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Epiandrosterone Glucuronide as a Sign to Indicate Natural Hormone Doping

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INTRODUCTION

One difficulty of dope analysis stems from the origin of the dope agents. As the detection of synthetic anabolic steroid became relatively easy and since there was no procedure available that could differentiate certain exogenous natural hormones, several physiological steroids, its esters and the precursors became a popular substitute of synthetic anabolic agents. For steroid abusers, the main purpose of the use of the free or the various ester form of T and DHT is not just a performance enhancement because the products were proven to be less anabolic and more androgenic than synthetic anabolic steroids. This paper refers to the re-investigation of the metabolism of T and DHT by means of stable isotope methodology. 19,19,19-trideuteromethylated tracers were applied to adult male volunteers and the urinary steroid profiles were measured after fractionation of the conjugates.

The formation of 3 β -hydroxy metabolites e.g. 5 α -androstane-3 β ,17 β -diol and epiandrosterone in the glucuronide fraction appeared to be the important sign of the exogenous application of T and DHT. Major part of these steroids were usually sulfo conjugated, and no significant amount of 3 β -hydroxy steroid glucuronides present in the urine of hCG stimulation test samples. The difference of the metabolism between the exogenous steroids and the endogenously secreted steroids became clear by our studies.

MATERIALS AND METHODS

The stable isotope studies were performed according to the similar protocol that reported by Shinohara et. al..[1] 19,19,19-trideuteromethylated androgens were synthesized and

gifted by Shinohara at Tokyo College of Pharmacy. Random urine specimens were collected before and after application of any of the steroids to a healthy male volunteer after informed consent agreement. Triethylamino-hydroxypropyl sephadex LH-20 (TEAP-LH20) was synthesized in Tokyo laboratory and was used for the fractionation of steroids into free, gluco- and sulfo conjugated steroids.[2] The other sample preparation and GC/MS analysis were done by our published procedures.[3]

RESULTS AND DISCUSSION

A. TESTOSTERONE:

Testosterone is believed to be sustain an athlete by preventing muscle losses through a period of few weeks prior to a competition when the athlete in the off synthetic anabolic steroid cycle.

Random urine specimens were collected for 3 days following oral administration of 5mg of 19,19,19-trideuterotestosterone (d3-T) after an informed consent agreement. The typical MS chromatogram is shown in figure-1. D3-T application resulted in the urinary excretion in large amount of d3-T (ion m/z:435), d3-5 α /5 β -androstane-3 α ,17 β -diols (ion m/z:349), d3-androsterone (ion m/z:437) and d3-etiocholanolone (ion m/z:437) in the glucuronide fraction. The formation of d3-ET due to d3-T administration was not observed. The results of our d3-T excretion study were in agreement with those of the previous study using 16,17-dideutero-T by Donike et.al..[4] In addition, the metabolism of exogenous T into minor compounds became clear by our application studies. It was confirmed that d3-T administration may lead to the excretion of d3-DHT and d3-epiandrosterone (EA) (ion m/z:437) in the glucuronide fraction

B. 5 α -DIHYDROTESTOSTERONE:

DHT is the active metabolite of T and its androgenic activity is 2-3 times more potent than the parent. In humans, DHT is mainly produced from T by 5 α -reductase in androgen-dependent target tissues, and it is required for normal male sexual differentiation. Because 5 α -reduction is essentially irreversible, undesired side effects due to the formation of estradiol by aromatase may be avoided when DHT is administered

