Tudela E, Deventer K, Geldof L, Van Eenoo P

Detection and identification of glucuronidated stanozolol metabolites by liquid chromatography–tandem mass spectrometry

Clinical Chemistry, Microbiology and Immunology, DoCoLab, Ghent University (UGent), Zwijnaarde, Belgium

Abstract

A wide range of methodologies have been described for the detection of stanozolol abuse, focussing on different metabolites. The most recently published studies were focussed on the detection of glucuronide metabolites. In this sense and as an extension of the previously described method by our research group for the detection of 3´-hydroxystanozolol glucuronide, the target compounds 4β-hydroxystanozolol glucuronide and 16β-hydroxystanozolol glucuronide were investigated. Due to the lack of commercially available reference standards, the compounds were in vitro synthesized from their corresponding aglycones using human liver microsomes. The application to a real positive samples and excretion study highlight the use of glucuronides in contrast to the corresponding aglycones and confirm that 3´-hydroxystanozolol glucuronide is so far the longest-term hydroxystanozolol metabolite for detection of stanozolol abuse.

Introduction

Stanozolol is one of the most frequently detected anabolic steroids in doping control samples [1]. The metabolism of this peculiar anabolic steroids which presents a pyrazol moiety fused to the A-ring of the androstane framework, has been extensively studied [2,3]. In humans, the main metabolic products excreted in urine as glucuronidated conjugates are 3´-hydroxystanozolol, 4β-hydroxystanozolol, 16β-hydroxystanozolol and 4,16-dihydroxystanozolol. In the past, detection of the aglycones was achieved by enzymatic hydrolysis followed by GC-MS or LC-MS.
Recently, the detection of intact 3´-hydroxystanozolol glucuronide (3STANG) has been described as a sensitive alternative to the detection of the aglycones [4]. Recently, other glucuronidated stanozolol metabolites, non-commercially available, including stanozolol-N-glucuronide, 17-epistanozolol-N-glucuronide and 16β-hydroxystanozolol-O-glucuronide have been investigated and have been also proven to be good target compounds for identification of stanozolol abuse. The authors synthesised in house the standards following a chemical pathway [5].
Unfortunately, chemical synthesis is not readily accessible to most routine doping control laboratories. Therefore an interesting alternative to overcome the problems associated with chemical synthesis, like diastereoselectivity of the reactions [6], is enzyme-assisted synthesis [7].
Herein we report a study of the detection of glucuronidated stanozolol metabolites including 4β-hydroxystanozolol glucuronide (4STANG) and 16β-hydroxystanozolol glucuronide (16STANG) to complete the development of an analytical method for the detection of hydroxystanozolol glucuronides metabolites.

Experimental

Chemical and Reagents
3´-hydroxystanozolol glucuronide (3STANG) and the internal standard (I.S.) d3-epitestosterone glucuronide (ETGd3) were purchased from the National Measurement Institute (Pymble, Australia). The I.S. prostanozol (17β-hydroxy-5α-androst-2-eno[3,2-c]-pyrazole, PROSTAN) was obtained from TRC (Toronto, Canada). LC-MS grade methanol and water were purchased from Biosolve (Valkenswaard, Netherlands). Ammonium acetate (NH_4OAc) was obtained from Sigma (St. Louis, MO, USA) and hydrochloric acid (HCl), ammonium hydroxide (NH_4OH) and acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany). Oasis® MCX LP extraction cartridges (3 cm^3, 60 mg) were purchased from Waters (Milford, MA, USA).

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For the in vitro synthesis of glucuronide stanozolol metabolites, pooled human liver microsomes (HLM) from 20-30 donors (452161) and uridine diphosphate-glucuronosyltransferase (UGT) reaction mix solutions A (25 mM uridine 5´-diphospho-glucuronic acid, UDPGA, in water; catalog number 451300) and B (250 mM Tris-HCl, 40 mM $MgCl_2$, 0.125 mg mL^{-1} alamethicin in water; catalog number 451320) were obtained from BD Gentest (Erembodegem, Belgium).

Sample Preparation
The sample preparation was performed according to the previously described procedure for the detection of 3STANG [4].

