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# Detection of dihydrotestosterone gel, oral dehydroepiandrosterone, and testosterone gel misuse through the quantification of testosterone metabolites released after alkaline treatment

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**The natural occurrence of endogenous anabolic steroids together with their availability in different administration forms makes the detection of their misuse a great challenge for doping control laboratories. Nowadays, the detection of endogenous steroids abuse is performed by the analysis of the steroid profile. Recently, androst-1,4-dien-3,17-dione (1,4-AD), androst-4,6-dien-3,17-dione (4,6-AD), 17β-hydroxy-androst-4,6-dien-3-one (6-T), and androst-15-en-3,17-dione (15-AD) have been described as testosterone (T) metabolites released after basic treatment of the urine. In the present work, the usefulness of these metabolites has been evaluated detecting the use of three different forms of endogenous steroids in a single dose: dihydrotestosterone gel (DHT), oral dehydroepiandrosterone (DHEA), and T gel. After the independent administration of these endogenous steroids, a rise in the value of several of the ratios calculated between the tested metabolites was noticed. For DHT, a small increase was observed for the ratios 1,4-AD/15-AD, 6-T/15-AD and 4,6-AD/15-AD although only for one volunteer. Better results were obtained for oral DHEA and T gel where an increase was observed in all volunteers for several of the tested ratios. The detection time in which the misuse can be detected (DT) has been evaluated using two different approaches: (1) comparison with population based reference limits, and (2) comparison with individual threshold levels. The obtained DTs were compared with the results of previously published markers for the misuse of such substances. When using basic released metabolites, shorter DTs were obtained for DHT, similar DTs for DHEA, and the detectability was substantially improved for T gel. Copyright © 2011 John Wiley & Sons, Ltd.**

**Keywords:** doping analysis; DHT; DHEA; testosterone gel; mass spectrometry

## Introduction

Testosterone (T) is an endogenous anabolic androgenic steroid (AAS) which was purified and synthesized in 1935. Since then, analogous compounds either naturally present in the body or new synthetic steroids have been marketed and introduced in sports as anabolic agents.[1] AAS are misused by athletes in order to increase lean body mass. For this reason, AAS are included in the list of prohibited substances published by the World Anti-Doping Agency (WADA).[2]

The detection of endogenous AAS misuse is a great challenge for doping control laboratories due to their natural presence in the body and availability as different substances and administration forms (dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), testosterone (T) or androstendione administered orally, intramuscularly or topically). Natural occurrence of these substances make that anti-doping control laboratories have to differentiate between endogenous AAS exogenously administered.

The gold-standard for this differentiation is the analysis by gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS).[3,4] However, this analysis is time-consuming and from a practical point of view cannot be applied, as a screening method, to the large number of samples managed in the doping control field. Therefore, GC/C/IRMS is only used as a confirmatory tool. Before applying this confirmation, screening methodologies able to discern between negative and suspicious samples are required.

The screening for endogenous AAS is normally based on the determination of the steroid profile and the comparison with established reference limits (RLs). Only when the RLs are exceeded are these samples considered suspicious. The main problem of this approach is that the RLs are based on population

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statistics which encompass large inter-individual variations. Nevertheless, the urinary concentrations of some steroids can show individual and ethnical variations, making the establishment of a unique RL difficult. That is the case of T, detection of which was proposed in 1983 by the ratio between the urinary testosterone and to its isomer epitestosterone (T/E) excreted as glucuronide.[5] In recent years, a T/E threshold of 4 was established by WADA.[6] Samples with a T/E value above this limit are considered suspicious for T misuse. Due to a genetic polymorphism a part of the population have T/E basal values far below the threshold limit, whereas other subjects are always above this threshold.[7,8] In the last few years, in order to overcome the limitations exhibited for the RLs, the establishment of subject-based or genotype-based reference ranges has been proposed as a suitable tool.[9–11]

T is the most reported endogenous AAS.[12] It is available in different forms like pills, injectable solutions, or gels. The application of the gel is approved in T replacement therapy in men with low T conditions. The principal problem is the poor absorption through the skin. Only 9–14% of the total administered T is absorbed, making it an ideal preparation to administer microdoses for cheating athletes.[13] As a consequence, changes in the steroid profile are low which leads to additional problems to detect T gel misuse with the actual available methods.[13–15] Besides the T/E, other ratios like 5α-androstane-3α,17β-diol (5ααβ-Adiol)/E and androsterone/E have been reported for long-term T gel administration detection.[15]

