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Distinguish Endogenous and Exogenous Epitestosterone Using GC/C/IRMS with TLC Purification

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Abstract

Epitestosterone is banned by the IOC as a masking agent. A urine sample with a concentration of epitestosterone over 200 ng/ml should be considered as an offence and followed by investigations using the procedures for the case with T/E > 6. But how to deal with the analytical results of investigation is not clear. According to the IOC rules the isotope ratio measurement could be used to draw a definitive conclusion.

This paper presents a GC/C/IRMS method with TLC purification for the urine extract. After hydrolysis with β-glucuronidase the urine samples were extracted with n-heptane in the presence of solid buffer. The residue of the extract was divided into two parts, one part was for TLC purification (chloroform:acetone = 90:10) of Androsterone and Etiocholanolone, after acetylation of the other part, TLC (ethyl acetate:toluene = 60:30) was used to purify testosterone, epitestosterone and pregnanetriol. After the purification with TLC all residues of the spot were dissolved in 50μl n-hexane of which 2 μl was injected into GC/C/IRMS and the delta ‰ values were measured.

1. Introduction

In the Olympic Movement Anti-doping Code it is so described for epitestosterone case that in Chapter II Article 4:3. "An epitestosterone concentration in the urine greater than 200 nanograms per milliliter will be investigated by studies as in Article (I.C.I b) of Appendix B for testosterone." and that in Appendix B..."MASKING AGENTS: bromantan, diuretics (see above), epitestosterone, probenecid, ...".

Based on the Anti-doping Code for dealing with epitestosterone cases we should answer two questions: 1) How will the conclusion be drawn from the investigation - Should the statistics be based on T/E ratios or on the concentrations of epitestosterone or other

endogenous substance? 2) How will the conclusion be drawn with the isotope ratio measurement - Which substance δ‰ values are to be measured and how to purify the samples?

In this paper we present a sample preparation method with TLC (thin layer chromatograph) for purification and two epitestosterone cases as examples of application of this method. Combined with TLC purification and GC separation, androsterone (A), etiocholanolone (Etio), testosterone (T), epitestosterone (E), 5α-androstane-3α, 17β-diol (5α-diol), 5β-androstane-3α, 17β-diol (5β-diol), DHEA and other endogenous were separated very well. The routine screen procedure IV was used for estimating the T/E ratios and the concentrations of epitestosterone.

2. Experimental

2.1 Urine samples

Blank urines were obtained from volunteers who did not take any medications 7days before and during the time urine samples were collected. Some urine samples were from doping control routine performed in our laboratory.

2.2 Reagents

The following reagents and standards were obtained from Sigma (St. Louis, MO, USA): testosterone, epitestosterone, androsterone, etiocholanolone, pregnanetriol (PT), and β-glucuronidase from E. coli. (125,000 units). 5α-Androstane-3α, 17β-diol, 5β-androstane-3α, 17β-diol and DHEA were obtained from Steraloids Inc. (Wilton, NH, USA).

Solid phase extractions were carried out on the home made columns filled with reverse phase octadecyl silica from Unican (Switzerland). All solvents and other reagents for solid buffer were of analytical grade purity.

TLC plates (20 x 20 mm) were Silica GF254 with 0.20-0.25 mm thickness and made by Qiangdao Chemicals, China

2.3 Instruments

2.3.1 GC-MS analysis for checking the purity of the prepared samples

GC/MS analyses of the standard solution of acetylated epitestosterone and the acetylated epitestosterone from the purification of the extracted urine samples were performed on a TSQ-7000 GC/MS/MS (Finnigan, USA) with a Hewlett-Packard (HP) 5890 series II plus gas chromatograph and a HP-1 fused-silica capillary column (17 m x 0.25 mm I.D. x 0.11 μm film thickness). The oven temperature was as our routine procedure IV (180°C—3.3°C/min→231°C—30°C/min→310°C for 2 min). Samples (1μl) were injected in the split mode (10:1) using helium as carrier gas with a flow rate on column of 0.8 ml/min at 180°C.

