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Androsterone, 5α-Androstandiol and Testosterone for GC/C/IRMS ----- Discussion with Case Analysis

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INTRODUCTION

Normally androsterone was used for isotope ratio measurement because it is the metabolite from a lot of endogenous steroids and is easier to be detected due to its high concentration. Testosterone could be direct metabolized to 5α-Androstandiol through 5α-dihydrotestosterone.

Androsterone (An), 5α-Androstandiol (5α-AD) and Testosterone (T) were analyzed with GC/C/MS for some cases using Pregenentriol (PT) as an internal marker. All data reported in this paper are the results in our routine. Based on the reference to the Sydney Laboratory for Olympic Games 2000, the criteria for the evidence of administration of a steroid preparation that may occur naturenally are the ratios of δ‰ values of one endogenous steroid to PT greater than 1.15. In our laboratory the criteria for a peak to be recognized are the signal of it greater than 0.15 V because the variation becomes larger if the response of the volts is too small. Our main idea is: the more larger than the criteria, the better sensitivity of the data. All cases reported in this paper are selected from our routine results during the last two years. In some cases, the ratios of δ‰ values of T/PT were greater than 5α-AD/PT, which were lager than An/PT. But in other cases, T and/or (5α-AD) could not be detected due to its low concentration. In most cases, An/PT showed the lowest sensitivity.

The aim of this paper is focused on discussion whether An is a suitable marker in our routine and which one is the first choice for GC/C/MS.

EXPERIMENTAL

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 ml 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.

[1] The corresponding author

TLC Purification

The layer of the n-heptane phase was concentrated then applied onto a TLC plate. On the left side of the same plate, a standard mixture was spotted. The plate was developed with 50 ml of Chloroform : Acetone (9:1 v/v) . After developing, the plate was separated and the standard mixture part was colored with sulfuric acid. The R_f values were calculated. On another part of the plate, every target part of silica was scratched off and eluted with 3 ml methanol.

Acetylation

The methanol phase was evaporated under gentle nitrogen. The residue was derivatized with 100ml 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-MS. It is expected that in fact the acetylation introduces some shifts for the δ‰ values of androsterone, testosterone, PT and so on, but the ratio of them could only be affected by the acetylation feebly.

GC/C/IRMS Measurement

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 0.32m 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 and each extract injected both twice.

Calibration of GC/C/IRMS

The δ‰ value was the standard symbol for results of isotope ratio measurements and defined as:

$$Rx(\delta 13C\%o) = \frac{R_{sample} - R_{PDB}}{R_{PDB}} x1000$$

Where PDB was an international standard (calcium carbonate from a fossil, *Belemnitella Americana*) obtained from the Pee Dee formation in South Carolina and R_x refers to the $^{13}C/^{12}C$ of the sample or international standard. ‰ was per mil. The δ‰ value of the standard carbon dioxide used as a reference gas was calibrated in the National Institute for Standard based on the formula mentioned above.

RESULTS

The following pictures show the major results of the GC/C/IRMS measurements, such as the background, the peak shape, the chromatograms of standards and the substances extracted from urine samples (urine from excretion study or from routine samples) etc. respectively. No significant effect from the matrix was found. The reproducibility for all measurements was quite good and reported in our previous paper.

The variation of the δ‰ values in a day is listed in the following tables:

Satndards	An.	Etio.	5α-diol	5β-diol	DHEA	PT	T	E
Level (μg/μL)	0.500	0.500	0.500	0.500	0.500	1.000	0.500	0.500
Mean of δ‰	-36.68	-26.56	-37.50	-36.50	-34.40	-21.80	-31.06	-37.41
SD of δ‰	0.35	0.22	0.22	0.44	0.52	0.35	0.41	0.14
cv%	0.12	0.05	0.05	0.20	0.27	0.12	0.17	0.02

In the period of several months, under the same chromatographic and mass spectrometric conditions, the δ‰ showed the coefficient of variation in a very small range listed in the following table.

