Pieter Van Renterghem[1], Pierre-Edouard Sottas[2], Martial Saugy[2], Frans Delbeke[1], Lance Brooker[3], Michael Polet[1], Wim Van Thuyne[1], Hans Geyer[4], Wim Van Gansbeke[1], Wilhelm Schänzer[4], Peter Van Eenoo[1]

Summary of the Alternative Steroid Profiling WADA project

DoCoLab, University of Ghent, Ghent, Belgium[1]; Laboratory for Doping Analyses, University Center of legal Medicine, Lausanne, Switzerland[2]; ASDTL, National Mesurement Institute, Sydney, Australia[3]; Institute of Biochemistry, German Sport University, Cologne, Germany[4]

Abstract

Using a comprehensive GC/MS method the usefulness of naturally occurring minor steroids metabolites was investigated for the detection of misuse with small doses of various formulations of endogenous steroids in sports. For 24 endogenous steroids, precursors and steroid metabolites the reference ranges were established and applied upon excretion urines.
It was concluded that decision limits based upon population statistics were inadequate to detect the misuse of small amounts of steroids and steroid gels. Several steroid metabolites were investigated for best detection sensitivity and maximal detection times with respect to their population thresholds.
Minor steroid metabolite ratios were investigated in a longitudinal way and implemented as potential biomarkers within the context of the adaptive Bayesian model as used in the Biological Passport. Using this individual approach, detection accuracy and detection times could be further improved.
According to the traditional WADA (TD2004) criteria for screening, 11% of excretion urines were identified with atypical steroid profiles of which 95% was confirmed by IRMS until 7 days after administration. Screening with the Alternative Steroid Profiling strategy led to an additional 14% more atypical steroid profiles of which 84% could be confirmed by IRMS analysis applying compound specific $\Delta\delta^{13}C$ criteria.
This study proves the usefulness of minor steroid metabolites in steroid profiling as well as the relevance of direct individual monitoring of steroid profiles of athletes in the biological passport concept.

Introduction

Steroid profiling is an informative and versatile method to detect the use of endogenous steroids in sports. Several administration studies with endogenous steroids reported that minor steroid metabolites provided specific information on the administered steroid. Hence, their role in detection strategies could be interesting.
In this project, the basic idea was that these minor steroid metabolites could contribute beneficially to steroid profiling and increase the specificity and sensitivity of current screening methods. Therefore, their occurrence in a population of negative urines was studied to establish reference ranges and evaluate them in administration urines. Longitudinal profiles were investigated with respect to the biological passport. This alternative steroid profiling (ASP) approach was finally compared with IRMS confirmation.

Experimental

Extended steroid profiling
A GC/MS SIM method was developed and validated for the quantification of a wide range of steroid metabolites (Table 1) [1].
Reference limits for GC/MS
2000 Male + 1000 female left-over blank routine samples were screened with the extended steroid profiling method. Refval software calculated the 97.5%, 99% and 99.9% reference limits with non-parametric statistics.

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Administration Studies
6 male volunteers administered with small doses of: Testosterone undecanoate (40 mg), Testosterone gel (100 mg), Dihydrotestosterone gel (250 mg), Dehydroepiandrosterone (50 mg).
All excretion urines were submitted to extended Steroid Profiling.
Population based evaluation
Population-based reference limits were used for evaluation of the post-administration urines and to determine detection times. ROC-analysis was applied to select the most sensitive steroid metabolites.
Individual Evaluation
The Adaptive Bayesian Model of Biological Passport was adopted for the steroid ratios (marker) that best responded upon administration. Detection sensitivity and specificity for all possible steroid ratios were evaluated with ROC-analysis. Determination of detection times using individual-based referencing. Eventually, a selection of best biomarkers was made.
IRMS Analysis
An IRMS method was developed and validated that monitored Andro, Etio, 5α-androsta, 5ααβ-Adiol and 5βαβ-Adiol. Pregnanediole (PD) was used as endogenous reference compound. In-house 99% reference ranges were obtained using 52 blank urine samples: 27 men and 25 females of which 19 on hormonal contraceptives. Finally, we compared the traditional steroid profiling, alternative steroid profiling and IRMS.

