Research Article

Drug Testing and Analysis

Received: 29 June 2010 Revised: 1 September 2010 Accepted: 5 September 2010 Published online in Wiley Online Library: 1 December 2010

(www.drugtestinganalysis.com) DOI 10.1002/dta.206

# Subject-based steroid profiling and the determination of novel biomarkers for DHT and DHEA misuse in sports

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**Doping with natural steroids can be detected by evaluating the urinary concentrations and ratios of several endogenous steroids. Since these biomarkers of steroid doping are known to present large inter-individual variations, monitoring of individual steroid profiles over time allows switching from population-based towards subject-based reference ranges for improved detection. In an Athlete Biological Passport (ABP), biomarkers data are collated throughout the athlete's sporting career and individual thresholds defined adaptively. For now, this approach has been validated on a limited number of markers of steroid doping, such as the testosterone (T) over epitestosterone (E) ratio to detect T misuse in athletes. Additional markers are required for other endogenous steroids like dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA). By combining comprehensive steroid profiles composed of 24 steroid concentrations with Bayesian inference techniques for longitudinal profiling, a selection was made for the detection of DHT and DHEA misuse. The biomarkers found were rated according to relative response, parameter stability, discriminative power, and maximal detection time. This analysis revealed DHT/E, DHT/5$\beta$-androstane-3$\alpha$,17$\beta$-diol and 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol/5$\beta$-androstane-3$\alpha$,17$\beta$-diol as best biomarkers for DHT administration and DHEA/E, 16$\alpha$-hydroxydehydroepiandrosterone/E, 7$\beta$-hydroxydehydroepiandrosterone/E and 5$\beta$-androstane-3$\alpha$,17$\beta$-diol/5$\alpha$-androstane-3$\alpha$,17$\beta$-diol for DHEA. The selected biomarkers were found suitable for individual referencing. A drastic overall increase in sensitivity was obtained.**

**The use of multiple markers as formalized in an Athlete Steroidal Passport (ASP) can provide firm evidence of doping with endogenous steroids.** **Copyright © 2010 John Wiley & Sons, Ltd.**

**Keywords:** biomarkers; steroid doping; dihydrotestosterone; dehydroepiandrosterone; comprehensive steroid profiling; Bayesian inference; doping analysis

## Introduction

Naturally occurring steroids have hypertrophic capacities for muscle tissue and can accordingly increase the athlete's strength. Therefore, these performance-enhancing substances are listed as prohibited compounds by the World Anti-Doping Agency (WADA).[1] Their detection has subsequently been implemented in routine procedures of doping control laboratories. Nowadays, endogenous steroids are believed to be among the most widespread doping substances misused in sports. In 2008, more than 40% of the total adverse analytical findings and atypical findings reported by WADA-accredited testing laboratories were attributed to endogenous steroids.[2]

The detection of misuse with naturally occurring steroids remains challenging since analytical screening methods cannot differentiate between synthetic and natural compounds. The costly and laborious confirmation analysis with gas chromatography/combustion/isotope ratio mass spectrometry has been applied since the end of the 1990s to differentiate exogenous from endogenous steroids in suspicious urine samples, but today availability of synthetic steroids with the same C13/C12 ratio as endogenous ones can seriously limit this approach.[3]

Supplementation with endogenous steroids alters basal steroid concentrations. In the early 1990s, population-based reference ranges were established for testosterone (T), epitestosterone (E), androsterone (Andro), etiocholanolone (Etio), 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol (5$\alpha\alpha\beta$-Adiol), 5$\beta$-androstane-3$\alpha$,17$\beta$-diol (5$\beta\alpha\beta$-Adiol) and a number of steroid ratios i.e. T/E, Andro/Etio, Andro/T.[4,5] These reference values were applied to screen for outlying values characteristic of doping with steroids. At that period, the studies mainly focused on the administration of T.

To defeat doping control tests in the early 1990s, athletes started to use new endogenous steroids which alter the steroid profile in different ways than T.[6] 5$\alpha$-dihydrotestosterone (DHT or androstanolone), a direct metabolite of T, was suited for this purpose as it leaves the T/E ratio unchanged just after administration. Indeed, the enzymatic 5$\alpha$-reductase conversion of T to DHT is considered as an irreversible process.[7] Moreover, DHT was known to be a 3-times more potent androgen than its precursor as it shows greater affinity for the androgen receptor

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complex.[8] Increased androgenic effect can lead to aggressive behaviour which can be beneficial in some sport disciplines like combat sports.[9] In addition, unlike T, DHT is non aromatizable which avoids its users from having undesirable estrogenic effects such as gynaecomastia.[10] These reasons provided DHT as a good candidate for improvement of physical and psychological condition in sports. As DHT misuse was investigated[11] and detection criteria were established in the mid 1990s,[6,7,12,13] the endogenous 5$\alpha$-androstane-3$\beta$,17$\beta$-diol was marketed as a prohormone of DHT to obtain the same effects and was sold as a nutritional supplement.[14,15]