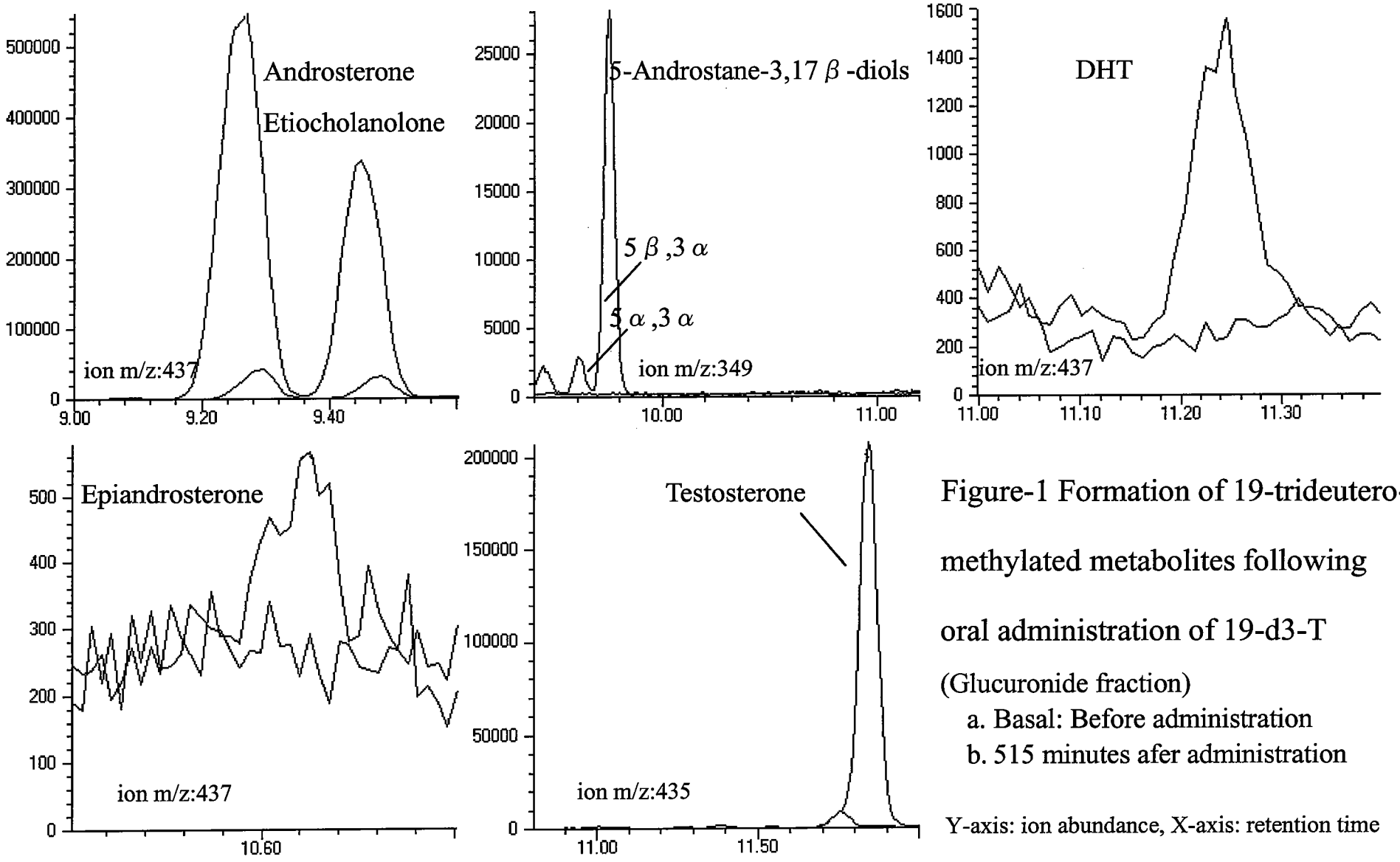

Figure-1 Formation of 19-trideutero-methylated metabolites following oral administration of 19-d3-T

(Glucuronide fraction)

