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Influence on the alternative steroid profile, part I

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Abstract

To date, knowledge of steroid profiling has gone beyond a mere T/E ratio evaluation. It was shown that incorporation of minor metabolites could contribute to the sensitivity and specificity steroid profiling methods. On the other hand, the evaluation of such steroid profiles is not straightforward to correctly judge alterations. In this respect, a steroidomic model was presented that was trained to recognise normal and abnormal steroid profiles. However, we must realise that steroid profiles can be influenced by numerous confounding factors, among which genetic background and the use of ethanol.
We show the implications of these confounders on the minor steroid metabolites in the framework of alternative steroid profiling as well as their impact on the results of a steroidomic model.

Introduction

The emerging introduction of the steroidal module of the biological passport for the first time provides anti-doping scientists with the systematic storage of longitudinal data of steroid profiles [1]. Using the adaptive Bayesian model these longitudinal data shall be compared with subject-based threshold which are more sensitive compared to population statistics [2]. Whereas traditional steroid profiles have been studied since the 90s, widening the scope of steroid profiles towards minor steroid metabolites like 4-OH-androstenedione [3]and 7β-OH-DHEA [4]proved to be interesting strategies to increase specificity. Evaluation of the resulting alternative steroid profiles with a steroidomic model [5]caused an increase of the sensitivity to doping with endogenous steroids. Hence, a combined approach can be very promising in the detection of misuse with testosterone (T) and other endogenous steroids.
As a consequence, these sensitive thresholds have the risk to be exceeded by small changes in the profile that cannot be attributed to doping. For this reason it is important to investigate and quantify these possible alterations of steroid profiles due to confounding factors:
Genetic polymorphisms of UGT2B17 explained the major intra-individual variance of the T/E ratio [6], particularly in the low range; high T/E values could be attributed to CYP17 [7]. Other polymorphisms in UGT2B7 and UGT2B15 were related to different glucuronidation patterns of E, Andro & 5ααβAdiol [8,9]and T & DHT [7], respectively.
The use of alcohol can cause altered T/E and Andro/T ratios [10].

Experimental

Study design genotyping:
49 healthy male volunteers were asked to provide ten blank urine samples in a 5 month period and a blood sample for genotyping for P-450c17α (CYP17), UGT2B7, UGT2B15 and UGT2B17 according to the methods described elsewhere [7]. All volunteers gave written consent. This sample collection study was approved of the local ethical committee of the University Hospital of Ghent (B670201110846). The volunteers were asked to mention if alcohol, medication or drugs were used at least 2 days prior to the production of each urine specimen. In total 487 steroid profiles were analysed using GC/MS [11].

Study design alcohol:
Ten volunteers provided written consent to participate an administration study with alcoholic beverages until a dose of 2 g/kg bodyweight was reached. The high dose was to achieve maximal alteration of the monitored markers. In 4h the

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volunteers aimed to drink personalised doses of jenever shots containing 20% alcohol. Before administration, volunteers were asked to provide five blank urine samples before which they abstained from any alcoholic beverage for at least three days. During the first 8h of the ethanol administration, the volunteers where asked to provide blood samples at 0, 1.5, 3, 4.5, 6 and 8h as well as to conduct hourly breath analyses. Urine was collected at 0, 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 60, 72h. Until 72h after the start of the experiment, the volunteers were asked to abstain from alcohol. Urine samples were stored frozen until analysis. The determination of blood alcohol concentration (BAC) was outsourced and analysed with a standard enzyme test.

Analytical
EtG-EtS
EtG and EtS measurements were performed with a LC-MSMS vantage (Thermo, Bremen). The mobile phase was 25/75 mixture of 0.2 mM aqueous NH4Ac/acetonitrile. 25 µL of urine samples were diluted 30 times with the mobile phase. 50 µg/mL EtG-D5 and EtS-D5 were spiked as internal standard. After centrifugation the 4 µL of the diluted samples was injected onto a Microsorb 100-3 CN reverse-phase column (100*4.6 mm, 3 µm). HPLC separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) and eluted with an isocratic run.
Between 5.2 and 5.9 mins of the 9.7 min. program, 100% ACN eluted.
MS/MS detection was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in negative mode. The ESI-MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source temperature, 350 °C; sheat gas pressure, 30 psi; auxiliary gas pressure, 10 psi; tube lens offset, 20V for EtS and EtS-D5 transitions, 78V for the EtG transitions and 110V for the EtG-d5 transitions. Dwell time for both glucuronides was 100ms, for the sulphate 50ms.

