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Longitudinal monitoring of steroid profiles – Improvements to the analytical procedure

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Background

In recent years, there has been an increasing focus within the anti-doping community on the concept of applying subject-based thresholds for the detection of prohibited substances or methods. This concept is already being implemented in the field of haematological analysis, and the possible application of subject-based thresholds for the urinary testosterone to epitestosterone (T/E) ratio has been discussed by Sottas *et al*. [1]. In comparison with the current, population-based threshold of T/E = 4, subject-based thresholds have been shown to significantly increase the possibility of detecting testosterone abuse [2]. This approach may be expanded to include any other analyte or ratio showing a smaller intra-individual than inter-individual variability. In addition to testosterone and epitestosterone, several endogenous steroids are of particular interest [3] if they are monitored longitudinally, and it is plausible that subject-based upper and lower thresholds may be applied to steroid ratios other than T/E.

In order for this approach to be effective, initial testing for endogenous steroids must be performed by a quantitative procedure which is properly validated and which has a well defined measurement uncertainty.

Experimental

Sample preparation

Based on the commonly used extraction procedure for anabolic steroids in urine [4, 5], an analytical procedure for the quantitative determination of selected endogenous steroids in urine was developed and validated.

Quantification of testosterone (T), epitestosterone (E), androsterone (Andro), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5a-diol), 5β-androstane-3α,17β-diol (5b-diol) and dehydroeipandrosterone (DHEA) was performed by use of a five-point calibration curve. The calibrators were prepared by addition of the target analytes to Surine artificial urine (Cerilliant, Texas, USA). The calibration ranges were chosen to allow quantification of each selected compound within the concentration range observed in the majority of normal samples. Deuterated analogues (National Measurement Institute, Sydney, Australia) of each target steroid were used as internal standards, with the exception of DHEA. Additionally, d_3-dihydrotestosterone glucuronide was added to all samples, and the ratio of d_3-DHT to d_3-T was monitored to assure adequate hydrolysis.

Instrumental analysis

The GC-MS analysis was performed on an Agilent 6890 gas chromatograph coupled to an Agilent 5973N mass spectrometer. The column was a J&W Ultra 1 capillary column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 µm. The carrier gas was helium at a constant flow of 0.6 ml/min. The initial column temperature was 180 °C, increasing by 3.3 °C/min to 231 °C, then increasing by 30 °C/min to a maximum of 330 °C/min, which was maintained for 2 min. The mass spectrometer was operated in selected ion monitoring (SIM) mode, and two ions were acquired for each of the target analytes.

Population study

The procedure was applied to investigate the intra-individual variability of the steroid profile in a group of male athletes over a period of time. Ten football players (age 17 to 28 years, average 24) at national level took part in the study, in which morning urine samples were collected every 14 days over a period of six months. All test subjects declared no previous or current use of anabolic-androgenic steroids or other medications which might interfere with the steroid profile, and all samples collected at the start of the experiment were tested negative for prohibited substances.

Results and discussion

Validation results

The results of the method validation are summarised in Table 1.

Analyte	***Target m/z***	**Internal standard**	**Linear range (ng/mL)**	**LOQ (ng/mL)**	**Intermediate precision (RSD, %)**
Testosterone	432	d3-T	2 – 200	1	4.1
Epitestosterone	432	d3-E	2 – 200	1	4.0
Androsterone	434	d3-Andro	100 – 10 000	90	3.8
Etiocholanolone	434	d5-Etio	100 – 10 000	80	4.3
5α-Androstane-3α,17β-diol	241	d3-5a-diol	10 – 1000	10	3.6
5β-Androstane-3α,17β-diol	241	d5-5b-diol	10 – 1000	7	4.2
Dehydroepiandrosterone	432	d3-E	2 – 200	3	12.2

Table 1: Summary of results from the method validation for the quantification of endogenous steroids in urine.

It was shown that the calibration curves were linear within the chosen range for all compounds. The limits of quantification are comparable to what has previously been reported [6]. The intermediate precision was shown to be in the range of 4 % for all compounds, except for DHEA. It is probable that the analytical precision for DHEA can be improved considerably if d3-DHEA (Steraloids, Rhode Island, USA) is used as internal standard.

Population study results

All concentrations were corrected to a specific gravity of 1.020, and the concentration ratios of T/E, Andro/Etio and 5a-diol/5b-diol were plotted as a function of time for each test subject. The results show that for all three ratios, the intra-individual variability is significantly smaller than the inter-individual variability. For the T/E ratio (Figure 1), the intra-individual coefficient of variation is between 10 % and 20 %. This is in accordance with the belief that T/E in males generally varies by less than 30 % [7, 8].