From 1996 onwards, DHEA has been marketed as a freely available food supplement. Together with androstenedione (Adion) and andostenediol, DHEA became an over-the-counter product in the United States. Elsewhere in the world, DHEA could be obtained via the Internet or as pharmaceutical preparations from local drug stores with a medical prescription. DHEA is known to be the most abundant androgen circulating in the body and its sulfate ester acts as a reservoir or buffer for steroid genesis of androgens. As the high concentrations decline during lifespan, it was believed that DHEA had anti-ageing effects [16] and was marketed accordingly. Although DHEA is generally claimed to be a weak androgen, the anabolic nature of DHEA has been proven using DNA micro arrays to compare its genomic expression profile with that of DHT.[17] DHEA metabolises via Adion to T and is therefore considered a prohormone of T.[18,19]

Both DHT and DHEA have been misused by athletes. Consequently, several groups have reported on the detection of both DHT [6,7,20] and DHEA[21–24] misuse and proposed decision criteria based upon population statistics.

Although it was already known in the 1990s that subject-based reference ranges are more reliable than population-based reference ranges for androgens and their ratios,[25] it is only in 2007 that an evaluation method was proposed for this purpose.[26] In that model, Bayesian inference techniques are used to switch the focus from comparison with a population to the determination of individual reference values as the number of individual records grows.[27] Today, following the Athlete Biological Passport (ABP) paradigm, individual records comprising results from blood and/or urine analyses acquired in different doping control laboratories are stored over time to reveal the biological response of doping or effects of a medical condition requiring a closer examination. The Athlete Steroidal Passport (ASP) is the endocrine module of the ABP that collects steroid profiling data obtained from urine samples.[28] The ASP records some steroids and metabolites which are routinely monitored in steroid profiling for doping control purposes. The ASP currently includes T, E, Andro, Etio, 5$\alpha\alpha\beta$-Adiol, 5$\beta\alpha\beta$-Adiol, DHEA, and DHT. In addition to the T/E ratio, relevant steroid ratios such as Andro/Etio, 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol, 5$\alpha\alpha\beta$-Adiol/Etio, and Andro/T are also evaluated. In its present form, the ASP remains mainly operational for the alteration of the steroid profile induced by different routes of T administration.

Minor steroid metabolites are important markers for specific determination of the misuse in sports with other naturally occurring steroids, for example, 7-keto-dehydroepiandrostenendione,[29] DHEA [18,22] and Adion [18,30,31] which can be easily purchased as food supplements. Together with traditionally monitored steroids, these hydroxylated steroid metabolites provide important information on how the steroid profile is altered after misuse of particular endogenous steroids. Hence, additional markers may provide useful information regarding the administration of other steroids like DHT and DHEA. This work aims to identify novel biomarkers for DHT and DHEA administration in sports, with a selection among a large set of potential markers based on the results obtained by the Bayesian framework of the ASP.

## Materials and Method

### Analysis

Five ml of urine was supplemented with internal standards 17$\alpha$-methyl-testosterone, (16,16,17$\beta$-d3)-testosterone and (16,16,17$\beta$-d3)-epitestosterone enzymatically hydrolyzed with $\beta$-glucuronidase from E. Coli at pH 7.4, 56 °C for 2.5 h. Liquid-liquid extraction was performed with 5 ml diethyl ether at pH 9.4 for 20 min. The extract was dried under nitrogen after which derivatisation with a mixture of MSTFA/$NH_4I$/ethanetiol (1150/3/6, v/w/v) was performed at 80 °C for 1 h. The GC–MS analysis was performed on an Agilent 6890 GC system coupled to a 5975B VI MSDmass spectrometer from Agilent Technologies (Palo Alto, CA, USA). The validation of the method is described previously.[32]

### Steroid profiling data

In a clinical trial carried out during 3 weeks, 6 healthy male volunteers (24.7 $\pm$ 1.0 years, 79.7 $\pm$ 9.5 kg) were administered with 250 mg dermal application of DHT at the first day of week 2 and with 50 mg oral DHEA at the first day of week 3. In the pre-administration week, blank urines were collected for longitudinal profiling. All volunteers delivered 3 urine samples a day excepting for the day of administration where 6 urines were collected. A total of 396 urine specimens were used in this study. Detailed information about the administration protocol can be found elsewhere.[19]

