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Alternative Steroid Profiling: Reference ranges for urinary endogenous steroids in a caucasian population of athletes

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Introduction

At the level of the laboratories, doping control samples are anonymous which currently does not allow for the use of individual reference ranges. Therefore, monitored endogenous steroids are compared to population based reference ranges. A few of such reference ranges are included in the WADA technical document on endogenous steroids [1]. Currently, routine screening methods for anabolics contain a limited number of endogenous steroids. This can result in a lack of specificity of traditional steroid profiling methods regarding a variety of prohormones and recombinant steroids available on the prohormone market like testosterone (T), dehydroepiandrosterone (DHEA), androstenedione (Adion), dehydroepiandrosterone (DHT),…[2] Another difficulty is the big inter-individual variation in the urinary levels of these endogenous steroids. These fluctuations in urinary steroid parameters can be attributed to inter-individual effects (e.g.gender, genotype, ethnic descent) and intra-individual factors (e.g. menstrual cycle, pregnancy, circadian rhythm, disease, age or even personal influences such as diet, drugs or exercise) [3]. These variations reduce the sensitivity of the current steroid profiling methods and the assessed decision limits.

Therefore, an extended steroid profiling method has been developed including several minor metabolites and additional concentration ratios to provide improved screening of the steroid profile. Based upon a database of more than 3000 doping samples, the reference ranges of these extra parameters have been set.

Experimental

Method: A steroid profiling method containing 29 steroids and metabolites has been developed and validated [4]. The monitored oxygenated and hydroxylated metabolites are listed in table 1.

Table 1: The monitored compounds

	Compound	Abbreviaton
1	Androsterone	Andro
2	Etiocholanolone	Etio
3	Testosterone	T
4	Epitestosterone	E
5	5α-Androstane-3α,17β-diol	5ααβ-Adiol
6	5β-Androstane-3α,17β-diol	5βαβ-Adiol
7	5α-Androstane-3β,17β-diol	5αββ-Adiol
8	Dehydroepiandrosterone	DHEA
9	Dihydrotestosterone	DHT
10	Androstenedione	Adion
11	11β-OH-Androsterone	11β-OH-Andro
12	11β-OH-Etiocholanolone	11β-OH-Etio
13	3α,5-cyclo-5α-androstan-6β-ol-17-one	5cyclo
14	4-OH-Testosterone	4-OH-T
15	4-OH-Androstenedione	4-OH-Adion
16	4β-OH- Dehydroepiandrosterone	4β-OH-DHEA
17	6β-OH-Androsterone	6β-OH-Andro
18	6β-OH-Etiocholanolone	6β-OH-Etio
19	6-oxo-Androstenedione	6-oxo-Adion
20	6α-OH-Testosterone	6α-OH-T
21	6α-OH-Androstenedione	6α-OH-Adion
22	7β-OH- Dehydroepiandrosterone	7β-OH-DHEA
23	7α-OH- Dehydroepiandrosterone	7α-OH-DHEA
24	7α-OH-Testosterone	7α-OH-T
25	7-keto- Dehydroepiandrosterone	7-keto-DHEA
26	16α-OH-Etiocholanolone	16α-OH-Etio
27	16α-OH-Androsterone	16α-OH-Etio
28	16α-OH- Dehydroepiandrosterone	16α-OH-DHEA
29	16α-OH-Androstenedione	16α-OH-Adion

In order to detect the low urinary concentrations of some metabolites, 5 ml of urine was submitted to enzymatic hydrolysis with E. Coli to screen for the free and glucuronidated steroid fractions. Liquid-liquid extraction with diethyl ether and derivatisation step using MSTFA/NH_4I/ethanethiol were applied before injection into the GC/MS.

Sample Collection: This study was approved by the ethical committee of the university hospital of Ghent (EC UZG 2005/33). More than 3000 left-over urine samples from routine screening were reanalysed using the extended steroid profiling method with consent of the Belgian doping authorities. All samples were tested at least three months earlier with current routine screening methods and stored until reanalysis at -20°C. Samples without adverse analytical finding were selected as reference population consisting out of at least 2000 male and 1000 female healthy athletes.

