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Combined profile of androgen glucuro- and sulfoconjugates in post-competition urine of sportsmen: a simple screening procedure using gas chromatography–mass spectrometry

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Abstract

An analytical screening procedure has been developed for the estimation of total androgen conjugates in post-competition urine, using gas chromatography–mass spectrometry with computerized data acquisition and concentration calculation. Rapid acid-catalyzed methanolysis is a key feature of the method, which allows simultaneous cleavage of glucuronides and sulfates. Analytical data generated by this method for testosterone and epitestosterone are in accordance with our previous results obtained by more accurate isotope dilution mass spectrometry. The usefulness of the ratio of testosterone glucuronide–total epitestosterone as an aid for a better discrimination between physiologically high and pharmacologically high ratios of testosterone glucuronide–epitestosterone glucuronide, which was demonstrated previously, has been confirmed here.

Keywords: Androgen; Testosterone; Epitestosterone

1. Introduction

It has been one of the brightest merits of the late Manfred Donike, at the head of his team, to have first introduced in 1983 the ratio of urinary glucuronides of testosterone to epitestosterone (TG/EG) as an, up to now, unchallenged criterion for disclosure of illicit testosterone self-administration by sportsmen [1]. Since a threshold value of TG/EG=6 has been adopted by the Medical Commission of the International Olympic Committee and is applied in its accredited laboratories, some cases have been described of healthy men with physiologically high TG/EG levels (between six and ten), without any supply of exogenous testosterone. Therefore, some complementary criteria have been suggested, such as the ratio of TG/luteinizing hormone [2], the ratio of serum testosterone/17-hydroxyprogesterone [3] and the ketoconazole test [4].

One of us has demonstrated that, taking into account the urinary sulfoconjugate excretion, and in particular the epitestosterone sulfate (ES) concentration within the ratio TG/(EG+ES), significantly better discrimination between physiologically high and pharmacologically high ratios of TG/EG could be attained [5]. Pursuing this, we now wish to report on a simple screening procedure for total (glucuro-,

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sulfo- and non-conjugated) urinary androgens using gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Urine collection

Post-competition urines were collected from 90 sportsmen, aged 24±6 years (mean±S.D.), under conditions which comply with official French and international regulations applicable to the doping control procedure. Only urine samples with normal specific gravity (≥1.005), measured with a KEM Model D-110 Specific Gravity Meter (Kyoto Electronics, Kyoto, Japan), were included in the analytical protocol. A post-race urine sample from a cyclist, which was addressed to the laboratory in conformity with the chain of custody applicable in official doping analysis, was found to be positive for testosterone administration.

2.2. Chemicals and reagents

Testosterone (T) (17β-hydroxy-4-androsten-3-one), testosterone sulfate (TS), testosterone glucuronide, epitestosterone (E) (17α-hydroxy-4-androsten-3-one), dihydrotestosterone (DHT) (17β-hydroxy-5α-androstan-3-one), dehydroepiandrosterone (DHA) (3β-hydroxy-5-androsten-17-one), dehydroepiandrosterone sulfate, 5-androstene-3β,17α-diol (5A3β17α), 5-androstene-3β,17β-diol (5A3β17β), 5-androstene-3β,17β-diol 3-sulfate, 5α-androstane-3α,17β-diol (5α3α17β), 5β-androstane-3α,17β-diol (5β3α17β), androsterone (And) (3α-hydroxy-5α-androstan-17-one), androsterone sulfate, androsterone glucuronide, etiocholanolone (Etio) (3α-hydroxy-5β-androstan-17-one) and epiandrosterone (EpiA) (3β-hydroxy-5α-androstan-17-one) were obtained from Sigma (St. Louis, MO, USA). Epitestosterone 17-sulfate was prepared as described previously [5]. The internal standard, 6α-methyltestosterone (6MeT) (6α-methyl-17β-hydroxy-4-androsten-3-one), was a gift from Syntex Research (Mexico City, Mexico). Trimethylchlorosilane (TMCS), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and trimethyliodosilane (ITMS) came from Fluka (Buchs, Switzerland). Organic solvents (Carlo Erba, Milan, Italy) were of analytical grade and were used without further treatment.

