Research Article

Drug Testing and Analysis

Received: 1 July 2009 Revised: 11 August 2009 Accepted: 17 August 2009 Published online in Wiley Interscience: 26 October 2009

(www.drugtestinganalysis.com) DOI 10.1002/dta.56

Combination of liquid-chromatography tandem mass spectrometry in different scan modes with human and chimeric mouse urine for the study of steroid metabolism

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Anabolic steroids are among the most frequently detected compounds in doping analysis. They are extensively metabolized and therefore an in-depth knowledge about steroid metabolism is needed. In this study, a liquid chromatography tandem mass spectometry (LC-MS/MS) method based on a precursor ion scan with a uPA-SCID mouse with humanized liver (a chimeric mouse) was explored for the detection of steroid metabolism. Methandienone was used as a model compound. The application of the precursor ion scan method in positive human samples and chimeric mice samples after methandienone administration allowed the detection of most steroid metabolites without any structural restriction. Three hitherto unreported metabolites were found using this approach. These metabolites were characterized using LC-MS/MS and feasible structures were proposed. The structure of one of them, 6-ene-epimethandienone, was confirmed by the synthesis of the reference compound. A selected reaction monitoring (SRM) method for the specific detection of all these metabolites has been developed. The application of this method to several human and chimeric mouse samples confirmed that more than 80% of the steroid metabolites were found in both samples. Only metabolites that are poorly detectable by LC-MS/MS were not detected in some urine samples. The metabolic nature of the unreported metabolites was also confirmed. A global strategy for the detection of steroid metabolites combining both human and chimeric mouse urine is proposed.

Keywords: anabolic steroids; chimeric mice; doping analysis; metabolites; methandienone; precursor ion scan; uPA-SCID

Introduction

The list of prohibited substances in the field of doping is published yearly by the World Anti-Doping Agency (WADA) and covers several classes of substances.[1] Of these, anabolic agents are the most frequently detected compounds.[2] For this reason, doping-control laboratories have made important efforts to enhance detection of this group of analytes.[3]

Target anabolic steroids and metabolites are usually detected by gas chromatography tandem mass spectrometry (GC-MS).[4–6] These methods normally involve liquid-liquid extraction followed by a derivatization step. The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) is becoming more important for the urinary detection of anabolic steroids because it avoids the derivatization step and facilitates the detection of some anabolic steroids like tetrahydrogestrinone (THG), stanozolol or trenbolone.[7–9] Some LC-MS/MS methods have been developed for the urinary detection of several target anabolic steroids, achieving the required sensitivity.[10–11]

Generally, anabolic steroids are metabolized in humans[12] and therefore analytical methods have to focus mainly on the detection of metabolites. In this way, research providing additional information about steroid metabolism is of great value because (1) these metabolites prove that the steroid was administered and passed through the body and (2) new metabolites can facilitate the detectability of the steroid misuse for a longer time period.[3,13–16]

For these reasons, several methodologies based on precursor ion scan and neutral loss have been developed for the detection of compounds with a steroid-like structure, which can be useful for the detection of steroid metabolites.[17–23] The open detection of steroids almost without any restriction in their structure has been reported by precursor ion scan of three specific ions.[17] Some of these methodologies have been successfully applied to the detection of anabolic steroid metabolites and previously unreported metabolites for fluoxymesterone,[21] stanozolol[22] and methyltestosterone[23] have been detected recently. These methods normally use human urine samples declared positive for steroid misuse after the detection of steroid metabolites. However, the use of human volunteers is not always easy for steroids that are not of pharmaceutical quality and therefore alternatives

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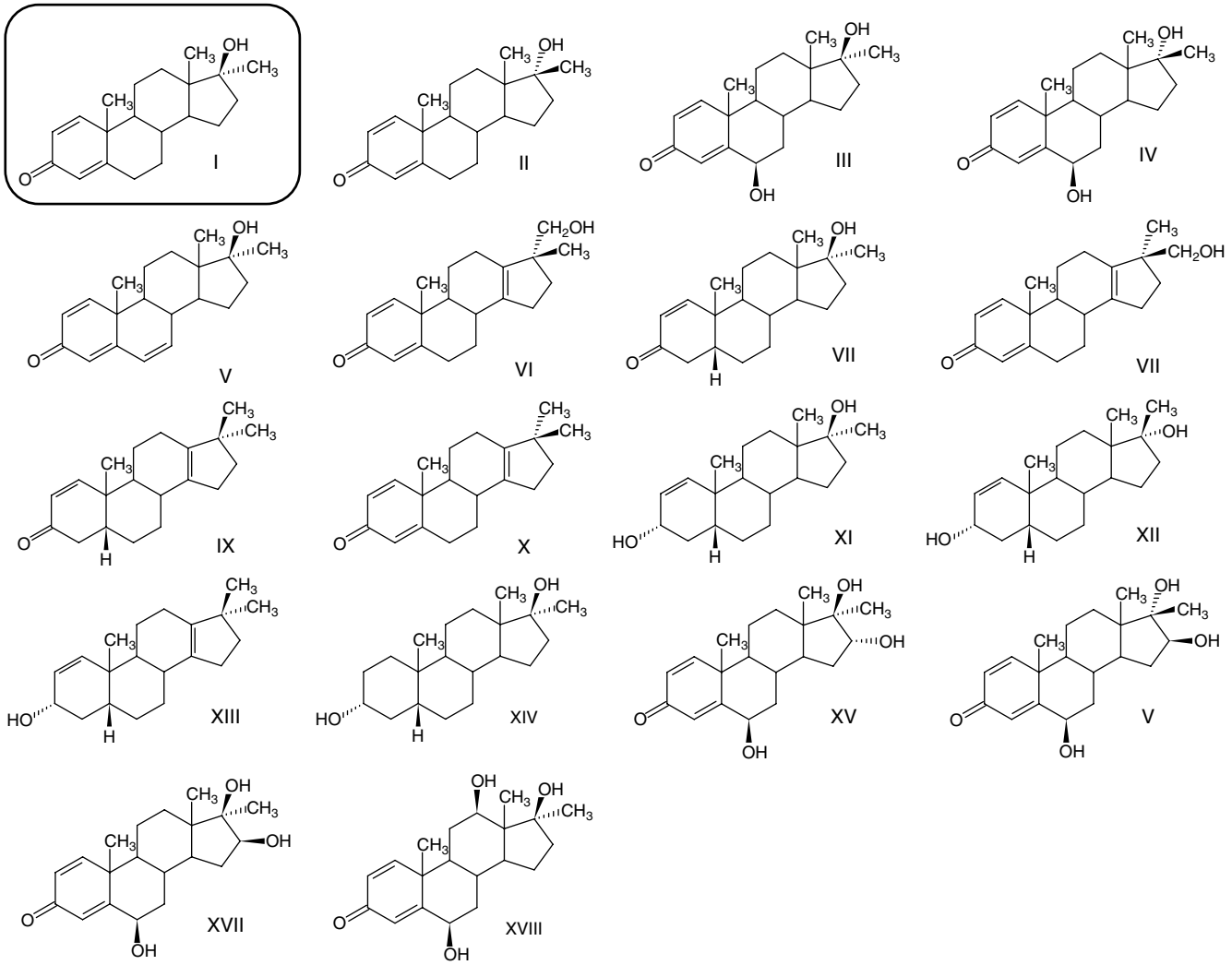


Figure 1. Chemical structures of methandienone metabolites reported in literature.

are required. Indeed, obtaining an ethical committee approval for administering black-market steroids, designer steroids and even pharmaceutical grade steroids that have been removed from the legal market to human volunteers, is virtually impossible.