Regarding the location of the sample collection from the approving NADO in Belgium, it was assumed that the reference population was Caucasian. Urine samples from athletes performing high risk sports like bodybuilding and weightlifting and from disabled athletes were not included. (We feared that their medication might cause unwanted interferences during analysis.)

Data analysis: The acquired data were adjusted for density of 1.020 using

$$C_{1.020} = \frac{(1.020-1)}{(\text{density}_{sample}-1)} \times C_{sample}$$

Samples with densities below 1.004 were omitted to avoid too big correction factors. Table 2 enumerates the steroid ratios that were assessed.

Table 2: Steroid ratios

	ratios
1	Andro/Etio
2	T/E
3	Andro/11β-OH-Andro
4	Etio/11β-OH-Etio
5	Adion/E
6	DHT/E
7	5ααβ-Adiol/5βαβ-Adiol
8	11β-OH-Andro/11β-OH-Etio
9	(Andro/Etio)/(11β-OH-Andro/11β-OH-Etio)
10	Etio/Andro
11	5βαβ-Adiol/5ααβ-Adiol

Male (n=2027) and female (n=1004) samples were treated in separate databases. Statistical outliers were confirmed via GC/C/IRMS and rejected from the reference population. The results of the Kolmogorov-Smirnov to verify the normality of the distribution of the steroid profile parameters were negative. A non-parametric statistical approach was applied to calculate the reference ranges. For this purpose, we used the software package 'REFVAL' which was recommended by the Expert Panel on Theory of Reference Values of the International Federation of Clinical Chemistry (IFCC) [5]. The 97.5%, 99% and 99.9% percentile reference values and their respective 95% confidence intervals were calculated.

Excretion studies: Three male and healthy volunteers aged between 23 and 30 took one capsule of 6-oxo® containing 100 mg of 6-oxo-Adion in the morning. Urine samples were collected before (0h) and 2, 4, 6, 8, 10, 12, 24, 30, 36 and 48h after administration.
One male volunteer (aged 31) was administered with 7-keto®, a nutritional supplement containing 25mg 7-keto-DHEA per tablet. The volunteer provided one urine sample prior to

and 13 samples 2, 4, 6, 9, 12, 24, 30, 36, 48, 72, 96, 120 and 144h after intake of 2 tablets 7-keto®. All urines were stored at -20°C until analysis.

Results

The results of the reference range calculations from a male and female population are presented in tables 3 until 6. All tables present three upper reference limits (URL) at different levels (97.5%, 99% and 99.9%) together with their respective 95% confidence intervals (CI). Not all URL's of all steroid profile parameters could be established because of a lack of statistical data for the REFVAL-processing which required enough values to calculate higher level URL's.

Of all monitored steroids, three steroids were not detected above the limit of detection (2.5ng/ml): 6-oxo-Adion, 7-keto-DHEA and 4-OH-T. Due to coeluting peaks causing poor quantification, URL's of 4β-OH-DHEA and 7β-OH-DHEA could not be established. Nevertheless, both metabolites can be detected qualitatively and elevated concentrations can be observed.

Table 3: 97.5%. 99% and 99.9% upper reference limit (URL) and respective 95% confidence intervals (CI) of steroid concentrations for a male caucasion population (n=2027)