Substance	An.	T.	PT
Mean of δ‰	-35.67	-31.03	-20.35
SD of δ‰	0.45	0.60	1.17
cv%	0.20	0.36	1.36

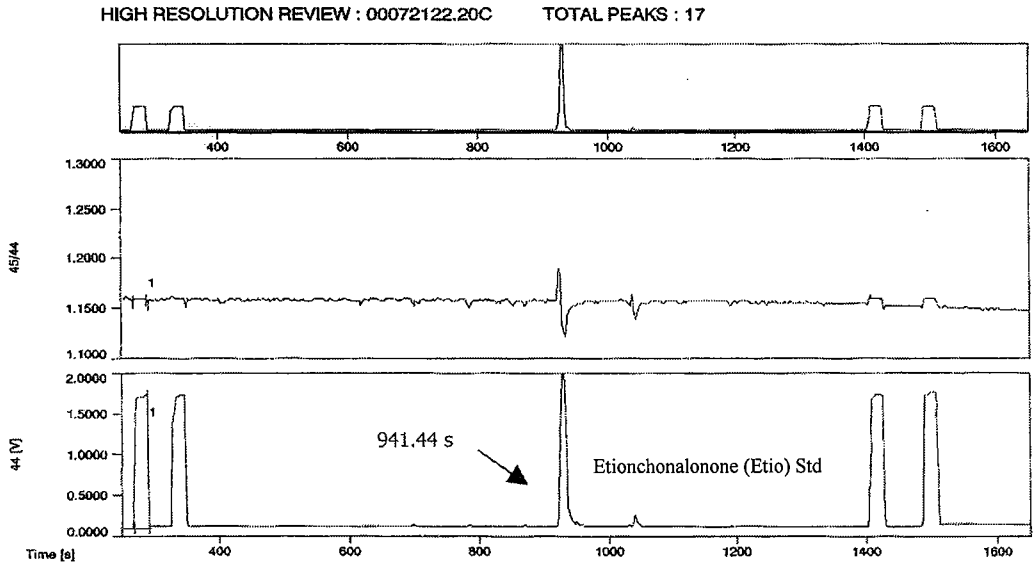
DISCUSSION WITH CASES

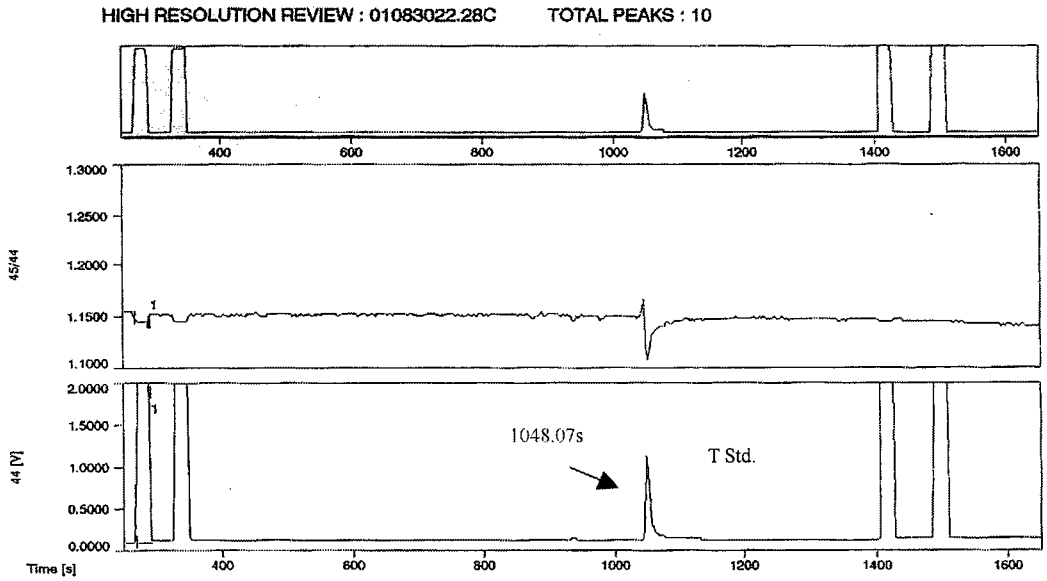
Case 1