The use of *in vitro* cultures is one of the most useful approaches for this purpose. Moreover, *in vitro* studies are potentially useful for the establishment of the effect and mechanism of doping substances.[24] The metabolism of several steroids has been studied by *in vitro* systems.[25–28] In this way, some metabolites of norandrostenedione,[25] THG,[26] gestrinone and other related steroids[27] have been described using these systems. Despite the satisfactory results, the model still has important drawbacks such as the limited number of metabolic pathways reported.

Recently, a new model based on the administration of steroids to a mouse with human hepatocytes (chimeric mouse) has been developed. This model involves the transplantation of primary human hepatocytes into uPA-SCID mice.[29] These immune-deficient mice suffer from a transgene-induced liver disease and therefore the transplanted human hepatocytes can repopulate the diseased liver without the risk of graft rejection. The occurrence of known human steroid metabolites in the chimeric mouse urine has been tested for some steroids, showing the suitability of the model.[30–31] In these studies, several target human metabolites were also detected in the mouse urine after steroid administration. Moreover, the chimeric mouse model can be used for the confirmation of the metabolic nature of compounds detected in human urine. Indeed, this methodology was successfully applied after administration of stanozolol[22] and methyltestosterone[23] to unequivocally confirm the presence of metabolites for these steroids. However, its applicability for the detection of completely unknown steroids without any structural restriction has not been tested yet.

The aim of this study was to explore the capabilities of the use of the different scan modes by LC-MS/MS in combination with human and chimeric mouse urine for the detection of anabolic steroid metabolites. Methandienone was selected as a case study (1) because it is one of the most frequently detected anabolic steroids in sports doping[2] and (2) due to the already existing knowledge about its metabolism. The metabolism of methandienone has been studied during the last 30 years.[13–16,32–37] These studies showed that methandienone is extensively metabolized and several compounds have been reported for the urinary detection of its misuse (Figure 1). Several metabolic and degradation pathways have been described

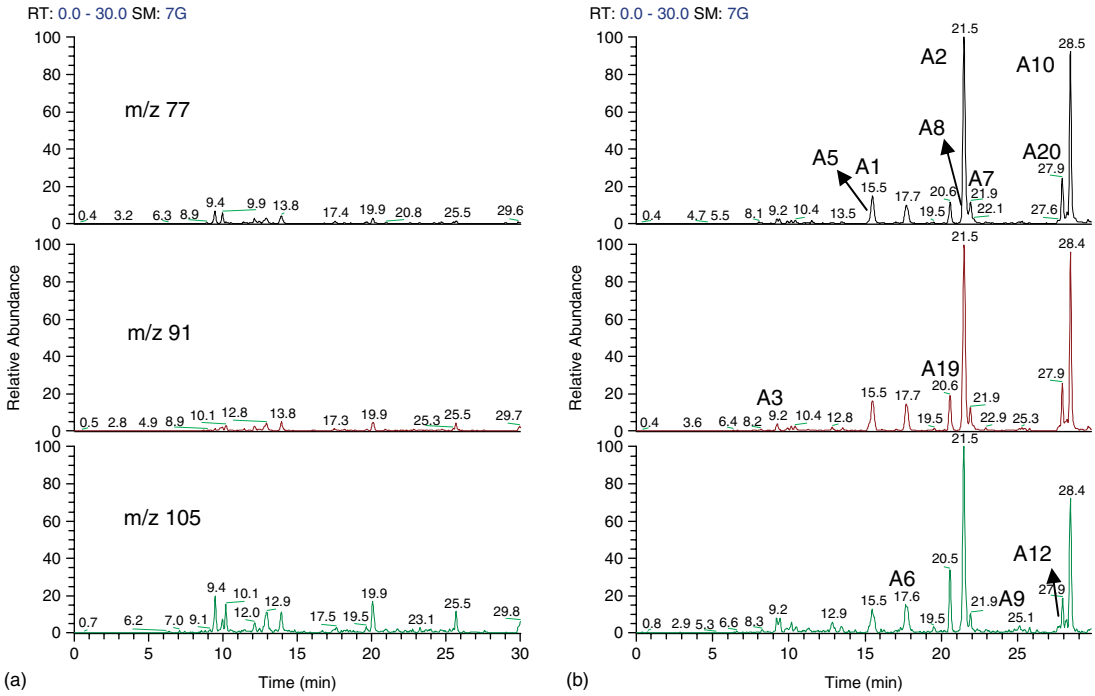


Figure 2. LC-MS/MS chromatograms obtained in the application of the PI method to (a) negative human urine sample and (b) human urine sample declared positive for methandienone metabolites.

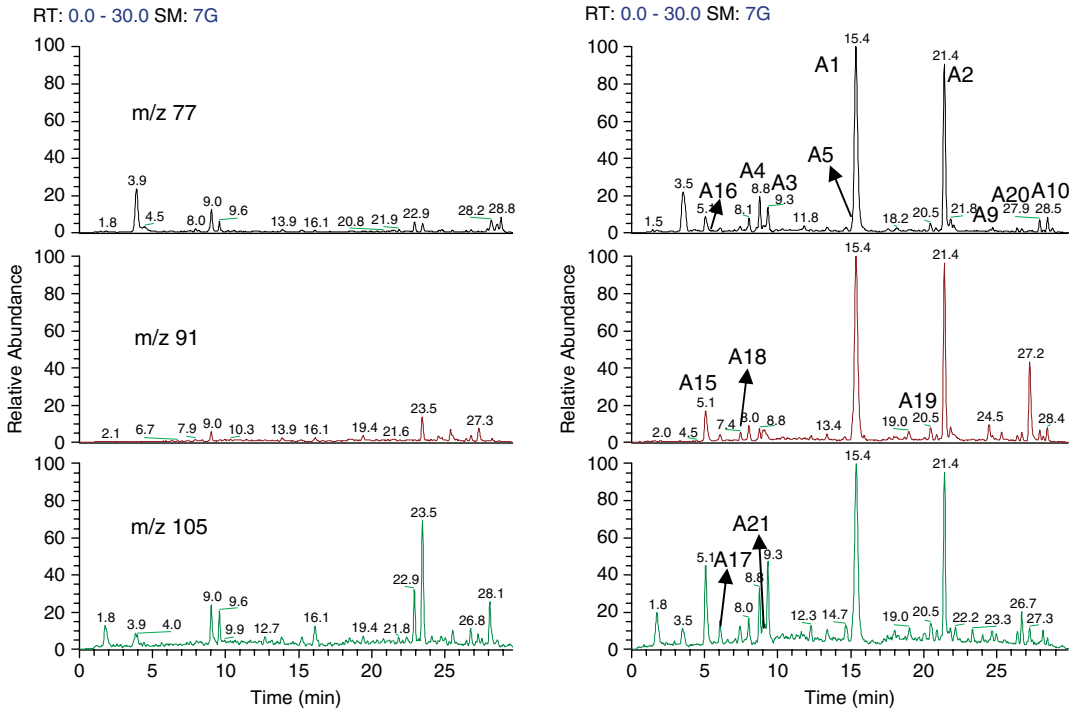


Figure 3. LC-MS/MS chromatograms obtained by the application of the PI method to (a) chimeric mouse urine collected before methandienone administration and (b) chimeric mouse urine collected after methandienone administration.

for methandienone, such as A-ring reduction, 3-reduction, 6-, 12- and 16-hydroxylations and dehydratation involving a 18-methyl migration or epimerization. A new long-term metabolite involving a dehydratation and hydroxylation in C_{18} has recently been described.[13,15] The use of this metabolite could improve considerably the detection period of methandienone misuse. The goal of the study is to explore the capabilities of the proposed model to detect steroid metabolites. Methandienone has been selected as a model compound because a large number of metabolites for this steroid have been described. The model can be validated if it allows for the detection of most of the reported metabolities.