2.3.2 GC-C-IRMS analysis for measuring the δ‰ values

The δ‰ value 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 0.32 mm 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°C and the Ox. Reactor at 940 °C. Split mode was used with a ratio of 1:5. For separating the acetylated testosterone and epitestosterone the oven temperature program was: 180°C—3.3°C /min→231°C— 30 °C/min→310°C (5min) and for free androsterone and others: 180°C (1 min)—5°C /min→310°C (2 min). Each sample was extracted twice and each extract injected also twice.

2.4 Samples extraction for GC-C-IRMS

Urine sample 5 ml was applied onto the C18 column then eluted with methanol. The methanol phase was evaporated to dryness using a rotary evaporator. To the residue 1 ml of buffer (pH 6.8) and 100 μl of β-glucuronidase from E. coli. (125, 000 units in 3 ml buffer, pH 6.8) were added and vortexed. The solution was kept 37°C for deconjugation overnight. The deconjugated sample was extracted with n-heptane. The n-heptane was evaporated to about 50 μl then applied to TLC silica (F 254) plate for purification.

2.5 TLC purification and acetylation

After the prepared sample and a standard mixture were applied onto the TLC plate, the plate was developed with a solution of Chloroform : Acetone (90 : 10) for about 12 – 14 cm. The standard mixture was spotted both on the left and right side of the plate and the samples between the standard and 2.5 cm was between each spot.

After TLC development, two columns with the standard were cut off for color reaction. The two glass plates with the developed standards were sprayed with the color reagent (sulfuric acid : methanol 1:1) and kept 100°C for 10 min. The colors of all endogenous steroids were light violet. Testosterone and epitestosterone showed spots visible under the ultra-violet lamp on the F 254 plate. The two visualised glass plates with the standards were put back together with the remaining non-visualised glass plate. The areas for different substances were marked with pencil based on the positions of different standards. All silica in the marked areas was scratched off and eluted with 3ml methanol. The methanol phase was evaporated under gentle nitrogen. The residue was derivatized with 100μl 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 70 μl of n-heptane for injection into GC-C-MS.

3. Results and Discussion

3.1 Separation of steroids by TLC

Some different solvent systems were used for separation of common endogenous steroids. From Table 1 and Fig.1 it can be seen that acetylated testosterone and epitestosterone can be separated from other common endogenous steroids using the solvent mixture ethyl acetate : toluol 2:1. Acetylated testosterone, epitestosterone and 5α-androstane-3α, 17β-diol are well separated by GC-MS.

From Fig.2 and Table 2 it was found that the non-derivatised common endogenous are well separated by chloroform : acetone = 9:1. Comparison of the thin layer chromatograms of acetylated and non-derivatized endogenous steroids, the solvent system of chloroform : acetone (9:1) for non-derivatized endogenous steroids was selected for purification of urine samples, which were suspicious for doping with epitestosterone.

Table 1 R_f values of acetylated endogenous steroids

Substance	Ethyl acetate:toluol 2:1
Androsterone	0.91
Etiocholanolone	0.91
5α-diol.	0.93
5β-diol	0.93
DHEA	0.92
pregnantriol	0.90