DHEA is available as a supplement in tablet, capsule, liquid, and sublingual forms. It is a weak endogenous androgen that may be used by athletes with the aim of increasing levels of more active androgens such as T and DHT.[16] According to WADA's statistics, DHEA is the second, most reported endogenous AAS.[12] The detection of DHEA administration has been difficult due to incomplete understanding of DHEA metabolism.[17–19] The administration of DHEA has shown to have minimal effects on urine T/E ratio.[17] Therefore several markers have been proposed for the detection of DHEA administration like the concentrations of DHEA or its 3α,5-cyclo metabolites, or the measurement of several ratios like 16α-hydroxy-DHEA/E, 7β-hydroxy-DHEA/E or DHEA/E.[18,20]

DHT is considered more potent than its precursor T. DHT binds with greater affinity to the androgen receptor and dissociates from the complex more slowly.[21] The enzymatic 5α- reductase promotes the conversion of T to DHT.[21] Several ratios like those between DHT and E or between 5ααβ-Adiol and 5β-androstane-3α,17β-diol (5βαβ-Adiol) were postulated in order to detect its misuse.[20,22,23]

Recently, the usefulness of four compounds released after alkaline treatment of the urine, androst-1,4-dien-3,17-dione (1,4-AD), androst-4,6-dien-3,17-dione (4,6-AD), 17β-hydroxy-androst-4,6-dien-3-one (6-T) and androst-15-en-3,17-dione (15-AD) have been characterized and a quantitative method has been developed.[24,25] The application of these metabolites for the detection of oral T undecanoate administration has been demonstrated.[26]

The goal of this study is to evaluate the variations observed for markers based on these four metabolites released after basic treatment after a single dose administration of DHT gel, oral DHEA, and T gel. Two statistical approaches are evaluated, based either on population reference limits or individual thresholds, and the results compared with previously published markers.

## Experimental

### Chemicals and reagents

Androst-4,6-dien-3,17-dione (4,6-AD) and 17β-hydroxy-androst-4,6-dien-3-one (6-T) were obtained from Steraloids Inc. (Newport, USA). Androst-1,4-dien-3,17-dione (1,4-AD) was purchased from NMI (Pymble, Australia), T, epitestosterone, methandienone (used as internal standard for liquid chromatography tandem mass spectrometry (LC-MS/MS)) and methyltestosterone (used as internal standard for GC-MS) were obtained from Sigma-Aldrich (St Louis, MO, USA). The β-glucuronidase preparation (from *Escherichia coli* type K12) was purchased from Roche Diagnostics (Mannheim, Germany).

Analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate and *tert*-butyl-methyl ether, and ammonium iodide were obtained from Merck (Darmstadt, Germany). The derivatization reagent *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and 2-mercaptoethanol was from Sigma-Aldrich (St Louis, MO, USA). Diethyl ether was purchased from Fisher scientific (Leicestershire, UK).

Acetonitrile and methanol (LC gradient grade), formic acid, ammonium formate (LC/MS grade) and cyclohexane were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

### LC-MS/MS instrumentation

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates, Milford, MA, USA) for the chromatographic separation. Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 °C and the source temperature to 120 °C.

The LC separation was performed using an Eclipse Plus $C_{18}$ column (50 x 2.1 mm i.d., 1.8 μm) (Agilent, Palo Alto, CA, USA), at a flow rate of 300 μl/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: at 0 min., 45%; at 1 min., 45%; at 3 min., 50%; at 10 min., 50%; at 11 min., 95%; at 11.5 min., 95%; at 12 min, 45%; at 15 min., 45%.

Analytes were determined by a SRM method including two transitions for each compound.[22] The most abundant and specific transitions (285 > 121 for 1,4-AD, 285 > 149 for 4,6-AD, 287 > 151 for 6-T and 287 > 95 for 15-AD) were selected for quantitative purposes. For 15-AD a semi-quantification was made using 6-T as a reference compound due to the impossibility of a commercial reference standard.

### GC-MS instrumentation

The GC-MS analysis was performed on an Agilent 6890 GC system coupled to a 5975B VI MSD mass spectrometer from Agilent Technologies (Palo Alto, CA, USA). The instrument was equipped with a

17 m J&W Ultra1 column (internal diameter 0.2 mm, film thickness 0.11 μm) (Palo Alto, CA, USA). The GC temperature program was: at 0 min, 120 °C; then 70 °C/min up to 177 °C, then 4 °C/min up to 231 °C, then 30 °C/min up to 300 °C, then 2 min to 300 °C. The temperatures of other instrument parts were 270 °C for the injector, 250 °C for the transfer line, 230 °C for the ion source and 150 °C for the quadrupole; 0.5 μl were injected in splitless mode.