	Men (n=2027)					
Compounds	**97,5% URL (ng/ml)**	**95% CI (ng/ml)**	**99% URL (ng/ml)**	**95% CI (ng/ml)**	**99,9% URL (ng/ml)**	**95% CI (ng/ml)**
5Cyclo	31.3	22.9 - 41.5	41.5	25.0 - 43.8	/	/
Androsterone	6700	6390 - 6860	7910	7320 - 9090	11600	10100 - 11800
Etiocholanolone	4920	4660 - 5290	6200	5800 - 6790	9800	7800 - 10200
Testosterone	103	96.3 - 114	128	119 - 145	184	167 - 193
Epitestosterone	88.9	80.5 - 96.6	112	97.6 - 125	172	160 - 187
5α-Androstane-3α.17β-diol	155	143 - 168	199	181 - 221	405	286 - 416
5β-Androstane-3α.17β-diol	416	394 - 445	516	473 - 643	1190	955 - 1260
5α-Androstane-3β.17β-diol	21.3	17.5 - 29.7	38.3	21.7 - 368	/	/
DHEA	116	109- 123	141	132 - 160	242	186 - 244
DHT	21.5	15.2 - 26.1	26.4	17.0 - 38.6	/	/
Androstenedione	22	17.5 - 28.3	30.5	25.7 - 36.4	/	/
11β-OH-Androsterone	2750	2580 - 2850	3244	3000 - 3700	5160	4780 - 5990
11β-OH-Etiocholanolone	910	842 - 964	1060	985 - 1250	1870	1360 - 1930
7α-OH-DHEA	21.6	20.1 - 24.0	25.6	24.0 - 29.4	43.8	30.6 - 43.8
6β-OH-Androsterone	20.6	19.5 - 22.2	23.8	21.5 - 26.8	39	22.4 - 39.0
6β-OH-Etiocholanolone	90.1	82.3 - 99.6	120	105 - 134	209	144 - 254
7α-OH-Testosterone	18.8	16.4 - 20.4	22.8	21.1 - 24.2	/	/
16α-OH-Etiocholanolone	320	293 - 361	453	387 - 562	957	797 - 1300
16α-OH-Androsterone	317	276 - 337	393	360 - 465	685	524 - 737
6α-OH-Androstenedione	18.3	8.03 - 19.1	/	/	/	/
6α-OH-Testosterone	24.2	10.2 - 28.3	/	/	/	/
4-OH-Androstenedione	20.4	17.8 - 22.7	25	21.6 - 28.1	36.5	26.1 - 36.5
16α-OH-DHEA	36.5	33.0 - 40.8	46.3	41.7 - 52.4	75.6	54.9 - 87.3
16α-OH-Androstenedione	17.4	16.1 - 18.7	19.7	17.9 - 23.7	/	/

Table 4: 97.5%. 99% and 99.9% upper reference limit (URL) and respective 95% confidence intervals (CI) of steroid ratios for a male caucasion population (n=2027)

	Men (n=2027)					
Ratios	**97.5% URL**	**95% CI**	**99% URL**	**95% CI**	**99.9% URL**	**95% CI**
Andro/Etio	3.64	3.38 - 3.75	4.39	4.01 - 4.62	5.61	5.32 - 5.84
T/E	4.33	3.93 - 4.52	5.11	4.70 - 5.58	6.34	5.84 - 6.78
Andro/11β-OH-Andro	37.6	32.5 - 41.2	45.8	42.6 - 48.9	91.9	57.5 - 92.4
Etio/11β-OH-Etio	22.6	21.2 - 24.8	30.0	25.4 - 32.2	45.7	35.7 - 49.8
Adion/E	1.09	0.89 - 1.63	1.66	1.24 - 3.36	/	/
DHT/E	1.03	0.82 - 1.47	2.53	1.88 - 3.59	/	/
5ααβ-Adiol/5βαβ-Adiol	1.69	1.55 - 1.88	2.05	0.98 - 2.53	4.39	2.75 - 4.39
11β-OH-Andro/11β-OH-Etio	11.8	10.4 - 12.6	15.4	13.1 - 16.9	23.5	17.3 - 26.0
(Andro/Etio)/(11β-OH-Andro/11β-OH-Etio)	1.72	1.51 - 2.05	2.49	2.12 - 3.45	4.8	4.34 - 4.87
Etio/Andro	2.04	1.78 - 2.18	2.58	2.29 - 2.92	4.05	3.28 - 4.31
5βαβ-Adiol/5ααβ-Adiol	8.34	7.71 - 9.34	11.4	10.6 - 12.9	18.9	16.8 - 18.9

Table 5: 97.5%. 99% and 99.9% upper reference limit (URL) and respective 95% confidence intervals (CI) of steroid concentrations for a female caucasion population (n=1004)

