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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

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Boldione (1,4-androstadien-3,17-dione) is included in the list of prohibited substances, issued by the World Anti-Doping Agency (WADA). Endogenous production of low concentrations of boldione has also been reported. The objective of this study was to assess boldione metabolism in humans. Detection of boldione metabolites was accomplished by analysis by liquid chromatography coupled to tandem mass spectrometry of urine samples obtained after administration of the drug and subjected to different sample preparation procedures to analyze the different metabolic fractions (free, glucuronides, sulpfates and released in basic media). In addition to boldione, eight metabolites were detected in the free fraction. Four of them were identified by comparison with standards: 6β-hydroxy-boldenone (M3), androsta-1,4,6-triene-3,17-dione (M5), (5α)-1-androstenedione (M6) and (5α)-1-testosterone (M8). Metabolite M7 was identified as the 5β-isomer of 1-androstenedione, and metabolites M1, M2 and M4 were hydroxylated metabolites and tentative structures were proposed based on mass spectrometric data. After β-glucuronidase hydrolysis, five additional metabolites excreted only as conjugates with glucuronic acid were detected: boldenone, (5β)-1-testosterone (M9), and three metabolites resulting from reduction of the 3-keto group. Boldenone, epiboldenone, and hydroxylated metabolites of boldione, boldenone and 1-testosterone were detected as conjugates with sulfate. In addition, boldione and seven metabolites (boldenone, M2, M3, M4, M5, M7 and M9) increased their concentration in urine after treatment of the urine in alkaline conditions. In summary, 15 boldione metabolites were detected in all fractions. The longer detection time was observed for metabolite M4 after alkaline treatment of the urine, which was detected up to 5 days after boldione administration.

Keywords: boldione; anabolic agents; metabolism; LC-MS/MS

Introduction

The use of anabolic androgenic steroids (AAS) can increase muscular strength and lean body mass in athletes.[1] For this reason, they are included in list of prohibited substances yearly published by World Anti-Doping Agency (WADA).[2] AAS is the group of substances largely detected in the anti-doping control field.[3] Therefore, doping control laboratories have to develop analytical strategies suitable for the detection of their misuse. AAS are extensively metabolized in the human body and the parent drug, if present, is detected in urine at very low concentrations.[4–6] Thus, the urinary detection of AAS metabolites is normally used as marker for AAS misuse. In order to establish the best marker to be included in screening methods, metabolic studies are necessary.[7]

Metabolic studies of AAS were traditionally performed by gas chromatography coupled to mass spectrometry (GC-MS).[5,6,8] The occurrence of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) opened several alternatives for metabolic studies such as the use of precursor ion or neutral loss scan methods,[9–11] and the possibility of the direct detection of phase II metabolites.[12–15]

Boldione (1,4-androstadien-3,17-dione) is a prohormone marketed in preparations for human and animal consumption.[16–18] It is an orally active precursor of the AAS boldenone. For this reason, boldione misuse can be screened by detection of boldenone metabolites.[6] Boldione has also been found to be endogenous in humans at low concentrations.[19] In addition, boldione is released after alkaline treatment of the urine samples and its concentration is increased after testosterone administration, indicating that boldione results from a phase II metabolite of testosterone which is hydrolyzed in alkaline conditions.[20,21]

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This phase II metabolite has been recently identified as a conjugate with cysteine.

Limited data on boldione metabolism in humans has been published in the literature.[6,16,22] Using GC-MS, Uralets and Gillette[16] identified 5β-reduced metabolites excreted as glucuronoconjugates: 5β-androst-1-en-3α-ol-17-one as the major metabolite, (5β)-1-testosterone, 5β-androst-1-en-3α,17β-diol and (5β)-1-androstenedione. These metabolites were also previously identified as metabolites of boldenone.[23] Hydroxylated boldione, 5β-androst-1-en-6β-ol-3,17-dione and another unidentified metabolite appeared after solvolysis of the sulfate conjugates.[16] Boldenone was not detected in that study. However in a second study using LC-MS/MS, boldione, boldenone, and two minor hydroxylated metabolites, not fully characterized, were described after administration of a boldione tablet to a healthy volunteer.[22] All these metabolites were detected in urine up to 48 h after administration of the drug. Boldione was also identified as a minor metabolite of boldenone, obtained after treatment of the urine with potassium carbonate.[23]

The objective of this work was to study boldione metabolism by LC-MS/MS in order to take advantage of this technology for the detection and identification of new phase I and phase II metabolites that could improve the detection of boldione misuse. Methods for the detection of new metabolites have been developed and applied to samples from excretion studies. In this paper, detection of metabolites excreted free, conjugated with glucuronic or sulfate, together with the metabolites released after alkaline treatment of the urines is described. Additionally, the usefulness of these metabolites for doping control analysis will be evaluated by determining their retrospectivity after boldione administration. In a second part of the study,[24] the detection and characterization of metabolites of boldione conjugated with cysteine and *N*-acetylcysteine is described.