Helium was used as carrier gas which was under constant pressure of 84.9 kPa. The instrument was operated in full scan mode for qualitative purposes between the *m/z* 50 and 650. For steroid quantification, selective ion monitoring (SIM) was used with a dwelltime of 20 ms for all monitored ions.[27] Additionally, *m/z* 272 was monitored to screen for possible presence of the mono-trimethylsilyl derivatives of androsterone and etiocholanolone which is an indication of incomplete derivatization by the MSTFA/$NH_4I$/ethanethiol (1150/3/6, *v/w/v*) mixture.

### Sample preparation

The method used for LC-MS/MS determination was based on the method described and validated elsewhere.[25] Briefly, after addition of 50 μl of ISTD solution (methandienone, 1 μg/ml), 2.5 ml of urine were basified by addition of 300 μl of 6 M KOH. The mixture was heated at 60 °C for 15 min, followed by a liquid-liquid extraction with 6 ml of *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 μl of a mixture of water:acetonitrile (1:1, *v/v*). Finally, 10 μl were directly injected into the LC-MS/MS system.

For GC-MS, the procedure is based upon the methods developed elsewhere.[27] After addition of 50 μl of ISTD solution (methyltestoterone at 2 μg/ml), 1 ml of phosphate buffer (0.1 M $Na_2HPO_4{\cdot}_2H_2O$/$NaH_2PO_4{\cdot}H2O$, pH 7) was added, and hydrolyzed with 50 μl of β-glucuronidase during 2.5 h at 56 °C. After cooling, 200 mg of NaHCO3/$K_2CO_3$ (2/1, *w/w*) buffer were added. Liquid-liquid extraction was performed by addition of 5 ml of freshly distilled diethyl ether. The tubes were centrifuged at 2700 rpm for 5 min and the organic layer was separated, dried over anhydrous $Na_2SO_4$ and evaporated under oxygen free nitrogen (OFN). The dry residue was derivatized with 100 μl MSTFA/$NH_4I$/ethanethiol (1150/3/6, *v/w/v*) for 1 h at 80 °C.

### Urine samples

For the excretion studies, the following single doses (one per week) were administered to three male volunteers (age 23–26 years): A therapeutic preparation of 50 mg DHEA ingested orally; a topical testosterone application (Androgel, Laboratories Besins International, Paris, France) and a topical application of DHT (Andractim, Besins International, Brussels Belgium) applied on upper arm, chest, thorax and abdomen in doses containing 100 mg and 250 mg active compound, respectively. All participants gave their written informed consent and the study was approved by the Ethical Committee of the Ghent University Hospital (B67020064707).

Urine samples collected before administration (five samples) and at 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, and 72 h after DHT gel, DHEA or T gel administration were analyzed. The urine were stored at −20 °C until analysis

### Data analysis

Two different approaches were applied in order to evaluate the usefulness of these markers for the detection of DHT gel, DHEA, and T gel administration and other previously published markers.

First, a comparison was performed between the results obtained after administration and the population based RLs previously published.[26,28] In this approach, a sample is considered suspicious if the value of the marker is higher than the RL.

Secondly, individual thresholds (IT) for the tested markers for each volunteer after the analysis of five samples before the administration were established, followed by the application of the equation: Mean ± 3*Standard deviation (SD).[9] In this approach, a sample is considered suspicious if the value of the marker is higher than the IT.

The detection time (DT) was defined as the maximum time after the application of the AAS in which the sample is considered suspicious, i.e. the values of the marker is above the upper RL in the first approach or above the upper IT in the second. In order to calculate the DTs, the excretion studies obtained after the administration of a single dose have been used.

## Results

### Population-based reference limits (RLs)

*Basal values*

Twelve ratios can be calculated between the four metabolites released in alkaline conditions. Some of them were multiplied by a factor in order to obtain data easier to interpret. Basal values normally below the RL previously established[26] were obtained.

**Table 1.** Detection times (DT) obtained using both the 97.5% reference limit (RL)[24,26] and individual threshold (IT) for the ratios within compounds released after alkaline treatment and ratios previously reported for DHT administration

| | | DHT | | | | | |
|---|---|---|---|---|---|---|---|
| | | DT (h) (with RL) | | | DT (h) (with IT) | | |
| | | Volunteer | | | Volunteer | | |
| Marker | 97.5% RL | 1 | 2 | 3 | 1 | 2 | 3 |
| 5ααβ-Adiol/5βαβ-Adiol | 1.6 | - | 24 | - | - | 72 | 72 |
| DHT/5βαβ-Adiol | 0.4 | - | - | - | 8 | 24 | 72 |
| DHT/E | 1.0 | - | 24 | 72 | 12 | 24 | 72 |
| 4,6-AD/1,4-AD (x1/100) | 1.8 | 10 | - | - | 10 | - | - |
| 1,4-AD/15-AD (x10) | 14.1 | - | - | - | 12 | - | - |
| 6-T/15-AD | 15.6 | - | - | - | 12 | - | - |
| 4,6-AD/15-AD (x10) | 7.7 | - | - | - | 12 | - | - |