	Women (n=1004)					
Compounds	**97.5% URL (ng/ml)**	**95% CI (ng/ml)**	**99% URL (ng/ml)**	**95% CI (ng/ml)**	**99.9% URL (ng/ml)**	**95% CI (ng/ml)**
5Cyclo	/	/	/	/	/	/
Androsterone	5910	4960 - 6600	7280	6520 - 8700	11300	8870 - 11300
Etiocholanolone	5130	4570 - 5750	6460	5670 - 7180	8880	7770 - 8880
Testosterone	29.4	24.9 - 32.3	35.7	31.2 - 51.6	/	/
Epitestosterone	28.5	24.7 - 33.8	45.3	38.5 - 51.0	/	/
5α-Androstane-3α.17β-diol	69.7	56.5 - 80.9	94.8	77.0 - 139	/	/
5β-Androstane-3α.17β-diol	212	183 - 237	283	234 - 362	444	385 - 444
5α-Androstane-3β.17β-diol	14	12.0 - 18.8	18.8	14.4 - 19.7	/	/
DHEA	119	108 - 130	165	128 - 186	231	182 - 231
DHT	20.5	15.1 - 21.1	/	/	/	/
Androstenedione	19.7	16.0 - 25.6	25.6	19.0 - 31.7	/	/
11β-OH-Androsterone	2400	2180 - 2580	2890	2530 - 3570	5720	3600 - 5720
11β-OH-Etiocholanolone	1120	971 - 1200	1410	1150 - 1980	/	/
7α-OH-DHEA	28.0	25.5 - 32.7	35.9	30.5 - 42.6	/	/
6β-OH-Androsterone	19.3	16.6 - 32.7	/	19.1 - 34.9	/	/
6β-OH-Etiocholanolone	92.9	74.2 - 108	121	102 - 142	/	/
7α-OH-Testosterone	10.5	9.67 - 12.4	14.3	12.4 - 16.1	/	/
16α-OH-Etiocholanolone	384	323 - 475	555	450 - 766	1230	749 - 1230
16α-OH-Androsterone	325	300 - 383	554	380 - 835	1920	864 - 1920
6α-OH-Androstenedione	/	/	/	/	/	/
6α-OH-Testosterone	/	/	/	/	/	/
4-OH-Androstenedione	22.6	20.9 - 23.9	27.5	23.2 - 29.1	/	/
16α-OH-DHEA	55.6	45.0 - 72.9	77.8	59.5 - 80.9	/	/
16α-OH-Androstenedione	24.4	22.8 - 25.7	26.9	24.3 - 30.8	/	/

Table 6: 97.5%. 99% and 99.9% upper reference limit (URL) and respective 95% confidence intervals (CI) of steroid ratios for a female caucasion population (n=1004)

	Women (n=1004)					
Ratios	**97.5% URL**	**95% CI**	**99% URL**	**95% CI**	**99.9% URL**	**95% CI**
Andro/Etio	2.70	2.38 - 2.83	3.05	2.82 - 3.40	4.33	3.34 - 4.33
T/E	2.65	2.46 - 3.51	3.51	2.65 - 4.00	/	/
Andro/11β-OH-Andro	26.9	20.9 - 32.5	36.1	28.9 - 42.8	/	/
Etio/11β-OH-Etio	22.4	20.6 - 25.2	27.4	23.0 - 35.0	/	/
Adion/E	1.68	1.43 - 2.23	2.28	1.58 - 2.63	/	/
DHT/E	/	/	/	/	/	/
5ααβ-Adiol/5βαβ-Adiol	1.33	1.09 - 1.45	1.58	1.41 - 2.09	/	/
11β-OH-Andro/11β-OH-Etio	9.43	8.34 - 11.3	13.5	10.6 - 16.4	/	/
(Andro/Etio)/(11β-OH-Andro/11β-OH-Etio)	1.41	1.25 - 1.62	1.85	1.67 - 2.10	/	/
Etio/Andro	2.78	2.58 - 2.99	3.61	2.96 - 4.65	5.70	4.67 - 5.67
5βαβ-Adiol/5ααβ-Adiol	15.4	13.4 - 17.7	19.9	16.7 - 22.0	/	/

The extended steroid profiling method was applied to the samples from the 6-OXO® and 7-KETO® excretion studies. After ingestion of one capsule of 6-oxo-Adion food supplement, 5 of the monitored steroids showed an increased urinary concentration: 6-oxo-Adion, 6α-OH-Adion, 6α-OH-T, 6β-OH-Andro and 6β-OH-Etio. The elevated excretion profiles are presented in figure 2.