Experimental

Chemicals and reagents

1,4-adrostadien-3,17-dione (boldione), 5α-androst-1-en-3,17-dione ((5α)-1-androstenedione), 17β-hydroxy-5β-androst-1-en-3-one ((5β)-1-testosterone) and 17β-hydroxy-5α-androst-1-en-3-one ((5α)-1-testosterone) were obtained from NMI (Pymble, Australia). Androsta-1,4,6-triene-3,17-dione (ATD) was purchased from Steraloids, Inc. (Newport, RI, USA). 17β-hydroxy-1,4-androstadien-3-one (boldenone) and 17β-hydroxy-17α-methylandrost-4-en-3-one (methyltestosterone) were obtained from Sigma (Steinheim, Germany). 6β-hydroxy-boldenone was obtained from RIVM (Bilthoven, the Netherlands).

Tert-butyl methyl ether (TBME, HPLC grade), ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC-MS grade), potassium carbonate, sodium hydroxide, di-sodium hydrogen phosphate, sodium hydrogen phosphate, sodium chloride, ammonia hydroxyde, ammonium chloride, and potassium hydroxyde (all analytical grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (HPLC grade) was obtained from Fluka, Sigma-Aldrich (Steinheim, Germany). β-glucuronidase from *Escherichia coli* was obtained from Roche Diagnostics (Mannheim, Germany). Milli Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

Sample preparation

Four independent sample preparation procedures were applied to urine samples in order to evaluate the different metabolic fractions.

Free fraction

For the analysis of unconjugated fraction, after adding methyltestosterone (internal standard, ISTD) to urine samples (5 ml) at 100 ng mL^{-1} concentration, pH was adjusted by addition of 250 μl of 25% K_2CO_3 solution. Liquid-liquid extraction (LLE) was performed by adding 6 ml of TBME and shaking at 40 mpm for 20 min. After centrifugation (3000 g, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The extracts were reconstituted with 150 μL of a mixture of deionized water:acetonitrile (1:1, *v/v*) and aliquots of 10 μl were analyzed by LC-MS/MS.

Combined fraction (free and glucuronide conjugates)

Methyltestosterone (ISTD) at a final concentration of 100 ng mL^{-1} and 1 ml of sodium phosphate buffer (1 M, pH 7) were added to urine samples (5 ml). Enzymatic hydrolysis was performed by adding 30 μL of *β*-glucuronidase from *E. coli* and incubating the mixture at 55 °C for 1 h. After the sample reached room temperature, 250 μl of 25% K_2CO_3 solution were added and the mixture was extracted with 6 ml of TBME by shaking at 40 mpm for 20 min. After centrifugation (3000 g, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The extracts were reconstituted with 150 μl of a mixture of deionized water:acetonitrile (1:1, *v/v*) and aliquots of 10 μl were analyzed by LC-MS/MS.

Direct detection of sulfated metabolites

Sulfate conjugated metabolites were directly extracted from the urine samples using a liquid-liquid procedure described elsewhere.[25] Briefly, to 5 ml of urine sample methyltestosterone (ISTD) was added at 100 ng mL^{-1}, and it was alkalinized by addition of 100 μl of a buffer (5.3 M ammonium chloride solution adjusted to pH 9.5 with ammonia hydroxide solution). Then, sodium chloride (1 g) was added to promote salting-out effect and the samples were extracted with 8 ml of ethyl acetate by shaking at 40 mpm for 20 min. After centrifugation (3000 g, 5 min), the organic layers were evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 150 μl of a mixture of deionized water:acetonitrile (9:1, *v/v*) and aliquots of 10 μl were analyzed by LC-MS/MS.

Metabolites released after alkaline treatment

Labile conjugated metabolites hydrolysed in alkaline conditions were extracted as previously described.[21] After adding methyltestosterone (ISTD) at 100 ng mL^{-1}, urine samples (5 ml) were directly alkalinized by addition of 300 μL of potassium hydroxyde 6 M (without previous enzymatic hydrolysis). Incubation was carried out during 15 minutes at 60 °C. After cooling to room temperature, a liquid-liquid extraction was performed by addition of 6 ml TBME. After centrifugation (3000 g, 5 min), the organic layers were evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 150 μl of a mixture of deionized water:acetonitrile (1:1, *v/v*) and aliquots of 10 μl were analyzed by LC-MS/MS.

LC-MS/MS analysis

Chromatographic separations were carried out on a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA, USA) using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 55 °C. The mobile phase consisted of deionized water with 1 mM ammonium formate and 0.01% formic acid (solvent A) and methanol with 1 mM ammonium formate and 0.01% formic acid (solvent B). Different gradient elution and flow-rates were used depending on the experiment and they are described in the next paragraphs.

The LC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation, Milford, MA, USA) with an electrospray (Z-spray) ionization source. Source conditions were fixed as follows: lens voltage, 0.2 V; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1200 L/h. In electrospray ionization (ESI) positive mode the capillary voltage was set at 3 kV, whereas in negative ESI mode the capillary voltage was set at 2.5 kV. High-purity nitrogen was used as desolvation gas and argon was used as collision gas.

For the open detection of metabolites in the free and combined fractions and metabolites released after alkaline treatment, the following gradient pattern was used: from 0 to 1.5 min, 30% B; from 1.5 to 8 min, to 55% B; from 8 to 15 min, 55% B; from 15 to 29.5 min, to 95% B; during 1 min, 95% B; from 30.5 to 31 min, to 30% B; from 31 to 34 min, 30% B, at a flow rate of 0.2 ml min^{-1}. Data acquisition was performed in precursor ion scan mode (Method 1, Table 1).

