported as "inconclusive" unless the ratio measured for the metabolite(s) is below –28 per mil based on non- derivatised steroid", may need to be supplemented with another value, not only below –28 per mil but above a certain value also.

5) Recently some other resources for testosterone, such as microbial production[7], came into the world, more careful studies on isotope ratio measurement have to be carried out.

Tab. 2 the δ values (in per mil) of some endogenous steroids

Time after Ad.	Androsterone	Etiocholanolone	5α-AD	Pregnenetriol
0 hour	-26.18	-26.65	-26.12	-25.68
7 hours	-26.25	-26.90	-27.46	-26.03
12 hours	-24.43	-26.48	-26.55	-26.45
Standard	-35.06	-25.46	-37.41	-26.70

Code	Androsterone	5α-AD	Pregnenetriol	Remark
IOC2002 10-4	-31.29	-28.89	-26.67	T/E > 6
01111512	-29.23	--	-25.40	T: -32.47
03120105	-36.51	-36.93	-27.71	Etio.: -36.93

Tab. 3 The δ values of some other steroids

Chemical name of the steroids	δ values in per mil
5α-Androst-1-ene-3β, 17β-dione	-20.52
5α-Androst-1-ene-3β, 17β -diol acetate	-20.01
Dihydrotestosterone (DHT)	-20.40

REFERENCE

1) Olympic Movement Anti-Doping Code 1999

2) WADA TD2004EAAS

3) J. Biol. Chem., 162, 601(1946); 166, 345(1946)4) Helv. Chin. Acta, 30, 1037, 1256(1947); 26, 562, 705, 721(1943); 27, 821(1944)5) U.S.pat. 3,030,358(1962); 2,403,683(1946)6) J. Am. Chem. Soc., 83, 1478(1961); 81, 427, 1513(1959); 74, 1871, 2126, 4974(1952); 71, 2443(1949);70, 1454, 882(1948)

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