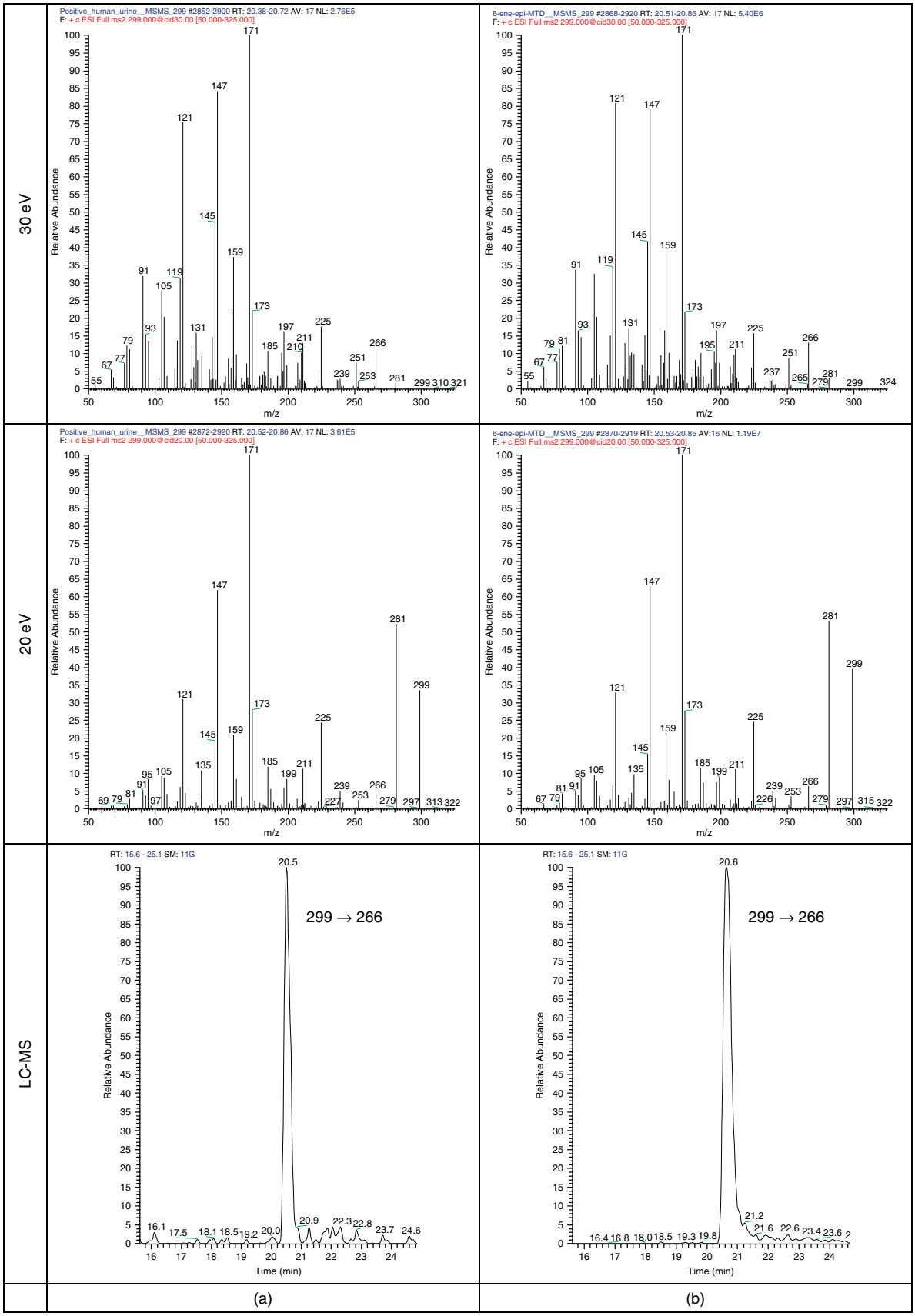


Figure 4. SRM chromatogram (bottom) and CID spectra (centre and top) for (a) synthesized 6-ene-epimethandienone and (b) human urine sample declared positive for methandienone metabolites.