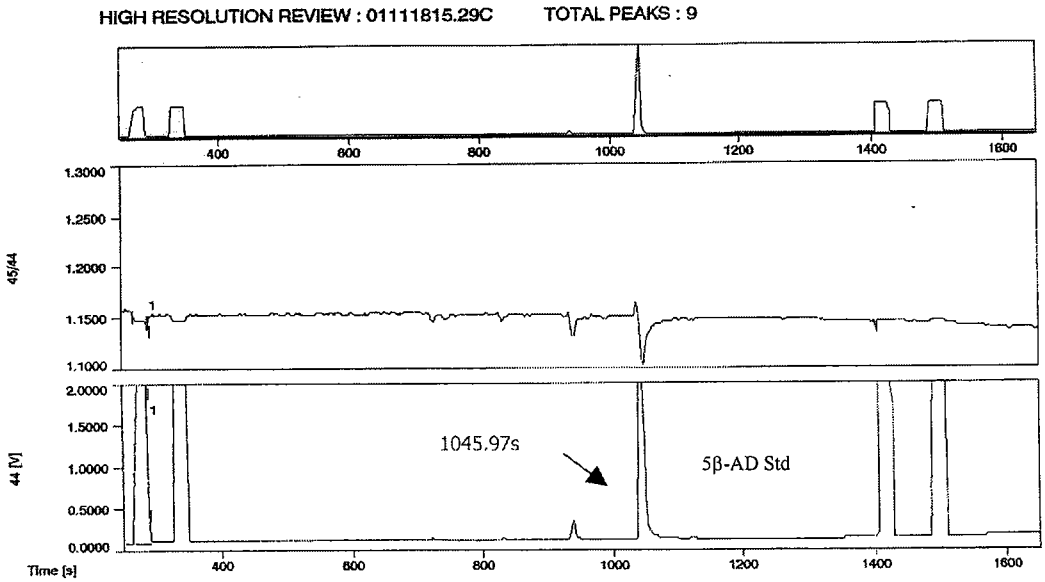
Laboratory code 01122401 with a elevated T/E ratio[1]

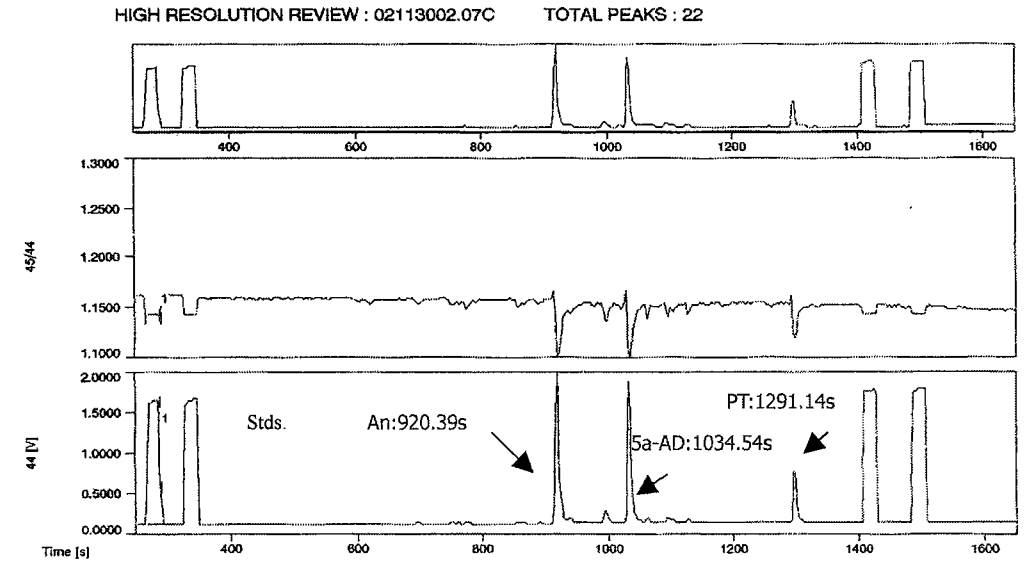
The following table shows the analytical results with GC/C/IRMS