For the untargeted detection of sulfate conjugated metabolites, the gradient elution was as follow: from 0 to 0.5 min, 15% B; from 0.5 to 9 min, to 50% B; from 9 to 14 min, 50% B; from 14 to 16.4 min, to 90% B; from 16.4 to 17 min, 90% B; from 17 to 17.5 min, to 15% B; from 17.5 to 20 min, 15% B at a flow rate of 0.2 ml min^{-1}. Data acquisition was performed in precursor ion scan of ion at *m/z* 97 (Method 2, Table 1).

For the targeted detection of metabolites, the following gradient pattern was used: from 0 to 0.6 min, 30% B; from 0.6 to 5 min, to 55% B; from 5 to 7 min, 55% B; from 7 to 11 min, to 95% B; from 11 to 12 min, 95% B; from 12 to 12.3 min, to 30% B; from 12.3 to 15 min, 15% B at a flow rate of 0.3 ml min^{-1}. Data acquisition was performed in selected reaction monitoring (SRM) mode (Table 2).

Table 1. Precursor ion scan methods for the untargeted detection of boldione metabolites (cone voltage, CV; collision energy, CE)

	Precursor ion (m/z)	ESI	CV (V)	CE (eV)	Mass range (*m/z*)
Method 1	77	+	25	50	250-400
	91	+	25	45	250-400
	105	+	25	45	250-400
Method 2	97	-	60	30	350-475

Excretion study samples

Urine samples obtained from an excretion study involving the administration of a single dose of 200 mg boldione to a healthy volunteer (male, Caucasian, 26 years, 65 kg) were obtained. The clinical protocol was approved by the local ethical committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). The urine samples were collected before administration (3 samples) and up to 15 days after administration at the following periods: 0–2 h, 2–3 h, 3–6 h, 6–8 h, 8–10 h, 10–12 h, 12–24 h; spot urines at 26 h, 30 h, 32 h, 36 h, 38 h, and 72 h; and daily spot morning urines from day 5 to day 15 after administration. Urine samples were stored at -20 ºC until analysis.

Results and discussion

Detection and characterization of metabolites excreted free and conjugated with glucuronic acid

Untargeted detection of boldione metabolites was accomplished by LC-MS/MS analysis of post-administration urine samples which were subjected to different sample preparation procedures to

Table 2. SRM method (cone voltage, CV; collision energy, CE) and retention times (RT) of the target detection of the boldione metabolites

Metabolite	RT (min)	ESI	Precursor ion (*m/z*)	Product ion (*m/z*)	CV (V)	CE (eV)
Boldione	6,1	+	285	121	30	25
Boldenone	6,7	+	287	121	30	25
M1	2,6	+	317	121	30	25
M2	3,4	+	303	121	30	25
6β-hydroxy-boldenone (M3)	3,7	+	303	121	30	25
M4	4,1	+	301	121	30	20
			283	97	30	25
ATD (M5)	5,9	+	283	97	30	25
(5α)-1-androstenedione (M6)	8,0	+	287	185	30	25
(5β)-1-androstenedione (M7)	8,2	+	287	185	30	25
(5α)-1-testosterone (M8)	8,8	+	289	187	30	25
(5β)-1-testosterone (M9)	8,9	+	289	187	30	25
M10,M11	9/9,3	+	271	81	30	25
M12	9,7	+	273	81	30	20
Boldenone sulfate	4,9	-	365	350	60	30
epiboldenone (M13) sulfate	5,4	-	365	350	60	30
M4 sulfate	2,6/3,1	-	379	97	60	30
M2,M3 sulfates	3,8/4,3	-	381	80	60	30
M14 sulfate	5,5	-	383	97	60	30

analyze the different metabolic fractions. Independent aliquots of urine were used to extract free fraction and combined fraction. Extraction of glucuronides can be done after extraction of free metabolites. However, small amounts of organic solvent remaining in the aqueous phase can hamper the glucuronidase activity. Therefore, separate aliquots were used to minimize this effect. A MS/MS method based on precursor ion scan of ions at *m/z* 77, 91, and 105 in positive mode were applied to detect metabolites excreted in the free fraction, in the combined fraction (free and glucuronide conjugates) and in the fraction of metabolites released after alkaline treatment. This method allows for the detection of compounds of steroidal nature.[9–11] After evaluating the information of all precursor ion scan methods, and contrasting the results between pre- and post-administration samples, different peaks were selected (Table 3). These peaks were considered as potential boldione metabolites.

Regarding the free fraction (Table 3), in addition to unchanged boldione, eight potential metabolites were detected. In positive ion mode, all of them were ionized by formation of the $[M+H]^+$ ion, indicating the presence of a conjugated 3-keto function.[9] Some of the metabolites were identified by comparison with standards: metabolite M3, 6β-hydroxy-boldenone; metabolite M5, ATD; metabolite M6, (5α)-1-androstenedione; and metabolite M8, (5α)-1-testosterone. For the rest of metabolites no standard was commercially available.

