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Determination of urinary androgenic glucuronides by gas chromatography-mass spectrometry

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Introduction

Doping control rules forbid the use of androgenic steroids in sports. Like other steroids, androgens are predominantly excreted in urine as their water-soluble conjugates. The conjugates are formed by substitution of 3- or 17-hydroxy groups for sulfate or glucuronide (G) in human liver (metabolism of phase II)[1]. The methods described in the literature for screening and confirmation of endogenous and exogenous anabolic androgenic steroids (AAS) include an enzymatic hydrolysis step. The aglycones (steroids) are derivatized and analyzed by GC/MS[2].The influence of the hydrolysis step in all procedures is well documented in the literature as having some problems such as incomplete hydrolysis of some conjugates, formation of artifacts, different enzyme activities (most of them due to matrix effects) and inhibition of the enzyme by some compounds. Recently Thevis *et al.* (2001)[3] described the synthesis and GC/MS characterization of glucuronide (G) conjugates of anabolic steroids (AAS-G) and the main fragmentation as well. The aim of this study was to determine the AAS-G by GC/MS in male volunteer's urine (n=3, 22.5 + /- 0.7 years) who have been treated with Androstat 150® (norandrostenedione, norandrostenediol, androstenediol and androstenedione) according to the ethical protocol approved by the HUCFF/UFRJ n° 068/02.

Experimental

Stock solutions were prepared in methanol at a concentration of 1000 ng/μL. These solutions were further diluted to yield for the preparation of the working solution standards at concentration levels of 10 ng/μL e 0,1 ng/μL. The solutions were sealed and frozen at –20°C until use.

Sample preparation: 5 mL of urine were added to an Amberlite XAD-2 column. The column was washed with 2 mL of water and the adsorbed fraction (AAS-G) was eluted with 3 x 1.5 mL of methanol. The methanolic eluate was evaporated to dryness under vacuum. The derivatization of

the final residue was done using 100 μL of a mixture of MSTFA–NH_4I–2-mercaptoethanol (1000:2:6, v/w/v) and heated at 60°C[4].

Instrumental conditions: 3 μL of the derivatives were injected into GC/MS. The capillary column was a HP1 (100% methylsilicone), 16m x 0.20mm i.d., d_f = 0.11μm. Injection mode: Split (1:10); Pulse pressure (50 psi per 0.80 min), constant flow 1mL/min. Temperature program: 180°C rate 20°C/min to 320°C (hold time 5 min)[3]. Injector temperature, 325°C. Mass spectra were obtained in scan mode, 70 eV. Mass range 50-800 Da.

Results

Analysis of glucoconjugated steroids from standard solution:

Firstly a working standard solution containing the analytes was analysed to check retention time and to evaluate the fragmentation pattern with the aim to select diagnostic ions. As the substances in the commercial formulation are prohormones, like androstenedione which is metabolized in endogenous compounds leading to increase and alteration in their ratio, spectra of the glucuronide endogenous conjugates were also obatined. In regards to the fragmentation parttern of endogenous compounds, the AAS-G-TMS studied showed similar patterns as described by Thevis *et al.* (2001)[3] (Figure 1a-c). This result is confirmed by the presence of the ions at m/z 204, 217, 305 and 375 in all mass spectra. In addition the loss of the ion at m/z 322 (significant in the spectrum of the steroid glucuconjugates ions) was also observed, due to the opening of the ring of the glucuronic acid moiety. However, the ratio between the main ions (Table 1) are little different from that observed by the authors[3], which could be due to our GC-MS conditions. Standards of norandrosterone-G and noretiocholanolone-G were also analyzed.

Analysis of glucoconjugated steroids from excretion study urine:

According to Schänzer *et al.* (2000)[5] the main metabolites of prohormones of norandrostenedione are norandrosterone and noretiocholanolone as expected. They also described the presence of 16α-hydroxynorandrosterone and 3α-hydroxyestr-4-en-17-one. The presence of these steroids in urine was seen in the total fraction (unconjugated and glucuronide fraction after hydrolysis with β-glucuronidase[2] (Figure 2 a-b) and using the method proposed in this study (conjugated fraction) (Figure 2 c-d). In Figure 3 the mass spectra of norandrosterone-G and noretiocholanolone-G are illustrated. According to Goudreault et al. (2001) the main metabolites of androstenedione, a biosynthetic precursor of testosterone are 6α-hydroxyandrosterone and 6β-hydroxyetiocholanolone, which were, therefore, tentative detected as conjugates (Figure 4).

