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Urinary steroid profiles after use of testosterone by different routes of administration - how do we detect doping with low doses?

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Abstract

Changes in the urinary steroid profile may be an indication of the use of testosterone or its precursors. The concentrations and ratios of selected steroids show a large inter-individual variation, and the screening approach relying on population based criteria lacks the discriminating power to detect the administration of testosterone in low doses. Genetic variation is the most important cause of inter-individual variability in the excretion of testosterone metabolites. The enzyme UGT2B17 is important in the glucuronidation of testosterone. A deletion in the gene encoding for this enzyme causes a reduced excretion of testosterone glucuronide. The aim of this study was to investigate how the steroid profile is affected in subjects with different *UGT2B17* genotypes after dermal application of testosterone gel and injection of testosterone enanthate.
Ten male volunteers were included in the study. Urine samples were collected prior to the administration of testosterone and during the applications. In addition a blood sample was collected for the determination of their *UGT2B17* genotype. The urinary steroid profiles were analysed by GC-MS. Selected samples were also analysed by isotope ratio mass spectrometry (IRMS).
The results show that the T/E ratio was affected by administration of testosterone, but only to a limited extent for dermal preparations. Even in individuals with small changes in T/E ratio following testosterone administration, analysis by IRMS confirmed the presence of testosterone and metabolites of exogenous origin.

Introduction

Changes in urinary steroid profile may indicate the use of testosterone or its precursors. The steroid concentrations and selected ratios show a large inter-individual variation. Genetic polymorphism is the most important cause of inter-individual variability in the excretion of testosterone metabolites [1]. The enzyme UGT2B17 is important in the glucuronidation of testosterone. A deletion in the gene encoding for this enzyme results in reduced excretion of testosterone glucuronide [1]. The aim of this study was to investigate if the steroid profile is affected in subjects with different *UGT2B17* genotypes after dermal application of testosterone gel and injection of testosterone enanthate in low doses. The disclosure of such administration was also investigated by isotope ratio mass spectrometry (IRMS).

Experimental

Ten male volunteers, aged 21-29 years, were included in the study. Prior to the administration of testosterone, five urine samples were collected from each participant, in order to establish their baseline steroid profiles. In addition, a blood sample was collected for the determination of their *UGT2B17* genotype. Testosterone gel (50 mg/day) was applied once daily for seven consecutive days. Urine samples were collected daily during the application period and for the following seven days. After an eight week wash-out period, the participants received a single intramuscular injection of testosterone enanthate (125 mg). Urine samples were collected for 14 days. The urinary steroid profiles were analysed by GC-MS by the established steroid screening procedure modified by introducing five point calibration curves for the selected endogenous steroids.

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A volume of 2.5 mL of urine was hydrolysed by *E.coli* glucuronidase. Free and hydrolysed endogenous steroids were extracted by tert-butyl methyl ether at pH 9,25 followed by derivatisation with MSTFA:NH_4I:ethanethiol (1000:2:6). For samples with a specific gravity less than 1.010 the sample volume was increased to 5 mL. The concentrations were normalised to a specific gravity of 1.020. In routine doping analysis, samples that show a T/E ratio > 4 are usually submitted to IRMS analysis, as recommended in the WADA technical document on reporting and evaluation of endogenous steroids, TD2004 EAAS [2]. In this study selected samples were analysed regardless of the T/E ratio, in order to determine whether an adverse finding could be issued according to the criteria defined in the WADA-document. The sample preparation for IRMS included HPLC cleanup and acetylation of selected steroids. The blood samples were analysed by PCR at the Department of Growth and Reproduction, Rigshospitalet, University of Copenhagen, for the determination of the *UGT2B17* genotype. The study was approved by the Regional Ethical committee (REK).

Results and Discussion

The genotypes of the participants are listed in Table 1.