After analysis of the combined fraction, which includes compounds excreted in free form and compounds excreted as glucuronoconjugates, five additional metabolites not present in the free fraction were detected (Table 3). Thus, these additional metabolites are only excreted as conjugates with glucuronic acid. Two of the metabolites, boldenone and metabolite M9 ((5β)-1-testosterone) were identified by comparison of the analytical data with pure standards of the compounds. These two steroids, which have been reported as glucuronoconjugates in humans, are the commonly used markers for boldenone misuse detection.[24] Boldenone and metabolite M9 were ionized by formation of the $[M+H]^+$ ion. However, for metabolites M10, M11, and M12 the ion $[M+H\text{-}H_2O]^+$ was formed instead of the ion $[M+H]^+$, suggesting that the conjugated 3-keto function is not present in their structures[9] (Table 3). This fact was confirmed by the acquisition of the scan where other adducts like $[M+Na]^+$ and $[M+NH_4]^+$ supported the assignation of the observed ion as $[M+H\text{-}H_2O]^+$.

Table 3. Potential boldione metabolites detected using the precursor ion scan method in the free fraction (A), additional metabolites detected in the combined fraction (B), and metabolites which increase their concentration after alkaline treatment (C). Retention times (RT) were obtained with the gradient described for the open detection approach

Fraction	Metabolite	MM (Da)	$[M+H]^+$	RT (min)	Detection time
A	M1	316	317	4,5	24 h
	M2	302	303	5,9	10 h
	6β-hydroxy-boldenone (M3)	302	303	6,1	38 h
	M4	300	301	4,1	38 h
	ATD (M5)	282	283	9,3	38 h
	Boldione	284	285	9,6	38 h
	(5α)-1-androstenedione (M6)	286	287	12,5	72 h
	(5β)-1-androstenedione (M7)	286	287	12,8	38 h
	(5α)-1-testosterone (M8)	288	289	14,0	72 h
B	Boldenone	286	287	10,4	72 h
	(5β)-1-testosterone (M9)	288	289	14,7	72 h
	M10	288	271[a]	16,8	38 h
	M11	288	271[a]	18,1	72 h
	M12	290	273[a]	18,9	8 h
C	M2	302	303	5,9	72 h
	6β-hydroxy-boldenone (M3)	302	303	6,1	72 h
	M4	300	301	4,1	5 days
	ATD (M5)	282	283	9,3	72 h
	Boldione	284	285	9,6	-[b]
	Boldenone	286	287	10,4	72 h
	(5β)-1-androstenedione (M7)	286	287	12,8	72 h
	(5β)-1-testosterone (M9)	288	289	14,7	72 h

[a]For metabolites M10 to M12, ion $[M+H\text{-}H_2O]^+$ was formed instead of $[M+H]^+$.

[b]Boldione was detected in every sample after alkaline treatment.

Structures of some of the metabolites (boldenone, M3, M5, M6, M8, and M9) have been confirmed by comparison of the data obtained in excretion study samples with authentic materials of the metabolites. For metabolites not commercially available as standards, structures have been proposed based on LC-MS/MS analysis using product ion scan methods. The ions produced for each metabolite were compared with those obtained for the parent drug and the commercially available standards. For the ultimate confirmation of these structures, the use of pure standards is needed. The structures of the metabolites detected are depicted in Figure 1 and their collision induced dissociation (CID) mass spectra of $[M+H]^+$ ion of the metabolites are shown in Figures 2 and 3.

In Figure 2, CID mass spectra of the steroids available as standards are presented. Spectra of boldione, boldenone, and M3 are governed by a product ion at *m/z* 121, formed by cleavage of the B ring characteristic of 1,4-diene-3-keto structure.[26,27] CID mass spectra of ATD (M5) is dominated by the ion at *m/z* 147, characteristic of steroids with a 1,4,6-triene structure. On the other hand, metabolites with 1-ene-3-keto structure (M6, M8, and M9) showed a characteristic loss of 84 Da (*m/z* 203 for M6; *m/z* 205 for M8 and M9), and a loss of 102 Da corresponding to an additional loss of water (*m/z* 185 for M6, and *m/z* 187 for M8 and M9).[26,28,29]

Metabolite M2

The molecular mass (MM) of metabolite M2 is 302 Da, indicating a hydroxylation and a reduction when compared to boldione. The product ion spectra for M2 contained the same ions that the obtained for M3 (6β-hydroxy-boldenone) but with different abundances suggesting that they are isomers (Figure 3). Therefore, 6-hydroxylation combined with the reduction of the 17-keto group was found to be the most feasible metabolic pathway for obtaining M2 (Figure 1). Both α and β alternatives are possible for C6 and C17. Since M3 is a 6β,17β-metabolite, the stereochemistry for M2 might be 6α,17β, 6β,17α or 6α,17α.

Metabolite M4

The MM of metabolite M4 is 300 Da, indicating a hydroxylation with respect to boldione. In addition to $[M+H]^+$ ion, another ion at *m/z* 283 ($[M+H\text{-}H_2O]^+$ was observed in the MS spectra. The product ion spectrum of M4 (Figure 3) is very similar to that obtained for ATD (Figure 2) suggesting that ATD ($[M+H]^+ = 283$) is formed after a loss of water from M4. Therefore, C6 and C7 are the most feasible positions for the hydroxylation. 6-hydroxylation is one of the common metabolic biotransformations of compounds