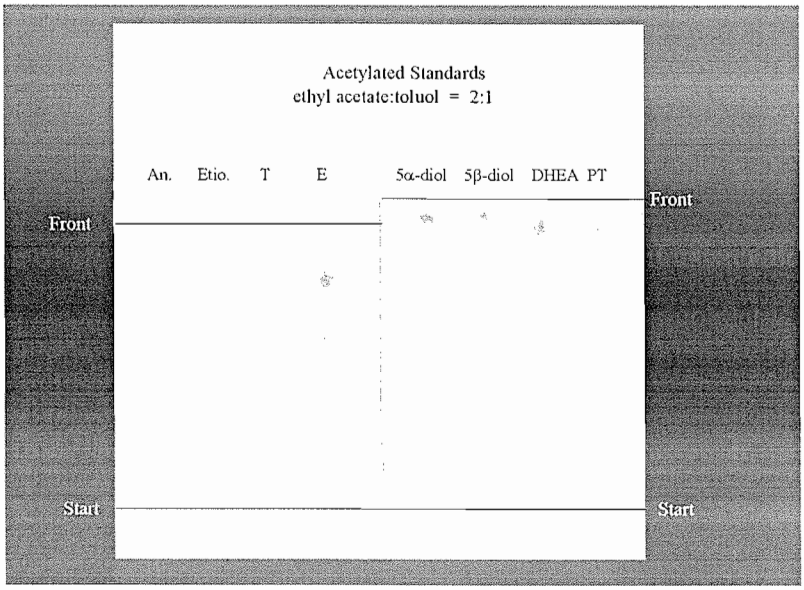

Fig.1 Thin layer chromatogram of acetylated endogenous steroids

Table 2 R_f values of some common endogenous steroids

Substance	chloroform : acetone (9:1)
Androsterone	0.69
Etiocholanolone	0.58
5α-diol.	0.49
5β-diol	0.34
DHEA	0.71
pregnantriol	0.04