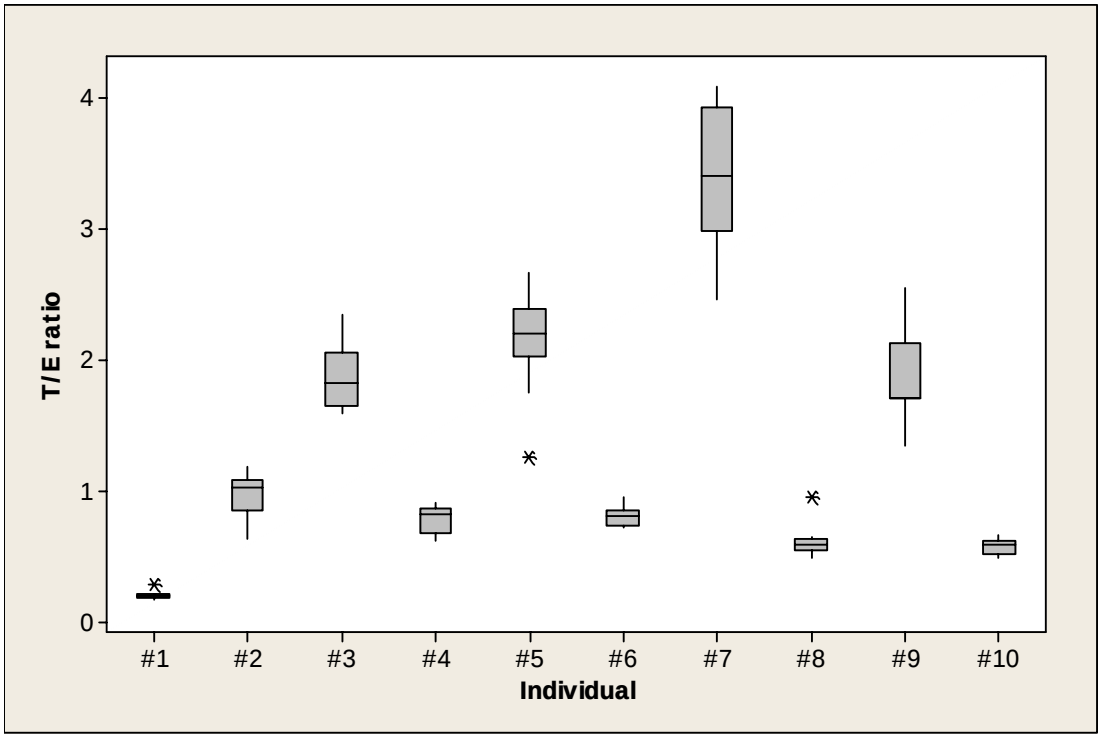

Figure 1: Urinary T/E ratios for ten test subjects over a time period of six months (n = 12).

The ratio of Andro/Etio (Figure 2) shows a pattern of variation similar to what was observed for T/E. The intra-individual coefficient of variation is between 9 % and 20 %.