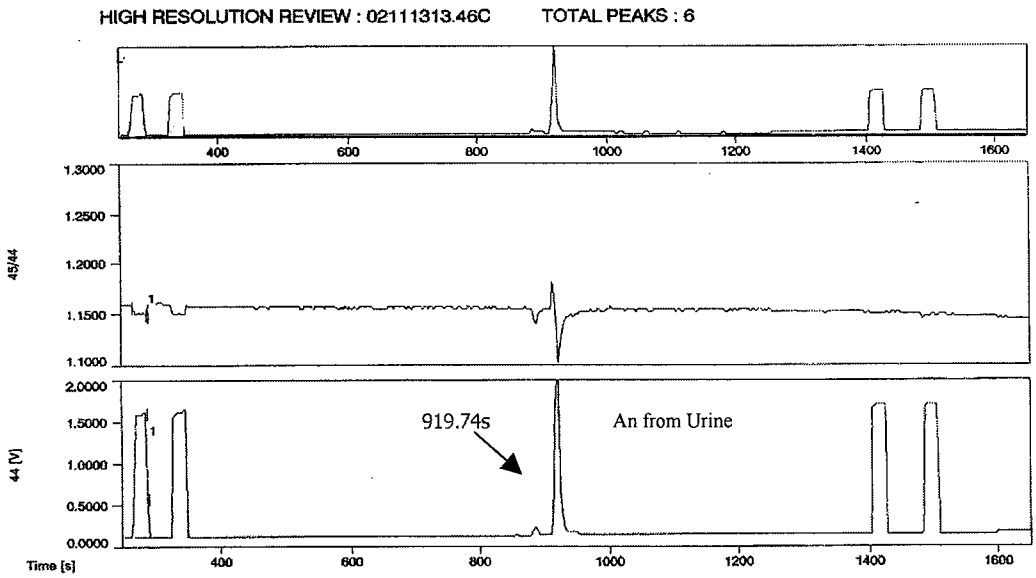
Delta Value	Ratio to PT
An: -27.90	An/PT: 0.97 < 1.15
5α-AD: -35.57	5α-AD/PT: 1.24 > 1.15
T: -37.30	T/PT: 1.30 > 1.15
PT: -28.68	

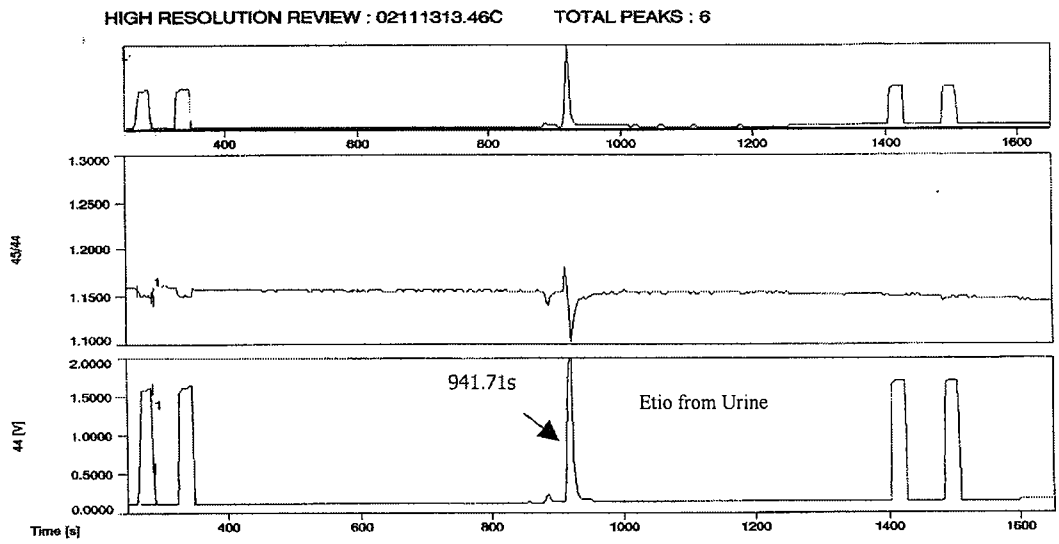

HIGH RESOLUTION REVIEW : 00072122.20C
TOTAL PEAKS : 17
45/44
44 [V]
941.44 s
Etionchonalonone (Etio) Std
Time [s]