Results and Discussion

Reference ranges [2]
The reference ranges of male athletes are given in Table 1.
Suspicious samples were verified by IRMS and removed from statistics. Risk sports for steroid use e.g. power sports were avoided. Care was taken for correct quantification as adjusted according to specific gravity.
Population-based Evaluation [3]
Post-administration profiles were compared with the corresponding 97.5% and 99% reference limits for all volunteers. 40 mg oral T resulted in maximal detection times of 24h were obtained with T, T/E, Etio and the androstanediols. 100 mg dermal T had little impact on the steroid profile. Slight elevations of T and T/E were detected in 2/6 volunteers. After DHT-gel application, maximal detection time was 54h using DHT, DHT/E and 5α-Adiol. Not all volunteers reached the given reference limits. 50 mg oral DHEA was detected for 60h with 5β-Adiol.
In ROC analysis (Figure 1), the detection sensitivities (at high specificity) could exceed those of traditional metabolites. 4-OH-Adion and 16α-OH-DHEA were preferred as biomarkers for T and DHEA administration, respectively.
Longitudinal approach with an Adaptive Model [4,5]
Longitudinal evaluation of steroid ratios in addition to the T/E ratio was proposed; steroid ratios had good doping sensitivity, ROC-performance and detection windows. Per administered preparation, the steroid ratios with best biomarker qualities and detection times are given in Table 2.
IRMS [6]
It was noticed that the use of contraceptives was a discriminating factor rather than sex for IRMS. The established reference limits are given in Table 3. Using the compound specific IRMS criteria, 86% of the ASP-positives or 14% of the post-administration samples were additionally confirmed; using WADA (TDEAAS2004) IRMS criterion Δ>3, 25% fewer positives which were additionally picked up by ASP could be confirmed. IRMS detection times were similar to those of ASP.

Conclusions

The alternative longitudinal steroid profiling strategy contributed by proposing new sensitive biomarkers, which are steroid ratios based upon minors steroid metabolites. These were studied for implementation in an adaptive Bayesian Model in the Biological Passport. In such doping cases residing in a probabilistic framework, these additional markers will contribute to the evidence of guilt. A confirmation procedure indicated that up to 86% of the samples detected by this technique could be verified showing altered IRMS values. Using ASP, more than twice as many samples were identified for administration of endogenous steroids as with the current criteria. This sensitivity is also illustrated by very similar detection times of the ASP-method and IRMS.