**Table 2.** Detection times (DT) obtained using both the 97.5% reference limit (RL)[24,26] and individual threshold (IT) for the ratios within compounds released after alkaline treatment and ratios previously reported for DHEA administration

| DHEA | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | DT (h) (with RL) | | | DT (h) (with IT) | | |
| | | Volunteer | | | Volunteer | | |
| Marker | 97.5% RL | 1 | 2 | 3 | 1 | 2 | 3 |
| 5βαβ-Adiol/5ααβ-Adiol | 8.3 | - | 72 | 72 | 30 | 72 | 72 |
| 16α-OH-DHEA/E | 2.3 | 4 | 24 | 48 | 8 | 30 | 30 |
| 7β-OH-DHEA/E | 8.4 | 2 | 6 | 12 | 8 | 30 | 12 |
| DHEA/E | 6.7 | - | 10 | 6 | 8 | 30 | 6 |
| 6-T/1,4-AD (x1/10) | 2.9 | All | 4 | 4 | 12 | 12 | 4 |
| 4,6-AD/1,4-AD (x1/100) | 1.8 | 72 | 4 | 4 | 12 | 4 | - |
| 6-T/15-AD | 15.6 | - | - | - | 12 | 24 | 12 |
| 4,6-AD/15-AD (x1/10) | 7.7 | - | - | - | 12 | 12 | 8 |
| 1,4-AD/15-AD (x10) | 14.1 | - | - | - | - | 10 | 12 |

The main exception were the ratios 4,6-AD/1,4-AD and 6-T/1,4-AD for volunteer 1. For this volunteer, the mean of the basal values were 1.7 (4,6-AD/1,4-AD) and 3.3 (6-T/1,4-AD) which are close to the RLs (1.85 and 2.9, respectively). These values together with the standard deviation (0.13 and 0.28, respectively) made that some basal values exceeded the RL.

Similar situation was observed for some previously reported markers. Regarding T/E, basal values for volunteer 1 were below the limit established by WADA of 4 (mean 1.7, standard deviation 0.4); however, higher T/E basal values were observed for the other volunteers. For volunteer 2, one basal value was above this limit (mean 3.8, standard deviation 0.8) and for volunteer 2, basal values always above the WADA's threshold were found (mean 5.1, standard deviation 1.4). Similarly, basal values for 5βαβ-Adiol/5ααβ-Adiol and 16α-hidroxy-DHEA/E were close to or even higher than the RL for volunteers 2 and 3. Basal values below the RL were found for the rest of markers.

*DHT gel*

From the 12 potential ratios between the basic released metabolites, 4 of them (4,6-AD/1,4-AD, 1,4-AD/15-AD, 6-T/15-AD, 4,6-AD/15-AD) increased after DHT gel administration only in one of the volunteer (volunteer 1). Nevertheless, none of these ratios increased above the RL.

Regarding previously reported markers, values above the RL were obtained for DHT/E for volunteers 2 and 3 with DTs higher than 24 h for all of them (Table 1). Interestingly, this marker did not provide successful results for volunteer 1.

*DHEA*

Five out of the twelve potential ratios (6-T/1,4-AD, 4,6-AD/1,4-AD, 6-T/15-AD, 4,6-AD/15-AD and 1,4-AD/15-AD) increased after DHEA administration. The use of RLs for some of these markers like 4,6-AD/1,4-AD and 6-T/1,4-AD resulted in DTs of 4 h for volunteers 2 and 3 (Table 2), whereas most of the samples were above the RL for volunteer 1 due the high basal value.

Among previously described markers, the use of 7β-hydroxy-DHEA/E allowed for the detection of DHEA misuse in the three volunteers with DTs between 2 h and 12 h. Similar DTs were observed with DHEA/E although it was not able to detect DHEA misuse for volunteer 1. The longest DTs (72 h) were observed with 5βαβ-Adiol/5ααβ-Adiol although these satisfactory results could not be obtained in volunteer 1. For 16α- hydroxy -DHEA/E relatively high DTs (between 4 h and 48 h) were observed. However, as explained before, it has to be taken into account that several samples were above the RL for volunteer 2 and 3 due to the high basal values for these markers.