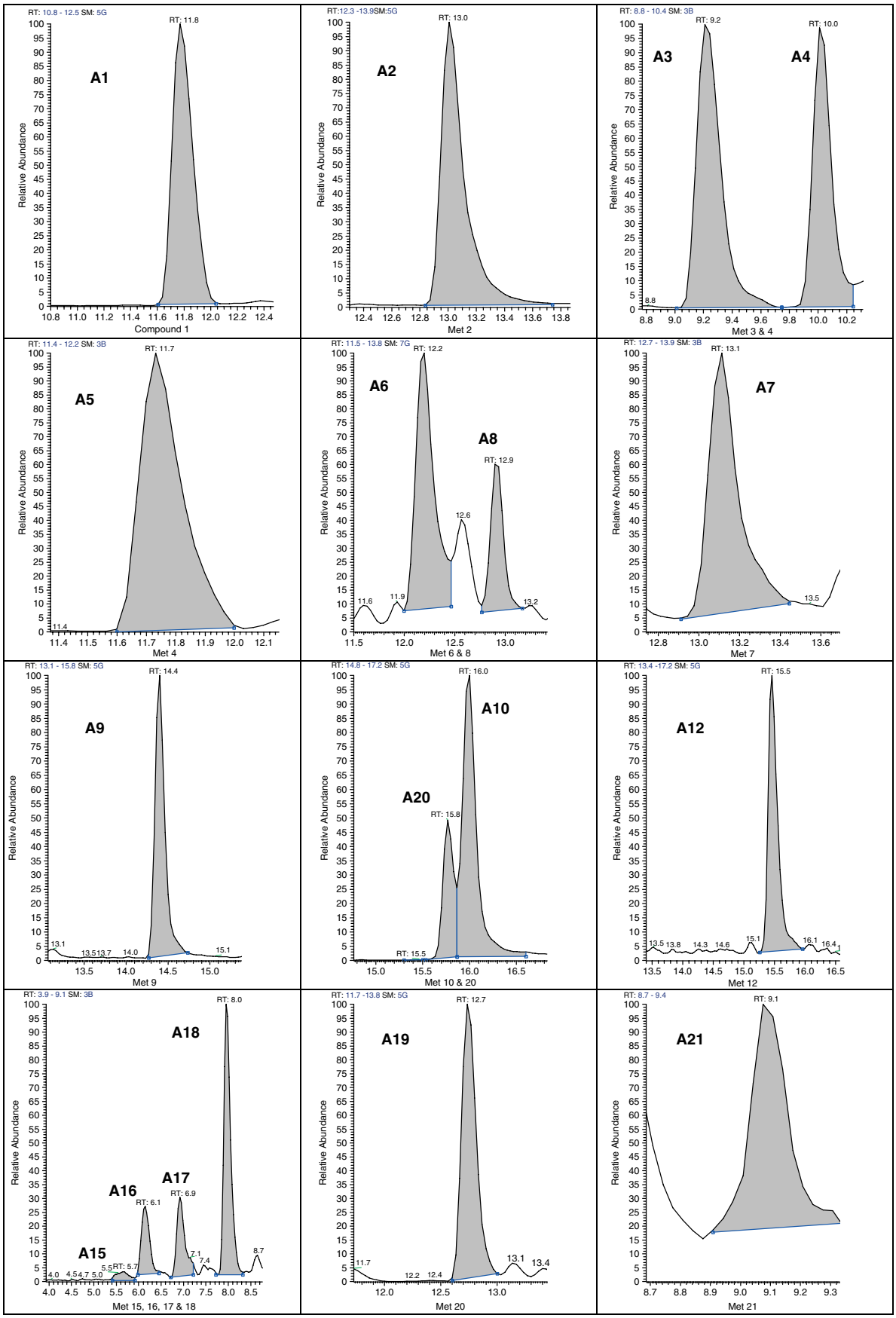


Figure 5. LC-MS/MS chromatograms obtained by the application of the SRM method to a human urine sample declared positive for the presence of methandienone metabolites (for metabolite assignment see Table 3).

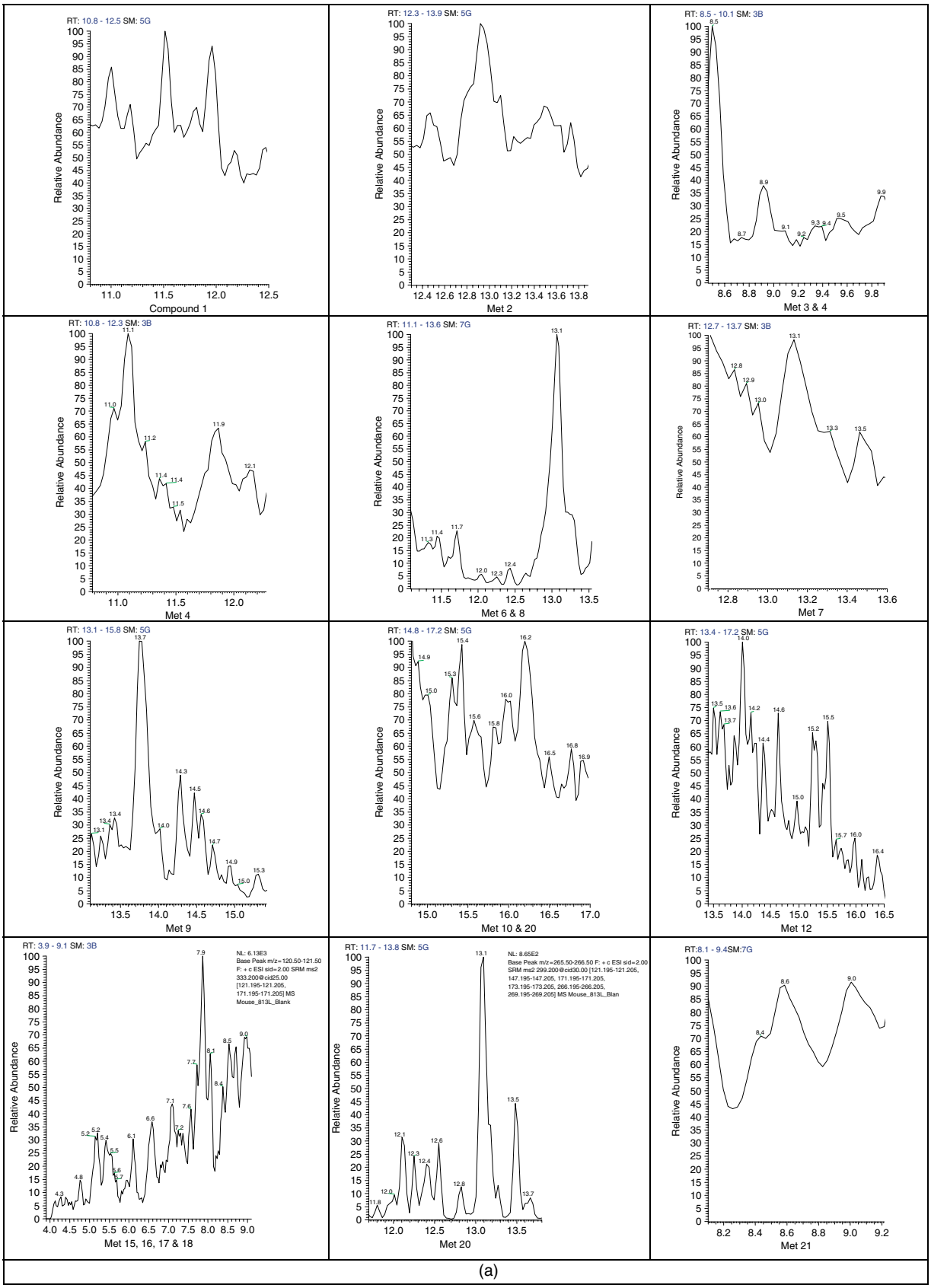
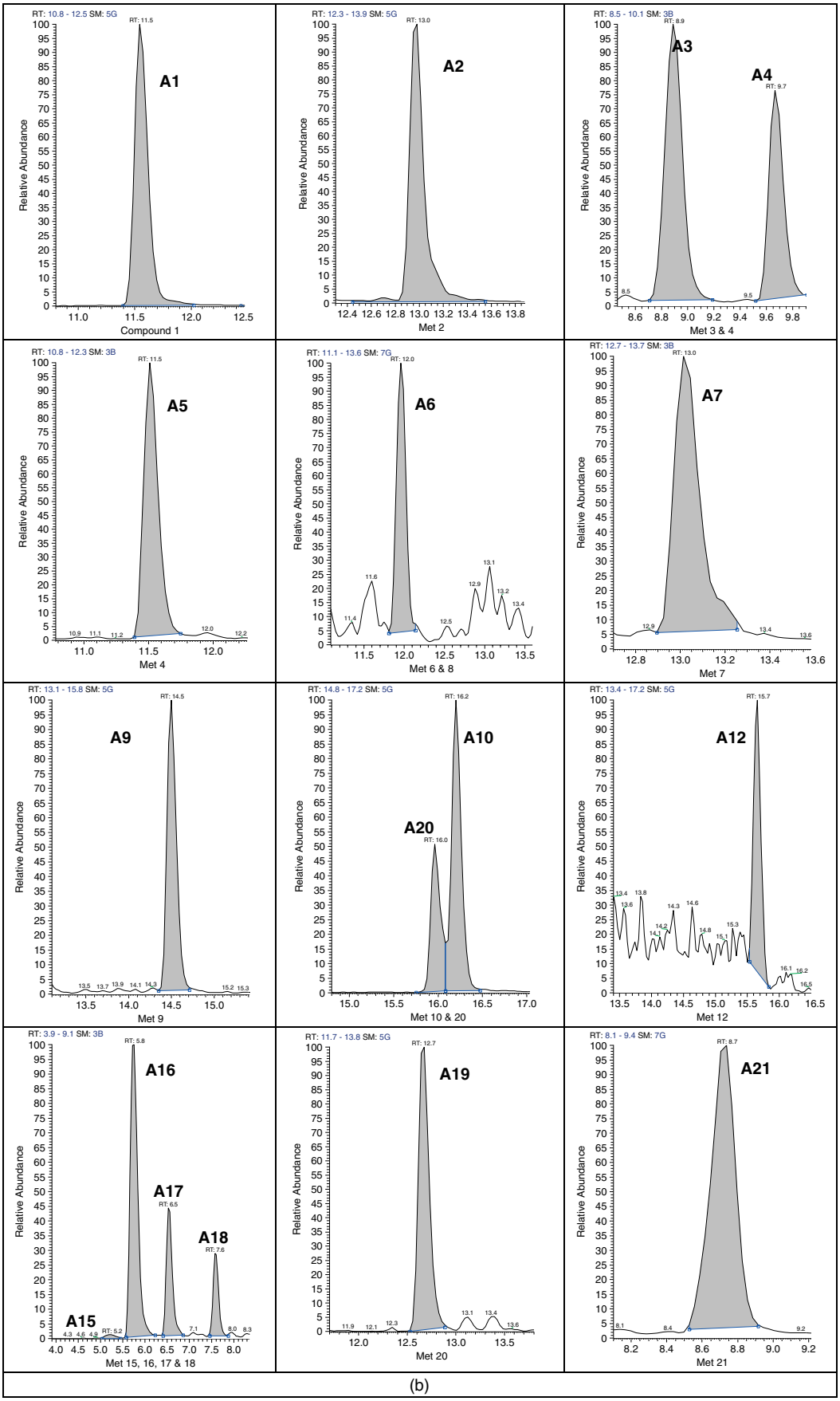