Care was taken for correct quantitation: Five-point calibration curves were used for all monitored steroid metabolites and all samples were manually checked for correct integration of chromatographic peak shape. All obtained concentrations were corrected for specific gravity according to the WADA technical document.[33]

All possible combinations of steroid ratios were calculated from 24 steroid concentrations which include the traditionally monitored steroid metabolites in doping control and minor steroid metabolites; for example, testosterone (T), epitestosterone (E), androsterone (Andro), etiocholanolone (Etio), 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol (5$\alpha\alpha\beta$-Adiol), 5$\beta$-androstane-3$\alpha$,17$\beta$-diol (5$\beta\alpha\beta$-Adiol), 5$\alpha$-androstane-3$\beta$,17$\beta$-diol (5$\alpha\beta\beta$-Adiol), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), 4-androstenedione (Adion), 11$\beta$-OH-androsterone (11$\beta$-OH-Andro), 11$\beta$-OH-etiocholanolone (11$\beta$-OH-Etio) and minor steroid metabolites: 4-OH-androstenedione (4-OH-Adion), 3$\alpha$,5cyclo-5$\alpha$-androstane-6$\beta$-ol-17-one (5cyclo), 7$\alpha$-OH-testosterone (7$\alpha$-OH-T), 7$\alpha$-OH-dehydroepiandrosterone (7$\alpha$-OH-DHEA), 7$\beta$-OH-dehydroepiandrosterone (7$\beta$-OH-DHEA), 6$\alpha$-OH-androstenedione (6$\alpha$-OH-Adion), 6$\alpha$-OH-testosterone (6$\alpha$-OH-T), 16$\alpha$-OH-androsterone (16$\alpha$-OH-Andro), 16$\alpha$-OH-etiocholanolone (16$\alpha$-OH-Etio), 16$\alpha$-OH-dehydroepiandrosterone (16$\alpha$-OH-DHEA), 16$\alpha$-OH-androstenedione (16$\alpha$-OH-Adion) and 6$\beta$-OH-etiocholanolone (6$\beta$-OH-Etio). From the 24 steroid concentrations, all possible ratios were generated ($23 \times 24 = 552$). Together with the 24 concentrations, a total of $552 + 24 = 576$ potential biomarkers are evaluated.

2014 samples from male athletes tested in our laboratory resulting in a negative finding were reanalyzed following the same method.[32] Population statistics were derived for all 576 biomarkers from these 2014 samples.[34]


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### Selection criteria

In a first step, each of the 576 biomarkers was rated for their response to doping with DHT and DHEA, for parameter stability and for the ratio of intra- to inter-individual variation. The response to doping was assessed as the maximal post-administrative increase of the parameter value relative to baseline and was averaged for all volunteers (mean maximum relative response: $\overline{MRR}$). For these calculations, the baseline values were computed as the mean of the pre-administration samples per volunteer. The parameter stability was assessed as the mean coefficient of variance ($\overline{CV}$) of the pre-administration data from all volunteers. In this first step, the ratio $\frac{\overline{MRR}}{\overline{CV}}$ is taken as a first selection criterion to measure the discriminative power of the biomarker to detect misuse with DHT or DHEA. A higher $\frac{\overline{MRR}}{\overline{CV}}$ indicates a better biomarker.

In 1974, the ratio of intra- to inter-individual variation was used to evaluate the use of population-based and subject-based threshold limits by Harris *et al*:[35]

$$r = \sqrt{\frac{E\sigma^2}{Var\mu_i}} \quad (1)$$

where $E\sigma^2$ is defined as the average of the intra-individual variances and $Var\mu_i$ as the variance of the individual mean values assessed on the pre-administration samples. The *r*-values could be interpreted that when $r < 0.6$, a large majority of individual is insensitive for population-based reference ranges and hence, subject-based reference ranges are preferred as a sensitive measure.

In a second step, the parameters necessary for the application of the adaptive Bayesian model were computed from pre-administration data[26,27] and individual reference limits were assessed. In detail, the intra-individual means and CVs were estimated for all 576 markers for all six volunteers using the data collected in the week before administration. In the adaptive model, 'mean' and 'CV' are variables whose (prior) distributions represent inter-individual differences.[27] Here, a log-normal distribution was assumed for all biomarkers, with the geometric mean (GM) and standard deviation (GSD) calculated from the 'mean' and 'CV' obtained from our cohort (n = 6).

A Receiver Operating Characteristic (ROC) analysis was then performed to evaluate the discriminative power of all biomarkers. In detail, the sensitivity was defined as the true positive rate (TPR) and was calculated within 84 h after administration. The specificity or 1 minus the false positive rate (1-FPR) was obtained during the pre-administration week and after 6 days post-administration. The ROC-analysis was performed by varying the threshold for levels of specificity equal to 99.9; 99.75; 99.5; 99; 97.5; 95; 90; 75; and 50%. The area under the curve (ROC-area) was calculated to determine the classification performance (accuracy) of the biomarkers.[36] All calculations were performed on Matlab version 7.7.0.