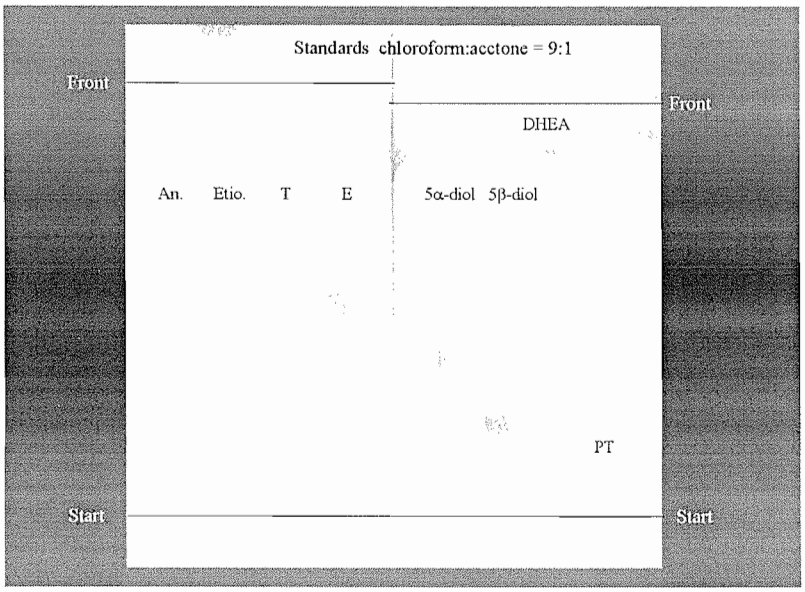

Fig. 2 Thin layer chromatogram of some common endogenous steroids

3.2 The purity of epitestosterone after TLC purification

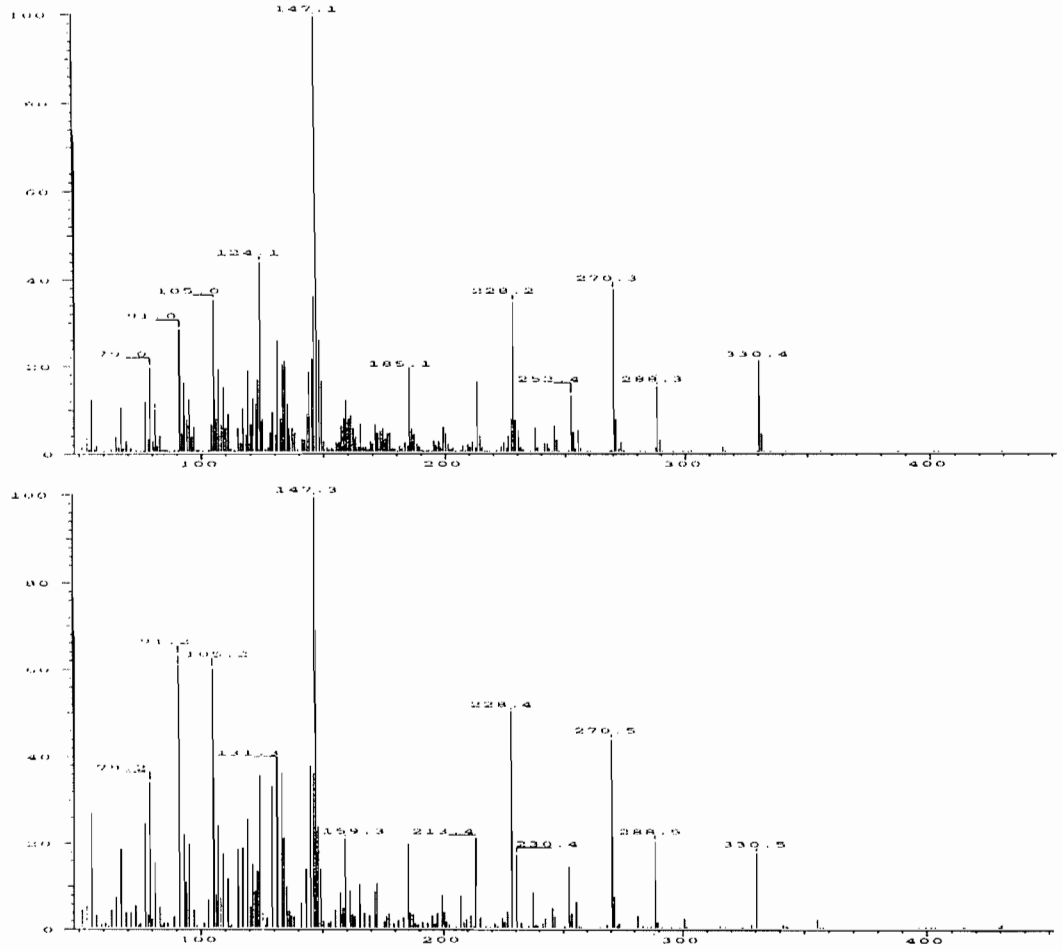

Fig. 3 The mass spectra of acetylated epitestosterone standard (top) and one from urine sample after TLC purification (bottom)

The epitestosterone purified by TLC was checked by GC-MS and showed identical mass spectrum to that of the standard. These two mass spectra were showed in Fig. 3.

The GC/C/IRMS showed also very purified isotope ratio chromatograms, which were presented in the Fig. 4.