Conclusion

The AAS-G-TMS studied showed similar fragmentation as described in the literature[3]. This result is confirmed by the presence of the m/z 204, 217, 305 and 375 fragments in all mass

spectra and the loss of the ion m/z 322. The present approach permitted identification of the main metabolites of norandrostenedione, norandrostenediol, androstenediol and androstenedione in their G form in urine by GC/MS. The retention time of the metabolites were in the range of 5-12 min. The next phase will be to improve the methodology to detect the sulfoconjugated metabolites. It was possible to obtain the same mass spectral behaviour in real urine samples as presented for synthetic standards by Thevis et al.[3].

Acknowledgement

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Reference

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2.Geyer H, Schänzer W, Mareck-Engelke U, Nolteersnting, E., Opfermann, G. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, editors. *Recent advances in doping analysis (5).* Sport and Buch Strauss 1998: 99-102.

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4.Donike M. and Zimmermann J. *J. Chromatogr.* 1980, 202, 43.

5.Schänzer W, Breidbach A., Geyer H, van Kuk C. Nolteernsting E. and Thevis M. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, editors. *Recent advances in doping analysis (8).* Sport and Buch Strauss 2000:155.

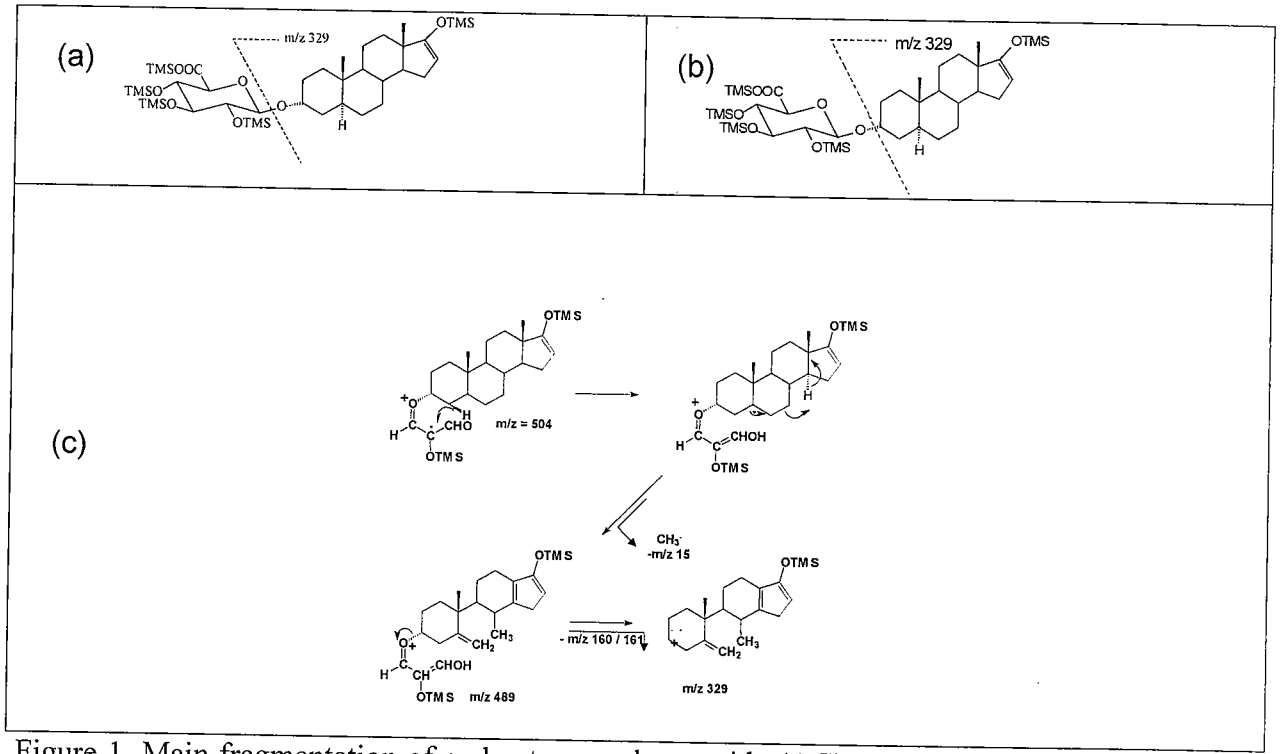

Figure 1. Main fragmentation of androsterone-glucuronide (AG) a) between the glucuronic acid moiety (oxygen) and the steroid (C3), b) between the oxygen and the C1 of the glucuronic acid moiety and c) between the C2 and the C1 from the glucuronic acid moiety (resulting in ring opening).