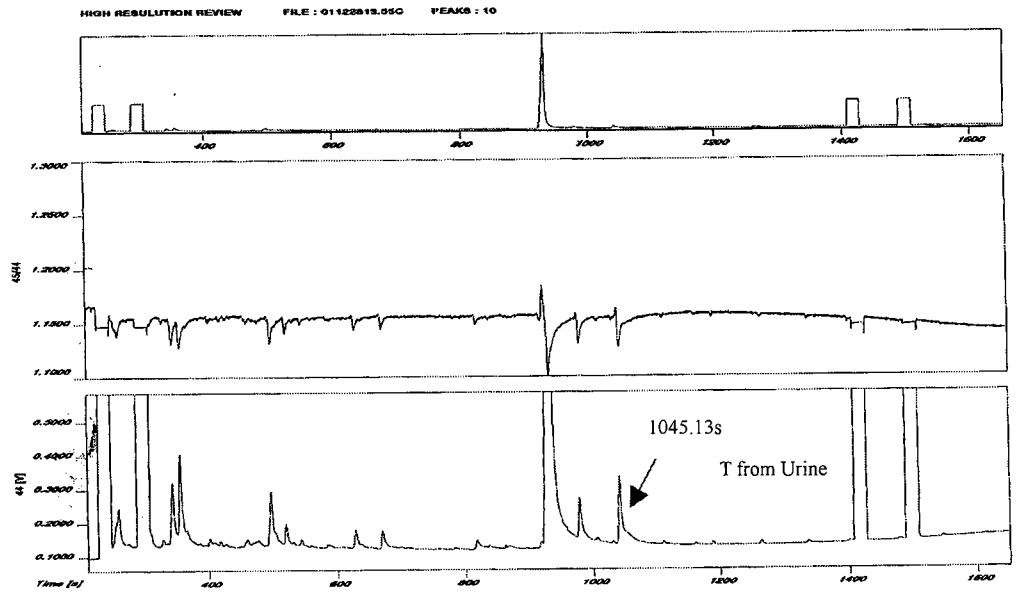

HIGH RESOLUTION REVIEW : 01083022.28C
TOTAL PEAKS : 10
45/44
44 [V]
1048.07s
T Std.
Time [s]