Figure 6a. LC-MS/MS chromatograms obtained by the application of the SRM method to chimeric mouse urine collected (a) before methandienone administration (blank) and (b) chimeric mouse urine collected after methandienone administration (for metabolite assignment see Table 3).



(b)

Figure 6b. (*Continued*).

Experimental

Chemicals and reagents

Methandienone metabolites are depicted in Figure 1. Methandienone (I), epimethandienone (II), 6β-hydroxy-methandienone (III), 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one (X) and 17β-methyl-5β-androsta-1-ene-3α,17α-diol (epimetenediol, XII) and 17α-methyl-5β-androsta-3α,17β-diol (XIV) were purchased from NMI (Pymble, Australia). 17β-Hydroxy-17α-methyl-5β-androst-1-en-3-one (VII) was bought from Steraloids (London, UK). 17 α -Hydroxymethyl-17 β -methyl-18-norandrosta-1,4,13-trien-3-one (VI) and 17β-Hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one (VIII) were synthesized at the Institute of Biochemistry, German Sport University Cologne (Germany).

17-Epimethyltestosterone (17α-hydroxy-17β-methylandrost-4-en-3-one) was synthesized at the Institute of Biochemistry, German Sport University Cologne (Germany) as described by Schänzer *et al*.[38] The β-glucuronidase preparation (type *E. Coli* K12) was purchased from Roche (Mannheim, Germany). Analytical grade potassium carbonate, sodium hydrogen carbonate and diethyl ether were obtained from Merck (Darmstadt, Germany).

HPLC-grade methanol and HPLC-grade water were purchased from Acros (Geel, Belgium) and Fischer Scientific (Loughborough, UK), respectively. Ammonium acetate and acetic acid were from Sigma (St Louis, MO, USA) and Merck, respectively.

Stock solutions were prepared by dissolving the reference material in methanol. Working solutions at 1 µg/mL were obtained by diluting adequate amounts of stock solutions in water : methanol (9 : 1).

Instrumentation

LC-MS/MS studies

A HPLC Finnigan Surveyor MS pump Plus (Thermo, San Jose, USA) was interfaced to a Finnigan TSQ Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo) using the electrospray interface. Twenty microlitres of sample were injected into the system using a Finnigan Surveyor autosampler Plus (Thermo). The LC separation was performed using an Omnispher C_{18} column (100 × 2 mm i.d., 3 µm) (Varian, Sint-Katelijne-Waver, Belgium), at a flow rate of 250 µL/min using a ChromSep guard column (10 × 2 mm i. d., 5 µm) (Varian).

Aqueous and methanolic solutions both with ammonium acetate (1 mM) and 0.001% acetic acid were selected as mobile-phase solvents. Nitrogen was used as sheath gas, ion sweep gas and auxiliary gas at flows of 70, 2 and 5 arbitrary units, respectively. Spray voltages of 4000 V and 3000 V were used in the positive and negative ionization modes, respectively. The capillary temperature was set at 300 °C and the CID source at 2 units. The collision gas pressure was 1.5 units.

Human samples

Positive doping test samples for methandienone were provided by other doping control laboratories, the International Olympic Committee (IOC) and WADA or were anonymous left-over samples from a non-challenged positive doping control test. Blank urine samples were used in order to check the specificity of the method.

uPA-SCID mouse samples

Chimeric mice were produced as described before.[29] Briefly, uPA$^{+/+}$-SCID mice were transplanted within two weeks after birth with primary human hepatocytes (chimeric mice). uPA-SCID mice that were not transplanted with human hepatocytes served as a control group (non-chimeric mice).

A phosphate buffer saline (PBS) suspension of methandienone (50 µg in 100 µL) or a placebo solution was administered to three mice via oral gavation according to a double-blind study protocol. The mouse urine was collected using metabolic cages especially designed for small rodents (Tecniplast, Someren, Netherlands). The design of the metabolic cages enables the urine and the faeces to be separated perfectly. Blank urine samples were collected 24 hours prior to dosage. Afterwards, urine was collected every 24 hours and stored at −20 °C awaiting analysis. The mice had *ad libitum* access to water and powdered food. The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University (ECD 06/09).

Sample preparation

The unconjugated fraction was analysed by adjusting the pH of the urine (5 mL for human urine and 0.5 mL for chimeric mouse urine) to 9.2 by addition of approximately 0.3 g sodium hydrogen carbonate : potassium carbonate (2 : 1 w : w). Liquid-liquid extraction was performed by adding 5 mL diethyl ether and rolling during 20 min. The sample was then centrifuged and the organic layer was separated and evaporated under oxygen-free nitrogen (OFN) at 40 °C.

For the total (conjugated + unconjugated) fraction, 1 mL phosphate buffer (pH 7) and 50 µL of the β-glucuronidase solution were added to 5 mL urine. The sample was hydrolysed during 2.5 h at 56 °C. After cooling to room temperature, extraction was performed as described for the unconjugated fraction.

For LC-MS/MS analysis, the residue was dissolved into 150 µL of methanol : water (50 : 50, v : v) and 20 µL were directly injected into the system.

Precursor ion analysis (QqQ)

A precursor ion scan method selecting the product ions at m/z 77, 91 and 105 was used.[17] The collision energy was 50 V for m/z 91 and 105 and 55 V for m/z 77. The peak width was set at 0.7 Da and the scan rate at 0.4 s/scan. A gradient program was used, the percentage of organic solvent was linearly changed as follows: 0 min, 30%; 1.5 min, 30%; 8 min, 55%; 15 min, 55%; 29.5 min, 95%; 30.5 min, 95%; 31 min, 30%, 34 min 30%.