Figure 1 presents the increased metabolite profiles after intake of 7-keto-DHEA. 7-keto-DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7α-OH-T were abundantly present in the post-administration period.

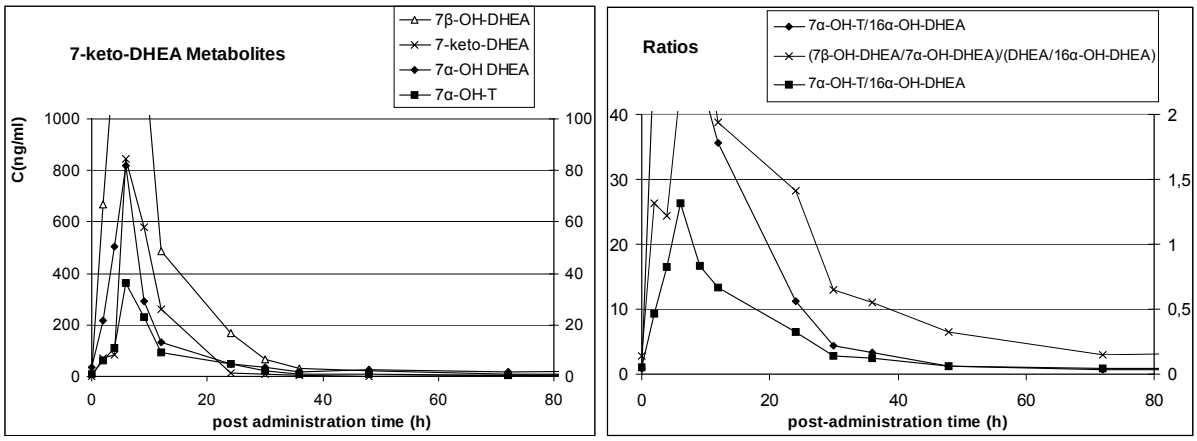

Figure 1: One volunteer excretion profiles of 7-keto-DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7α-OH-testosterone, 7β-OH-DHEA/16α-OH-DHEA, (7β-OH-DHEA/7α-OH-DHEA)/(DHEA/16α-OH-DHEA) and 7α-OH-T/16α-OH-DHEA after intake of the food supplement containing 7-keto-DHEA.