Subject	*UGT2B17* genotype	Baseline T/E ratio	Testogel application T/E ratio > 4 Number of samples	Testosterone enethate injection T/E ratio > 4 Number of samples
1	del/del	0,41	0	0
2	ins/ins	0,94	0	2
3	ins/ins	0,70	0	4
4	ins/ins	1,73	4	9
5	ins/ins	1,87	4	9
6	ins/del	1,79	0	2
7	ins/del	0,75	0	0
8	ins/ins	0,71	0	2
9	ins/ins	0,70	0	8
10	ins/ins	1,91	1	8

Table 1: Genotype, average T/E ratio measured at five consecutive days prior to the study and number of samples exceeding the population based threshold of T/E > 4, in each part of the study.

As shown in Figures 1 and 2, the genotype affects the excretion of testosterone glucuronide. Subject 1 (del/del) excreted lower amounts of testosterone glucuronide, subjects 6 and 7 (ins/del) showed both T/E ratio and testosterone glucuronide excretion in the same range as the ins/ins subjects (data not shown). After application of testosterone gel, only three of the ten participants reached a T/E ratio higher than 4, in one or more samples. None of the samples collected from subjects 1 (del/del) and 7 (ins/del) showed a T/E ratio > 4. However, the T/E ratio increased in all subjects after both injection and gel application, as expected, to a larger extent after testosterone enanthate injection than after use of testosterone gel. This was caused by an increased excretion of testosterone glucuronide.

Some of the subjects (3 of 10) showed a slightly elevated excretion of androsterone and etiocholanolone after the injection (not shown). The 5a/5b-androstanediol ratio is sometimes used to indicate transdermal application of testosterone. In this study, only four subjects showed an increase in this ratio (20-30 %, not shown), none exceeded a value of 2. However subject 1 (del/del) had a higher 5a/5b-androstanediol ratio (2.6), before and during gel application than the participants with ins/del and ins/ins genotypes. This is in accordance with results published by Juul et al [3].

The results from the IRMS analysis show testosterone and 5b-androstanediol of exogenous origin (Δδ > 3 ‰) after 6 days of gel application and from the day following the testosterone enanthate injection (Table 2). Androsterone is affected only to a limited extent and does not exceed a Δδ-value of 3 ‰ at any time. The same applies to etiocholanolone (results not shown). Similar results were obtained on samples from subjects 5 and 9 (results not shown).

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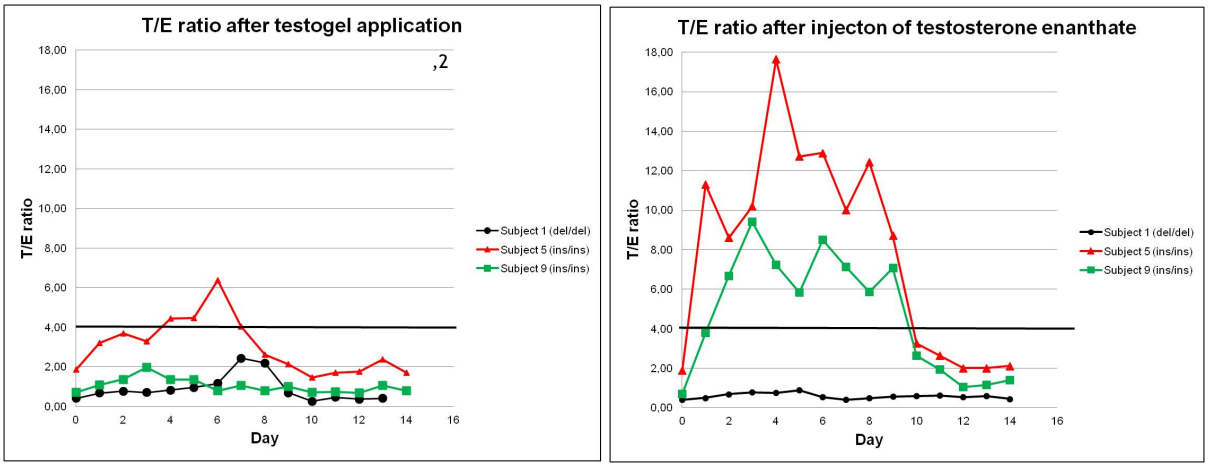

Figure 1: T/E ratio in samples collected from subject 1,5 and 9 after application of testosterone gel and testosterone enanthate. Application of gel started on day 0 and was stopped on day 7. In the second part of the study one injection of testosterone enanthate was administered on day 0.