SRM analysis

A SRM method using the triple quadrupole instrument was developed for the LC-MS/MS detection of methandienone metabolites. Two transitions were selected for each metabolite (Table 1). The acquisition time for each transition was set at 50 ms. The same column and mobile phase compositions were used as in precursor ion scan analysis. A gradient program was used; the percentage of organic solvent was linearly changed as follows: 0 min, 25%; 1.5 min, 25%; 15 min, 95%; 18 min, 95%; 19 min, 25%; 22 min, 25%.

Table 1. SRM method for the detection of methandienone metabolites

Analyte	TLV (V)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
A1	110	301	121	25
			149	15
A2	110	301	121	25
			149	15
A3	90	317	121	25
			147	25
A4	90	317	121	25
			147	25
A5	110	299	147	25
			171	25
A6	110	299	147	25
			269	20
A7	110	303	201	20
			145	25
A8	110	299	147	25
			269	20
A9	110	285	201	20
			186	35
A10	110	283	121	30
			147	15
A12	130	269	105	35
	70	322	269	15
A15	110	333	121	25
			171	25
A16	110	333	121	25
			171	25
A17	110	333	121	25
			171	25
A18	110	333	121	25
			171	25
A19	110	299	171	25
			266	25
A20	110	283	121	30
			147	15
A21	110	315	121	25
			171	25

TLV: Tube Lens Voltage.

Proposed structures of metabolite

The CID product ion spectra of each metabolite were acquired with the triple quadrupole mass analyser at two different collision energies: 20 V and 30 V. Two mass regions were studied in depth: all ions close to the precursor ion (product ions with m/z $\geq$ M-100) and the most abundant ions at high collision energy. The number of ions and their characteristics was related to the structure of the metabolite according to a previous study.[39] The product ion spectra were acquired in a m/z range between 30 and 400 using a peak width of 0.7 Da at a scan time of 0.5 s/scan.

Synthesis of 6-ene-epimethandienone

17-Epimethyltestosterone was dehydrogenated in a low mg scale with chloranil in refluxing i-amyl alcohol within 1.5 hours. After filtration of the mixture, the filtrate was evaporated to dryness and the products were purified by liquid-liquid extraction with n-pentane and potassium hydroxide solution (0.1 N). Evaporation of the organic layer yielded 17α-hydroxy-17β-methylandrosta-1,4,6-trien-3-one (63%) besides some only partially dehydrogenated 17α-hydroxy-17β-methylandrosta-4,6-dien-3-one (33%, yields determined from peak areas in GC-MS chromatogram). Structure identification was performed by mass spectrometry of underivatized steroids and TMS derivatives of the products.

Application to spiked samples

Negative human urine was spiked at 5 and 50 ng/ml and negative chimeric mouse urine at 50 and 500 ng/ml with a mixture containing the methandienone metabolites. Appropriate amounts of the methanolic standard mixture at 1 µg/ml were added in a tube. After evaporation, the mixture was reconstituted in the respective negative urine.

The spiked samples were analysed using both the precursor ion scan (PI) and the SRM methods. The limit of detection (LOD), defined as the peak with a S/N higher than 3, was calculated by extrapolation of the signal obtained for the lowest spiked sample.

Ion suppression was calculated as the difference between the area values obtained for a solvent and a negative urine sample both spiked at the highest concentration tested and extracted as described above.

Results and Discussion

Precursor ion scan and SRM analysis of spiked urine samples

Methandienone was selected for study due to the extensive knowledge about its metabolism and to the large number of metabolites commercially available. Up to eight reported metabolites and the parent drug were available in our laboratory.

For the non-target detection of methandienone metabolites, a PI method based on the acquisition of the ions at m/z 77, 91 and 105 was evaluated.[17] Moreover, for the target detection of available compounds, a SRM method was developed based on a previously published method.[11]

The analytical characteristics of both methods were checked by the analysis of urine samples (from both human and chimeric mouse) spiked with the parent drug and the eight available metabolites. The extraction recoveries for all tested compounds were higher than 70% in both human and chimeric mouse urine. Similar ion suppression values were obtained for both types of urines (Table 2). Although the sample volume used in chimeric mouse was 10 times lower, the higher number of interferences in this sample can be responsible for these similar values. Only for epimetenediol (analyte XII) was the ion suppression clearly higher in the mouse urine.

All the spiked analytes were detected by the PI method in human urine except for analyte XIV, which does not contain any ionizable moiety (oxo groups or hydroxyl groups in α position from a double bond) and therefore cannot be detected by LC-MS/MS.[40] The ion detected for every compound was the $[M+H]^+$ except for analyte XII for which $[M+H-2H_2O]^+$ was obtained (Table 2). That is common for the application of this PI method to steroids containing hydroxyl groups close to a double bond.[17] The LOD obtained for this approach in human urine (calculated as the peak which can be detectable in the PI chromatogram with S/N higher than 3 for the TIC) ranged from 5 to 50 ng/ml (Table 2). In the case of 6-hydroxy-methandienone (metabolite III)

Table 2. Results of the application of PI and SRM method to both human and chimeric mouse urine samples spiked with reference standard

	Ion suppression (%)		PI method					SRM method		
						LOD (ng/ml)			LOD (ng/ml)	
Analyte	Human	Mouse	*Retention time (min)*	*Precursor ion (m/z)*	*Specie*	Human	Mouse	*Retention time (min)*	Human	Mouse
I	59	63	15.5	301	$[M+H]^+$	10	100	11.8	0.5	5
II	48	48	21.5	301	$[M+H]^+$	5	50	13.0	0.2	2
III	55	71	9.4	317	$[M+H]^+$	50	250	9.2	2	25
VI	57	59	17.6	299	$[M+H]^+$	20	200	12.2	1	15
VII	45	48	21.9	303	$[M+H]^+$	40	500	13.1	2	15
VIII	34	35	21.4	299	$[M+H]^+$	15	200	12.9	1	10
X	62	53	28.4	283	$[M+H]^+$	5	50	16.0	0.5	5
XII	7	37	27.6	269	$[M+H-2H_2O]^+$	50	500	15.6	4	50
XIV	*n.d.*	*n.d.*	*n.d.*	–	–	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

Table 3. Results of the application of PI and SRM methods to human methandienone positive samples and mouse urine samples collected after methandienone administration

	PI method				SRM method		
Peak	*Precursor ion*	*Specie*	*Human*	*Mouse*	*Human*	*Mouse*	**Assignment**[a]
A1	301	$[M+H]^+$	√	√	10/10	3/3	I
A2	301	$[M+H]^+$	√	√	10/10	3/3	II
A3	317	$[M+H]^+$	√	√	10/10	3/3	III
A4	317	$[M+H]^+$	×	√	10/10	3/3	IV[b]
A5	299	$[M+H]^+$	√	√	10/10	3/3	V[b]
A6	299	$[M+H]^+$	√	×	10/10	3/3	VI
A7	303	$[M+H]^+$	√	×	10/10	3/3	VII
A8	299	$[M+H]^+$	√	×	6/10	1/3	VIII
A9	285	$[M+H]^+$	√	√	10/10	3/3	IX[b]
A10	283	$[M+H]^+$	√	√	10/10	3/3	X
A11	–	–	×	×.	–	–	XI
A12	269	$[M+H-2H_2O]^+$	√	×	8/10	1/3	XII
A13	–	–	×	×.	–	–	XIII
A14	–	–	×	×.	–	–	XIV
A15	333	$[M+H]^+$	×	√	5/10	3/3	XV[b,c]
A16	333	$[M+H]^+$	×	√	8/10	3/3	XVI[b,c]
A17	333	$[M+H]^+$	×	√	10/10	3/3	XVII[b,c]
A18	333	$[M+H]^+$	×	√	8/10	3/3	XVIII[b,c]
A19	299	$[M+H]^+$	√	√	10/10	3/3	Unreported
A20	283	$[M+H]^+$	√	√	10/10	3/3	Unreported
A21	315	$[M+H]^+$	×	√	6/10	3/3	Unreported