Following transitions were applied for
EtG
221 → 74.4 (collision energy CE 18eV)
221 → 84.4 (CE 20eV)
221 → 158.9 (CE 16eV)
EtG-D5
226 → 74.4 (CE 18eV)
226 → 84.4 (CE 20eV)
EtS
124.9 → 63.3(CE 45eV)
124.9 → 79.3 (CE 37eV)
124.9 → 96.4(CE 17eV)
124.9 → 124.9(CE 5eV)
EtS-D5
129.9 → 79.3 (CE 37eV)
129.9 → 97.4 (CE 17eV)

Results and Discussion

Genetics:
Four genetic polymorphisms were checked whether they affected various steroid profile markers significantly. For the UGT2B17 enzymes significant differences were found in T, DHT and 5βαβ-Adiol. 8.1% (4/49) showed a homozygous deletion for UGT2B17. Besides the known bimodal effect on T/E distribution, also DHT/E and the 5α/β-Adiol ratio were significantly influenced (Figure 1). Where UGT2B17 polymorphism causes a bimodal distribution for the T/E ratio, this was not the case for 5α/β-Adiol and DHT/E but significant changes were noticed with the rest of the population (del/ins+ins/ins).
Among these del/del subjects, one was significantly different from the other three with a E concentration that was 30-40% lower causing upper outliers relative to the entire population of 5α/β-Adiol and DHEA/E (Figure 1). This explains why the mean is low in the box plots of the del/del subjects. Such difference could not be attributed to any polymorphism of the other monitored enzymes.
Other markers were not significantly affected by UGT2B17 polymorphism.

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Also no significant results were found with respect to polymorphisms UGT2B7, UGT2B15 and CYP17 on any of the markers of interest. No differences in the output of the steroidomic model [5] were experienced over the different genetic polymorphisms.