The detection time of doping substances is often cited as the major feature of a certain detection method or substance and is often well known among those who dope and their medical supervisors. As a consequence, long detection times can act as a deterrent for misuse by an athlete. Here, detection times were calculated as the time the biomarker present values outside the 99% percentile of the individual expected values.

**Table 1.** Markers ranked according to best response to stability ratio after administration of DHT

| Marker | $\overline{MRR}$ | $\overline{CV}$ | $\frac{\overline{MRR}}{\overline{CV}}$ | Rank |
|---|---|---|---|---|
| 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol | 2.9 | 0.17 | 17.8 | 1 |
| DHT/5$\beta\alpha\beta$-Adiol | 4.1 | 0.29 | 14.6 | 2 |
| DHT/E | 3.5 | 0.32 | 13.3 | 3 |
| DHT/4-OH-Adion | 3.8 | 0.31 | 12.7 | 4 |
| 5$\alpha\alpha\beta$-Adiol/7$\beta$-OH-DHEA | 3.0 | 0.26 | 12.2 | 5 |
| 5$\alpha\alpha\beta$-Adiol/T | 2.7 | 0.22 | 12.1 | 6 |
| 6$\alpha$-OH-Adion/7$\alpha$-OH-DHEA | 2.5 | 0.35 | 11.6 | 7 |
| DHT/T | 3.1 | 0.28 | 11.5 | 8 |
| 5$\alpha\alpha\beta$-Adiol/E | 2.9 | 0.26 | 11.2 | 9 |
| 16$\alpha$-OH-Andro/16$\alpha$-OH-Etio | 1.3 | 0.13 | 11.1 | 10 |
| Andro/Etio | 1.4 | 0.15 | 10.5 | 14 |
| DHT | 4.4 | 0.43 | 10.4 | 16 |

**Table 2.** Markers ranked according to best response to stability ratio after administration of DHEA

| Marker | $\overline{MRR}$ | $\overline{CV}$ | $\frac{\overline{MRR}}{\overline{CV}}$ | Rank |
|---|---|---|---|---|
| 16$\alpha$-OH-DHEA/E | 18.3 | 0.271 | 67.6 | 1 |
| 7$\beta$-OH-DHEA | 24.3 | 0.417 | 58.3 | 2 |
| 16$\alpha$-OH-DHEA/6$\beta$-OH-Etio | 21.4 | 0.373 | 57.4 | 3 |
| 7$\beta$-OH-DHEA/E | 16.5 | 0.290 | 56.9 | 4 |
| 16a-OH-DHEA/11$\beta$-OH-Andro | 18.9 | 0.349 | 54.3 | 5 |
| 7b-OH-DHEA/6$\beta$-OH-Etio | 19.6 | 0.383 | 51.1 | 6 |
| DHEA/E | 12.6 | 0.260 | 48.6 | 7 |
| 16$\alpha$-OH-DHEA | 25.7 | 0.537 | 47.9 | 8 |
| 7$\beta$-OH-DHEA/11$\beta$-OH-Andro | 16.3 | 0.346 | 47.2 | 9 |
| 16$\alpha$-OH-DHEA/11$\beta$-OH-Etio | 22.6 | 0.489 | 46.2 | 10 |
| 7$\beta$-OH-DHEA/7$\alpha$-OH-DHEA | 8.5 | 0.264 | 32.2 | 20 |
| DHEA | 16.1 | 0.516 | 31.3 | 23 |
| 5cyclo | 10.9 | 0.644 | 16.9 | 62 |

## Results and Discussion

The combination of all monitored steroids leads to 552 steroid ratios which were evaluated together with the 24 steroid concentrations to find the best biomarkers for DHT and DHEA. Therefore, a selection of best biomarkers was made according to the inherent biomarkers' effect on the volunteer ($\frac{\overline{MRR}}{\overline{CV}}$) and the diagnostic performance associated with the chosen threshold limits obtained from the ROC-analysis. When these criteria remained inconclusive, individual detection times were considered to make a final selection of the best biomarkers constituted from the monitored steroid metabolites.

### Relative response and parameter stability

The $\overline{MRR}$, $\overline{CV}$ and $\frac{\overline{MRR}}{\overline{CV}}$ were computed for all 576 markers. The 10 best biomarkers according to $\frac{\overline{MRR}}{\overline{CV}}$ are ranked in descending order in Table 1 and Table 2 for DHT and DHEA, respectively.