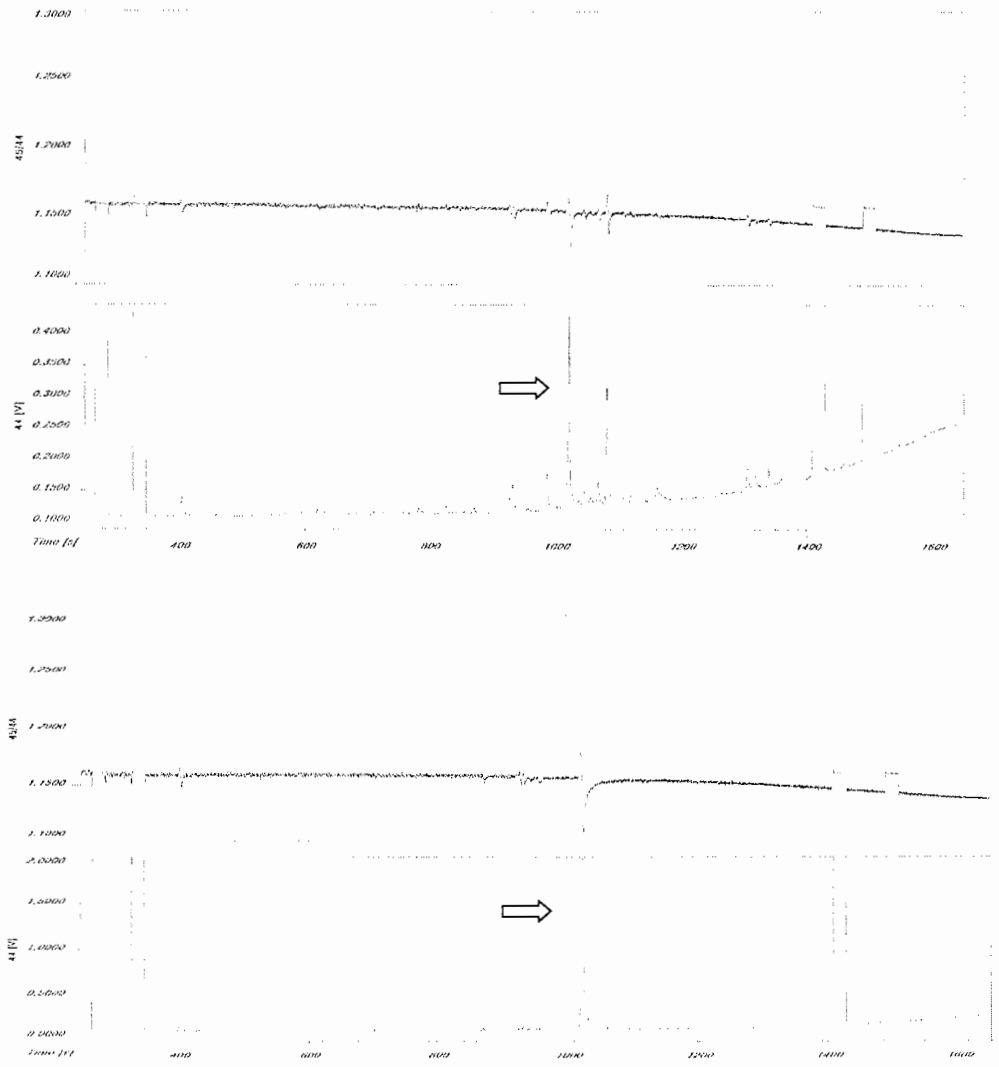

Fig. 4 Isotope ratio chromatograms of acetylated epitestosterone standard (lower) and one from urine sample after TLC purification (top)

3.3 Variation of isotope measurement with TLC purification

The variation of GC/C/MSD with TLC procedure for Standards was tested and listed in the following Table 3.

Table 3 The variation of isotope measurement with TLC procedure: 1: In a day, 2: Between the days, 3: Different the amount of acetylated epitestosterone injected on the column in a day.

1

Substance	Mean Value	Standard Deviation	n
Acetylated Epitestosterone	- 36.30	0.126	8
Androsterone Sdandard	- 33.87	0.238	8

2

Substance	Mean Value	Standard Deviation	n
Acetylated Epitestosterone	- 35.88	0.540	8

3

Substance	Mean Value	SD	Ampl. Range
Acetylated Epitestosterone	- 36.06	0.45	from 0.150 to 3.17 V (5 points)

From the Table 3 the following points could be found: 1) even with TLC purification procedure, the isotope measurements showed very good reproducibility; 2) in a very wide range the δ‰ values were almost identical. The values of 0.150 to 3.17 V corresponded about 70 ng to 1500 ng acetylated epitosterone injected on the column in a day. The 0.150 V was set by our laboratory as the minimum response, lower than which the peak would not be accepted.