2.3. Gas chromatography–mass spectrometry

Selected-ion monitoring was performed with a Hewlett-Packard 5890 gas chromatograph combined with a 5970 mass-selective detector and Unix data processing. The ion source was operated in the electron impact mode with 70 eV electron energy. The following ions were used for quantification: m/z 432.4 [M^+] for T, E and DHA; m/z 434.4 [M^+] for DHT, 5A3β17α, 5A3β17β, And, Etio and EpiA; m/z 241.2 [M^+-CH_3-2(Me_3SiOH)] for 5α3α17β and 5β3α17β and m/z 446.4 [M^+] for 6MeT. Gas chromatography was performed on a HP-1 (methylsilicone, 0.11 μm film thickness) fused-silica column (17 m×0.22 mm I.D.), operated with 105 kPa pressure of ultra-high-purity helium, and with the following oven-heating program: initial temperature 170°C, 3.0°C/min up to 230°C, 30°C/min up to 300°C and held for 3.67 min. The injector and interface were heated at 280°C and 1 μl of the sample was injected in the split mode (ratio, 10:1) using a Hewlett-Packard 7673 autosampler.

2.4. Sample preparation

Aliquots (4 ml) of urine were supplemented with 6MeT (800 ng dissolved in 40 μl of methanol) and solid phase extraction was performed with C_{18} cartridges (500 mg, J.T. Baker, Noisy-le-Sec, France) using a Benchmate II Workstation (Zymark, Hopkinton, MA, USA). Conditioning, loading and elution of the cartridge was as follows: 2 ml of methanol, 2 ml of water, urine sample aspiration, rinsing with 3 ml of water and finally elution with 4 ml of methanol. The latter eluate was divided in two equal parts, one being used for methanolysis and the other for enzymatic hydrolysis.

2.4.1. Methanolysis of glucuronides and sulfates

After evaporation of the solvent, the residue was taken-up in 1 ml of methanol containing 1 *M* TMCS and methanolysis was performed at 55°C for 1 h.

The solvolysis mixture was then evaporated at 60°C and three drops of methanol followed by 0.5 ml of phosphate buffer (0.1 *M*, pH 6.5) were added to the residue. Extraction was performed with 2.5 ml of a mixture of *n*-pentane–diethyl ether (1:1, v/v) by mixing thoroughly for 1 min. Phase separation was promoted by centrifugation when necessary, and the aqueous phase was then discarded by aspiration into a pasteur pipette. The organic phase was dried with approximately 50 mg of anhydrous sodium sulfate, was transferred into a dry tube and evaporated to dryness. Derivatization was performed with 50 μl of a mixture of 0.3% ITMS in MSTFA at 60°C for 20 min. Finally, samples were transferred into conical vials prior to loading in the autosampler.

2.4.2. Enzyme hydrolysis of glucuronides

The other methanolic extract was evaporated, dissolved in 1 ml of phosphate buffer (0.1 *M*, pH 6.5) and incubated at 55°C for 1 h with ten international units of β-glucuronidase from *Escherichia coli* (Sanofi, Diagnostics Pasteur, Marnes-la-Coquette, France). Further work-up involved extraction and derivatization as described in Section 2.4.1.

2.5. Calibration and quantification

Quantification was performed with a 50-μl volume of a calibration mixture composed of androsterone and etiocholanolone (2000 ng/ml), DHA (1000 ng/ml), androstenediols and epiandrosterone (400 ng/ml), androstanediols (100 ng/ml), testosterone and epitestosterone (80 ng/ml), DHT (40 ng/ml) and internal standard (6MeT, 200 ng/ml). Peak-areas were measured and concentrations, expressed as nanograms of free steroid/ml, were calculated by data processing with correction for internal standard losses.

2.6. Precision and recovery of standard additions of androgen conjugates

For all analytes, intra-assay variability (R.S.D., $n=6$) was in the 7 to 10% range and the corresponding inter-assay variability ($n=4$) was in the 11 to 17% range. Recovery was evaluated by quantification of standard additions of androgen conjugates made to a urine pool which had been analyzed previously for endogenous steroid levels, which were as follows: for androsterone glucuronide, 900 ng/ml; for androsterone sulfate 1050 ng/ml; for 5-androstene-3β,17β-diol sulfates, 216 ng/ml; for epitestosterone sulfate, 17.7 ng/ml; for testosterone sulfate, 13.0 ng/ml and for testosterone glucuronide, 16.3 ng/ml. Sulfates of androsterone, epitestosterone, testosterone, 5-androstene-3β,17β-diol and DHA were recovered with 95±6% yield (mean±S.D., $n=3$). Glucuronides of androsterone and testosterone were recovered with 92±8% yield. When methanolyzed samples were submitted to supplementary β-glucuronidase hydrolysis, no further increase of the yield of any free androgen could be evidenced.

3. Results

Urinary androgen concentrations of total conjugates and glucuronides, which were determined analytically, and sulfate concentration, which were calculated as the difference between the total conjugates and glucuronides, are listed in Table 1. The total and glucuroconjugate fractions also comprise non-conjugated parent compounds, but these represent less than 3% of total androgens [6], and thus remain within the experimental error of conjugate determinations. Glucuronide concentrations of 5-androstene-3β,17β-diol and epiandrosterone were negligible in comparison to the corresponding sulfates, and for dihydrotestosterone, only the glucuronide could be quantified, since glucuronide and total conjugate levels were similar.