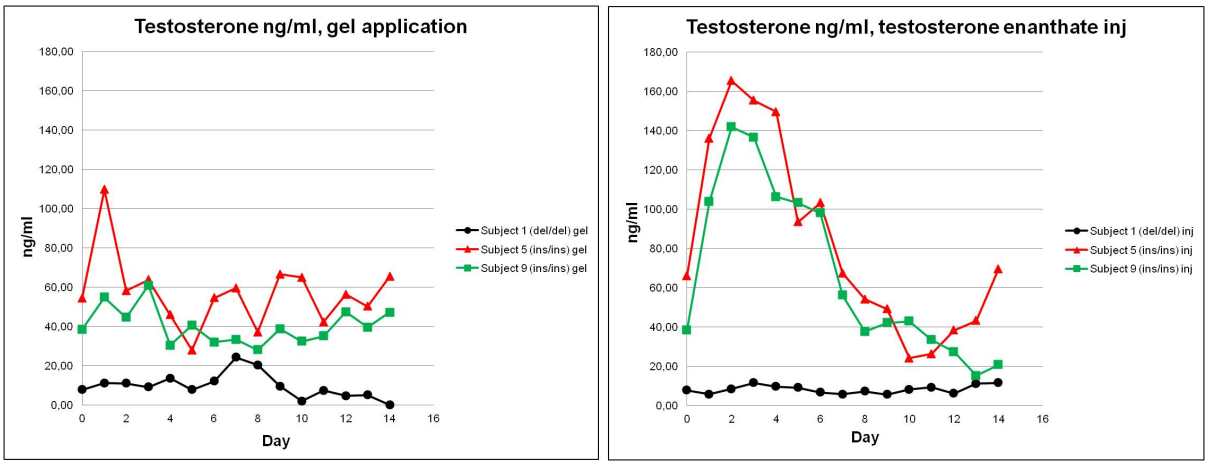

Figure 2: Concentration of testosterone, ng/mL, measured in samples collected from subjects 1,5 and 9 after application of testosterone gel and testosterone enanthate. The values are corrected using a specific gravity of 1.020. Application of gel started on day 0 and was stopped on day 7. In the second part of the study one injection of testosterone enanthate was administered on day 0.

Subject 1 (del/del) Testosterone gel application			
	Testosterone	5b-diol	Androsterone
Sample	Δδ (‰)	Δδ (‰)	Δδ (‰)
Day 0	0.5	1.5	0.3
Day 1	< LOD	1.3	0.7
Day 3	2.2	2.5	0.9
Day 6	3.9	3.8	1.4
Day 8	4.7	5.0	2.4
Day 10	< LOD	4.2	0.8

Subject 1 (del/del) Testosterone enanthate injection			
	Testosterone	5b-diol	Androsterone
Sample	Δδ (‰)	Δδ (‰)	Δδ (‰)
Day 0	0.5	1.5	0.3
Day 1	4.5	1.4	0
Day 2	< LOD	2.6	0.4
Day 3	3.0	4.7	1.9
Day 4	< LOD	3.5	0.7
Day 5	5.1	4.5	1.1
Day 6	4.4	2.9	0.5
Day 7	< LOD	1.6	0.4

Table 2: 13C/ 12C Δδ-values in selected samples collected from subject 1 (del/del). Pregnanediol was used as ERC.

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Conclusions

The approach relying on population based criteria for the selection of samples for IRMS analysis might give false negative results. This study shows that IRMS can be a very sensitive technique for detecting the use of low doses of testosterone. There will be a need for sensitive selection criteria to find suspicious samples, and steroid profiles should be a part of the Athlete Biological Passport. In subjects with the *UGT2B17* genotype del/del the excretion of testosterone glucuronide is low and constitutes an analytical challenge in the IRMS analysis. In this case it will be of importance to measure $^{13}C/^{12}C$ δ-values of 5a-androstanediol and 5b-androstanediol, in agreement with Piper et al [4].

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Acknowledgements

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