a. Basal: Before administration

b. 515 minutes afer administration

Y-axis: ion abundance, X-axis: retention time

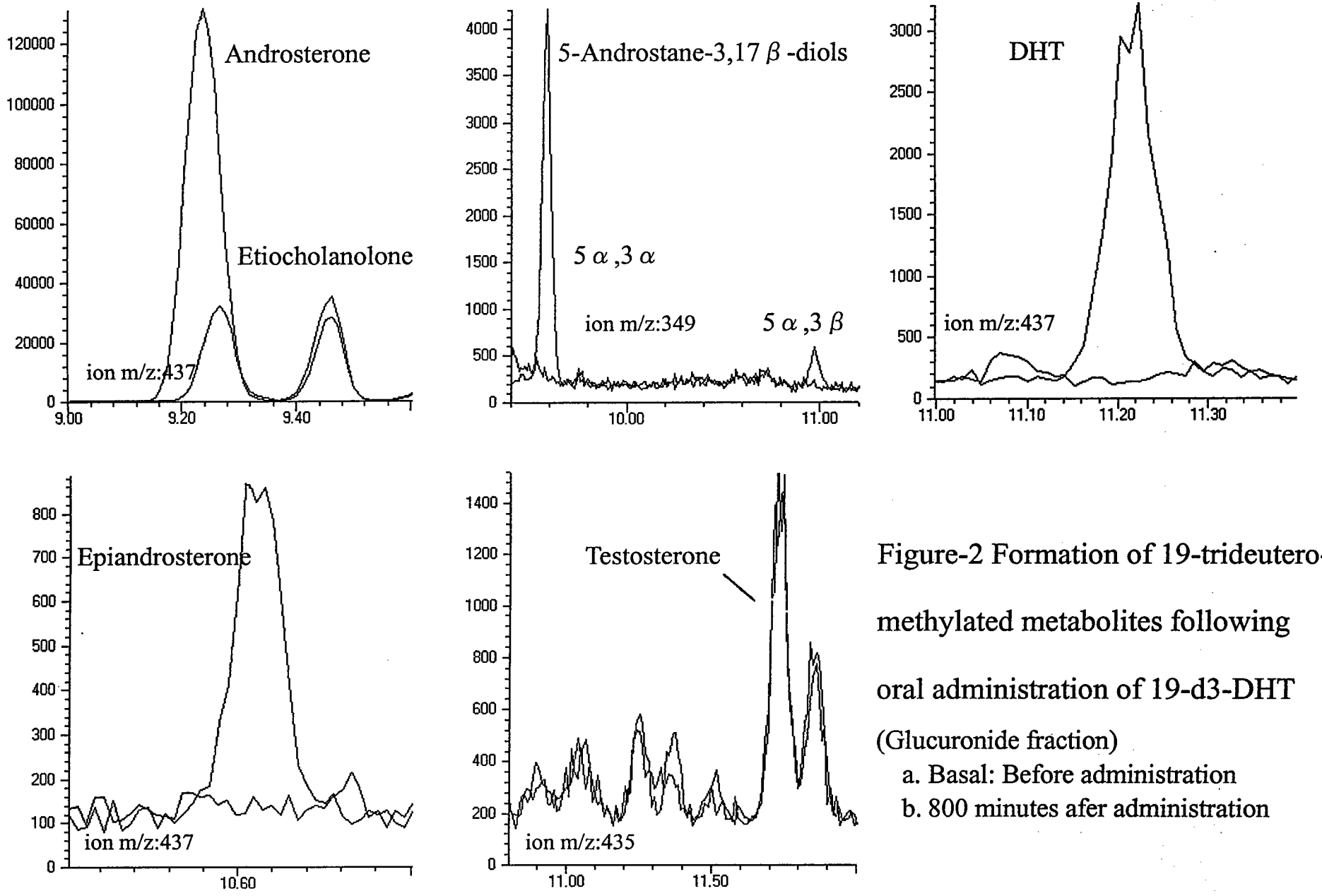

Figure-2 Formation of 19-trideutero-methylated metabolites following oral administration of 19-d3-DHT

(Glucuronide fraction)

a. Basal: Before administration

b. 800 minutes afer administration

as the substitute of T. The possible parameters to indicate DHT doping became available in 1992. Southan et. al. suggested from the results of their pilot study the use of concentration ratios of DHT/T and DHT/luteinizing hormone(LH) as the primary markers for DHT screening, and ratios of 5 α -androstane-3 α ,17 β -diol to the other isomer as the additional markers. [5)]

Figre-2 shows the partial results of our excretion studies on exogenous DHT using 19,19,19-trideutromethy dihydrotestosterone (d3-DHT). In this study, 1mg of d3-DHT was self administered orally by a healthy male adult after informed consent agreement. D3-DHT administration resulted in urinary excretion in large amount of d3-androsterone (ion m/z:437), d3-5 α -androstane-3 α ,17 β -diol (ion m/z:349) and d3-DHT (ion m/z:437). In contrast to the results from d3-T application studies, the excretion of 5 β -steroids, e.g. d3-etiocholanolone (ion m/z:437) and d3-5 β -androstane-3 α ,17 β -diol (ion m/z:349) did not increase. The minor metabolite of d3-DHT was d3-EA (ion m/z:437) and it was mainly excreted as sulfate, and another part of EA was excreted as the glucuronide as it was also observed in the d3-T application study. Our results show that the considerable inter-conversion between the 5 α -metabolites and the 5 β -steroids does not occur, thus the absolute concentrations of DHT and its 5 α metabolites and/or their ratios to non-5 α -steroids can be used as the conclusive evidence of DHT doping. Interestingly, the concentration of D3-EA-glucuronide was highly significant with that of DHT-glucuronide.