**Table 3.** Detection times (DT) obtained using both the 97.5% reference limit (RL)[24,26] and individual threshold (IT) for the ratios within compounds released after alkaline treatment and ratios previously reported for T gel administration

| T gel | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | DT (h) (with RL) | | | DT (h) (with IT) | | |
| | | Volunteer | | | Volunteer | | |
| Marker | 97.5% RL | 1 | 2 | 3 | 1 | 2 | 3 |
| T/E | 4* | - | All | All | - | - | - |
| 1,4-AD/15-AD (x10) | 14.1 | - | - | - | 12 | 10 | 10 |
| 6-T/15-AD | 15.6 | - | - | - | 10 | 10 | - |
| 4,6-AD/15-AD (x10) | 7.7 | - | - | - | 12 | 10 | - |

* WADA threshold value.

*T gel*

Four ratios between metabolites released after alkaline treatment increased after T gel administration. However, none of these markers (1,4-AD/15-AD, 6-T/15-AD, 4,6-AD/15-AD and 4,6-AD/1,4-AD) showed values above their RL (Table 3). In volunteer 1, some samples collected after T gel were above the RL established for 4,6-AD/1, 4-AD (97,5% RL = 1.8) probably due to the high basal values for this volunteer.

Similar effect was observed for T/E. After T gel administration, several samples were above the WADA's threshold for T/E in volunteer 2 and all of them were above this limit in volunteer 3 due to the elevated basal values for these volunteers.

**Individual threshold (IT)**

*DHT gel*

As already explained, variations in the values of ratios between basic released metabolites were only observed after DHT gel administration for volunteer 1. In this case, DTs between 10 h and 12 h were obtained by the use of IT of the four selected markers (1,4-AD/15-AD, 6-T/15-AD 4,6-AD/15-AD and 4,6-AD/1,4-AD) (Table 1, Figures 1d, 1e, and 1f). Remarkably, none of these markers allowed for the detection of DHT gel misuse in volunteers 2 and 3.

Regarding the previously published markers for the DHT detection,DTs between 8 h and 72 h were reported using DHT/5βαβ-Adiol and DHT/E for all volunteers (Figures 1b and 1c). DTs of 72 h were reported using 5ααβ-Adiol/5βαβ-Adiol for volunteer 2 and 3 although this marker did not provide satisfactory results for volunteer 1.

*DHEA*

After DHEA administration, five of the tested markers exhibited an increase in their values. Markers giving longer DTs were found to be 6-T/15-AD, 4,6-AD/15-AD, 6-T/1,4-AD and 4,6-AD/1,4-AD (Figures 2e, 2g, 2h, 2f). Two of the markers (6-T/15-AD and 4, 6-AD/15-AD) allowed for the detection of DHEA administration in all three volunteers whereas the other three gave satisfactory results for two of the volunteers (Table 2). DTs between 8 h and 24 h were obtained with these markers.

Similar DTs (between 6 h and 30 h) were found when using some ratios proposed in the literature for DHEA misuse: 16αOH-DHEA/E, 7βOH-DHEA/E and DHEA /E (Figures 2a–2d). The longest DTs were obtained using the ratio 5βαβ-Adiol/5ααβ-Adiol (Table 2).

*T gel*

Three of the potential markers allowed for the detection of T gel administration. Whereas the use of 1,4-AD/15-AD allowed the detection of T gel misuse in all volunteers (Table 3), the use of 6-T/15-AD and 4,6-AD/15-AD made possible the detection of T gel administration in volunteer 1 and 2 (Figures 3c and 3d). DTs between 10–12 h were obtained by the use of these markers. The marker 1,4-AD/4,6-AD which was found to be the most useful for the detection of oral administration of testosterone[26] also increased for all volunteers but it only exceeded the threshold value in one of them.

No significant changes in the common marker for T misuse the T/E were observed after T gel administration (Figure 3a), all the values being below the established IT.