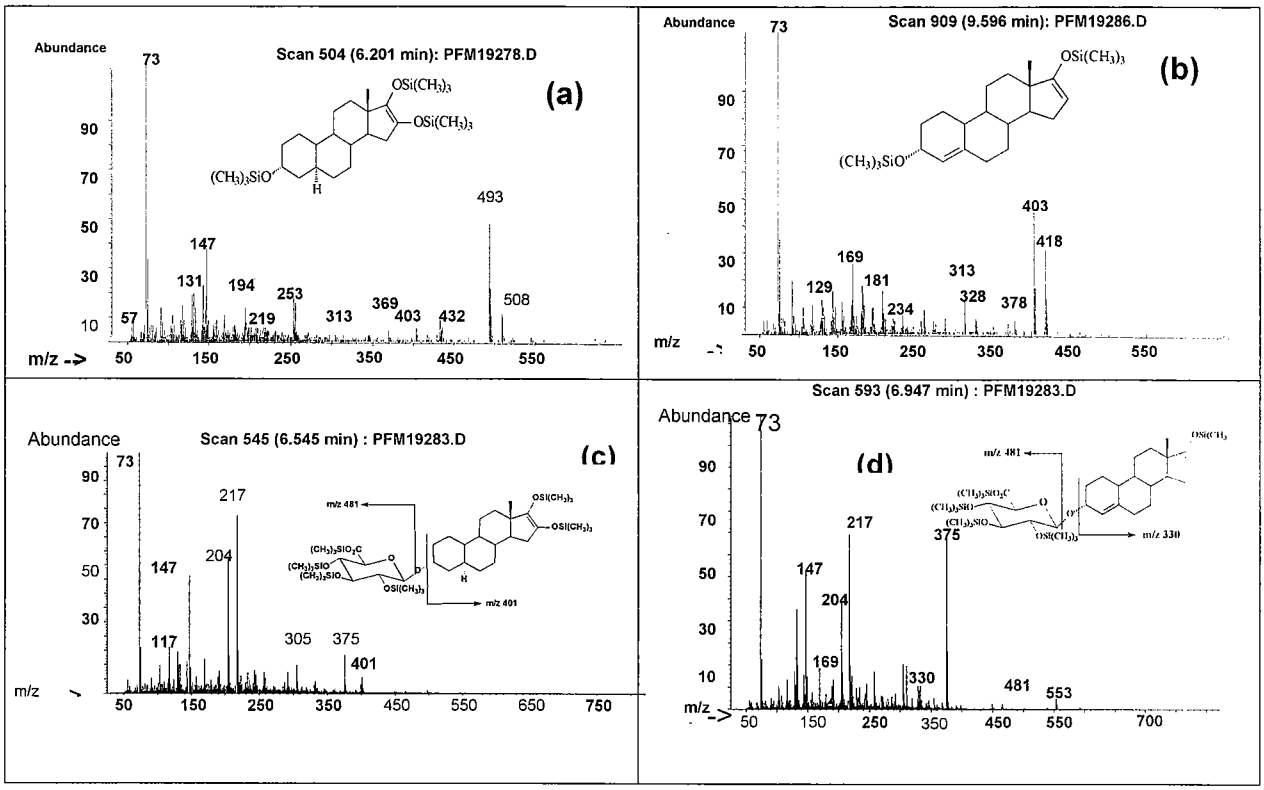

Figure 2. GC/MS spectra compatible with the tentative identification of (a) 16α-hydroxynorandrosterone, (b) 3α-hydroxyestr-4-en-17-one in hydrolyzed urine; (c) 16α-hydroxynorandrosterone and (d) 3α-hydroxyestr-4-en-17-one, glucuconjugated.

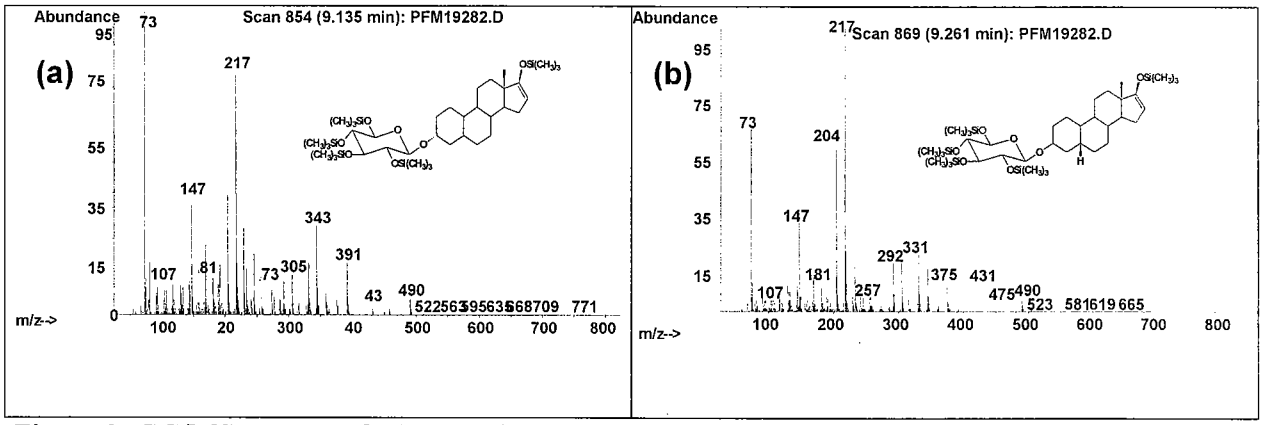

Figure 3. GC/MS spectra of (a) norandrosterone-G and (b) noretiocholanolone-G in urine.