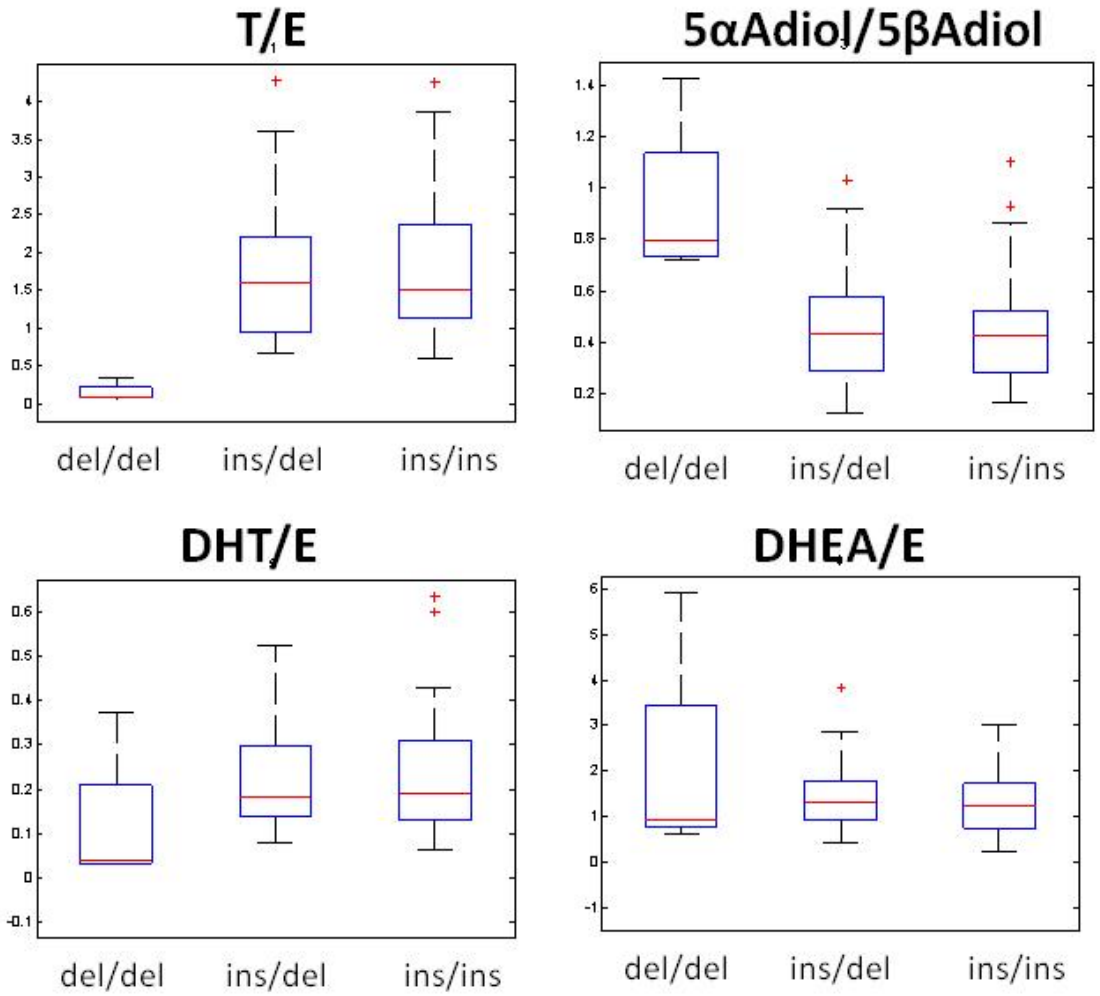

Figure 1: UGT2B17 polymorphisms in a pool of 49 subjects (n=4 del/del, n=23 ins/del and n=22 ins/ins) for the T/E 5αAdiol/5βAdiol, DHT/E, DHEA/E markers.

Validation EtG-EtS:
The EtG/EtS method was validated in a calibration range of 5-200 µg/mL. Linearity for both compounds was good (r^2>0.99). The matrix effects for EtG & EtS were assessed at -55% & -50% for the peak areas and 32% & 0% for the compound/IS ratios. Repeatability and reproducibility were lower than the respective thresholds of 2/3 RSD_{max} and RSD_{max} at low, medium and high level over the calibration range. Specificity was checked by analysing 13 blank urines which did not show any interference. The LOQ was assessed at 5 µg/mL.

Ethanol administration study:
Based upon a theoretical model, which was kindly provided by Thieme et al. [10], it was expected that a 2 g/kg dose of EtG would be cleared from the body after 24-32h whereas blood alcohol concentrations (BAC) would be detectable until 10h after the last drink (Figure 2). The maximal BAC was found 2h after the last drink. The experimental values followed the curve of the theoretical model and are presented in Table 1.

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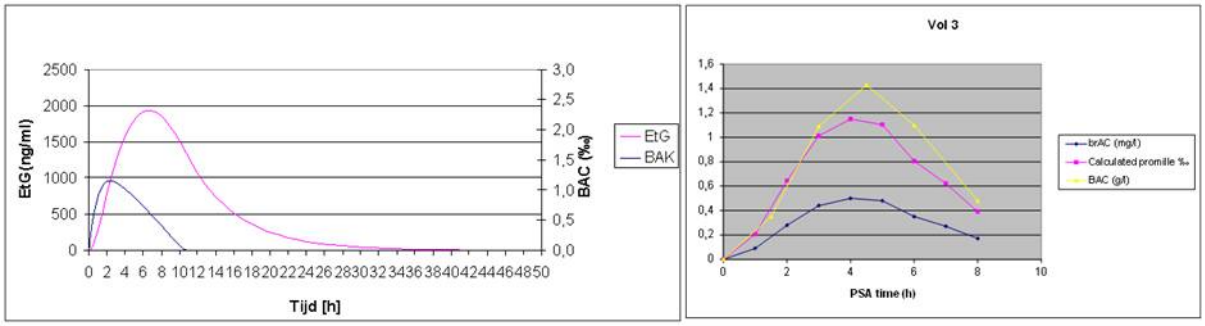

Figure 2: Theoretical model for the prediction of EtG in urine and the BAC (left) and the experimental data of the BAC and breath alcohol concentration (BrAC)(right)

Volunteer	1	2	3	4	5	6	7	8	9	10
weight(kg)	58	77	52	83	84	98	67	89	92	66
gender	m	m	f	m	m	m	f	m	m	m
dose(g/kg)	2,0	2,0	2,0	1,6	2,0	1,6	2,0	2,0	2,0	2,0
max BAC(g/L)	1,72	1,69	1,43	1,28	2,31	1,33	1,78	1,84	1,53	1,62

Table 1: Information on the volunteers, the doses they effectively consumed and the maximal measured BAC.