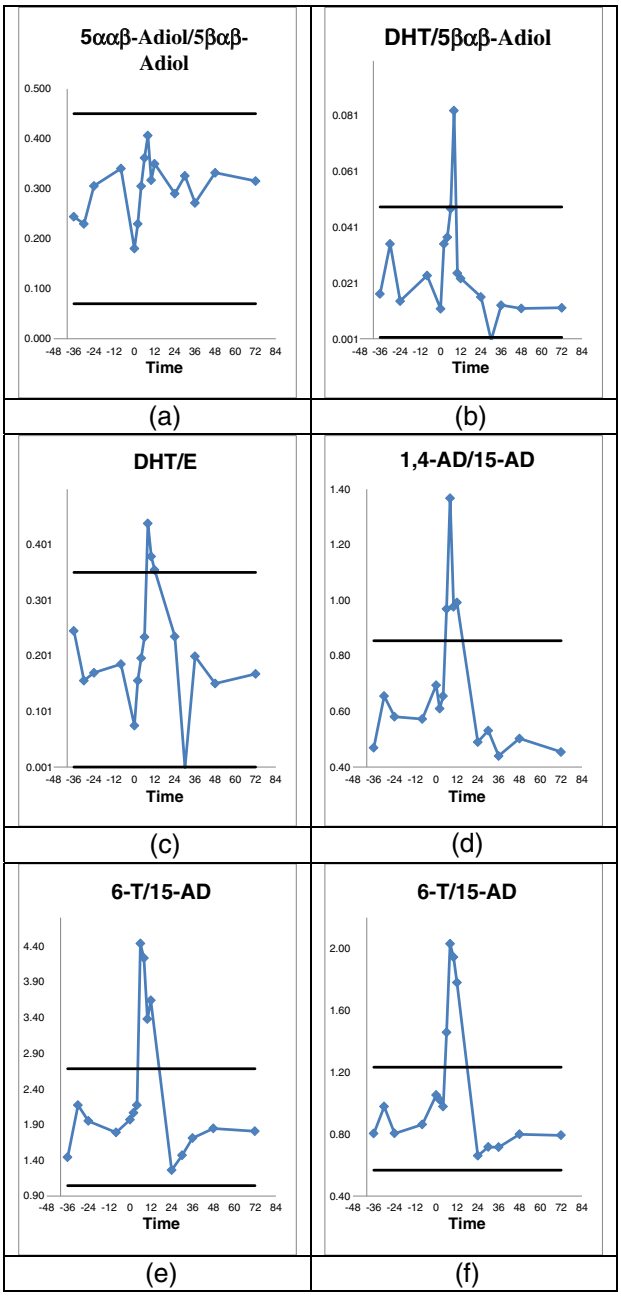


**Figure 1**. Results of DHT gel administration in volunteer 1: ratios observed for T metabolites released after alkaline treatment (d) 1,4-AD/15-AD; (e) 6-T/15; (f) 4,6-AD/15-AD and ratios previously published (a) 5ααβ-Adiol/5ααβ-Adiol; (b) DHT/5βαβ-Adiol; (c) DHT/E. Bold lines indicate individual thresholds (IT).

## Discussion

The usefulness of four T metabolites released after alkaline treatment has been evaluated for the detection of a single dose administration of DHT gel, oral DHEA, and T gel. Two alternative approaches for the detection of a suspicious sample have been tested: (1) the use of population based reference limits, and (2) the use of individual threshold limits.

The application of population based RLs for basic released T metabolites showed most of the limitations of this approach already reported for conventional enzymatic released metabolites. On the one hand, subjects having a basal value far below