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		Reference Ranges Concentrations (ng/ml) Men					
Compounds	**Abbreviation**	**97.5% RL**	**95% CI**	**99% RL**	**95% CI**	**99.9% RL**	**95% CI**
3α.5-cyclo-5α-androstan-6β-ol-17-one	5cyclo	31.3	22.9 - 41.5	41.5	25.0 - 43.8	/	/
Androsterone	Andro	6700	6390 - 6860	7910	7320 - 9090	11600	11000 - 11800
Etiocholanolone	Etio	4950	4660 - 5280	6200	5800 - 6790	9800	7800 - 10200
Testosterone	T	103	96.2 - 1140	128	119 - 146	185	167 - 193
Epitestosterone	E	88.9	80.5 - 96.6	113	97.5 - 125	172	160 - 187
5α-Androstane-3α.17β-diol	5ααβ-Adiol	155	143.5 - 169	199	181 - 221	405	286 - 416
5β-Androstane-3α.17β-diol	5βαβ-Adiol	416	394 - 445	517	473 - 643	1190	955 - 1260
5α-Androstane-3β.17β-diol	5αββ-Adiol	21.4	17.5 - 29.7	38.3	21.7 - 367	/	/
Dehydroepiandrosterone	DHEA	117	108 - 123	141	132 - 160	243	186 - 244
Dihydrotestosterone	DHT	21.5	15.2 - 26.1	26.4	17.0 - 38.5	/	/
Androstenedione	Adion	22.0	17.5 - 28.3	30.5	25.7 - 36.4	/	/
11β-OH-Androsterone	11β-OH-Andro	2750	2580 - 2850	3240	3000 - 3700	5160	4790 - 5990
11β-OH-Etiocholanolone	11β-OH-Etio	910	8420 - 9640	1060	985 - 1250	1870	1370 - 1930
7α-OH-Dehydroepiandrosterone	7α-OH-DHEA	21.6	20.1 - 24.0	25.6	24.0 - 29.4	43.8	30.6 - 43.7
6β-OH-Androsterone	6β-OH-Andro	20.6	19.5 - 22.1	23.8	21.5 - 26.8	39.0	22.4 - 39.0
6β-OH-Etiocholanolone	6β-OH-Etio	90.1	82.3 - 99.6	120	105 - 134	210	144 - 254
7α-OH-Testosterone	7α-OH-T	31.3	23.7 - 36.6	41.2	32.1 - 47.8	/	/
4β-OH-Dehydroepiandrosterone	4β-OH-DHEA	/	/	/	/	/	/
7β-OH-Dehydroepiandrosterone	7β-OH-DHEA	/	/	/	/	/	/
16α-OH-Etiocholanolone	16α-OH-Etio	320	293 - 361	454	387 - 562	957	797 - 1307
16α-OH-Androsterone	16α-OH-Andro	318	276 - 337	394	360 - 465	685	524 - 737
6-OXO-Androstenedione	6-OXO-Adion	/	/	/	/	/	/
7-keto-Dehydroepiandrosterone	7-keto-DHEA	/	/	/	/	/	/
6α-OH-Androstenedione	6α-OH-Adion	18.3	8.03 - 19.1	/	/	/	/
6α-OH-Testosterone	6α-OH-T	24.0	10.2 - 28.3	/	/	/	/
4-OH-Androstenedione	4-OH-Adion	20.4	17.8 - 22.6	25.0	21.6 - 28.1	36.5	26.1 - 36.5
16α-OH-Dehydroepiandrosterone	16α-OH-DHEA	36.5	33.0 - 40.7	46.3	41.7 - 52.4	75.6	54.9 - 87.3
4-OH-Testosterone	4-OH-T	/	/	/	/	/	/
16α-OH-Androstenedione	16α-OH-Adion	17.4	16.1 - 18.7	19.7	17.9 - 23.7	/	/

	Reference Ranges Ratios Men					
Ratios	**97.5% RL**	**95% CI**	**99% RL**	**95% CI**	**99.9% RL**	**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	37.5	32.5 - 41.2	45.7	42.6 - 48.9	91.9	57.4 - 92.3
Etio/11β-OH-Etio	22.7	21.2 - 24.8	30.0	25.4 - 32.1	45.7	35.7 - 49.7
Adion/E	1.09	0.89 - 1.62	1.66	1.23 - 3.35	/	/
DHT/E	1.03	0.82 - 1.47	2.53	1.88 - 3.59	/	/
5ααβAdiol/5βαβAdiol	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	2.11 - 3.45	4.80	4.33 - 4.87
Etio/Andro	2.04	1.78 - 2.18	2.58	2.28 - 2.92	4.05	3.28 - 4.31
5βαβ-Adiol/5ααβ-Adiol	8.34	7.71 - 9.34	11.4	10.6 - 12.8	18.9	16.7 - 18.8

Table 1: The 97.5, 99 and 99.9% reference limits (RL) and the respective 95% confidence intervals (CI) of some steroid concentrations and ratios in men.