Instrumentation
The liquid chromatographic system consist of a Finnigan Surveyor HPLC system interfaced to a TSQ Quantum Discovery MAX triple quadrupole mass spectrometer (Thermo, San Jose, USA) using the electrospray interface.
Separation was performed on a Sunfire C18 3.5 µm (2.1 mm x 50 mm) (Waters, Milford, Massachusetts, USA) at a flow rate of 250 µL min^{-1}. The temperature of the autosampler and column oven were 15 °C and 35 °C, respectively.
The aqueous and methanolic mobile phases consisted both of 1mM NH_4OAc and 0.1% HOAc. The percentage of organic solvent on the gradient program used changed as follows: 0 min, 40%; 0.5 min, 40%; 2 min, 55%; 8.9 min 70%; 9.0 min, 100%; 10 min, 100%; 10.10 min, 40%; 14 min, 40%.
Nitrogen was used as sheath gas and auxiliary gas at flows of 50 and 20 arbitrary units, respectively. Sample ionization was carried out in positive and negative modes using spray voltages of 4500 V in absolute value. The capillary temperature was set at 350 °C. The collision gas was argon (Air Liquide, Desteldonk, Belgium) with a collision gas pressure of 1.5 mTorr.

In vitro synthesis
For the synthesis of 4STANG and 16STANG the phase-II metabolism of the corresponding aglycones was simulated using human liver microsomes (HLM). The microsomal incubations were performed using 10 µg of the parent compound in ethanolic solution (2.5 µL). The reaction mixture additionally contained 171 µL of water, 20 µL of UGT reaction mix solution A and 50 µL of UGT reaction mix solution B. This mix was pre-incubated during 5 minutes at 37 °C. The reaction was initiated by addition of 6.5 µL of HLM, to obtain a final protein concentration of 0.5 mg mL^{-1}. The mixture was vortexed briefly and incubated at 37 °C. After 2 h, the incubation was stopped by adding 250 µL of ice-cold methanol at 4 °C (15 min). The samples were centrifuged afterwards for 5 min at 14,000 × *g* at 4 °C.

Results and Discussion

Mass spectrometry
The solutions obtained after the in vitro synthesis of 4STANG and 16STANG were used as stock solutions. Investigating full scan mass spectra in both positive and negative ionization showed several potential precursors for the SRM method. Negative ionization mode yielded an abundant deprotonated molecule $[M-H]^-$ (m/z 519) due to the acidity of the glucuronide moiety for both compounds. In positive mode an abundant protonated molecular ion was observed $[M + H]^+$ (m/z 521) due to the proton affinity of the pyrazol ring [8] present in both compounds.
Fragmentation of the 16STANG exhibit the loss of the glucuronide moiety in both positive (m/z 345) and negative (m/z 343) mode at low collision energies. For the fragmentation of the stanozolol skeleton higher collision energies, up to 50 eV, are necessary to yield the ion m/z 81, which has been described as being typically for the aglycone [9,10] in positive ionization (Figure 1).

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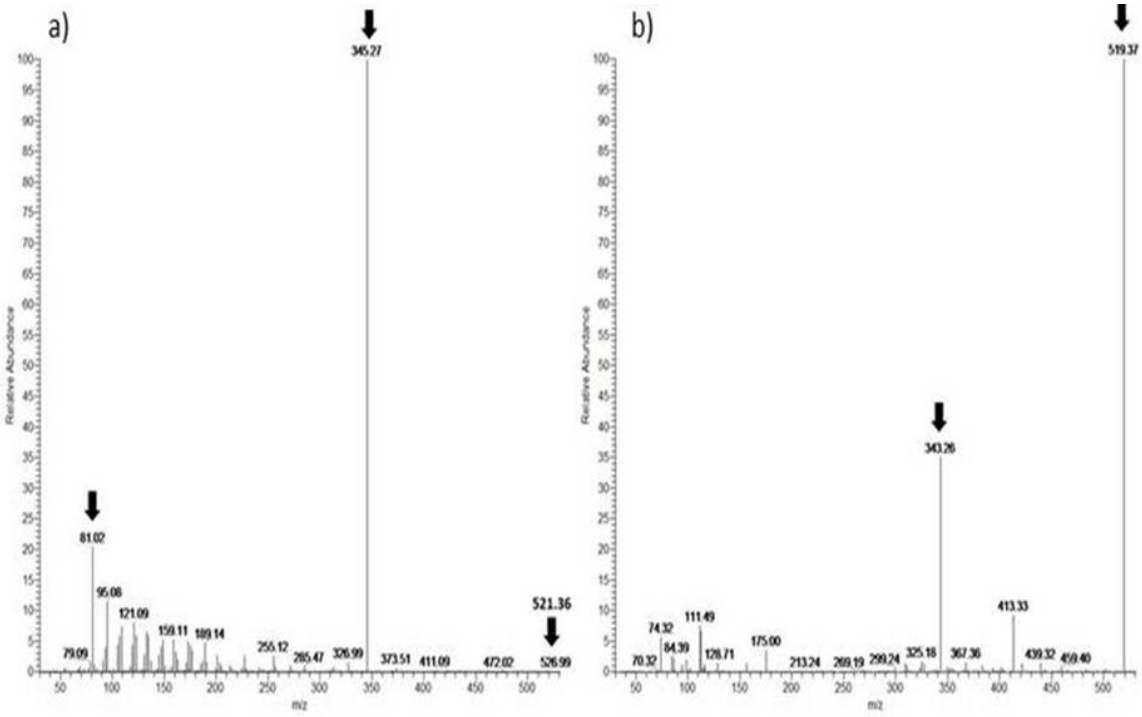