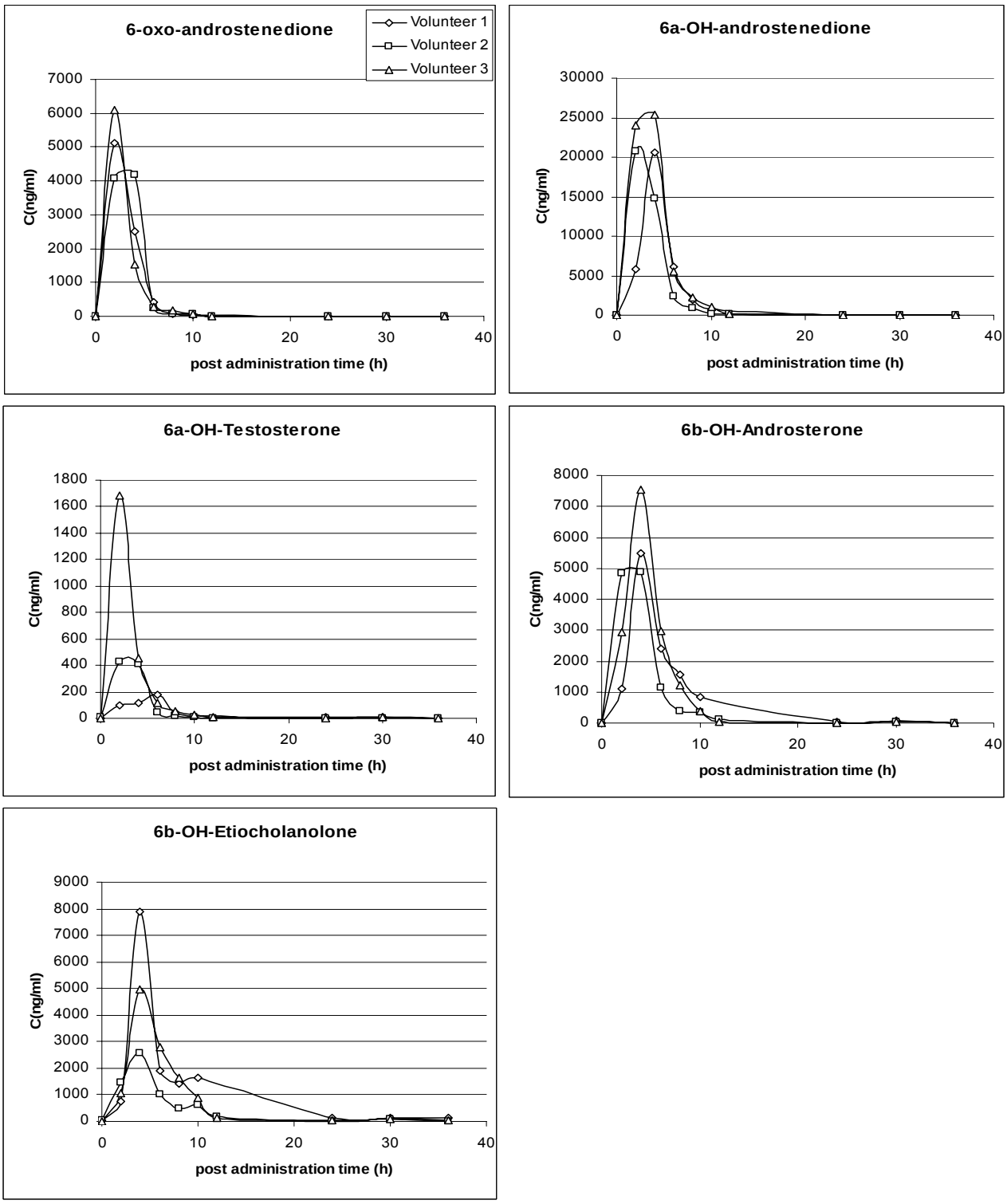

Figure 2: Post-administration profiles of 6-oxo-androstenedione, 6α-OH-androstenedione, 6α-OH-testosterone, 6β-OH-androsterone and 6β-OH-etiocholanolone after intake of the 6-oxo-androsterone food supplement in three volunteers.

Discussion

Reference Ranges: Three of the monitored steroid metabolites were not detected or only present in urinary concentrations lower than the LOD = 2.5ng/ml: 4-OH-T, 6-oxo-Adion and 7-keto-DHEA. A mere detection of one of these compounds in the glucuronide or free fraction can be considered as proof of exogenous origin of the compound or a precursor. Regarding the compounds that are abundantly present in negative urine it can be concluded that besides Andro and Etio their 11β-hydroxyl and 16α-hydroxy derivates also occur in very high concentrations in blank urine. This is reflected in high reference ranges in tables 3 and 5. On the other hand, the reference ranges of minor metabolites remain generally below 100ng/ml.

Comparing the reference ranges between men and women, table 7 lists the differences in 97.5% URL's for both sexes. Only for T and E, remarkable differences of more than 100% are noticed whereas the reference ranges of other steroids metabolites are likely more similar between males and females. Besides T and E, the URL's of the androstanediols also show differences between both sexes.

Table 7: Differences (%) between the 97.5% upper reference limits (URL) between men and women for compounds and ratios

Compounds	Difference between 97.5% URL sexes (%)	Ratios	Difference between 97.5% URL sexes (%)
Androsterone	12	Andro/Etio	30
Etiocholanolone	-4	T/E	48
Testosterone	111	Andro/11β-OH-Andro	33
Epitestosterone	103	Etio/11β-OH-Etio	1
5α-Androstane-3α.17β-diol	76	Adion/E	-43
5β-Androstane-3α.17β-diol	65	5ααβ-Adiol/5βαβ-Adiol	24
5α-Androstane-3β.17β-diol	41	11β-OH-Andro/11β-OH-Etio	22
DHEA	-2	(Andro/Etio)/(11β-OH-Andro/11β-OH-Etio)	20
DHT	5	Etio/Andro	-31
Androstenedione	11	5βαβ-Adiol/5ααβ-Adiol	-59
11β-OH-Androsterone	13		
11β-OH-Etiocholanolone	-21		
7α-OH-DHEA	-26		
6β-OH-Androsterone	7		
6β-OH-Etiocholanolone	-3		
7α-OH-Testosterone	0		
16α-OH-Etiocholanolone	-18		
16α-OH-Androsterone	-3		
4-OH-Androstenedione	-10		
16α-OH-DHEA	-41		
16α-OH-Androstenedione	-33		