Different ratios of testosterone and epitestosterone concentrations, which are characteristic criteria for disclosure of testosterone administration [5], are presented in Fig. 1.

Correlations between androgen metabolites have been calculated (Table 2). They demonstrate that testosterone and epitestosterone are less correlated in the glucuronide fraction than in the total conjugate and sulfate fractions. Moreover, testosterone, epitestosterone and DHA are very well correlated with either the corresponding precursors or metabolites.

Table 1
Concentrations of androgen conjugates measured in 90 post-competion urine samples and expressed as ng of free steroid/ml

	T	E	DHA	DHT	5α3α17β	5β3α17β	5A3β17α	5A3β17β	EpiA	And	Etio	And/Etio
Total conjugate												
Mean	37.8	54.4	830		46.7	152	444	272	199	5132	3450	1.66
S.D.	31.6	40.8	1518		26.2	124	290	251	240	2672	1991	0.66
Range	0.2–210	9.9–202	47–12 190		1.0–92.0	9.9–775	70.3–1492	9.2–1276	9.6–1720	1213–13 640	591–10 147	0.6–4.0
Glucuronide												
Mean	34.2	36.4	57	5.6	41.1	101	88.5			3111	1912	1.92
S.D.	30.8	28.8	42	6.5	30.6	106	65.1			1837	1246	0.91
Range	0.8–203	4.2–144	7.9–245	0.3–50.6	5.1–173	0.5–447	10.1–361			400–9566	105–5981	0.6–5.0
Sulfate												
Mean	7.2	19.8	746		35.7	60.8	352	252	194	2166	1578	1.61
S.D.	10.4	20.8	165		37.9	73.7	302	247	226	2100	1580	0.90
Range	0.1–54.4	0.5–101	12–12 079		0.5–214	0.5–382	0.5–1389	9.2–1077	9.6–1088	100–8234	50–7794	0.5–4.7

Sulfate concentrations were obtained by subtracting glucuronide concentrations from total conjugate concentrations.
T=testosterone, E=epitestosterone, DHA=dehydroepiandrosterone, DHT=dihydrotestosterone, 5α3α17β=5α-androstane-3α,17β-diol, 5β3α17β=5β-androstane-3α,17β-diol, 5A3β17α=5-androstene-3β,17α-diol, 5A3β17β=5-androstene-3β,17β-diol, EpiA=epiandrosterone, And=androsterone and Etio=etiocholanolone.

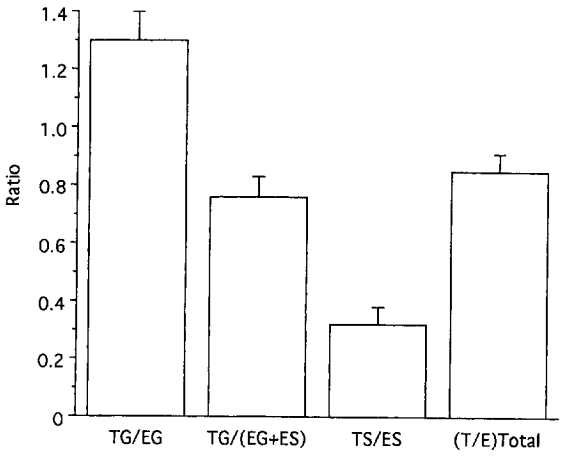

Fig. 1. Concentration ratios (mean±standard error) of glucuronides (G), sulfates (S) and total conjugates (Total) of testosterone (T) to epitestosterone (E) measured in 90 post-competition mictions.

4. Discussion

Quantification of urinary androgens in doping analysis has been almost exclusively performed on the glucuronides. This may be attributed to the preponderance of glucuronides in the overall screening procedure of prohibited substances in sport. Nevertheless, hepatic sulfoconjugation, which is of paramount importance in the metabolism of drugs and some steroids, results, as is the case with glucuronides, in a significant increase in the polarity with respect to the parent compound, thereby facilitating urinary excretion [7]. Moreover DHA sulfate, which is present in human plasma at a level that is higher than that of any other steroid hormone and more than 95% of it is secreted by the adrenal gland, serves as a metabolic intermediate in androgen and estrogen biosynthesis [8].

The testis also produces androgen sulfates, whereby epitestosterone sulfate secretion is of particular interest, due to its suggested contribution to the establishment of physiologically high urinary TG/EG ratios, through deficient excretion of epitestosterone glucuronide in favour of the corresponding sulfate [5,9].

The sum of all these enumerated reasons justified, in our opinion, the development of a GC–MS screening method for total androgen, deconjugated by adequate methanolysis.