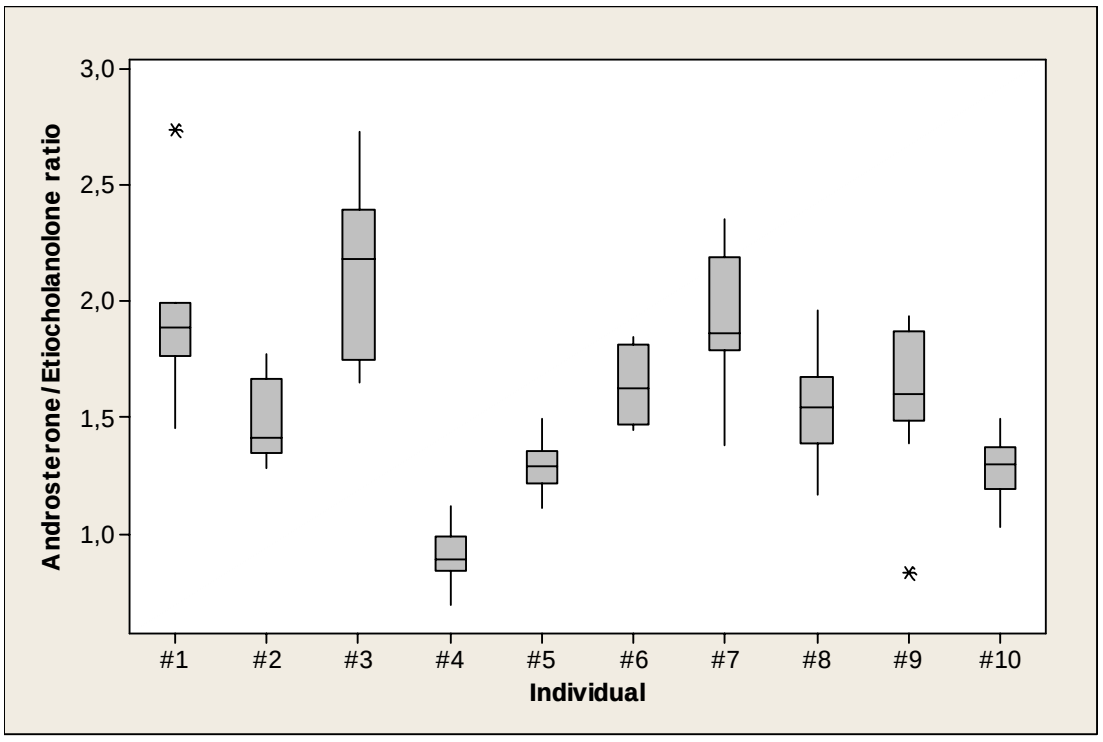

Figure 2: Urinary Andro/Etio ratios for ten test subjects over a time period of six months (n = 12).

The ratio of 5a-diol/5b-diol (Figure 3) shows a somewhat greater variability for several subjects, however, the intra-individual coefficient of variation does not exceed 30 %.

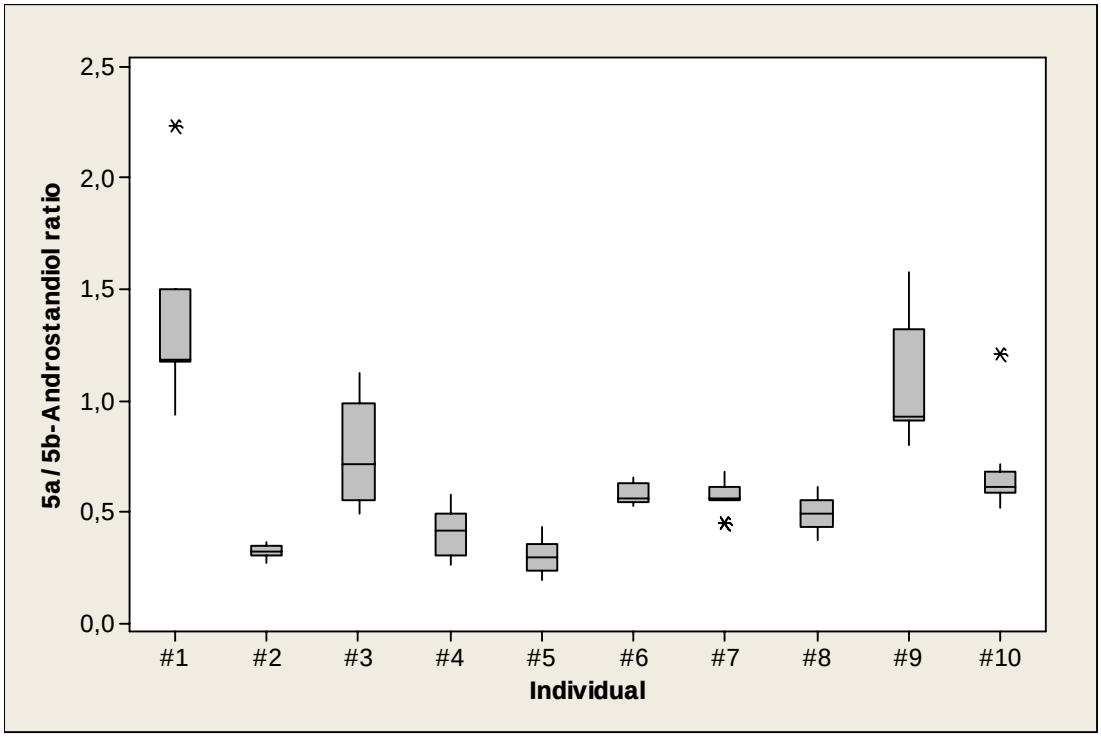

Figure 3: Urinary 5a-diol/5b-diol ratios for ten test subjects over a time period of six months (n = 12).

The significantly smaller intra- than inter-individual variability observed for T/E, Andro/Etio and 5a-diol/5b-diol forms a basis for the application of statistical tools to establish subject-based thresholds.

Conclusion

Various statistical models have previously been discussed for the prediction of normal longitudinal variability in the T/E ratio. Bayesian statistics, as proposed by Sottas *et al*. [2], can be used to generate subject-based upper thresholds for T/E, and the results of this study shows that statistical models may also be used to generate subject-based upper and lower thresholds for Andro/Etio and 5a-diol/5b-diol.

In comparison with the current, fixed decision limits, subject-based thresholds appear to provide greater selectivity and sensitivity in determining which samples should be subjected to further investigation.

References

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