Fragkaki A[1], Angelis Y[1], Kiousi P[1], Georgakopoulos C[2], Lyris E[1]

Detection of sulpho-conjugated urinary metabolites of methenolone by liquid chromatography/high resolution mass spectrometry

Doping Control Laboratory of Athens, Olympic Athletic Center of Athens, Athens, Greece[1]; Anti-Doping Laboratory of Qatar, Doha, Qatar[2]

Abstract

Methenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one) misuse in doping control is commonly detected by monitoring the parent drug and its metabolite (1-methylene-5α-androstan-3α-ol-17-one) excreted conjugated with glucuronic acid using gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) for the parent molecule, after hydrolysis with β-glucuronidase. The aim of the present study was the evaluation of methenolone metabolism as sulpho-conjugated metabolites by LC-high resolution (HR)MS and the estimation of the long-term detectability of its sulphate metabolites analyzed by LC-HRMSMS compared to the current practice for the detection of methenolone misuse used by the anti-doping laboratories. The results of the study reveal that the sulphate analogues of methenolone as well as of 1-methylene-5α-androstan-3α-ol-17-one, 3β-hydroxy-1α-methyl-5α-androstan-17-one and 16β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione were the major metabolites in the sulphate fraction. The time window for the detectability of the sulpho-conjugated methenolone metabolites by LC-HRMS is comparable with that of their hydrolyzed glucuronide analogues analyzed by GC-HRMS. Methenolone sulphate is reported for the first time as metabolite of methenolone.

Introduction

Methenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one) is a synthetic anabolic androgenic steroid (AAS) [1,2], which is frequently detected in human sports doping controls [3] and is misused for its lower hepatic toxicity compared to its 17α-alkylated analogs [4,5,6].
The detection of sulpho-conjugated metabolites has been described for endogenous steroids while their detection for exogenous AAS is evident using liquid chromatography tandem mass spectrometry (LC-MSMS) (e.g. boldione [7], boldenone [8], methyltestosterone [9] and methandienone [10]).
The aim of the present study was the evaluation of methenolone metabolism as its sulpho-conjugated metabolites by LC-high resolution (HR)MSMS and the estimation of the long-term detectability of its sulpho-conjugated analogues analyzed by LC-HRMSMS compared to the known analytes used by the anti-doping laboratories.

Experimental

Materials

All substances and reagents were of analytical grade. Two excretion studies from the inventory of the Doping Control Laboratory of Athens were used. The collection time for the first administration study was 0-298 h and 0-632 h for the second one.

Sample preparation

Direct detection of sulphate conjugates by LC-HRMS
Mefruside (50 µL from solution 21 µg/µL as internal standard, ISTD) was added to 5 mL of urine sample. The pH was adjusted to 9.5 by adding 1.4 g of $NaHCO_3$:Na_2CO_3 10:1. Sodium sulphate (3 g) was added to promote salting out effect and the

 ISBN 978-3-86884-040-7

sample was extracted with 5 mL of ethyl acetate by shaking for 20 min. After centrifugation (2000 rpm, 10 min), the organic layer was evaporated to dryness under a stream of nitrogen at 50°C. The dry extract was reconstituted with 100 µL of a mixture of solvent A:solvent B (80:20 v/v, solvent A: 5 mM ammonium formate in 0.01 % formic acid, and solvent B: mixture of acetonitrile/water (90:10 v/v) containing 5 mM ammonium formate and 0.01% formic acid) and aliquot of 5 µL was analyzed by LC-HRMS.

Detection of methenolone metabolites excreted conjugated with glucuronic acid by GC-HRMS was done according to Ref. [11].