After application of the DHT gel, moderate elevations were found due to the slow releasing effect and low bioavailability of the


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gel (estimated at 10% of the applied dose).[10] Highest responses ($\overline{MRR}$ up to 440%) were found for the steroid ratios involving the parent compound DHT followed by those containing 5$\alpha\alpha\beta$-Adiol. Hence, these results of a comprehensive statistic-based biomarker selection are in accordance with previous publications presenting the DHT/E and 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol ratios as best markers for most efficient detection of DHT misuse.[12,13] As the ratio of the 5$\alpha$- and 5$\beta$-epimers is featured by an excellent stability (CV = 17.8%), 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol shows the highest relative response to stability ratio. A large inter-individual variability was observed for the ratios 16$\alpha$-OH-Andro/16$\alpha$-OH-Etio and Andro/Etio: 4 volunteers showed no significant alteration after DHT-gel application while 2 volunteers with naturally high T/E ratios only showed a maximal variation of 70% for Andro/Etio and of 45% for 16$\alpha$-OH-Andro/16$\alpha$-OH-Etio. As a result, these 2 markers showed a limited response after a single DHT-gel administration and were not taken into consideration as best biomarkers regardless their relatively high $\frac{\overline{MRR}}{\overline{CV}}$ values.

The oral administration of 50 mg of DHEA had a more general influence on the steroid profile causing large relative responses up to 18 times the basal level of a range of monitored metabolites. The metabolites identified as the most sensitive to DHEA administration are 16$\alpha$-OH-DHEA and 7$\beta$-OH-DHEA as well as the parent drug. This confirms previous results.[19] Consequently, these metabolites are found multiple times among the parameters with highest $\frac{\overline{MRR}}{\overline{CV}}$ (Table 2). It can be noticed that the ratios with E in the denominator are amongst the parameters with highest $\frac{\overline{MRR}}{\overline{CV}}$ values. The latter have lower intra-individual CVs than the other parameters in Table 2: the average CVs of DHEA/E, 7$\beta$-OH-DHEA/E and 16$\alpha$-OH-DHEA/E are all lower than 30%. Dividing by E results in a stability gain of the marker between 13% and 27% compared to steroid concentrations. Since, steroid ratios involving E are highly ranked it is suggested that E is a more stabilizing factor relative to other metabolites as denominator in steroid ratio markers, analogous to the stability of T/E ratio.

Ratios containing known metabolites of DHEA including Andro, Etio, 5$\beta\alpha\beta$-Adiol and 5cyclo are also listed with corresponding rank to indicate their suboptimal combination of relative response and stability. The minor metabolite 5-cyclo has been proposed as a marker for DHEA administration.[22] Here, the relative response of 5cyclo to a 50 mg dose of DHEA was outperformed by a large number of other metabolites. 5cyclo was therefore not selected as an optimal biomarker for DHEA misuse.

### ROC-analysis

Since a high specificity is required in anti-doping, the ROC-areas were assessed for specificities exceeding 70% only. Table 3 and Table 4 show the 10 highest ROC-area values after DHT and DHEA administration, respectively. For DHT administration, only ratios involving DHT and 5$\alpha\alpha\beta$-Adiol in the numerator are markers producing elevated ROC-areas values. All other steroid ratios returned ROC-areas significantly smaller than those listed in Table 3. The ratios with DHT in the numerator had better discriminative power than those with 5$\alpha\alpha\beta$-Adiol.

In Table 4, biomarkers with 16$\alpha$-OH-DHEA in the numerator generally returned high ROC-area values after DHEA administration. Best discrimination was obtained for ratios including 16$\alpha$-OH-DHEA, 16$\alpha$-OH-Etio, Etio and 5$\beta\alpha\beta$-Adiol in the numerator and, again, E in the denominator. The good accuracies of the Etio, 16$\alpha$-OH-Etio and 5$\beta\alpha\beta$-Adiol versus E ratios could be associated with delayed excretion rates of 5$\beta$-steroids sulphate conjugates after DHEA administration as reported by Kazlauskas.[23] The generally longer elevated excretion profiles of these 5$\beta$-metabolites (see below) increase the classification performance expressed by the ROC-area. All ratios with E and 7$\alpha$-OH-DHEA in the denominator returned elevated ROC-areas. Surprisingly, 7$\alpha$-OH-DHEA, although reported as a less sensitive metabolite of DHEA,[18] occurred nevertheless several times in the denominator of markers having high ROC-area values.

