J. Wang, X. Liu, Y. Xu, M. Wu

Profiling urinary steroids by GC-MSD and confirming androstenedione administration through isotope ratio mass spectrometry

National Anti-Doping Laboratory, China Anti-Doping Agency, Beijing, China

Introduction

Androstenedione (androst-4-ene-3,17-dione) is an endogenous steroid hormone banned by the World Anti-Doping Agency (WADA). The method to distinguish the origin of endogenous steroid is isotope ratio mass spectrometry (IRMS) [1-3]. The IRMS analysis is initiated only when specified parameters of steroidprofile are over the threshold. Studies have revealed that in some situations, the parameters given in WADA Technical Document TD2004EAAS are not effective for triggering IRMS analysis and that will lead to a false negative result [4]. Very few specific studies on androstenedione had been carried out investigating the relationship between urinary steroidprofile and IRMS detection. The aim of this study was to investigate the alteration of steroidprofile and the detection of androstenedione doping with IRMS.

Materials and Methods

Twelve healthy young volunteers (Chinese, 6 males and 6 females) were recruited and each volunteer was orally administered a single dose of 100 mg of androstenedione. Urine samples were collected a day prior to the steroid administration (0 h) and after administration (1 h, 2 h, 3 h, 5 h, 7 h, 9 h, 12 h, 22 h, 30 h, 36 h, 48 h, 56 h, 60 h, 72 h, 95 h, and 132 h).

The analyses of steroid profiles using GC-MSD were performed as previously reported [5], and the concentrations were corrected to a specific gravity of 1.020 g/cm^3.

The urinary sample was hydrolyzed and extracted for IRMS analysis using the same method as GC-MSD analysis procedure. The analyte extracted was purified with HPLC on a Zorbax SB-C_{18} column (4.6×250mm, 5μm particle size). The mobile phase, H_2O/CH_3CN 70:30→0:100 within 18 minutes, was delivered at 1 mL/min. The collected fractions were dried under N_2 gas and dissolved in *tert*-butyl-methyl ether for injection. The carbon isotope

ratio measurements were performed using a Delta-V isotope ratio mass spectrometer (Thermo Fisher Scientific).

Results and Discussion

Time-profiles of An, Etio, 5α- diol, and 5β-diol concentration

Fig.1a shows that the mean concentrations peaked at 5 h and were over the threshold of 10,000 ng/mL from 2 - 12 h for An and from 2 - 22 h for Etio. There are no cut-off values of 5α- and 5β-diol concentrations for predicting steroid misuse. Subject Based Reference Ranges, which were defined as the values of mean + 3×SD pre-administration [6], were utilized as the upper limits of these parameters (Fig.1b). After ingestion, the concentrations were higher than the reference upper limit of 135.0 ng/mL for 5α-diol and of 282.5 ng/mL for 5β-diol in 11 subjects.

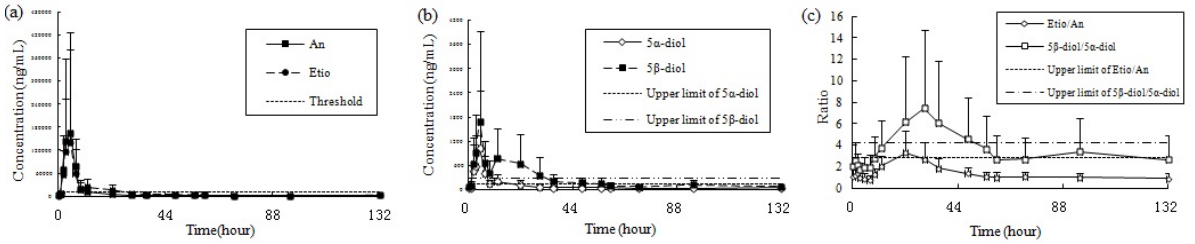

Figure 1. Time courses of mean urinary concentrations of: An and Etio (a); 5β-diol and 5α-diol (b); and mean ratios of Etio/An and 5β-diol/5α-diol (c).

Time-profiles of Etio/An and 5β-diol /5α-diol ratio

Androstenedione administration resulted in characteristic alterations of Etio/An and 5β-diol/5α-diol ratio (Fig.1c). The ratios were higher than the reference upper limit of 2.9 for Etio/An and of 4.4 for 5β-diol/5α-diol in 6 subjects after ingestion. Due to the slower clearance than 5α-diol, the concentration of 5β-diol remained high for a longer period than that of 5α-diol, and the 5β-diol/5α-diol ratio was an effective indicator in later period. The ratios of Etio/An and 5β-diol/5α-diol showed less variable in inter-individual than the concentrations of An, Etio, 5α- and 5β-diol.

Time-profiles of Testosterone and Epitestosterone concentrations

Depending on the extent of alternation in T concentration, subjects were classified into two categories: type A (with more alteration) and type B (with less alteration). The time courses of mean urinary concentrations of T and ET for type A and type B are shown in Fig. 2a, 2b. The T concentrations for type A (8 subjects, 4 males and 4 females) increased significantly

and were above the threshold of 200 ng/mL. Contrary to type A, the T concentrations for type B (4 subjects, 2 males and 2 females) was under 200 ng/mL throughout the experimental period. There was scarcely any difference between type A and type B in the changes of ET concentration.