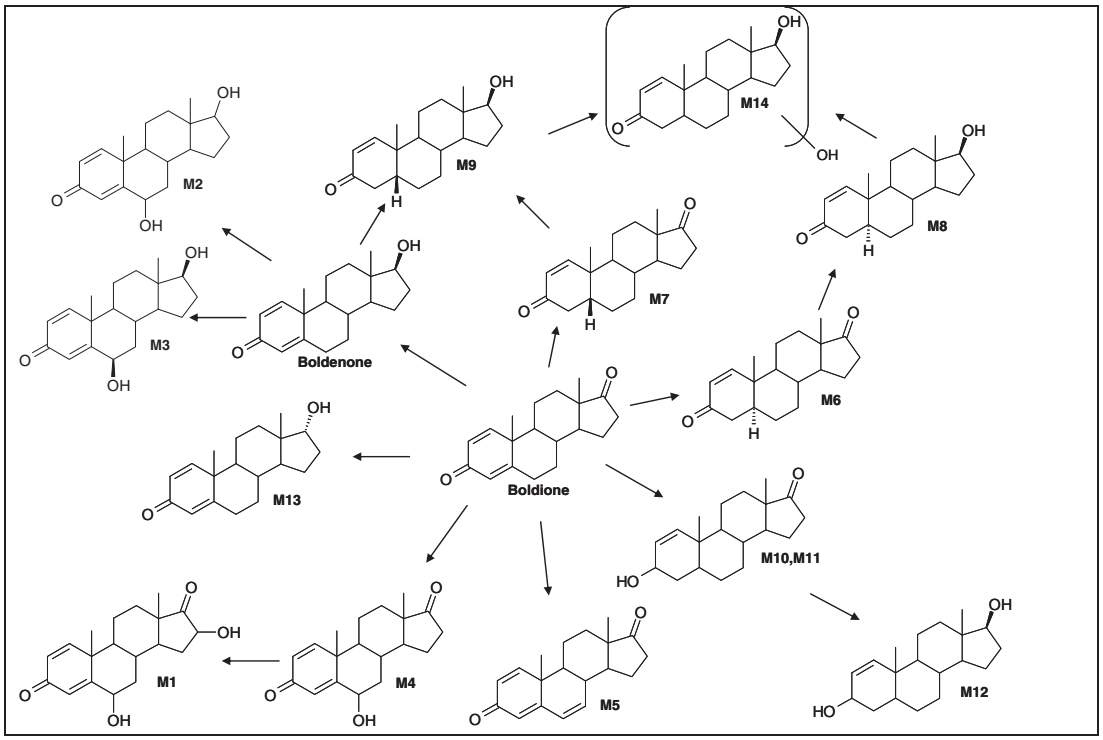

Figure 1. Proposed boldione phase I metabolic pathway.

of steroidal nature,[4] and androst-1,4-diene-6β-ol-3,17-dione was previously identified as boldenone metabolite.[23] Therefore, the most feasible structure for metabolite M4 is androst-1, 4-diene-6-ol-3,17-dione (Figure 1).

Metabolite M1

The MM of metabolite M1 is 316 Da, suggesting the addition of two hydroxyl groups. The position of the hydroxyl groups could not be proposed based on mass spectrometric data available (Figure 1). However, taking into consideration the common metabolic pathways for anabolic steroids,[4] hydroxylations in C6 and C16 are the most feasible possibilities. Therefore, androst-1,4-diene-6,16-diol-3,17-dione is the proposed structure for metabolite M1 (Figure 1).

Metabolites M7

Metabolite M7 has a product ion mass spectrum similar to M6, suggesting that they are isomers (Figures 2 and 3). M6 was identified as (5α)-1-androstenedione by comparison with a standard. Therefore, metabolite M7 is proposed to be (5β)-1-androstenedione. Metabolite M7 was also described by Uralets and Gillette[16] as boldione metabolite. Both metabolites were identified by Schanzer *et al.*[23] after alkaline treatment of the urines obtained after boldenone administration.

Metabolites M10, M11, and M12

As indicated above, for metabolites M10, M11 and M12 the ion $[M+H\text{-}H_2O]^+$ was formed and the ion $[M+H]^+$ was not present, indicating that the conjugated 3-keto function is not present in the structure of the metabolites. Metabolites M10 and M11 have an MM of 288 Da, indicating two reductions when compared to boldione. Identical product ion mass spectra were obtained for both compounds suggesting that they are isomers (Figure 3). Because the conjugated 3-keto function is not present, reductions in 3-keto group and Δ^4 are suggested (Figure 1).

M12 has an MM of 290 Da, indicating an additional reduction when compared to M10 and M11. Thus, an additional reduction of the 17-keto group is proposed (Figure 1).

Metabolites M10, M11, and M12 were described by other authors after administration of both boldione[16] and boldenone.[23]

Metabolites excreted as sulfo-conjugates

The direct extraction of sulfate conjugates was performed using a liquid-liquid procedure with ethyl acetate.[25] Free steroids are also extracted with ethyl acetate and, for this reason, a separate aliquot of urine was used to study sulfo-conjugates. A precursor ion scan method in negative mode of ion at *m/z* 97 was applied for the direct detection of metabolites conjugated with sulfate. Ion at *m/z* 97 is the hydrogensulfate anion, which is common to sulfates in negative mode.[12,30] After combining the information between pre- and post-administration samples, seven sulfated steroids were considered as potential boldione metabolites (Table 4). Boldenone sulfate was identified by comparison with a pure standard. Additionally, epiboldenone (M13) sulfate was identified as reported in a recent boldenone metabolism study conducted by our group.[12] In that study, boldenone and epiboldenone sulfates were described as minor metabolites of boldenone in humans.