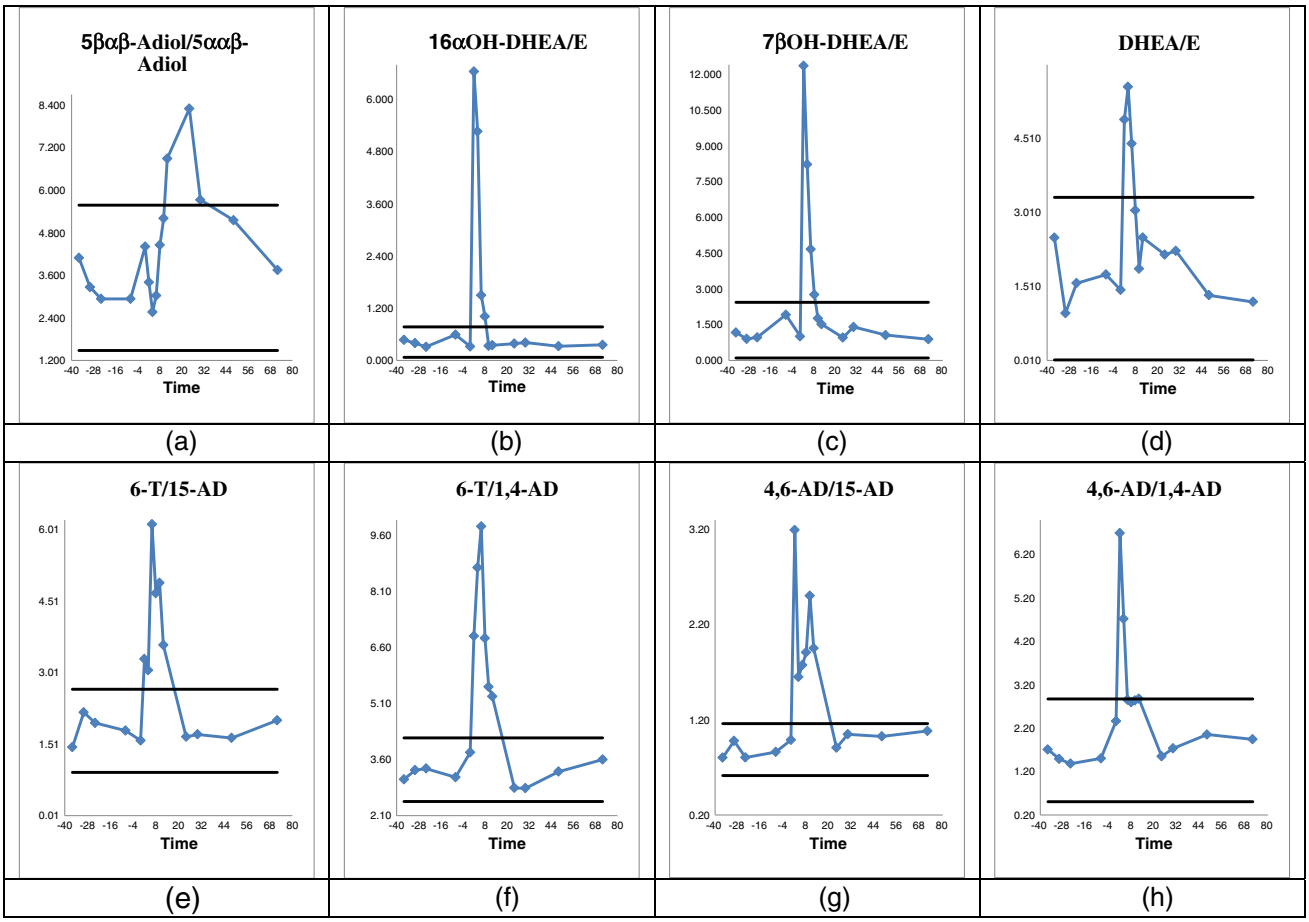


**Figure 2**. Results of DHEA administration in volunteer 1: ratios observed for T metabolites released after alkaline treatment (e) 6-T/15-AD; (f) 6-T/1,4-AD; (g) 4,6-AD/15-AD; (h) 4,6-AD/1,4-AD and ratios previously published (a) 5βαβ-Adiol/5ααβ-Adiol; (b) 16αOH-DHEA/E; (c) 7βOH-DHEA/E; (d) DHEA/E. Bold lines indicate individual thresholds (IT).

the threshold and exhibiting a significant increase cannot be detected by the RLs approach. On the other hand, subjects show a basal value higher than the established RL and therefore the RL is inadequate to distinguish between samples collected before and after administration. This fact is illustrated by the detection of DHEA administration by means of the 6-T/1,4-AD ratio (Table 2). Since the basal value for volunteer 1 (mean 3.3, standard deviation 0.28) is higher than the RL (2.9) all the samples from this subject would be considered as suspicious by this approach causing a risk of false positive findings. On the other hand, the low basal value for volunteer 2 (mean 1.3, standard deviation 0.30) made that the administration could be detected only in samples collected 4 h after DHEA intake. Similar results have been reported for the steroid profile[29] making the use of individual threshold limits necessary to improve detection sensitivity in the doping control.[9,10,15,23,30]

Similarly to what was observed with conventional metabolites, the efficacy of basic released T metabolites was improved by the use of IT limits. Using this strategy, valuable pieces of information can be extracted for the three AAS tested.

The formation of 1,4-AD, 4,6-AD and 6-T from DHT would imply the addition of two double bonds and the inherent loss of the 5α-hydrogen. Therefore, it is not expected that the amount of these metabolites would increase after DHT administration. In contrast, it seems more rational the use of ratios between 5α-containing metabolites and 5α-non-containing metabolites (5β or 4-ene metabolites) as DHT marker. This fact was confirmed after sublingual DHT administration, where the marker 5ααβ-Adiol/5βαβ-Adiol increased substantially.[23] Similar results were also observed after DHT gel administration in volunteers 2 and 3 where the use of 5ααβ-Adiol/5βαβ-Adiol, DHT/5βαβ-Adiol and DHT/E provide DTs higher than 24 h, whereas the markers based on basic released metabolites did not exhibit any increase (Table 1). An exception for this behaviour was volunteer 1, in which 5ααβ-Adiol/5βαβ-Adiol did not increase, thus showing an unexpected behavior for DHT. For this volunteer, DTs lower than 12 h were obtained using DHT/5βαβ-Adiol and DHT/E as markers. Similar DTs for volunteer 1 were observed when using markers based on basic released metabolites. The most satisfactory markers were those related with 15-AD, which might indicate a drop in 15-AD concentration after topical DHT administration. In any case, the use of basic released metabolites did not provide any substantial improvement in the detection of DHT administration.