Figure 1. Product ion mass spectra of 16STANG at: a) ESI+, 60 eV b) ESI-, 30 eV

In the case of 4STANG due to the formal allylic position of the hydroxyl group in 4, the loss of the glucuronide group is accompanied by the loss of water, one and even two molecules yielding the ions m/z 327 and 309 in positive ionization. The abundance of the ion m/z 309 at different collision energies is higher than the ion m/z 327. Both ions have been also described for the fragmentation of the 4-hydroxystanozolol [9,11]. At higher collision energies (50 eV), the fragmentation of the stanozolol framework gives the well-known ion m/z 145 [10,11]. No specific ions were found in negative ionization for 4STANG (Figure 2).

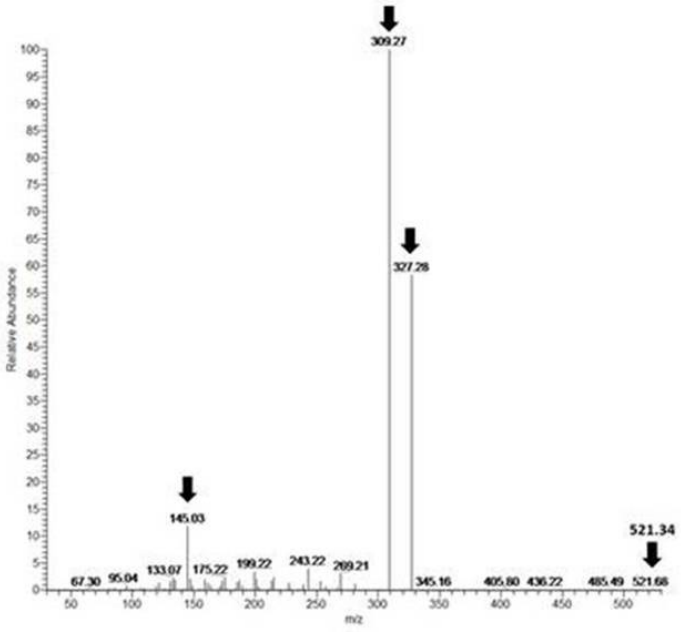

Figure 2. Product ion mass spectrum of 4STANG at 30 eV in positive ionization

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The selected ions for 4STANG and 16STANG were added to the previously described method for detection of 3STANG [4]. The mass spectrometric settings are displayed in Table 1.

Compound	Ionization Mode	Ion Transition	Collision Energy (eV)	Tube Lens (V)	Retention Time (min)
EpiTGd3	ESI+	468.0/97.0	30	110	8.51
PROSTAN	ESI+	315.2/81.0	40	82	8.18
3STANG	ESI+	521.4/345.3	40	107	8.82
		521.4/97.0	64	107	
4STANG	ESI+	521.4/309.3	20	107	8.19
		521.4/145.1	50	107	
16STANG	ESI+	521.4/345.3	40	107	6.42
	ESI-	519.4/343.3	30	134	

Table 1: Mass spectrometric settings

Chromatography

Injecting separately a stock solution of the commercially available 3STANG and the solutions obtained after the in vitro synthesis of 4STANG and 16STANG the retention of every metabolite was studied. The compounds are baseline separated, this is especially important for 3STANG and 16STANG which have common ion transitions. The most retained compound is 3STANG, followed by 4STANG and finally 16STANG elutes first. So no modifications of the previously described method for the detection of 3STANG [4] were necessary regarding chromatography.