HCG (Human Chorionic Gonadotropin)

Urine samples were collected before and after i.m. injection of 5,000U of hCG to a healthy male volunteer. The urinary hCG and the steroid profiles were analyzed. The maximum endogenous production of T was occur at 50 hours after hCG injection, and was about 5 times higher than the basal value. (figure-3) However, the urinary T/ET ratio remained within –50 to +200% of the basal since hCG also stimulated the production of ET.[3)] In all the specimen, no significant amount of EA nor 5 α - androstane-3 β ,17 β -diol was detected (<0.5 ng/ml) in our hCG stimulation test samples.

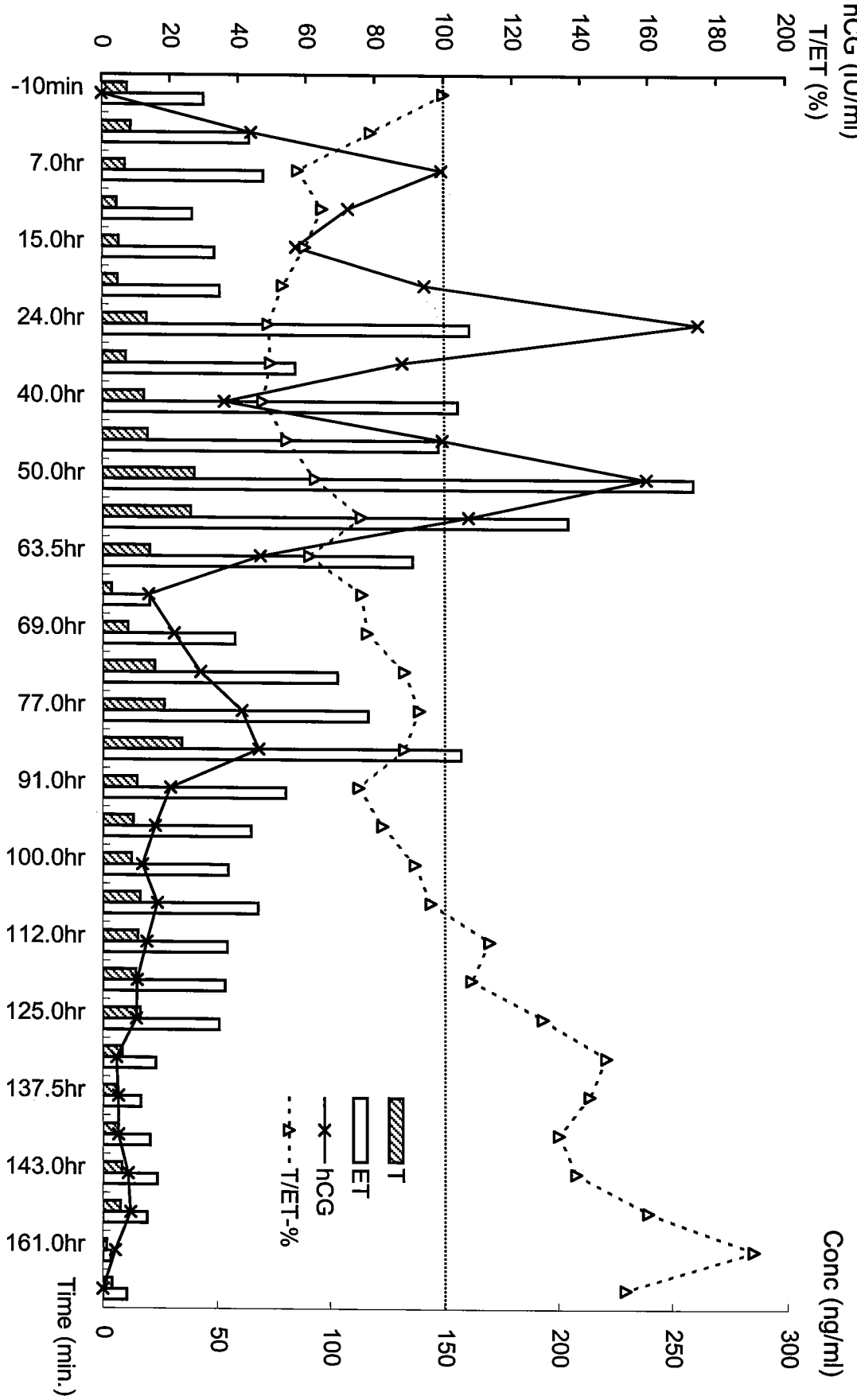

Figure-3 hCG application study (5,000 U i.m.)