3.4 Case 1

A concentration of epitestosterone over 200 ng/ml in a urine sample was notified by an International Federation, which carried out further investigation for the case. On the request of the National Federation further urine samples of the athlete concerned were collected under control. Some of these samples showed higher concentration of epitestosterone (over 200 ng/ml). The method presented here was used to determine the δ‰ values of epitestosterone and the results were listed below.

Table 4. δ‰o of Case 1

Resource	δ‰o	
	Epitestosterone	Pregnanetriol
Urine Sample	-26.67	-26.40
Standard	-35.37	-26.46

The δ‰o values of epitestosterone and pregnanetriol showed no evidence of exogenous application of epitestosterone in this case. Later the International Federation received further analytical results using GC/C/IRMS from other IOC accredited laboratories and concluded that the higher concentration of epitestosterone was naturally produced. The case was closed.

In the Table 5 and Table 6 the endogenous steroid profiles of further samples from the same athlete under controlled conditions are listed. The concentrations varied greatly but the value of the T/E ratio was constant. It was suggested that the longitudinal criteria for the investigation of elevated T/E ratios could be quite a good marker for such elevated concentration of epitestosterone.

Table 5 Variation in a Day of the Concentrations of Endogenous Steroids from the Athlete Concerned in Case 1 (in ng/ml)

Time of Collection.	pH	SG	T	E	T/E	Exercise
10:00	5.5	1.030	62.2	226.2	0.27	After 1.5 hr. Exercise
15:20	6.5	1.020	58.2	180.5	0.32	Before afternoon training
20:25	6.5	1.010	16.2	50.5	0.32	Before go to bed

Time of Collection.	An.	Etio.	5α-diol	5β-diol	11β-OH-An	11β-OH-Etio.
10:00	3240	4205	93.8	234.7	852.3	571.4
15:20	3313	3598	91.9	201.3	1112	625.7
20:25	859	854	19.5	43.8	301.1	139.9

Table 6. Variation between Days of the Concentrations of Endogenous Steroids from the Athlete Concerned in Case 1 (in ng/ml or mIU/ml)

Day of Collection.	Time of Collection.	pH	SG	T	E	T/E
Day 1	18.00	6.5	1.010	1.9	6.3	0.32
Day 4	13:10	6.5	1.020	125.5	500.3	0.26
Day 5	12:05	6.0	1.025	85.3	338.9	0.26
Day 6	15:45	6.0	1.025	61.4	279.6	0.23
Day 7	13:15	7.0	1.015	161.8	37.3	0.24

Day of Collection.	An.	Etio.	5α-diol	5β-diol	hCG	LH
Day 1	582	327	23.6	17.4	< 2	5.1
Day 4	4619	4072	149.4	291.2	< 2	0.58
Day 5	4344	3810	101.2	186.6	< 2	0.91
Day 6	4295	3614	84.7	127.9	< 2	0.96
Day 7	1649	1405	56.1	100.7	< 2	3.25

3.5 Case 2

In this case a concentration of epitestosterone over 600 ng/ml in the urine sample was found. The delta ‰ values of epitestosterone and some other endogenous substances were measured and are listed in the Table 7 and 8. The δ‰ ratio of acetylated epitestosterone to preganetriol was 1.31 which was much higher than our criteria of 1.15. The results showed that this elevated epitestosterone concentration was due to exogenous administration. Based on the isotope ratio measurement the final report was issued without further investigation. In hearing the athlete concerned admitted that she had used a kind of preparation to help hair growth. In the patent of this product epitestosterone was an ingredient.

Table 7. δ‰ of Case 2

Resource	δ‰	
	Epitestosterone	Pregnanetriol
Urine Sample	-34.66	-26.46
Standard	-35.37	-26.37

Table 8 The steroid profile of case 2 (in ng/ml)

ng/ml	An	Etio.	5α-diol	5β-diol	OH-A.	OH-Etio.	E	T
Mean	2183	2371	58.2	192.5	580.4	208.4	619.	75.7
Std.	21.4	25.0	2.4	3.8	3.3	5.2	7.3	1.0

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