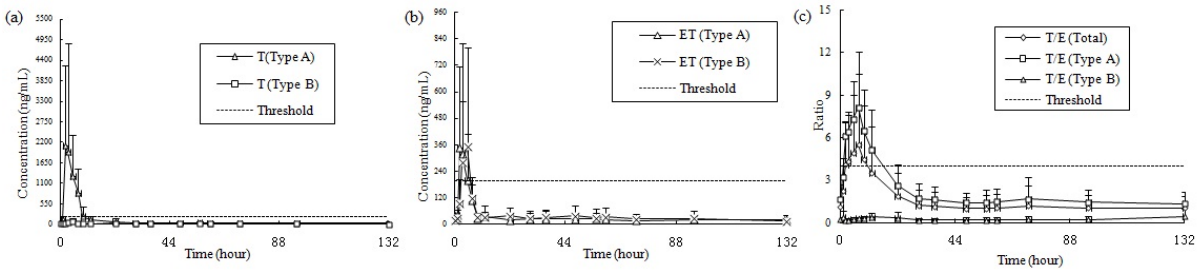

Figure 2. Time courses of: mean urinary concentrations of T for Type A and B (a); mean urinary concentrations of ET for Type A and B (b); and mean T/E ratios for Type A, Type B, and all subjects (c).

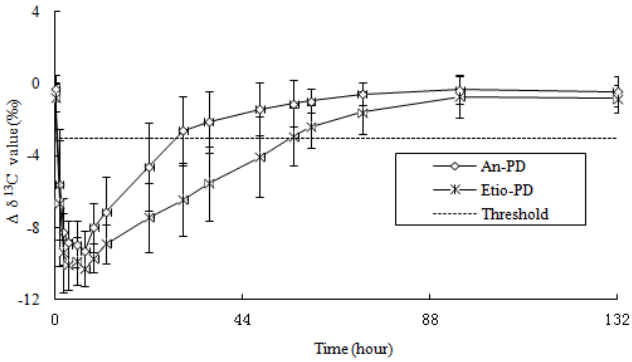

Figure 3. Time courses of $\Delta\delta^{13}$C-values of An-PD and Etio-PD.

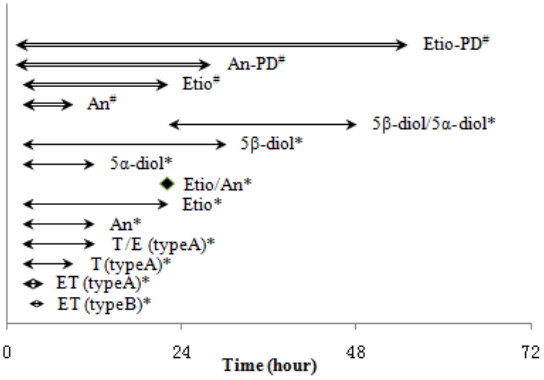

Figure 4. Time windows for indicators on GC-MSD and for analytes with positive results on IRMS. indicators on GC-MSD.
Analytes with positive results in IRMS analysis.

Time-profiles of Testosterone/Epitestosterone ratio

The different effects of androstenedione application on the T concentrations led to the different changes in T/E ratio between types A and B groups (Fig.2c). For type A, the T/E ratios increased significantly and were above 4:1. For type B, the T/E ratios remained under the threshold through the experimental period. The prominent inter-individual variability in T concentration or T/E ratio should be the consequence of polymorphism of UGT2B17 genotypes [7].

Time- profiles of $\Delta\delta^{13}$C-value

Fig.3 shows that $\Delta\delta^{13}$C-values of An-PD and Etio-PD could be detected as positive results between 28 and 55 h respectively after ingestion, but the concentrations of An and Etio could predict endogenous steroid abuse only for up to 12 and 22 h respectively. In addition, to confirm a positive finding, Etio could be detected more effectively than An in the later period.

The summary of time windows for steroid profile parameters (i.e. indicators) to suspect the misuse of endogenous steroids and for analytes with positive results in IRMS analysis is shown in Fig. 4.

Conclusion

The ingestion of androstenedione resulted in changes in the urinary steroidprofile. The rapid increases in the concentrations of An and Etio, as well as in T/E ratio for some subjects (type A) could provide indicators for initiating IRMS analysis when they were over the cut-off values. However, these indicators invalidated so rapidly that there was a period in which no current indicators could initiate IRMS analysis effectively while IRMS could yield positive determinations. Thus, more parameters of the urinary steriodprofile should be considered in the screening process to reduce the rate of false negatives. The concentrations of 5α-diol (>135 ng/mL) and 5β-diol (>282 ng/mL), and the ratios of Etio/An (>2.9) and 5β-diol/5α-diol (>4.4) could be served as additional indicators to infer androstenedione administration. Lastly, in detecting androstenedione doping with IRMS, Etio was a more effective target compound than An because of its slower clearance.

References

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