Product ion scan spectra in negative mode of steroid sulfates are characterized by the presence of a single ion at *m/z* 97, no other ions that could help on structural elucidation are formed.[12] For this reason, based on the molecular masses of the peaks detected, only tentative structures could be proposed for the other five metabolites (Table 4). Thus, two hydroxylated metabolites of boldione (M4), two hydroxylated metabolites of boldenone (M2 and M3), and one hydroxylated metabolite of 1-testosterone (M14) are

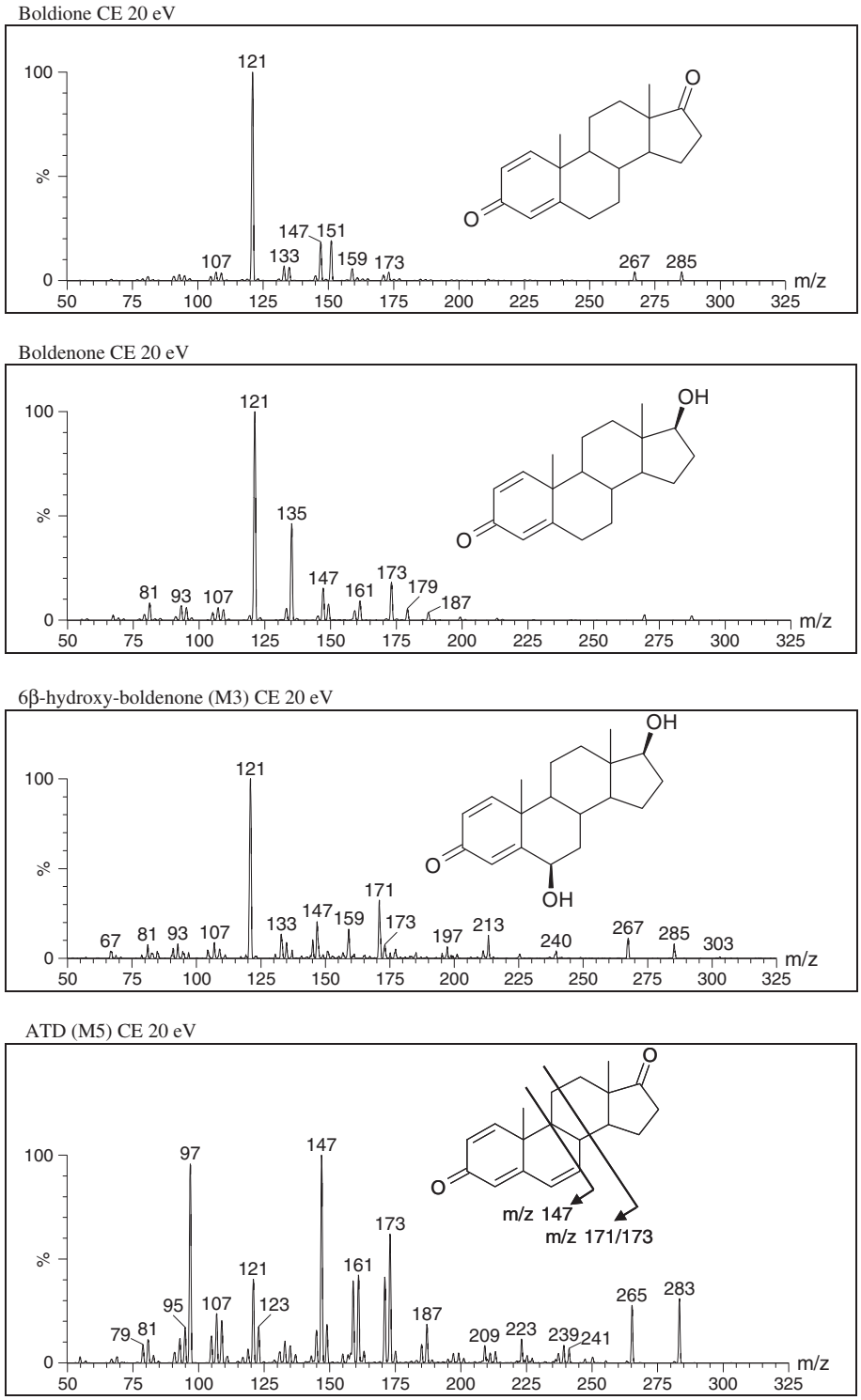

Figure 2. Product ion mass spectra of $[M+H]^+$ ion of boldione, boldenone, 6β-hydroxy-boldenone (M3), ATD (M5), (5α)-1-androstenedione (M6), (5α)-1-testosterone,(M8) and (5β)-1-testosterone (M9), available as standards.

excreted as sulfates. The presence of hydroxylated metabolites of boldione excreted in the sulfated fraction was also described by Uralets and Gillette.[16]

Metabolites released after alkaline treatment

It was seen that boldione, boldenone, and six of the metabolites detected in the free fraction (M2, M3, M4, M5, M7, M9) increased their concentration in urine after treatment of the urine in alkaline conditions (Table 3). It has been recently demonstrated that labile conjugates of testosterone metabolites released in alkaline conditions are conjugates with cysteine or *N*-acetylcysteine.[24] The formation of these conjugates implies the conjugation with glutathione of a phase I metabolite with a polyunsaturated carbonyl group, and subsequent transformation to cysteine or *N*-acetylcysteine conjugates in urine. According to the characterization