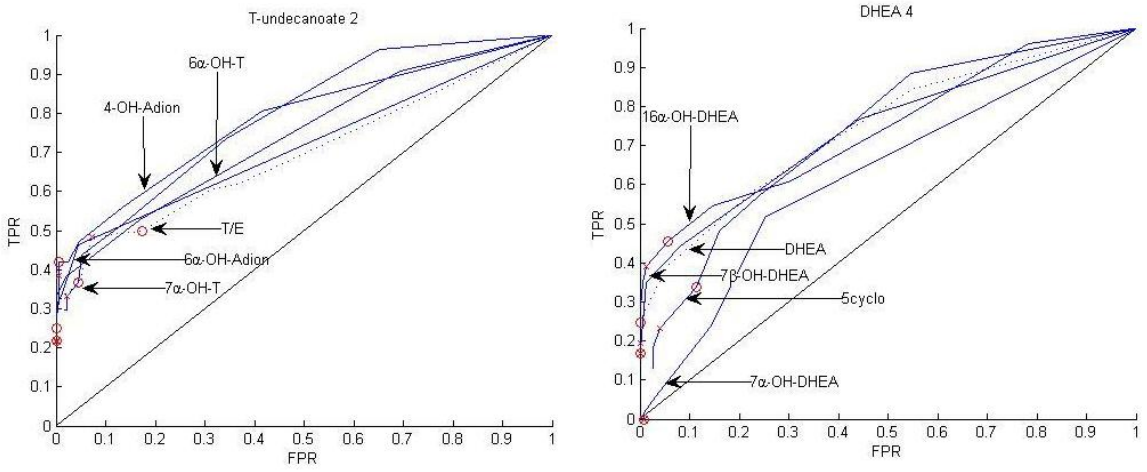

Figure 1: ROC-curves for minor steroid metabolites after T undecanoate and DHEA

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40 mg T undecanoate	250mg DHT gel	50mg oral DHEA
6α-OH-Adion/16α-OH-DHEA	DHT/E	DHEA/E
T/E	DHT/5βαβ-Adiol	16α-OH-DHEA/E
4-OH-Adion/16α-OH-Adion	5ααβ-Adiol/5βαβ-Adiol	7β-OH-DHEA/E
7α-OH-T/7β-OH-DHEA DHT/5βαβ-Adiol		5βαβ-Adiol/5ααβ-Adiol
Maximal detection time: 30h	Maximal detection time: 78h	Maximal detection time: 60h

Table 2: Selected biomarkers and maximal detection time for T undecanoate, DHT gel and DHEA

	marker	mean (‰)	99% RL (% min (‰)		max (‰)
δ-values	Etio	-23.7	-25.8	-25.4	-22
	Andro	-22.9	-25.1	-24.9	-21.3
	5βαβ-Adiol	-23.3	-25.8	-25.6	-21.2
	5ααβ-Adiol	-23.1	-25.8	-25.8	-21.2
	PD	-22.4	-24.5	-24.5	-21.1
Δ-values vs PD	Etio	1.28	2.64	0.32	2.92
	Andro	0.48	1.83	-0.8	2.08
	5βαβ-Adiol	0.84	2.27	-0.3	2.22
	5ααβ-Adiol	0.61	2.04	-0.6	1.93

Table 3: Statistics and 99% reference limits for males and females that are not on hormonal contraceptives

References

1. Van Renterghem P., Van Eenoo P., Van Thuyne W., Geyer H., Schänzer W., Delbeke F.T. (2008) J. Chromatogr. B 876, p. 225-235.
2. Van Renterghem P., Van Eenoo P., Delbeke F.T., Geyer H., Schänzer W. (2010) Steroids 75, p. 154-163.
3. Van Renterghem P., Van Eenoo P., Delbeke F.T. (2010) Steroids 75, p. 1047–1057.
4. Van Renterghem P., Van Eenoo P., Sottas P.E., Saugy M., Delbeke F.T. (2010) Drug Test. Anal. 2, p. 582–588.
5. Van Renterghem P., Van Eenoo P., Sottas P.-E., Saugy M., Delbeke F.T. (2011) Clin. Endocrinol. 75, p. 134–140.
6. Van Renterghem P., Polet M., Brooker L., Van Gansbeke W., Van Eenoo P. (2012) Steroids 77, p. 1050-1060.

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