[a] For structures see Figure 1.
[b] Tentative assignment.
[c] Metabolites XV, XVI, XVII and XVIII could not be differentiate by LC-MS/MS. √ Detected, × Not detected.

a high LOD was obtained due to the presence of an endogenous compound with m/z 316, which elutes at a similar retention time. Although the sensitivity reached with this PI approach does not fulfil WADA requirements for all target analytes,[41] it can be helpful for the detection of unexpected metabolites. In the case of chimeric mouse urine the LOD were normally 10 times higher (ranging from 50 to 500 ng/ml) due to the lower amount of urine used (500 µl). Higher doses therefore have to be administered to the chimeric mouse in order to have similar results.

By applying the SRM method, better LODs were obtained and concentrations below 4 ng/ml (human) and 50 ng/ml (chimeric mouse) were detectable (Table 2). Although the sensitivity of this method is higher, it is only applicable for the selected analytes.

Precursor ion analysis of human urine samples

The PI method was applied to several human urine samples declared positive for the presence of methandienone metabolites. In the positive urine samples up to 12 different peaks could be detected which were not present in the negative urine samples (Figure 2). These peaks can be considered as methandienone metabolites. The PI method allows for the identification of the precursor ion of the detected peaks (Table 3). Most of these

peaks could be assigned to previously reported metabolites. However, a direct assignment could not be performed for two of them (A19 and A20) with precursor ion at m/z 299 and m/z 283.

Precursor ion analysis of uPA-SCID mouse urine samples

The same PI method was also applied to chimeric mouse urine samples collected before and after methandienone administration. In the sample collected after administration 14 peaks were detected, which were not present in the sample collected before administration (Figure 3). While eight of these peaks (A1, A2, A3, A5, A9, A10, A19 and A20) were common to those detected in human urine, the other six (A4, A15, A16, A17, A18 and A21) were not detectable using the PI method in human urine. Four compounds (A6, A7, A8 and A12) that were detected in human could not be detected in the chimeric mouse urine (Table 3). Most of the detected metabolites could be assigned to a previously reported metabolite except for metabolites A19, A20 (also present in human) and A21 with precursor ion at m/z 315.

Full scan MS analysis of positive urine samples

The full scan spectra of every metabolite were studied in order to know the species predominantly formed in the ionization. In positive ionization mode, $[M+H]^+$ was the most abundant peak found for every compound due to the high conjugation of the keto group in position 3 for most of the metabolites. This was confirmed by the presence of adducts such as $[M+Na]^+$ or $[M+NH_4]^+$. The main exception for this behaviour was metabolite A12 (compound XII) where the most abundant ion was $[M+H-2H_2O]^+$. Several adducts such as $[M+NH_4]^+$ or $[M+NH_4-H_2O]^+$ were also present in the spectrum. These results were in agreement with a previous study about the ionization of anabolic steroids.[40] In negative ionization mode, only polyhydroxylated compounds exhibited signal as $[M+CH_3COO]^-$. This ion was also used for the confirmation of the molecular weight of each metabolite.

Product ion spectra of positive urine samples

The product ion spectra of every metabolite at different collision energies were acquired. The most abundant product ions are shown in Table 4.

At 20 V collision energy, most of the compounds exhibited several $[M + H - nH_2O]^+$ ions as main fragments. The number of molecules of water lost can be related to the number of oxygen atoms and the conjugation of the keto function.[39] Moreover, metabolites A6 and A8 were the only compounds where a loss of CH_2O was observed, indicating that no additional metabolites had the group – CH_2OH. Two metabolites (A7 and A9) exhibited a loss of 84 Da corresponding to a 1-ene-3-oxo structure.

At 30 V, most of the compounds exhibited the ion at m/z 121, which is common for 1,4-diene-3-oxo-anabolic steroids.[18,36,39,42] The absence of this ion in compounds A7, A9 and A12 confirmed that these compounds present a reduced A ring. The ions at m/z 147, 171 and 173 were also commonly observed, mainly for hydroxylated compounds or compounds with a double bond in position 6.

Based on the product ion spectra and the retention time, and after comparison with the data available in the literature, the identity of most of the metabolites was suggested (Table 4). Three of the metabolites found (A19, A20 and A21) could not be associated with previously reported metabolites. This illustrates the use of the approach for the detection of new steroid metabolites.

In order to propose a feasible structure for these three metabolites, the product ion spectra were studied. For A19, the occurrence of an odd electron ion (the product ion at m/z 266) indicated the presence of a highly conjugated system. This behaviour has also been reported for a new metabolite of methyltestosterone, 6-ene-epimethyltestosterone.[23] The presence of the ions 147, 171 and 173 also supported the structure 6-ene-epimethandienone. Comparison with the synthesized reference standard confirmed this assignment (Figure 4).

In the case of A20, a similar behaviour with A10 was observed. It can therefore be associated with an isomer. Since A10 is formed after the loss of a 17-sulphated metabolite,[12] another rearrangement from this specie as discussed in literature[38] can be proposed as mechanism for the occurrence of this compound. However, the comparison with the reference standard is necessary for the ultimate confirmation.

A21 can be related with an additional hydroxylation of A5, A6, A8 or A19. However, the product ion spectrum of A21 does not give additional information about the origin of this compound. Therefore, more research is needed in order to propose the structure of this metabolite.

A SRM method for the urinary detection of methandienone metabolites

A SRM method was developed for the target detection of the methandienone metabolites found. For this purpose, the most abundant transitions were selected for every compound (Table 1).

The selected precursor ion was $[M+H]^+$ for every metabolite except for metabolite A12 (epimetenediol) where two different precursor ions ($[M+H-2H_2O]^+$ and $[M+NH_4]^+$) were selected in order to increase the sensitivity of the secondary transition.

The two most abundant product ions were selected for each compound. In the case of A6, A8 and A19, product ions at m/z 269 and 266 were selected as shown in Table 1 in order to increase the specificity of the detection.

Application of the SRM method to human urine samples

The developed SRM method was applied to 10 positive human samples confirming the presence of all detected compounds (Figure 5). Most of the metabolites were present in all analysed samples. However, some of them (A8, A12, A15, A16, A18 and A21) were only detected in some of the samples (Table 3). That can be related to the excretion profile of each metabolite. Untimed samples were analysed (the time between the intake and the collection was unknown) so the concentration for some metabolites could be below the limit of detection of the method. The evaluation of excretion studies with samples collected during known periods can be helpful to corroborate this fact.