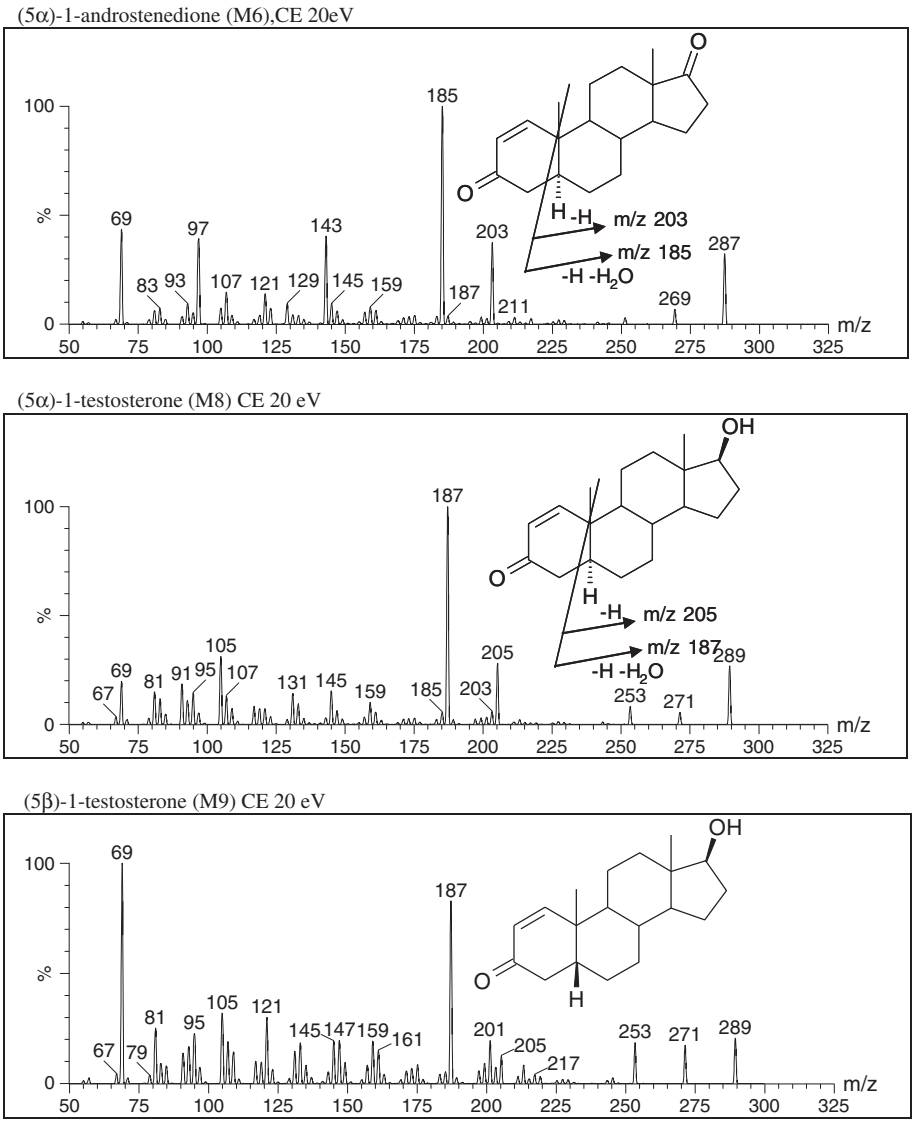

Figure 2. (Continued)

performed, metabolites M2, M3, M4, M5, M7, and M9 bear in their structure a polyunsaturated carbonyl group (Figure 1). Thus, they have an adequate structure for conjugation with glutathione. Therefore, our results suggest that boldione, boldenone and metabolites M2, M3, M4, M5, M7, and M9 besides being excreted by common metabolic pathways (unconjugated and/or conjugated with glucuronide or sulfate) are also excreted as conjugated to cysteine or *N*-acetylcysteine. Characterization of these conjugates is described in the second part of our study on boldione metabolism.[24]

Boldione phase I metabolism

In Figure 1, a representation of all phase I metabolites characterized for boldione in the present study is presented. These metabolites were detected in free form, and conjugated with glucuronic acid, sulfate, cysteine, and *N*-acetylcysteine.

The main metabolic biotransformations are: reduction in 17-keto group, in 3-keto group or in Δ^4; hydroxylations, in C6 and, probably, in C16; and 6,7-dehydrogenation. Reduction of the 17-ketone function results in boldenone and epiboldenone (M13). Epiboldenone was only detected as conjugated with sulfate. Boldenone is further hydroxylated to metabolites M2 and M3.

Reduction in Δ^4 of boldione results in the formation of 5α and 5β-isomers of 1-androstenedione ((5α)-1-androstenedione, M6, and (5β)-1-androstenedione, M7). Further reductions of the 17-keto group of M6 and M7 produce (5α)-1-testosterone (M8) and (5β)-1-testosterone (M9), respectively.