**Table 3.** Markers ranked according to highest ROC area for specificity >70% after DHT administration

| Marker | ROC-area (spec >70%) | Rank |
|---|---|---|
| DHT/5$\beta\alpha\beta$-Adiol | 0.191 | 1 |
| DHT/Etio | 0.186 | 2 |
| DHT | 0.186 | 3 |
| DHT/T | 0.172 | 4 |
| 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol | 0.172 | 5 |
| DHT/Andro | 0.165 | 6 |
| DHT/E | 0.164 | 7 |
| 5$\alpha\alpha\beta$-Adiol/T | 0.163 | 8 |
| DHT/4-OH-Adion | 0.158 | 9 |
| DHT/16$\alpha$-OH-Etio | 0.158 | 10 |

**Table 4.** Markers ranked according to highest ROC area for specificity >70% after DHEA administration

| Ranking | ROC-area (spec >70%) | Rank |
|---|---|---|
| 16a-OH-DHEA/11$\beta$-OH-Andro | 0.266 | 1 |
| DHEA/7$\alpha$-OH-DHEA | 0.252 | 2 |
| 16$\alpha$-OH-Etio/E | 0.251 | 3 |
| 7$\beta$-OH-DHEA/11$\beta$-OH-Andro | 0.249 | 4 |
| 16$\alpha$-OH-Etio/E | 0.246 | 5 |
| 16$\alpha$-OH-DHEA/Adion | 0.245 | 6 |
| Andro/E | 0.245 | 7 |
| 16$\alpha$-OH-DHEA/16$\alpha$-OH-Adion | 0.245 | 8 |
| DHEA/11$\beta$-OH-Andro | 0.243 | 9 |
| 7$\beta$-OH-DHEA/7$\alpha$-OH-DHEA | 0.242 | 10 |
| DHEA/E | 0.242 | 11 |
| 7$\beta$-OH-DHEA/E | 0.240 | 13 |
| 7$\beta$-OH-DHEA | 0.235 | 16 |
| DHEA | 0.224 | 30 |
| DHEA/T | 0.218 | 33 |

### Biomarkers selection

Unsurprisingly, markers with a good relative response to parameter stability ratio also present a high discriminative power after application of the adaptive Bayesian model. In particular, 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol showed excellent relative response to stability ratio as well as a good discrimination to detect DHT abuse. This is in contrast to Kickman *et al.*[7] who considered the ratio of the androstanediol epimers as a less sensitive marker after a moderate dose of percutaneous DHT. The lower response, however, is balanced by the self-adapting Bayesian model that optimally benefits from the high stability of 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol which allows for setting narrow personal reference limits. The DHT/5$\beta\alpha\beta$-Adiol ratio was also highly ranked. DHT/


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5$\beta\alpha\beta$-Adiol was already proposed by Donike and coworkers[12] as a useful marker since it does not suffer from low E concentrations which might lead to biased ratios and moreover, is independent from E as a masking agent. The DHT ratios to T, a precursor of DHT, and to E are additional markers that present a high response and high discriminative aptitude. The production of T and E is suppressed by the negative feedback on the testicular steroid synthesis[7,8] making both DHT/T and DHT/E suitable markers for the detection of DHT misuse. Since T is irreversibly metabolised to DHT, no extra T is formed after supplementation of DHT. The combination of a negative feedback mechanism on both T and E and no formation of T yield an unchanged T/E ratio after DHT administration.

The best parameters found here are in accordance with those already identified by Donike[12] and Kicman.[7] Donike also proposed the DHT concentration and Andro/Etio as significant determinants. However, in a longitudinal adaptive framework, the DHT/E ratio was preferred to the DHT concentration alone because the lower intra-individual CV's of steroid ratios enhance the efficiency of longitudinal steroid profiling. In this experiment, the dose of DHT was most probably too low to account for an effect on the Andro/Etio ratio. Also, inter-individual variations were high; with only one of the volunteers showing a raise in Andro/Etio exceeding his reference limits.[19]

As DHEA affects the steroid profile to a broader extend than DHT, the selection of good markers aims to include various sensitive metabolites that globally represent the changes induced by DHEA administration. In that respect, interesting markers to screen for DHEA misuse are 16$\alpha$-OH-DHEA/E and 7$\beta$-OH-DHEA/E.

16$\alpha$-OH-DHEA/11$\beta$-OH-Andro was not included in the selection despite a similar performance as 16$\alpha$-OH-DHEA/E: the former marker presented lower detection times for 4 volunteers than the latter.

The DHEA/E ratio is a good alternative to the concentration of the parent compound – currently one of the main indicators to DHEA misuse if a population-based threshold of 100 ng/ml is exceeded. Dehennin *et al.*[21] claimed that DHEA to E ratio was an ineffective parameter for doping control purposes in relation to population-based threshold values. However, for use in the ABP, our results indicate the appropriateness of DHEA/E when combined with a longitudinal approach that excludes its large inter-individual variations. Finally, 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol is an interesting biomarker since it provided the longest detection time in the case of 3 volunteers.