Some metabolites (A4, A15, A16, A17, A18 and A21) were not detected by the PI method in human urine samples but they were detectable by PI in chimeric mouse urine samples. The application of the sensitive SRM method allowed for the detection of these analytes in human urine (Figure 5), confirming that these compounds were also human metabolites of methandienone.

Application of the SRM method to mice urine samples

The application of the SRM method to the urine of three chimeric mice revealed that most of the compounds were detected

Table 4. Product ions obtained for every metabolite at two collision energies and proposed assignment

Analyte	Precursor ion (m/z)	Collision energy (V)	Product ions (m/z)	Assignment[a]
A1	301	20	283, 149, 121	I
		30	121	
A2	301	20	283, 149, 121	II
		30	121	
A3	317	20	299, 281, 225, 173, 147, 121	III
		30	147, 121	
A4	317	20	299, 281, 225, 173, 147, 121	IV[b]
		30	147, 135, 121	
A5	299	20	281, 225, 171	V[b]
		30	171, 147, 121	
A6	299	20	281, 269, 225, 173	VI
		30	173, 171, 147, 135, 121	
A7	303	20	285, 267, 219, 201	VII
		30	201, 145	
A8	299	20	281, 269, 225, 173	VIII
		30	173, 171, 147, 135, 121	
A9	285	20	267, 201	IX[b]
		30	201, 186, 145	
A10	283	20	173, 149	X
		30	121	
A11	–	–	–	XI
		–	–	
A12	269	20	105	XII
		30	105	
A13	–	–	–	XIII
		–	–	
A14	–	–	–	XIV
		–	–	
A15, A16, A17, A18	333	20	315,297,279,253,171	XV[b], XVI[b], XVII[b], XVIII[b]
		30	173, 171, 147, 121	
A19	299	20	281, 266, 225, 173	Unreported
		30	173, 171, 147, 121	
A20	283	20	173, 149	Unreported
		30	121	
A21	315	20	297,279,253,171	Unreported
		30	173, 171, 147, 145, 121, 119	

[a] For structures see Figure 1.
[b] Tentative assignment.

after methandienone administration (Figure 6). Therefore, the metabolic nature of the selected analytes was confirmed. Some metabolites (A8 and A12) were not detectable in every sample (Table 3). In the case of A8, this can be due to the low concentration expected for this metabolite. For A12, the low sensitivity and the higher ion suppression of this analyte in mouse urine can be the reason for this low occurrence (Table 2).

Some metabolites (A6, A7, A8 and A12) were not detected by using the PI method in chimeric mouse urine but they were detected after application of this method in human urine. The occurrence of these metabolites after application of the SRM method confirmed the metabolic nature of these compounds.

Proposed strategy for the detection of steroid metabolites

A general strategy can be defined for the detection of anabolic steroid metabolites by combination of both models (Figure 7). In a first step, the application of the PI method is necessary in order to provide information about possible metabolites. For some specific structures, other methods based on PI or neutral loss scan can also be useful.[18,21,22] In those cases where human urine is available it is advisable to start with this matrix due to the higher sensitivity reached (Table 2). Once potential metabolites are detected, the acquisition of the product ion spectrum facilitates the proposal of a feasible structure and the development of the SRM method. The application of the SRM method to chimeric mouse urine collected after the steroid administration is useful for the confirmation of the metabolic nature of the detected compounds.

However, human urine samples collected after steroid administration are not always available (mainly for ethical reasons). In these cases, the use of chimeric mouse is a suitable alternative for the investigation of steroid metabolism. The use of PI methods allows for the establishment of some potential metabolites. Afterwards, a SRM method can be developed by the study of the product ion spectra. This SRM method can be applied to routine

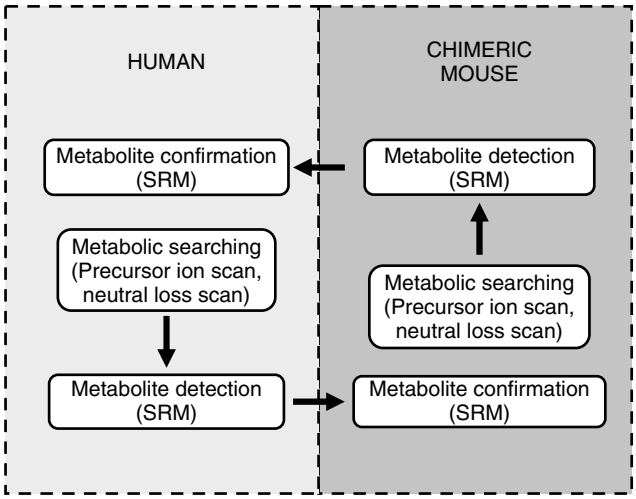


Figure 7. Proposed strategy for the detection of steroid metabolites and the confirmation of their metabolic nature using LC-MS/MS in different scan modes in combination with human and chimeric mouse urine.

human urine samples. Once a positive sample is found, it can be used for the study of possible additional metabolites.

Conclusions

The use of LC-MS/MS in different scan modes in combination with human and chimeric mouse urine is a powerful tool for the study of steroid metabolism. The results obtained by applying the SRM method revealed that both human and chimeric mouse exhibited similar target metabolites after methandienone administration. The application of the selected PI method allows for the detection of steroids without any specific structure – previous knowledge about the steroid metabolism is not necessary. Thus, the developed approach is potentially applicable to most of the anabolic steroids. The application of the PI method to both human and chimeric mouse after methandienone administration generated several differences mainly due to differences in sensitivity. Therefore, although the use of only one of the proposed models (human or chimeric mouse) provides valuable information about steroid metabolism, the combination of both models seems to be the best option for the comprehensive detection of steroid metabolites. A general strategy has been defined for the detection of anabolic steroid metabolites by combining both models.

The use of this approach allowed for the detection of more than 80% of the previously reported metabolites for methandienone. These results improve significantly those obtained with *in vitro* methodologies where only metabolites III and XIV were detected.[28] Other important metabolites were not found using this methodology, probably due to the lack of some cosubstrates. On the other hand, metabolites resulting from hydroxylation, A-ring metabolism, D-ring metabolism and B ring metabolism were detected using the approach with humans and chimeric mice. Only very minor metabolites or metabolites with a theoretic low ionization were not detected by this approach. Moreover, three previously unreported metabolites have been found. The structure of one of them (6-ene-epimethandienone) has been confirmed by the synthesis of the reference compound.

The described methodology is a promising tool for the detection of steroid metabolism. It can be helpful for the detection of previously unreported metabolites of known steroids. It can be used for the detection of anabolic steroid metabolites in those cases where human post-administration samples are not available – such as for designer steroids and prohormones. The main limitations of this tool are the low amount of urine available from chimeric mouse and the relatively high LODs obtained in this matrix. The administration of higher doses and/or the collection on subsequent days can minimize these drawbacks.

Acknowledgements

Postdoctoral grants by the Flemish Ministry of Culture, Youth, Sports and Media (PVE, KD) and Research Foundation – Flanders (FWO) (PM) are gratefully acknowledged. The technical assistance of Lieven Verhoye and Petra Premereur is also acknowledged. This study was supported financially by WADA, the Belgian State (IUAP P6/36-HEPRO), the Ghent University via the Special Research Fund (BOF; PhD Grant LL) and a Concerted Action Grant (01G00507), the German Federal Ministry of the Interior and the Manfred-Donike Institute for Doping Analysis e.V.

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