Acid-catalyzed methanolysis, which is an efficient procedure for the simultaneous cleavage of steroid glucuro- and sulfoconjugates, was first described by Tang and Crone [10], who used a mixture of 1 *M* acetylchloride in methanol, thus producing anhydrous hydrochloric acid by a strong exothermic reaction. We have found a similar property for TMCS, which has the advantage that it can be mixed with methanol without any special care. Concerning the mechanism of methanolysis of neutral steroid glucuronides and sulfates, it is most plausible that, with the mixture of 1 *M* TMCS in methanol, sufficient hydrochloric acid is released [11] to produce enough

Table 2
Significant correlations between androgen concentrations (Spearman's rank correlation coefficient with a significance level of $p<0.05$)

	Correlation coefficient
Total conjugates	
Testosterone vs. epitestosterone	0.46
Testosterone vs. 5α-androstane-3α,17β-diol	0.63
Testosterone vs. 5β-androstane-3α,17β-diol	0.72
Epitestosterone vs. 5-androstene-3β,17α-diol	0.71
Dehydroepiandrosterone vs. 5-androstene-3β,17β-diol	0.67
Dehydroepiandrosterone vs. epiandrosterone	0.68
5-Androstene-3β,17α-diol vs. 5-androstene-3β,17β-diol	0.74
Glucuronides	
Testosterone vs. epitestosterone	0.33
Sulfates	
Testosterone vs. epitestosterone	0.56

of the active intermediate $[CH_3OH_2]^+Cl^-$, which is the cleaving agent of both glucuronic ethers and sulfate esters.

As an internal standard, 6α-methyl-testosterone was retained rather than 17-methyltestosterone, because the latter compound has a tertiary alcohol function which dehydrates readily under methanolysis conditions in the 17-position. Similar dehydration occurred with 11β-hydroxy steroids, but all other androgens of endogenous origin were stable during methanolysis, as proven by the quantitative recovery mentioned in Section 2.

This large-scale analytical procedure produced results for both conjugates of testosterone and epitestosterone, which were in accordance with those obtained by more accurate isotope dilution–mass spectrometric analysis of 24-h urine collections of sporting adult male subjects [5,6].

The urinary DHA sulfate levels agreed with reported data [12,13], and their high inter-individual variability has to be related to similarly high variability of plasma concentrations [14]. Moreover, as DHA sulfate is a specific marker of adrenal secretory activity, it is plausible that DHA sulfate excretion may also reflect the stress status of an individual during a sports event. Other 3β-hydroxy-steroids are also preferentially excreted as sulfates and their excretions are significantly correlated. This can be observed for 5-androstene-3β,17β-diol (3-sulfate and 3,17-disulfate) and epiandrosterone sulfate, in accordance with published data [12]. 5-Androstene-3β,17α-diol was shown previously to be a potential precursor of epitestosterone [9] and this is corroborated by significant correlation both in the glucuronide and in the total conjugate fraction, where this androgen is present as disulfate [12,15].

Concerning androsterone and etiocholanolone, which are final androgen metabolites, the proportion of sulfates attains 42 and 46% of the total conjugates, respectively. These values are much higher than those reported for 24-h urine collections of sedentary men [16,17]. Whether this metabolic shift in favour of sulfate excretion is related to intense physical activity remains to be confirmed. Moreover, the androsterone–etiocholanolone ratio indicates a significant difference (Wilcoxon test, $P<0.002$) between corresponding glucuronides and sulfates, and this is the result of a higher proportion of etiocholanolone sulfate.

The average ratios of TG/EG, TG/(EG+ES) and TS/ES obtained by this rapid screening method are in good agreement with those generated by selective determination of glucuronides and sulfates separately [5]. The usefulness of the ratio TG/(EG+ES) for the discrimination between physiologically high and pharmacologically high ratios of TG/EG is confirmed by data from this study. Indeed, two subjects with TG/EG ratios of 5.8 and 7.3 had corresponding TG/(EG+ES) ratios of 2.2 and 1.9, respectively, well below the tentative threshold value of three, which was determined previously [5]. Conversely, a testosterone-positive subject with a TG/EG ratio of 75 had a TG/(EG+ES) ratio of 28.

In conclusion, this simple screening procedure allows easy experimental access to total conjugate levels of urinary androgens, and to sulfoconjugate concentrations by calculation, when routine screening of glucuronides has been performed previously. This is of particular interest for epitestosterone, its metabolic precursor 5-androstene-3β,17α-diol and for dehydroepiandrosterone. This simple procedure should be considered as complementary to the routine analysis of androgen glucuronides when TG/EG ratios exceed the official threshold in the range of six to ten.

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