### Detection times

All biomarkers were evaluated according to the duration of their supra-natural elevations towards the volunteer's own reference values. Using the adaptive model, individual thresholds were established which allow for the determination of detection times. The detection times of the 6 volunteers after DHT and DHEA application are shown in Table 5 for a set of markers (4 for DHT, 4 for DHEA) selected in terms of the criteria described earlier.

For DHT administration, DHT/5$\beta\alpha\beta$-Adiol is the marker that provided the longest detection time in 3 out of 6 volunteers. In volunteers 1 and 3, DHT/E and 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol provided the longest detection for 10 and 48 h, respectively. DHT/T does not give added value for longer detection for any of the volunteers and is therefore omitted from the final biomarker selection. Maximal detection times in this DHT study were obtained until 78 h. These detection times are comparable with those found after sublingual administration of 25 mg DHT,[13] the estimated bioequivalent of the amount active compound traversing the skin in this study. In contrast, the maximally obtained detection time according to regular population statistics was 48 h.[19] Using population statistics, the reference ranges were only exceeded for the markers DHT and 5$\alpha\alpha\beta$-Adiol in 2 respectively 4 out of 6 volunteers.[19] In contrast, the biomarkers selected in this study classified all volunteers as positive after DHT application. Moreover, multiple parameters exceeded the individual reference limit per volunteer as additional supporting evidence.

**Table 5.** Detection times using selected biomarkers after administration of 250 mg DHT gel and 50 mg DHEA in 6 subjects

| | Parameters | Detection times (h) 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| DHT | 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol | 0 | 24 | 30 | 60 | 54 | 24 |
| | DHT/5$\beta\alpha\beta$-Adiol | 8 | 48 | 24 | 78 | 72 | 24 |
| | DHT/E | 10 | 24 | 24 | 60 | 48 | 24 |
| | DHT/T | 0 | 24 | 24 | 60 | 48 | 24 |
| DHEA | 16$\alpha$-OH-DHEA/E | 8 | 60 | 54 | 30 | 12 | 36 |
| | 7$\beta$-OH-DHEA/E | 8 | 12 | 36 | 60 | 12 | 12 |
| | DHEA/E | 8 | 12 | 54 | 6 | 12 | 24 |
| | 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol | 30 | 30 | 60 | 24 | 54 | 60 |

Using the adaptive model, DHEA administration could be detected up to 60 h after ingestion.

In 4 out of 6 volunteers, 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol resulted in the longest detection. For the two other volunteers, detection until 60 h was established with 16$\alpha$-OH-DHEA and 7$\beta$-OH-DHEA. No other biomarkers resulted in longer detection than those displayed in Table 5. The previously performed population-based evaluation of the extended profile[19] gave rise to maximal detection times of 54 h in 2 volunteers compared to 5 in this study presenting equal or longer detection times.

### Appropriateness for longitudinal following

The intra-individual variation of most steroid ratios is smaller than the inter-individual variation. As shown for T/E and other steroid ratios, Donike *et al.*[37,38] established the difference of inter and intra-individual variation for a range of steroids and concentrations. By comparing both sources of biological variance, Harris *et al.*[35] established a test to quantify the appropriateness of subject-based testing. Therefore, the ratio of intra-individual to inter-individual variation according to Equation 1 was assessed for all parameters on data before administration. The *r*-values are listed in Table 6 for the same set of 8 biomarkers as selected before.

Except DHT/T, all suggested biomarkers for DHT and DHEA featured an *r*-ratio below 1.4 indicating that subject-based reference ranges are more reliable tools for these parameters than population-based references. For both administered steroids, 5 of the presented markers have a ratio of intra- to inter-individual variation below 0.6 which demonstrates that population statistics can be considered as an insensitive tool to monitor steroid variations in a person. Since DHT/T does not provide an added value regarding the detection times to the other selected markers for DHT detection nor is featured by a favourable *r*-value, it should be disposed from the selection of candidate biomarkers to detect DHT.