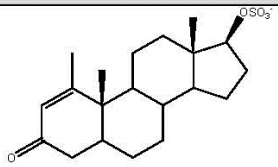
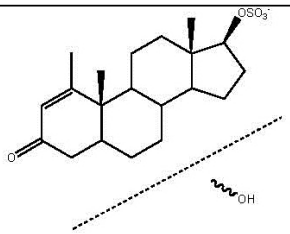
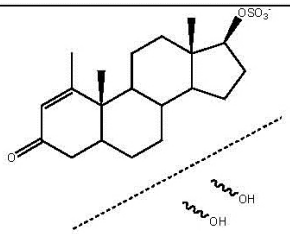
Identification of sulphate metabolites
LC fractionation of urine samples was performed in order to achieve metabolite characterization. Sulpho-conjugates were extracted from three replicates of 5 mL of a post-administration sample (and of a pre-administration sample, for comparison) according to the preparation procedure described above. The organic phases of the three replicates were combined, evaporated to dryness, reconstituted with 110 µL of a mixture ACN:H_2O 60:40 v/v, and injected in the HPLC Fraction Collector.
Fractions were collected every minute for 30 minutes. An analytical column (LiChrospher 100RP, 125 mm×4 mm i.d., 5 µm particle size) and an automatic injection system were used. Injection volume: 100 µL, flow rate: 1 mL/min and oven temperature: 40 °C. Mobile phase: mixture of solvent A, H_2O:CH_3CN (90:10 v/v) and solvent B, CH_3CN, starting at a proportion of 20% solvent B. A linear gradient was used, increasing from the initial proportion to 55% solvent B in 10 min, held for 5 min, and then increased to 100% solvent B in 10 min. Each fraction was evaporated to dryness under a stream of nitrogen and was reconstituted with 100 µL of a mixture of solvent A:solvent B (80:20 v/v, see *Direct detection of sulphate conjugates by LC-HRMS*) and 5 µL was analyzed by LC-HRMSMS.
Fractions containing metabolites were transferred into clean test tubes, evaporated to dryness under a stream of nitrogen at 40°C and then hydrolyzed to release free steroids of the sulpho-metabolites [12]. For GC-MS analysis, the dry extract was derivatized by adding 100 µL of a mixture of MSTFA/NH_4I/dithioerythritol (1000:2:6, v/w/v), incubated at 80°C for 30 min and transferred to a vial. Aliquot of 2 µL was injected.

Instrumental conditions
Instrumental conditions are described in Refs. [11,13].

Results and Discussion

Results and Discussion
Recovery as well as hydrolysis of the sulphate metabolites were initially checked and deemed satisfactory using 17β-boldenone sulphate and following the preparation protocols described above. Different metabolic pathways concerning the methenolone sulphate metabolism were initially considered, such as oxidations, reductions, hydroxylations and combinations of them. Samples collected before and after administration of methenolone from the two excretion studies of methenolone were examined. Fourteen different accurate m/z of $[M-H]^-$ ions (Table 1) and $[M+H]^+$ ions corresponding to potential methenolone sulpho-conjugated metabolites were investigated in post-administration samples using LC-HRMS in both negative and positive ionization modes, respectively. No signal was observed in positive ionization mode. No peaks were observed in pre-administration samples at the same RTs. In negative ionization mode, eight sulpho-conjugated methenolone metabolites were detected using LC-HRMSMS in targeted MSMS mode monitoring the fragments with m/z 96.9619 and 79.9534 from the product ion spectrum of each $[M-H]^-$ (Fig. 1). The product ions at m/z 96.9619 and 79.9534 correspond to the ions HSO_4^- and HSO_3^- (common for sulpho-conjugated metabolites) [14].
The identification was focused on the most abundant and with long-term rectrospectivity peaks. Four of them were considered significant taking into account their abundance and long-term detectability: S1 (two peaks), S3 and S6 (Table 1). No more consideration was given for metabolites S5, S10 and S11 (two peaks).

 ISBN 978-3-86884-040-7

ID	Sulphate structure	Metabolite	Molecular formula (negative mode)	[M-H]⁻ (m/z)	Mass error (ppm)
S1		Methenolone-sulphate (S1-a), or Methenolone+1ox+1red-sulphate (S1-b), or17-Epimethenolone sulphate	$C_{20}H_{29}O_5S^-$	381.1741	2.1 (for S1-a) 1.9 (for S1-b)
S2		Methenolone+1ox-sulphate, or Methenolone+2ox+1red-sulphate	$C_{20}H_{27}O_5S^-$	379.1585	
S3		Methenolone+1red-sulphate, or Methenolone+2red+1ox-sulphate	$C_{20}H_{31}O_5S^-$	383.1898	3.4
S4		Methenolone+2red-sulphate	$C_{20}H_{33}O_5S^-$	385.2054	
S5		Methenolone-OH-sulphate	$C_{20}H_{29}O_6S^-$	397.1690	5.7
S6		Methenolone-OH-1ox-sulphate, or Methenolone+2ox+1red-sulphate	$C_{20}H_{27}O_6S^-$	395.1534	2.9
S7		Methenolone-OH-2ox-sulphate	$C_{20}H_{25}O_6S^-$	393.1181	
S8		Methenolone-OH-1red-sulphate, or Methenolone+2red+1ox-sulphate	$C_{20}H_{31}O_6S^-$	399.1847	
S9		Methenolone-OH-2red-sulphate	$C_{20}H_{33}O_6S^-$	401.2003	
S10		Methenolone-diOH-sulphate	$C_{20}H_{29}O_7S^-$	413.1640	6.1
S11		Methenolone-diOH-1ox-sulphate	$C_{20}H_{27}O_7S^-$	411.1483	6.7 (for S11-a) 7.2 (for S11-b)
S12		Methenolone-diOH-2ox-sulphate	$C_{20}H_{25}O_7S^-$	409.1531	
S13		Methenolone-diOH-1red-sulphate	$C_{20}H_{31}O_7S^-$	415.1796	
S14		Methenolone-diOH-2red-sulphate	$C_{20}H_{33}O_7S^-$	417.1953	