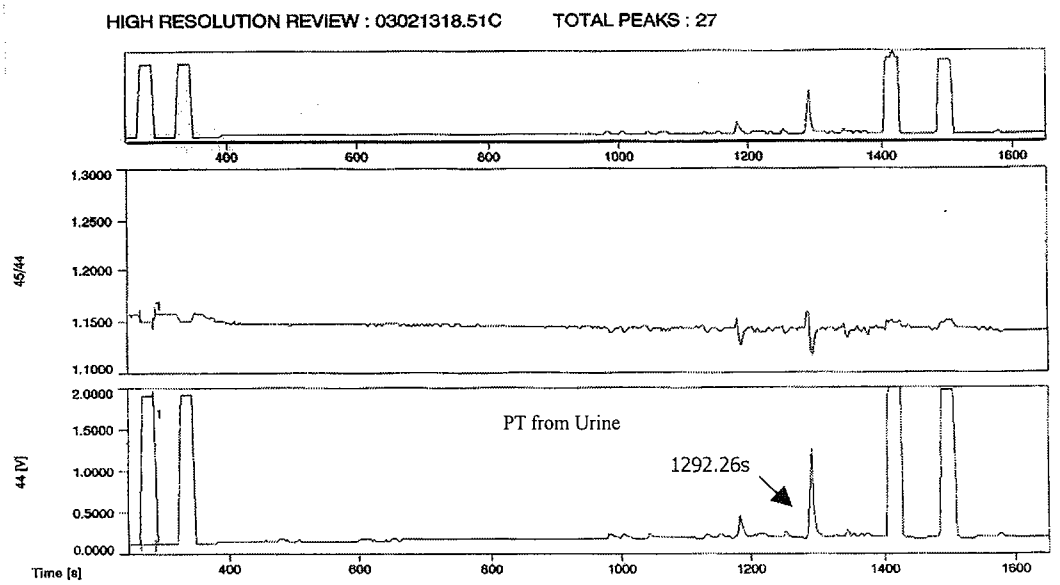

HIGH RESOLUTION REVIEW : 01111815.29C
TOTAL PEAKS : 9
45/44
44 [V]
1045.97s
5β-AD Std
Time [s]

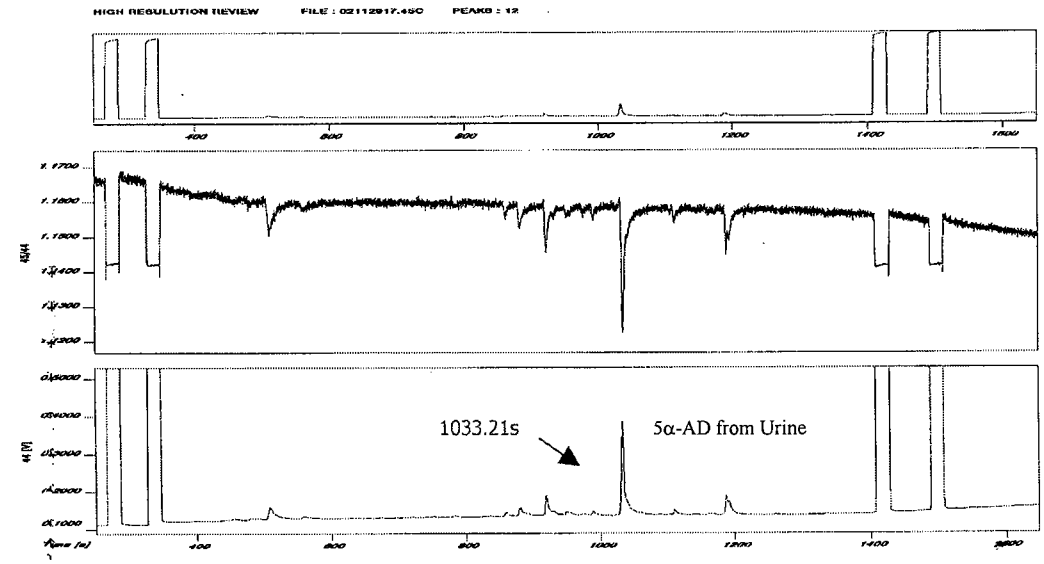

HIGH RESOLUTION REVIEW : 02113002.07C
TOTAL PEAKS : 22
45/44
44 [V]
Time [s]
Stds.
An:920.39s
PT:1291.14s
5a-AD:1034.54s