Comparable to the steroid concentrations of T and E, the URL's for the T/E ratio show considerable variations between the sexes. Remarkably, the URL's of the Adion/E and 5βαβ-Adiol/5ααβ-Adiol ratios are at least 40% higher for females than for males.
WADA's technical document on endogenous steroids [1] reports only the threshold values of 5 compounds and the T/E ratio (see table 8). Except for DHEA, the WADA threshold concentrations correspond with our 99.9% URL's. The DHEA limit concentration by WADA falls below the calculated 97.5% URL's in this study. The widely known T/E ratio threshold limit of 4 is just below the 99% URL for the male population.

	WADA TD2004
Compounds	**Thresholds**
Androsterone	**10000ng/ml**
Etiocholanolone	**10000 ng/ml**
Testosterone	**200 ng/ml**
Epitestosterone	**200 ng/ml**
DHEA	**100 ng/ml**
T/E	**4**

Table 8: The WADA thresholds according the technical document on endogenous steroids

6-oxo-Adion: Analysis of excretion urines after ingestion of 6-oxo-Adion revealed the increase of the parent steroid, 6α-OH-Adion, 6α-OH-T, 6β-OH-Andro and 6β-OH-Etio in figure 2. The first three metabolites were already mentioned by Van Thuyne [6] as markers for the use of 6-oxo-Adion. In the case of 3 volunteers, 6-oxo-Adion, 6α-OH-Adion and 6α-OH-T return to their baseline levels after 12h and administration could be suspected with the 99% URL until 12h and 24h post-administration. Via the presented method we can add 2 more sensitive markers: 6β-OH-Andro and 6β-OH-Etio. It appears that these latter metabolites remain clearly longer elevated than the markers presented by Van Thuyne [6] and only fall below their respective 99% URL after 36h after administration.

7-keto-DHEA: A one-volunteer excretion study with the food supplement 7-keto-DHEA results in a changed steroid profile. Obviously, the parent compound and its two reduced hydroxyl epimers were detected in large amounts whereas these minor metabolites normally occur in low concentration as appears from their reference values table 3 and 5. Screening with the extended steroid profile ended up in the detection of another metabolite: 7α-OH-T. Figure 1 shows the rise and fall of the excretion rates of 7-keto-DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7α-OH-T after ingestion of the substance. These parameters returned back to their baseline between 24h and 36h. 7-keto-DHEA had a detection time of 36h and thus accounts as direct proof of exogenous 7-keto-DHEA intake. The elevated concentrations of

7α-OH-DHEA and 7α-OH-T decreased after 12h under the respective 99% URL. Evaluation of 7β-OH-DHEA/16α-OH-DHEA, (7β-OH-DHEA/7α-OH-DHEA)/(DHEA/16α-OH-DHEA) and 7α-OH-T/16α-OH-DHEA shows that these ratios can stretch the effect of 7-keto-DHEA administration on the steroid profile even further until 48h.

Conclusion

A comprehensive screening method for steroid profiling was developed covering 29 steroid metabolites. Additionally, 10 steroid ratios were evaluated. Based upon a reference population of more than 3000 samples from healthy athletes the reference values were established at 3 different levels: 97.5%, 99% and 99.9%. Using the extended method and the reference ranges, 2 additional metabolites from 6-oxo-Adion were identified; 6β-OH-Andro and 6β-OH-Etio could stretch the detection time for 6-oxo-Adion administration until 36h. In the case of 7-keto-DHEA, 7α-OH-T can be regarded as additional specific metabolite. 7-keto-DHEA can be detected 36h after intake although steroid ratios indicate prolonged changes up to 48h of the steroid profile.

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