It was envisaged to also screen for EtS as a marker for alcohol misuse and compare it with EtG. Figure 3 shows the measured concentrations of EtS and EtG before and after ethanol intake. It can be seen that EtS concentrations are three times lower throughout the excretion profile. For all volunteers, an average factor of three was constant between the time of maximal concentration (10h) and 30h. Due to this difference in analytical sensitivity it was opted to prefer EtG as prime marker.

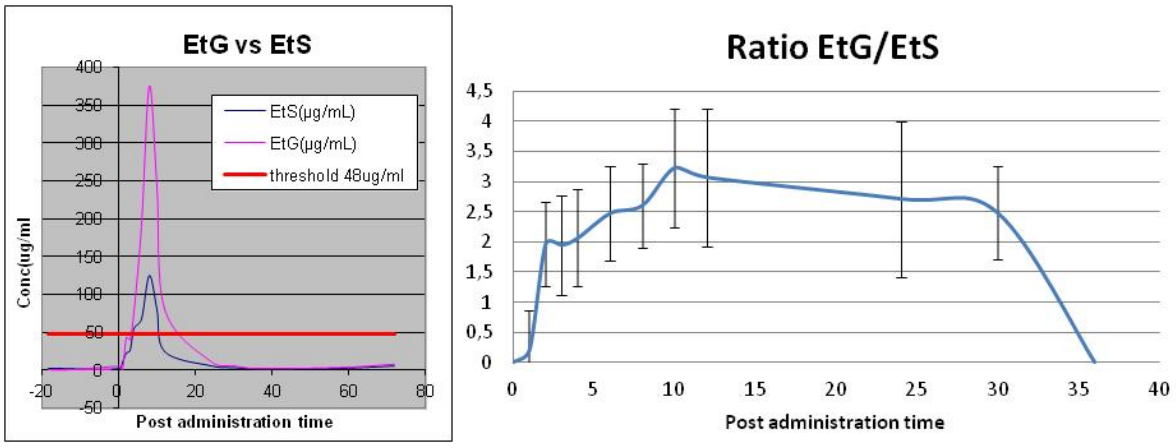

Figure 3; The left plot shows the EtG and EtS administration profiles of volunteer 1 together with the current EtG threshold proposed by Thieme et al. [10]. The right plot presents the average EtG/EtS ratios over all volunteers after administration.

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Increases of the T/E ratio were found up to 280% in females and 133% in males, the T/Andro ratio showed even increases up to 1700% in females and 280% in males, the 5α/βAdiol ratio showed increases up to 450% in females and 220% in males. After 24h all altered ratios were back to baseline levels. Nevertheless, with these high doses some EtG values did not exceed this threshold even the longitudinal profile was significantly changed. We found 12% records for T/E and 9% for T/Andro that showed alterations in the individual profile but where EtG did not exceed 48 µg/ml. Also taking into account that Thieme et al. [10] reported that much lower doses could alter steroid profiles, we argued that the proposed threshold of 48 µg/mL for EtG levels in men is to conservative. For the markers with the minor steroid metabolite, only 7β-OH-DHEA/E showed significant alterations after alcohol intake up to 330% in men.
Based the post-administration results, it was proposed to lower the EtG threshold in males from 48 to 20 µg/mL. Hence, the rates of misclassifications could be reduced from 12% to 6% for the T/E ratio and from 9% to 3% for T/Andro.
The score (ASPS) of the steroidomic model was subjective to changes of ethanol of which most did not exceed the proposed threshold of 0.79 [5] (Figure 4). In only one volunteer a maximal raise of the ASPS was found that significantly exceeded the ASPS limit.