Excretion urine samples

An excretion study was performed in a healthy volunteer (male) who took a single dose (2 mg stanozolol) of Winstrol® (Desma, Madrid, Spain). Urine samples were collected before the intake and at 1 h, 6 h, 8 h, 10 h, 12 h, 30 h, 36 h, 48 h, 60 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h, 240 h and 264 h after the administration. The study protocol was approved by the ethical committee (UZGent, Project B67020084191). Samples were stored frozen at -20 °C awaiting analysis. Additionally, two positive doping test samples, reported for the presence of stanozolol-metabolites, were reanalyzed using the described protocol [4].

In Figure 3 a chromatogram of a positive doping sample is shown. The three studied metabolites are present in the sample and the estimated concentration for 3STANG is 3.5 ng mL^{-1}. In this example, the concentration of stanozolol metabolites is very high and is not possible to conclude which is the best long-term target compound for doping-control purposes.

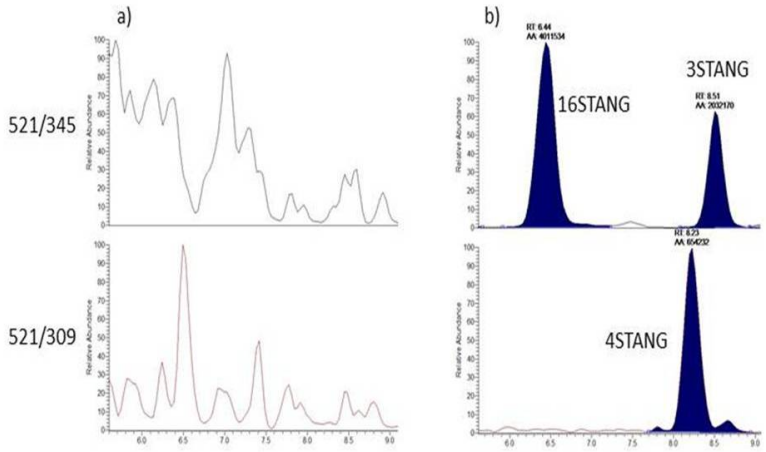

Figure 3. Chromatograms for: a) negative urine. b) Real adverse analytical finding for stanozolol

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An excretion study can give this information. In this sense, after the analysis of the excretion samples we observed that 4STANG were detected untill 120 h and 16STANG till 144 h after the administration of the single dose of stanozolol. However the detection time of 3STANG is longer (240 h) [4]. In all the cases, the ions fulfilled WADA identification criteria [12]. In Figure 4, excretion urines collected after 12 h, 120 h and 240 h after the intake of a single dose of 2 mg of stanozolol are shown.