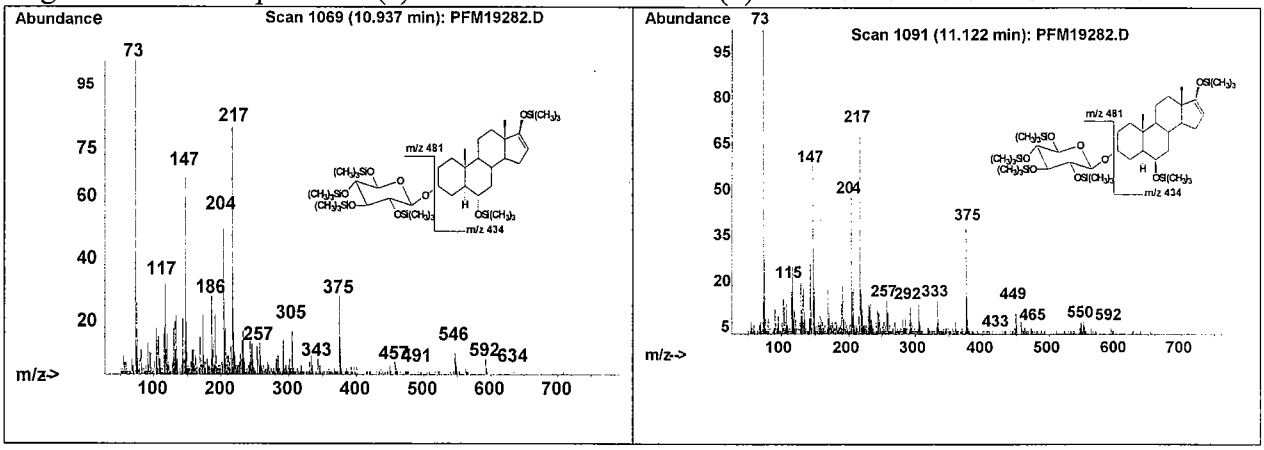

Figure 4. GC/MS spectra compatible with the tentative indentification (a) 6α-OH-androsterone-G and (b) 6β-OH-etiocholanolone-G in excretion urine (4 hours after administration).

Table 1. GC/MS selected fragments of silylated steroid glucuronide conjugates (intensity in % relative to base peak)[a,b]

Analytes	$M^{+\bullet}$	$[M^{+\bullet}]$ – m/z			m/z										
		249	322	322-15	465	449	375	Aglic+30	Aglic	305	292	Aglic-90	217	204	169
A-G	826	577 (<1)	504 (11)	489 (2)	465 (<1)	449 (1)	375 (7)	361 (1)	345 (35)	305 (20)	292 (19)	255 (8)	217 (100)	204 (52)	169 (11)
E-G	826	577 (<1)	504 (14)	489 (2)	465 (<1)	449 (3)	375 (15)	361 (<1)	345 (60)	305 (27)	292 (28)	255 (12)	217 (100)	204 (73)	169 (8)
EpiT-G	824	575 (<1)	502 (3)	487 (1)	465 (<1)	449 (1)	375 (7)	373 (3)	343 (42)	305 (6)	292 (2)	253 (1)	217 (46)	204 (49)	169 (12)
T-G	824	575 (<1)	502 (2)	487 (<1)	465 (<1)	449 (1)	375 (8)	373 (3)	343 (48)	305 (7)	292 (3)	253 (1)	217 (32)	204 (60)	169 (15)
6α-OH-G[c]	914	665 (<1)	592 (4)	577 (<1)	465 (<1)	449 (<1)	375 (30)	463 (<1)	433 (1)	305 (13)	292 (8)	343 (3)	217 (70)	204 (50)	169 (30)
6β-OH-G[c]	914	665 (<1)	592 (2)	577 (<1)	465 (3)	449 (7)	375 (36)	463 (<2)	433 (1)	305 (11)	292 (9)	343 (<1)	217 (66)	204 (50)	169 (15)

[a]A, androsterone; E, etiocholanolone; EpiT, epitestosterone; T, testosterone and G, glucuconjugate. [b] Results observed in processed urine.

[c]Tentative identification