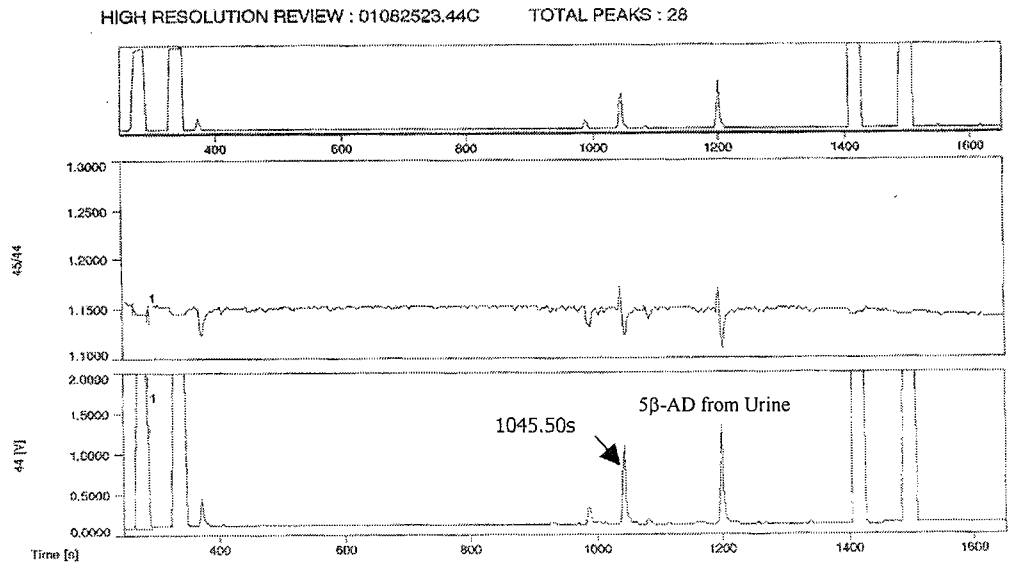

HIGH RESOLUTION REVIEW : 02111313.46C
TOTAL PEAKS : 6
45/44
44 [V]
Time [s]
919.74s
An from Urine


HIGH RESOLUTION REVIEW : 02111313.46C
TOTAL PEAKS : 6
45/44
44 [V]
Time [s]
941.71s
Etio from Urine


1045.13s
T from Urine


HIGH RESOLUTION REVIEW : 03021318.51C
TOTAL PEAKS : 27
45/44
44 [V]
Time [s]
PT from Urine
1292.26s


1033.21s
5α-AD from Urine



Wording in Analytical Report:

The ratio of T/PT showed that the testosterone for this elevated T/E ratio over 6 has an exogenous resource.

Discussion:

The T/E ratio of this case was 24±0.52 (n = 3 x 2) with a concentration of testosterone 303 ng/ml (s.g. = 1.023, pH = 6.5). This urine was collected from a female weight lifter during an out of competition control organized by Chinese Olympic Committee Anti-Doping Commission (COCADC). The ratio of An/PT is the smallest and far lower than the criteria though it was the substance measured with the highest concentration. From the very high concentration of testosterone in this case, it could be imaged that the urine collection be very short after the administration of testosterone preparation. The difference among these ratios may be due to the time close to the administration.

Case 2

WADA Proficiency Test with a Elevated T/E Ratio

Delta Value	Ratio to PT
An: -26.91	An/PT: 0.99 < 1.15
5α-AD: -29.89	5α-AD/PT: 1.10 < 1.15
5β-AD: -30.13	5β-AD/PT: 1.11 < 1.15
T: -31.90	T/PT: 1.17 > 1.15
PT: -27.16	

Discussion:

We did not have any information about the urine sample whether it was an artificial or natural urine sample. But from the analytical results we could have some

comments anyway. It showed us a very interesting rank of these ratios. The ratio of T/PT was strong to support the conclusion that the testosterone for this elevated T/E ratio over 6 had an exogenous resource[2]. But the others were not over the criteria, which may drive into a negative conclusion when only the metabolites of testosterone were measured with GC/C/IRMS. Though these ratios are so close to the criteria, among them the ratio of An/PT is still the smallest. It could be imaged that if the metabolite is in the lower flow river of the metabolism, this metabolite could be affected later than the metabolites in the upper flow river of the metabolism. Based on this hypothesis, androsterone could be always effected later by the administration of 5α-dihydrotestosterone, testosterone, dehydroepiandrosterone, androstenedion, 1-testosterone etc. such kinds of testosterone deriveatives.

Case 3

IOC Reaccreditation Test 2002 with a Elevated T/E Ratio[3].

The steroid profile for this urine sample (Lab code 02101404) from our laboratory in screen is in the following table.

Substance	M/z	Rt	rRt	Area	
Istd	446	15.24	1.00	653126	T/E > 6
testosterone	432	13.53	0.89	116526	
epitestosterone	432	12.72	0.83	13925	
androsterone	434	10.98	0.72	2923656	An/Eto = 2.3
etiocholanolone	434	11.13	0.73	1291591	
11β-OH-An	522	13.84	0.91	207220	11β-OH-An/ 11β-OH-Etio = 4.7
11β-OH-Etio	522	14.06	0.92	43408	
5α-And-diol	241	11.29	0.74	109707	5α-And-diol/ 5β-And-diol = 0.4
5β-And-diol	241	11.42	0.75	251768	

The results of isotope ratio measurement was in the following table[4].