The formation of the unusual 3 β -hydroxy steroid glucuronide after tritium labeled DHEA application was already reported in 1965 by Baulieu et. al.[6] and of 5 α -androstane-3 β ,17 β -diol after DHT administration was reported by Schänzer[7] in 1995. In the sulfate steroid fraction, the concentration of EA was highly correlated with DHEA sulfate (DHEA-S) but was less correlated with the concentration of T- and ET-glucuronide. The results suggested that EA-sulfate was formed from DHEA-S but not via testosterone pathway. On the other hand, EA-glucuronide was probably formed from exogenously administered steroids by 5 α -reductase and following 3 β -hydroxylation. (figure-4) Although the excretion of 3-hydroxy steroid glucuronides is minor the results are of importance when considering the difference of metabolism between endogenous and exogenous steroids. As the sensitivity of analytical instruments is improving year-by-year, such minor specific metabolites may be considered as evidence of doping.

CASE REPORT: Followed up Study of a DHT suspect.

The first case report of the DHT doping were published in the literature by Donike and our research team in 1995.[8),9)] A different approach from the previous report by Southan[5)] and Kicman[10)] was needed since in some cases DHT was abused in combination with T and Chinese traditional medicine, and certain parameters e.g. DHT/T could not be used because of the complexity of the steroid profiles.[9)] In several cases, the previous or the subsequent test results were available. Through the longitudinal studies, the doping scheme became apparent.

One world class female swimmer won the competitions and the urine samples were taken by the doping control officer of the games for three times. The test result indicated the sign of DHT doping when considered the urinary ratios of 5 α -steroid to non 5 α -steroid, e.g. A/E, 5 α /5 β -androstane diols, DHT/E, DHT/ET etc. The results were not declared positive but the athlete was followed by the further subsequent testing because absolute amount of the certain steroids were not high enough. Subsequent tests of the athlete were performed about 10 months later at the world championships. The sample information to follow the athlete by the laboratory test was given as the code number.