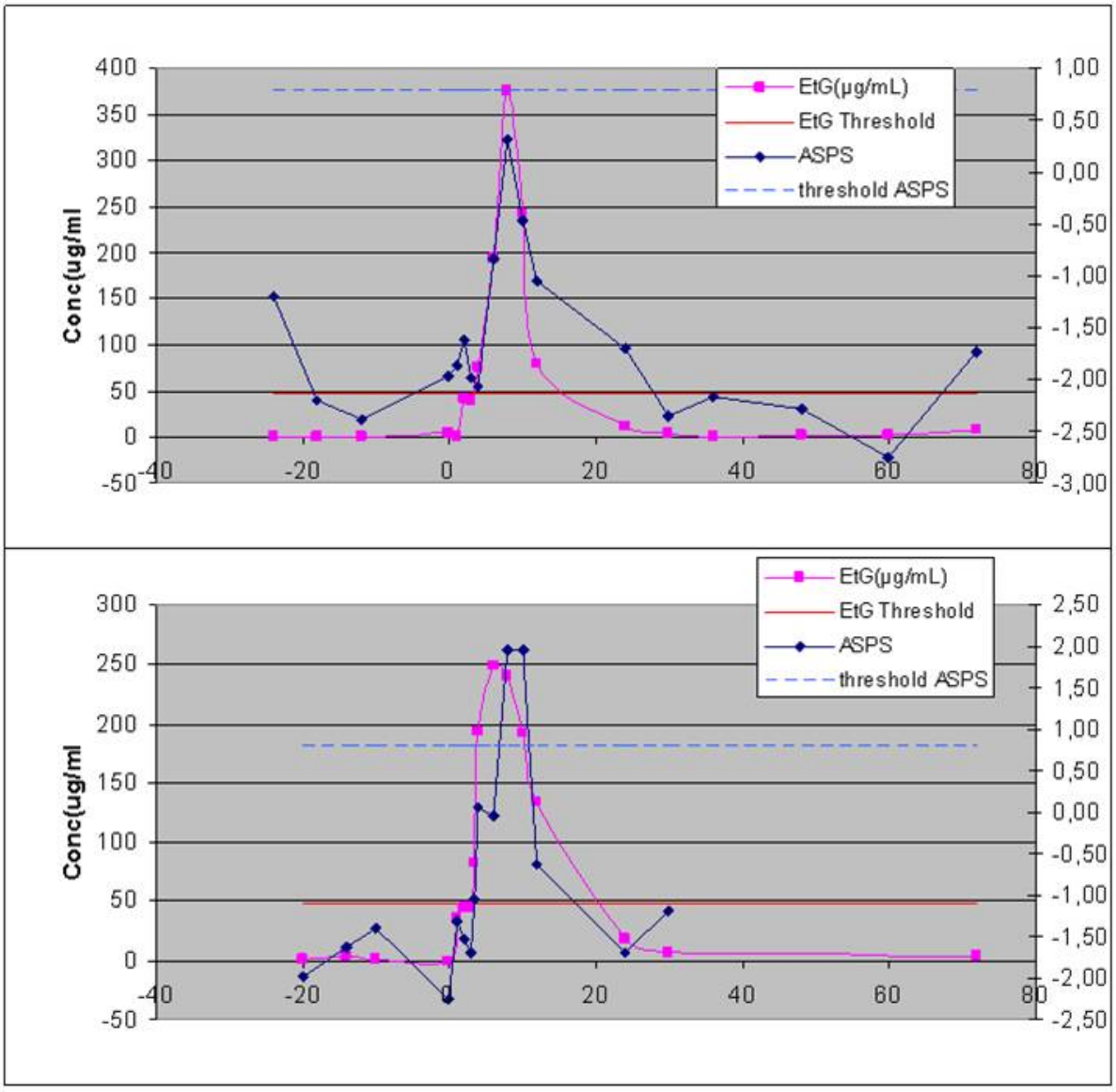

Figure 4: Post administration profiles of EtG and the abnormal steroid profile score (ASPS) with their respective threshold (48 µg/ml and 0.79)

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Conclusions

The influence of genetic polymorphisms and alcohol on the alternative steroid profile and steroidomic model was studied. With respect to the genetic polymorphisms we can conclude that only UGT2B17 polymorphism significantly affect markers T/E, DHT/E and 5α/βAdiol ratio. For polymorphisms of UGT2B15, UGT2B7 and CYP17 no conclusions can be drawn for doping control with respect to any monitored steroid profile marker. The steroidomic model was not influenced by genetic polymorphisms.
EtG is the preferred alcohol markers to find alterations in primarily T/E, T/Andro, 5α/βAdiol and 7β-OH-DHEA/E. It was proposed to lower that EtG threshold to 20 µg/mL. The steroidomic model is also changed after alcohol use.

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