Several ratios based on basic released metabolites were found to be useful for the detection of DHEA administration. Metabolites 4,6-AD and 6-T were involved in most of these ratios suggesting an increase in the concentration of these metabolites after oral DHEA administration. The formation of these metabolites from DHEA would imply either oxidative transformations from 5-ene to 4,6-diene and from 3-hydroxy to 3-oxo or conversion from DHEA to T and subsequent transformation from T to 4,6-AD and 6-T. Since 1,4-AD is also a direct T metabolite[24] and no increase of this metabolite is observed, the first hypothesis is

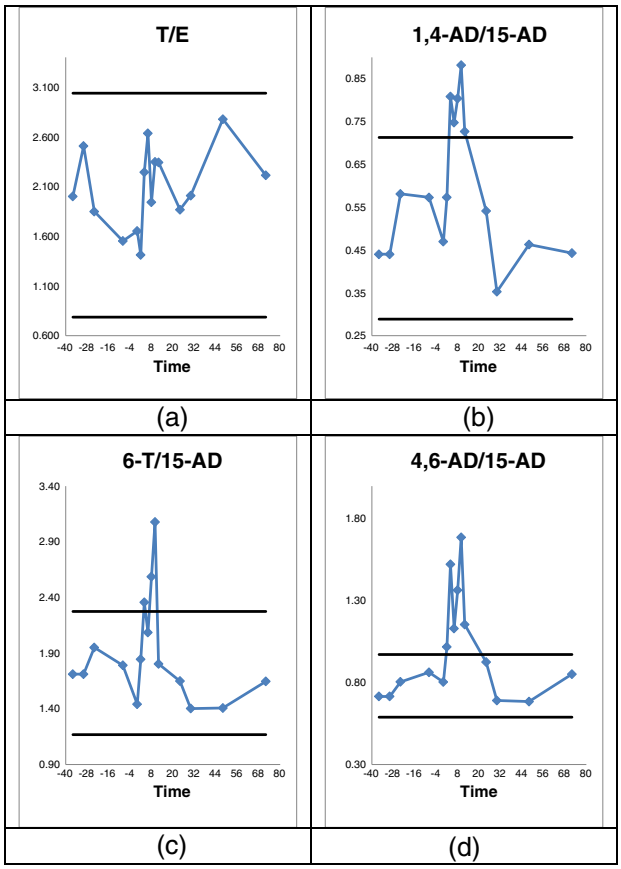


**Figure 3**. Results of T gel administration in volunteer 1: (a) T/E ratio and ratios observed for T metabolites released after alkaline treatment; (b) 1, 4-AD/15-AD; (c) 6-T/15-AD; (d) 4,6-AD/15-AD. Bold lines indicateindividual thresholds (IT).

more likely to occur. Using these metabolites, 6-T/15-AD was the best marker found for DHEA administration. DTs between 12 and 24 h were obtained (Table 2). These results were comparable to those obtained with previously reported markers. The longest DTs were obtained using 5βαβ-Adiol/5ααβ-Adiol although the increase of this ratio was less significant than the observed with the other ratios (Figure 2).

The four compounds evaluated in this study were reported as T metabolites and all of them increased after oral T intake being the highest increase for 1,4-AD and 15-AD.[24] After topic T administration, several markers based on these metabolites were found. Most of them were based on 15-AD suggesting either a decrease on 15-AD concentration or a simultaneous increase in the concentration of the other three compounds. These results differed drastically from those generated after an oral T administration where the highest increase was observed for 15-AD. A possible explanation could be the lack in the skin of the enzymes needed for 15-AD formation. The best marker was found to be 1,4-AD/15-AD which allows for the detection of T gel administration in all the volunteers with DTs higher than 10 h (Table 3; Figure 3). These results represent a significant advance in T gel detection since it could not be detected using previously reported markers like T/E. Currently, the main disadvantage of the use of these ratios is the absence of commercially available 15-AD. Once it is available, the usefulness of these results should be confirmed by using a quantitative method also for 15-AD.

Therefore, the use of basic released metabolites can be useful for doping control analysis mainly in the detection of oral DHEA and T gel administration. However, most of the useful markers involve the quantification of 15-AD. A semi-quantitative method was used for the quantification of this compound due to the lack of pure standard. It is expected to obtain more reliable results with a method including the 15-AD standard.

In the present study, excretion studies obtained after a single administration of drugs were evaluated. It has to be taken into account that this is not the most likely scenario when dealing with AAS users. Prolonged administrations of AAS are needed in order to obtain the expected benefits. The analysis of samples obtained after multiple doses have shown that some markers like T/E, 5ααβ-Adiol/E and androsterone/E which are not useful for single dose administration of T gel can be applicable for the detection of T gel after administration for longer periods.[13,15] In this sense, it will be valuable to test the applicability of the markers presented in this study for the evaluation of multiple administrations of endogenous AAS. The evaluation of these results would help in the consideration of these metabolites as complement of the analytes currently included in the steroid profile.

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