Delta Value	Ratio to PT
An: -28.87	An/PT: 1.08 < 1.15
5α-AD: -31.29	5α-AD/PT: 1.17 > 1.15
PT: -26.67	

Discussion:

The information about the urine sample was not available for us. But we would like to discussion with the analytical results. Due to the low concentration of testosterone, its delta value could not be measured correctly. But the T/E ratio and other ratios of endogenous steroids suggested that the urine sample be collected from

the excretion study with testosterone not with 5α-dihydrotestosterone or androstenedione etc. Only was the ratio of 5a-AD/PT greater than the criteria. This case also informed us that it would be very careful when only the androsterone and/or etiocholanolone is analyzed to exclude the possibility of administration with some exogenous preparation.

Case 4

Laboratory Code 01111552 with a Relative High Testosterone Concentration

In our routine we found the urine sample with above mentioned lab code showed a relative high concentration of testosterone. The following table listed the concentration of some endogenous steroids from screen in ng/ml.

An	Etio	11β-OH-An	11β-OH-Etio	5α-AD	5β-AD	T	E
8142	4632	4252	629	140	247	322	159

The T/E ratio is over the normal range of Chinese Han people with relative high concentration of testosterone. The isotope measurement was carried out with this sample. The result is in the following table.

Delta Value	Ratio to PT
An: -25.72	An/PT: 1.0 < 1.15
T: -24.92	T/PT: 0.97/1.03 < 1.15
PT: -25.75	
Blank urine An: -27.19	An/PT: 1.0 < 1.15
Blank urine PT: -26.92	

Wording in Analytical Report:

No evidence showed that the testosterone has been affected by an exogenous origin.

Discussion:

To drive a negative conclusion, both the substance itself and its metabolite should be checked with GC/C/IRMS.

Case 5

Lab Code 01111512 with a Elevated T/E Ratio The urine sample with the above mentioned lab code showed the following steroid profile obtained from confirmation procedure in ng/ml with n = 3 x 2 (treble extraction, each extract injected twice).

	An	Etio	5α-AD	5β-AD	11β-OH -An	11β-OH -Etio	T	E
Coc.	2404	2896	26	87	343	32	259	7.0
SD.	121	168	0.7	5.1	19	6	12	0.3

The urine sample was collected by COCADC in competition test for a female rowing player with S.G. 1.024 and pH 6.0. The concentrations of 5α-AD and 5β-AD were too low to be measured with GC/C/IRMS. Only androsterone and testosterone were analyzed with isotope measurement. The delta values and ratios are listed in the following table.

Delta Value	Ratio to PT
An: -29.23	An/PT: 1.15 = criteria
T: -32.47	T/PT: 1.27 > 1.15
PT: -25.40	
Blank urine An: -27.19	An/PT: 1.0 < 1.15
Blank urine PT: -26.92	

Wording in the Analytical Report

The isotope measurement showed that both androsterone and testosterone of this urine came with exogenous resources.

Discussion[5]

Though in this case the delta value of androsterone informed its exogenous origin, the ratio to PT of it was still smaller than that of T. With three-year experiences on GC/C/IRMS in our routine for the elevated T/E cases, few of them could be confirmed positive by the ratio of androsterone to PT only, while more of them showed the exogenous origin of testosterone with the ratio of testosterone or 5-androstanediol to PT only.

CONCLUSION

Neither Androsterone nor Etiocholanolone only could be the strong evidence for excluding the origin of testosterone. For the cases with an elevated T/E ratio the best information should be obtained from the isotope measurement of testosterone itself. Even the delta value of 5-AD could be better than that of androsterone and/or etiocholanolone because androsterone and etiocholanolone come from more different resources and in the lower flow river of the testosterone metabolism.

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