Abbreviations: ox; oxidation, red; reduction, OH; hydroxylation.

Table 1. Potential sulphate metabolites of methenolone

The identification of methenolone sulpho-conjugated metabolites was based on the isolation of each metabolite after HPLC fractionation and LC-HRMSMS analysis and the subsequent analysis by GC-MS after solvolysis and trimethylsilylation. The long-term detectability of the sulpho-metabolites was compared with the detection times after treatment of the analytes with β-glucuronidase of their gluco-conjugated analogues; the time window for the detection of methenolone sulpho-metabolites by LC-HRMS is comparable with that of the hydrolyzed glucuronide analogues analyzed by GC-HRMS (Table 2).

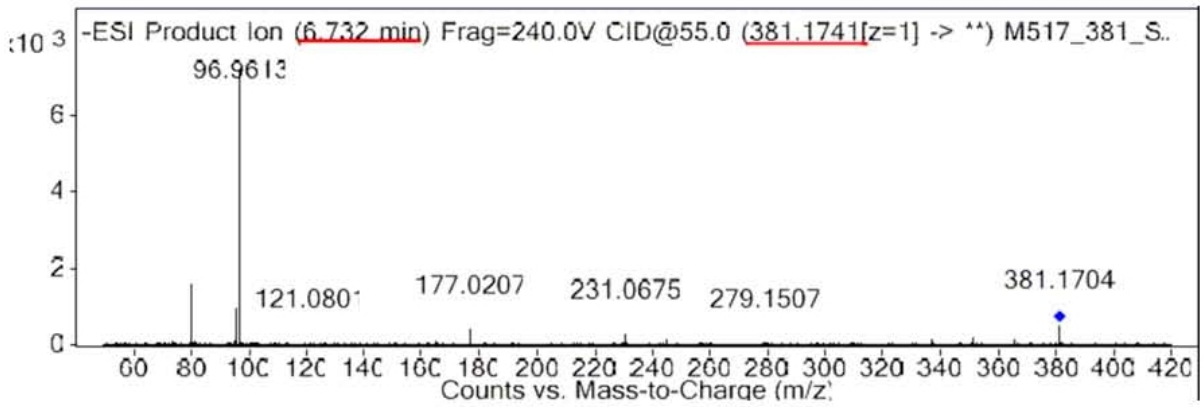


Figure 1. Product ion mass spectrum of [M-H]- ions for methenolone sulphate (S1-a) in ESI negative ionization mode at CE 55 eV in a post-administration sample of methenolone excretion study (detection time 1 day).

Analyte*	LC-HRMS analysis				GC-HRMS analysis			
			Detection time (days)				Detection time (days)	
	RT (min)	$[M-H]^-$ (m/z)	Excretion study 1	Excretion study 2	RT (min)	$[M]^+$ (m/z)	Excretion study 1	Excretion study 2
Metabolite S1-a	6.7	381.1741	12	6	8.9	446.3036	12	10
Metabolite S1-b	7.3	381.1741	12	3	8.3	446.3036	12	11
Metabolite S3	7.6	383.1898	11	10	-	-	-	-
Metabolite S6	6.2	395.1534	12	7	10.2	532.3224	12	11

* S1-a: methenolone sulphate, S1-b: 1-methylene-5α-androstan-3α-ol-17-one sulphate, S3: 3β-hydroxy-1α-methyl-5α-androstan-17-one sulphate, S6: 16β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione sulphate.

Table 2. Retention times, ions and detection times of methenolone sulpho-conjugated metabolites (LC-HRMS analysis, negative ionization mode), and of methenolone gluco-conjugated metabolites hydrolyzed with E.Coli and TMS-derivatized (GC-HRMS analysis). The detection times (in days) in post-administration samples of two excretion studies are also presented.