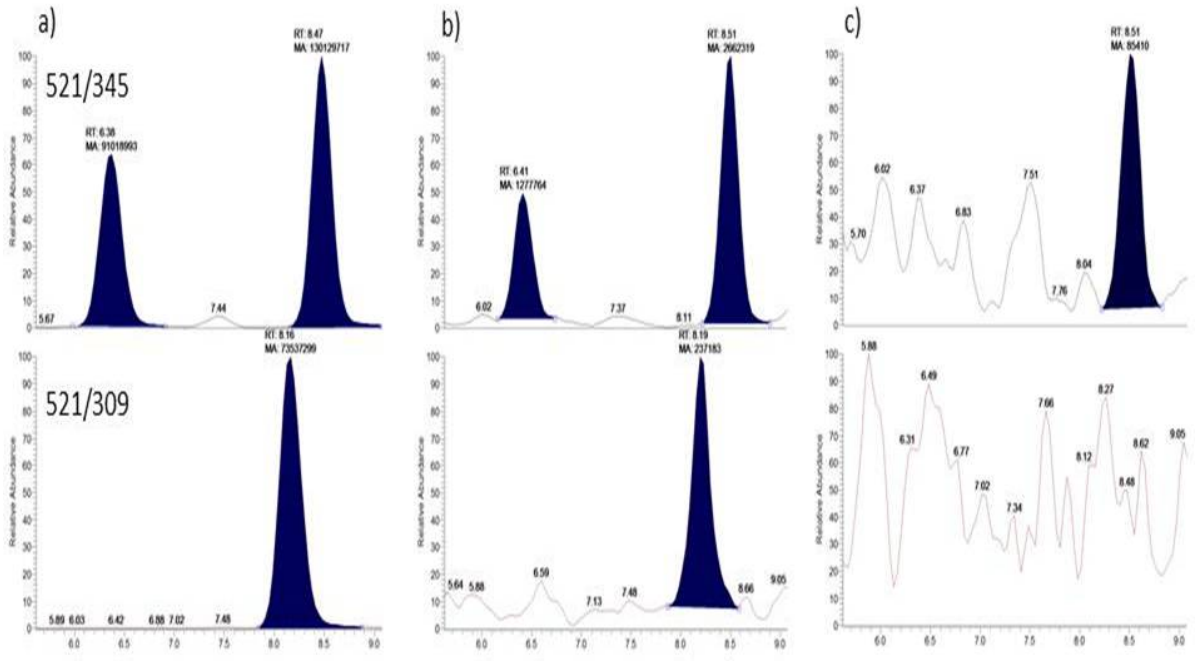

Figure 4. Chromatograms for excretion urines at: a) 12 h. b) 120 h. c) 240 h after the intake of a single dose of 2 mg of stanozolol

Another approach for the detection of the corresponding aglycones of hydroxystanozolol metabolites showed that 4β-hydroxystanozolol (4STAN) and 16β-hydroxystanozolol (16STAN) could be detected until 120 h [9]. This fact demonstrates that the detection of the corresponding phase-II metabolites is in all the cases at least equal or better than the detection of the corresponding aglycones.

Conclusions

The previously described method for the detection of 3STANG has been extended to the detection of other common hydroxystanozolol metabolites glucuronides 4STANG and 16STANG. The lack of commercially available glucuronides requires other alternatives like in vitro synthesis. The method has been applied to an excretion study and adverse analytical findings showing that the longest term metabolite, among the studied metabolites, for detection of stanozolol misuse is still 3STANG.

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References

1. World Anti-Doping Agency (WADA). 2011 Adverse Analytical Findings and Atypical Findings Reported by Accredited Laboratories. Montreal (2011) http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2011-Laboratory-Testing-Figures.pdf (access date 06.01.2013).
2. Masse R, Ayotte C, Bi H, Dugal R (1989) Studies on anabolic steroids. III. Detection and characterization of stanozolol urinary metabolites in humans by gas chromatography-mass spectrometry. *J Chromatogr B* **497**,17-37.
3. Schaenzer W, Opfermann G, Donike M (1990) Metabolism of stanozolol: identification and synthesis of urinary metabolites. *J Steroid Biochem* **36**,153-174.
4. Tudela E, Deventer K, Van Eenoo P (2013) Sensitive detection of 3'-hydroxy-stanozolol glucuronide by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1292**,195-200.
5. Schaenzer W, Guddat S, Thomas A, Opfermann G, Geyer H, Thevis M (2013) Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal*,Ahead of Print.
6. Sanaullah, Bowers LD (1996) Facile synthesis of 16,16,17-H-2(3) -testosterone, -epitestosterone and their glucuronides and sulfates. *J Steroid Biochem Mol Biol* **58**,225-234.
7. Kuuranne T, Pystynen KH, Thevis M, Leinonen A, Schanzer W, Kostiainen R (2008) Screening of in vitro synthesised metabolites of 4,9,11-trien-3-one steroids by liquid chromatography-mass spectrometry. *Eur J Mass Spectrom* **14**,181-189.
8. Kuuranne T, Vahermo M, Leinonen A, Kostiainen R (2000) Electrospray and atmospheric pressure chemical ionization tandem mass spectrometric behavior of eight anabolic steroid glucuronides. *J Am Soc Mass Spectrom* **11**,722-730.
9. Pozo OJ, Van Eenoo P, Deventer K, Lootens L, Grimalt S, Sancho JV, Hernandez F, Meuleman P, Leroux-Roels G, Delbeke FT (2009) Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids* **74**,837-852.
10. Thevis M, Makarov AA, Horning S, Schanzer W (2005) Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers. *Rapid Commun Mass Spectrom* **19**,3369-3378.
11. McKinney AR, Suann CJ, Dunstan AJ, Mulley SL, Ridley DD, Stenhouse AM (2004) Detection of stanozolol and its metabolites in equine urine by liquid chromatography-electrospray ionization ion trap mass spectrometry. *J Chromatogr B* **811**,75-83.
12. World Anti-Doping Agency (WADA). Technical Document – TD2010IDCR, Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry. Montreal (2010) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf (access date 13.08.2011).

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