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**Table 6.** Relation *r* of the intra- to the inter-individual variance for selected biomarkers for DHT and DHEA in urine

| | Biomarker | *r* |
|---|---|---|
| DHT | 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol | 0.27 |
| | DHT/5$\beta\alpha\beta$-Adiol | 0.43 |
| | DHT/E | 0.66 |
| | DHT/T | 1.80 |
| DHEA | 16$\alpha$-OH-DHEA/E | 0.45 |
| | 7$\beta$-OH-DHEA/E | 0.67 |
| | DHEA/E | 0.57 |
| | 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol | 0.41 |

**Table 7.** Sensitivities (%) of selected biomarkers for DHT and DHEA using the population-based and adaptive, subject-based approach established at specificity = 99%

| | | Sensitivity% (specificity >99%) | |
|---|---|---|---|
| | Best biomarker | Population-based | Subject-based |
| DHT | 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol | 10.0 | 42.1 |
| | DHT/E | 15.0 | 40.4 |
| | DHT/5$\beta\alpha\beta$-Adiol | 16.0 | 46.8 |
| DHEA | 16$\alpha$-OH-DHEA/E | 29.5 | 43.2 |
| | 7$\beta$-OH-DHEA/E | 22.0 | 36.8 |
| | DHEA/E | 30.0 | 37.9 |
| | 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol | 7.5 | 23.0 |

### Sensitivity

In Table 7, the sensitivities for the selected biomarkers are given using ROC analysis at a specificity level of 99% for both population-based and subject-based thresholds.

As such, the population-based approach, as used nowadays in anti-doping laboratories, resulted in sensitivities between 10% and 16% to detect doping with a DHT-gel. By applying the adaptive Bayesian approach of the ABP to the selected markers, a maximal sensitivity of 41% in a 4-day time window was reached after DHT administration. For the selected markers, an average increase in sensitivity of 29% was associated with the conversion from population- to subject-based threshold limit using the adaptive model.

Today, DHEA detection mainly relies on the population statistics for the concentration of DHEA in urine with a 100 ng/ml threshold prescribed by WADA.[33] In our study, this criterion yielded a sensitivity of 28.6% with a specificity of 98.7%. A 99% reference limit, calculated in previous work,[34] had a sensitivity of 22% without detecting any false positives in this experiment. Using a higher population-based threshold limit for urinary DHEA concentrations before submitting a urine sample to IRMS was also supported by Dehennin *et al*.[21] and Mareck *et al*.[24] Hence, the number of samples to be forwarded to IRMS for confirmation can be decreased knowing that 4.5% of a normal Caucasian population would most probably exceed the current 100 ng/ml screening threshold.[34] Additionally, in a population-based approach, the discriminative power could be slightly increased to 30% sensitivity for a 99% specificity level by applying the selected DHEA markers. The implementation of these markers in the longitudinal model of the ABP significantly improved the detection performance for 50 mg DHEA attaining 43% sensitivity within 4 days post-administration. An average increase of 13% sensitivity could be attributed to a switch from the population- to subject-based approach for the DHEA markers.

The lower levels of sensitivity of the 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol ratio could be attributed to its delayed elevated profile which can be expected after 8 and 12 h post-administration when the sampling rate in the protocol was diminished.

A larger increase in sensitivity was found for the DHT-gel application as the subject-based threshold is particularly efficient for minor changes in the steroid profile caused by long-acting steroid formulation as gels in contrast to the insensitivity of general reference limits. This was also concluded by Geyer *et al*.[39] for detection of T gel use.

These results indicate that a subject-based approach significantly increases the sensitivity in the detection of endogenous steroids like DHEA and DHT.

## Conclusion

A paradigm shift is taking place in doping analysis towards subject-based referencing for endogenous agents, the so-called Athlete Biological Passport. With respect to naturally produced anabolic steroids, the ABP was initially developed for the T/E ratio, a sensitive marker to detect T misuse in sports. This work contributes to the steroidal module of the ABP by studying the detection of other endogenous steroids like DHT and DHEA which do not necessarily alter the T/E ratio. For both steroids, a set of biomarkers was selected out of more than 500 steroid ratios to optimally increase the sensitivity in longitudinal testing after the administration of single low doses. Good candidate markers identified to detect DHT were DHT/E, DHT/5$\beta\alpha\beta$-Adiol and 5$\alpha\alpha\beta$-Adiol/5$\beta\alpha\beta$-Adiol and for DHEA administration DHEA/E, 16$\alpha$-OH-DHEA/E, 7$\beta$-OH-DHEA/E and 5$\beta\alpha\beta$-Adiol/5$\alpha\alpha\beta$-Adiol.

By considering several markers derived from an extended steroid profile, the probability of guilt can be significantly increased within the probabilistic framework of the ABP. By using the individual as his own reference, a single 250 mg administration with DHT-gel could be detected until 78h and one 50 mg oral dose of DHEA yielded detection times until 60h. The application of new biomarkers, suitable for and combined with longitudinal profiling, can improve the sensitivity as much as a factor of 4.

This suggests that longitudinal follow-up as performed nowadays in doping control following the ABP framework is suitable for the detection of single doses of DHT and DHEA, wherein multiple biomarkers offer complementary information to maximalize the detection times.

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