ISBN 978-3-86884-040-7

Conclusions

In the present study, the detection of sulpho-conjugated metabolites of methenolone was evaluated by LC-HRMSMS. HPLC fractionation, solvolysis of the fractions corresponding to the peaks of interest with subsequent analysis by GC-MS after TMS-derivatization, gave evidence that four sulpho-conjugated metabolites of methenolone are considered as a useful tool for detection of methenolone abuse: methenolone sulphate (S1-a), 1-methylene-5α-androstan-3α-ol-17-one sulphate (S1-b), 3β-hydroxy-1α-methyl-5α-androstan-17-one sulphate (S3) and 16β-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione sulphate (S6). The detection times of methenolone sulpho-metabolites by LC-HRMS is comparable with the detection times of their hydrolyzed glucuronide analogues analyzed using GC-MS by the anti-doping laboratories. Methenolone sulphate is reported for the first time as a metabolite of methenolone in human urine.

References

[1] Goudreault D, Massé R. Studies on anabolic steroids – 4. Identification of new urinary metabolites of methenolone acetate (Primobolan®) in human by gas chromatography/mass spectrometry. (1990) *J. Steroid Biochem. Molec. Biol.*, **37**, 137-154.
[2] Massé R, Goudreault D. (1992) Studies on anabolic steroids – 11. 18-hydroxylated metabolites of mesterolone, methenolone and stenbolone: new steroids isolated from human urine. J. Steroid Biochem. Mol. Biol. **42**, 399-410.
[3] World Anti-Doping Agency, 2013 Anti-Doping Testing Figures, Laboratory Report, https://wada-main-prod.s3.amazonaws.com/resources/files/WADA-2013-Anti-Doping-Testing-Figures-LABORATORY-REPORT.pdf (access date 25.10.2014).
[4] Palva IP, Wasastjerna C. (1972) Treatment of aplastic anemia with methenolone. *Acta Haemat.* **47**, 13-20.
[5] Lockner D. (1979) Treatment of refractory anemias with methenolone. *Acta Med. Scand*. **205**, 97-101.
[6] Sanchez-Medal L, Gomez-Leal A, Duarte L, Rico MG. (1969) Anabolic androgenic steroids in the treatment of acquired anemia. *Blood* **34**, 283-300.
[7] Gómez C, Pozo OJ, Fabregat A, Marcos J, Deventer K, Van Eenoo P, Segura J, Ventura R (2012) Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part I: Phase I metabolites excreted free, as glucuronide and sulfate conjugates, and released after alkaline treatment of the urine. *Drug Test. Analysis* **4**, 775-785.
[8] Gómez C, Pozo OJ, Geyer H, Marcos J, Thevis M, Schänzer W, Segura J, Ventura R. (2012) New potentials markers for the detection of boldenone misuse. *J. Steroid Biochem. Mol. Biol.* **132**, 239-246.
[9] Gómez C, Pozo OJ, Marcos J, Segura J, Ventura R (2013) Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids* **78**, 44-52.
[10] Gómez C, Pozo OJ, Garrostas L, Segura J, Ventura R. (2013) A new sulphate metabolite as a long-term marker of methandienone misuse. *Steroids* **78**, 1245-1253.
[11] Tsivou M, Kioukia-Fougia N, Lyris, et al. (2006) An overview of the doping control analysis during the Olympic Games of 2004 in Athens, Greece. *Anal. Chim. Acta* **555**, 1-13.
[12] Torrado S, Roig M, Farre M, Segura J, Ventura R. (2008) Urinary metabolic profile of 19-norsteroids in humans: glucuronide and sulphate conjugates after oral administration of 19-nor-4-androstenediol. *Rapid Commun. Mass Spectrom*. **22**, 3035-3042.
[13] Vonaparti A, Lyris E, Angelis YS, Panderi I, Koupparis M, Tsantili-Kakoulidou A, Peters RJB, Nielen MWF, Georgakopoulos C. (2010) Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high resolution time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom*. **24**, 1595-1609.
[14] Yi L, Dratter J, Wang C, Tunge JA, Desaire H. (2006) Identification of sulfation sites of metabolites and prediction of the compounds' biological effects. *Anal. Bioanal. Chem.* **386**, 666-674.

Acknowledgements

The financial support received from the World Anti-Doping Agency, WADA (reference no. 11A13CG) is gratefully acknowledged. The authors also thank Mrs. Stella Loui, Mrs. Fotini Chlapana and Ms. Ioanna Koutsouli for their technical assistance.