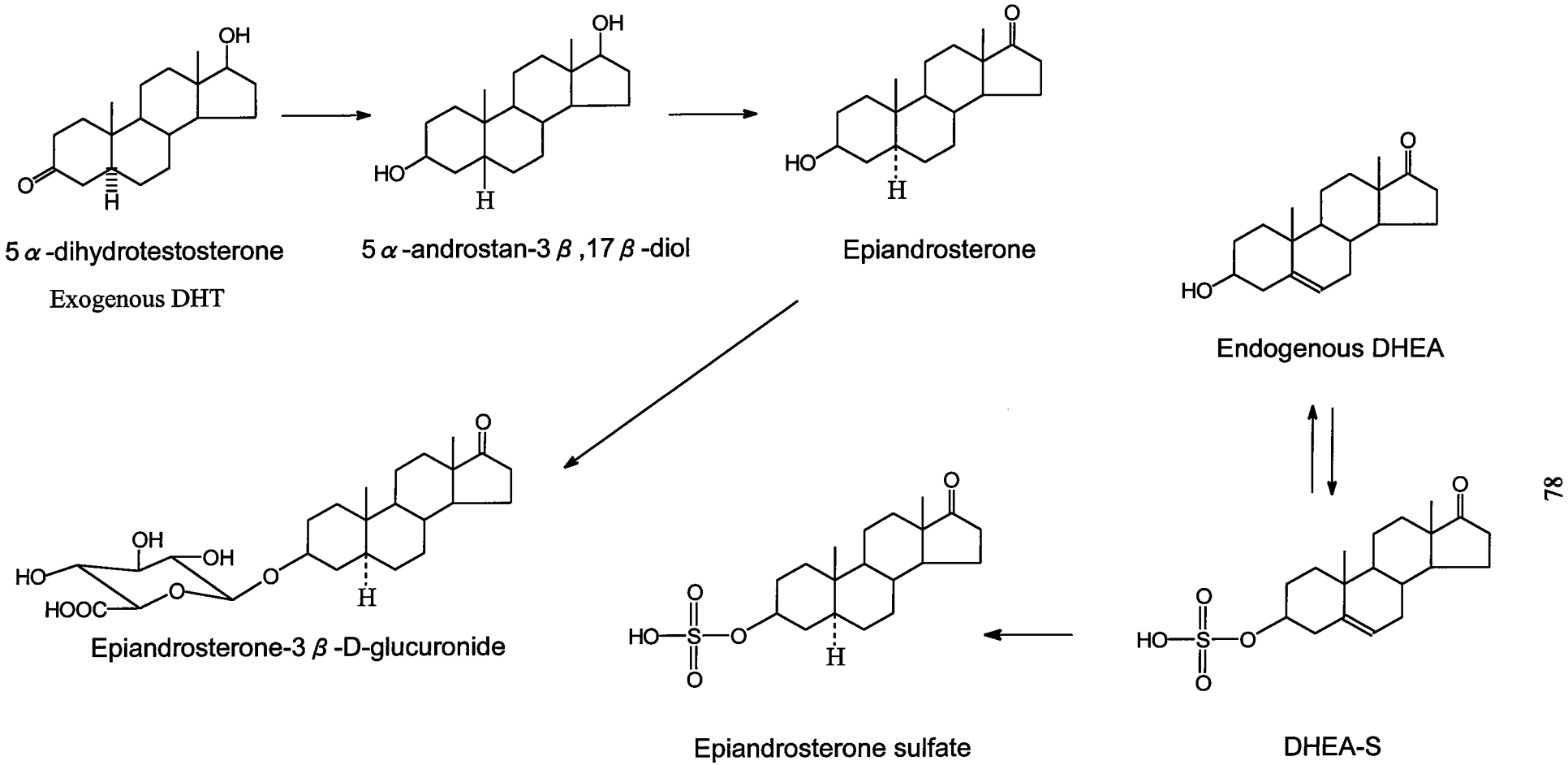

Figure-4 Proposed metabolism of the formation of epiandrosterone-sulfate and -glucuronide

In table-1, intra-individual variation of urinary steroid profiles of the swimmer was listed. When the results were compared between two series of in-competition testings, it was confirmed that the abnormalities of the urinary ratios of steroids fell within the threshold at the later tests. The most significant change was the absolute amount of DHT and its major metabolite 5 α -androstane-3 α ,17 β -diol. The concentration of DHT in the first series of urine samples was in normal range, but that in the later series of samples was extremely low in all the specimens. The ratios of 5 α - to 5 β -androstane-3 α ,17 β -diol was as high as 10.0 to 23.8 because of the high 5 α - androstane-3 α ,17 β -diol, and EA-glucuronide was also detectable in the first series of the specimens. However, the concentration of 5 α - androstane-3 α ,17 β -diol and the ratio between androstanediols was found to be normal in the later series of samples. In this case, the difference of the steroid profiles among specimens exceeded the limit of the intra-individual variations.[9), 11), 12)] Therefore, the abnormal steroid profiles in the previous test results were considered to be due to DHT doping.

SUMMARY

The major basis of a method for the detection of doping with naturally occurring steroids is the alteration of steroid profiles. This decision is made indirectly by statistical evaluation of the analytical results using the reference values as its threshold. The important information concerning conjugation of the steroid is lost when the hydrolysis is performed at the first step in the analysis. Our experiment confirmed the difference of the steroid metabolism between exogenously applied androgens and endogenously secreted one. The discrimination of doped and un-doped case could be made more clearly by considering the unusual EA-glucuronide as the sign of natural hormone doping.

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Table-1 Use of 3β-hydroxy steroid glucuronide as additional parameters for natural hormone doping

Presented parameters

Unit: ng/ml

Time	T	T/ET	A	A/E	DHT	DHEA	5α,3α A_2	5α/5β, 3α A_2	mDHT/ E	DHT/ ET	Decision
10/06/94	1.0	0.15	3240	**2.97**	5.8	37.5	250.9	**23.8**	5.3	0.9	±
10/07/94	1.3	0.14	3541	**2.38**	5.7	52.0	229.9	**14.2**	3.8	0.6	±
10/08/94	1.3	0.13	4101	**2.41**	2.7	65.3	147.0	**10.0**	1.6	0.3	±
08/24/95	0.9	0.24	1673	1.05	0.1	28.2	12.8	0.9	0.1	0.0	–
08/27/95	1.2	0.12	2941	1.11	0.1	51.3	28.4	0.9	0.0	0.0	–

Additional parameters

Time	EA	5α,3β A_2	Decision
10/06/94	**1.0**	**2.7**	+
10/07/94	**0.9**	**3.3**	+
10/08/94	**0.8**	**2.0**	+
08/24/95	ND	ND	–
08/27/95	ND	ND	–

T: Testosterone

ET: Epitestosterone

A: Androsterone

E: Etiocholanolone

DHT: Dihydrotestosterone

DHEA: Dehydroepiandrosterone

5α, 3α A_2: 5α-Androstan-3α, 17β-diol

5β, 3α A_2: 5β-Androstan-3α, 17β-diol

5α, 3β A_2: 5α-Androstan-3β, 17β-diol

EA: Epiandrosterone

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