1-testosterone is also formed through the reduction of boldenone in Δ^4. However, according to data described in the literature for boldenone metabolism,[23] only the 5β-isomer (M9) is produced. Hydroxylation of 1-testosterone results in the formation of metabolite M14, only detected as a sulfate.

Reduction of boldione in Δ^4 and 3-keto forms M10, M11 and M12, only excreted as conjugates with glucuronic acid. Finally, 6,7-dehydrogenation results in the formation of ATD.

Boldione and some of the metabolites (boldenone, M2, M3, M4, M5, M7, and M9) are detected after alkaline treatment of the urine and therefore they can be considered as excreted conjugated with cysteine or *N*-acetylcysteine. These metabolic pathways are more detailed described in the second part of this work.[24]

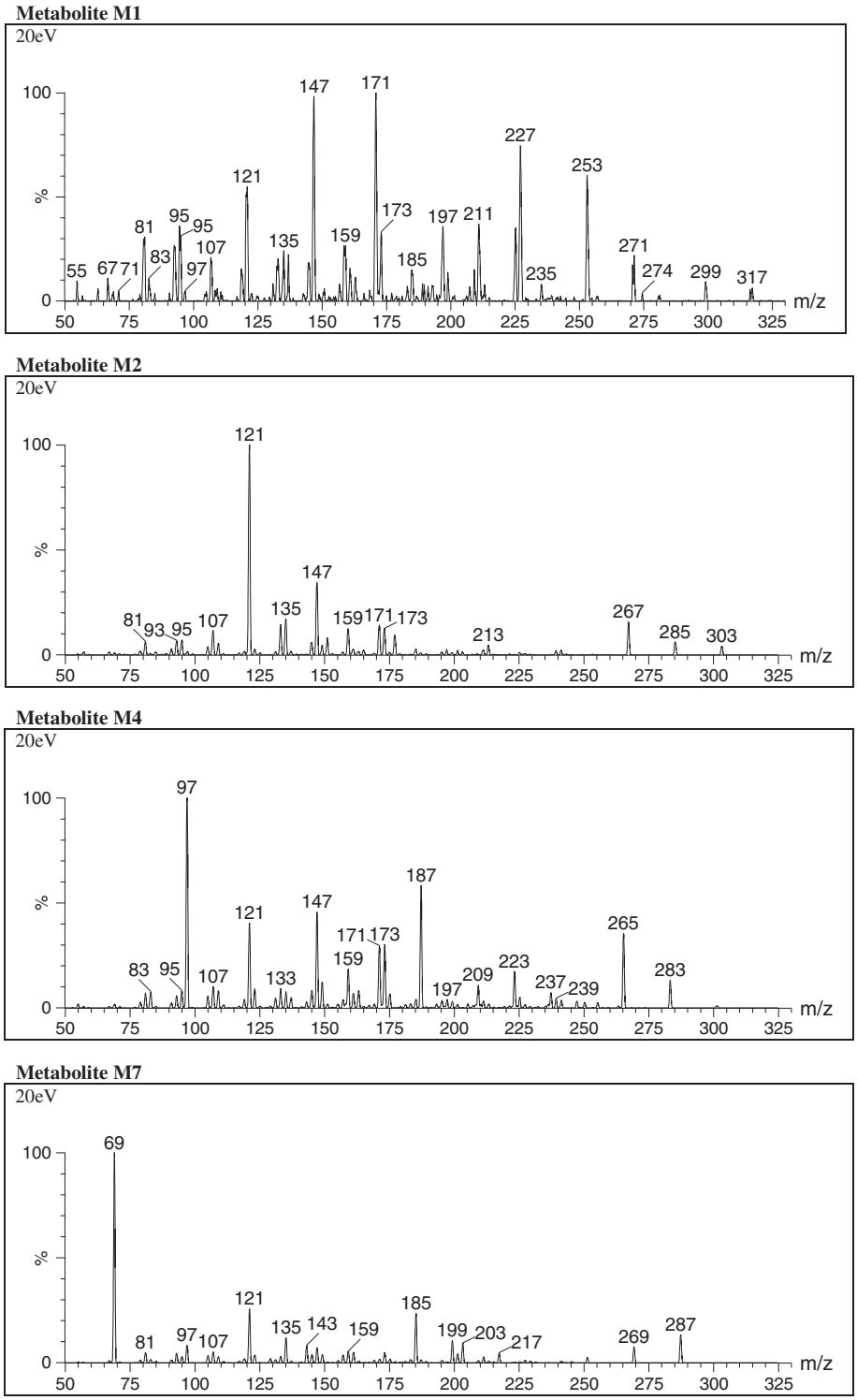

Figure 3. Product ion mass spectra of $[M+H]^+$ ion of M1, M2, M4 and M7, and product ion mass spectra of $[M+H\text{-}H_2O]^+$ ion of M10, M11 and M12.

Detection times

SRM method was developed to monitor all metabolites in urine samples (Table 2). The method was found to be selective for all the targeted analytes as no interferences were found in any of the three blank samples analyzed. Only boldione was found in every sample after alkaline treatment as previously described.[20,21] To evaluate the excretion profiles of the metabolites and their detection times, urine samples collected up to 15 days after the administration of an oral dose of boldione to a healthy volunteer were analyzed using this SRM method.

Detection times of each metabolite are listed in Tables 3 and 4. Detection times depend on the abundance of the metabolite and also on the signal intensity of each metabolite in the current

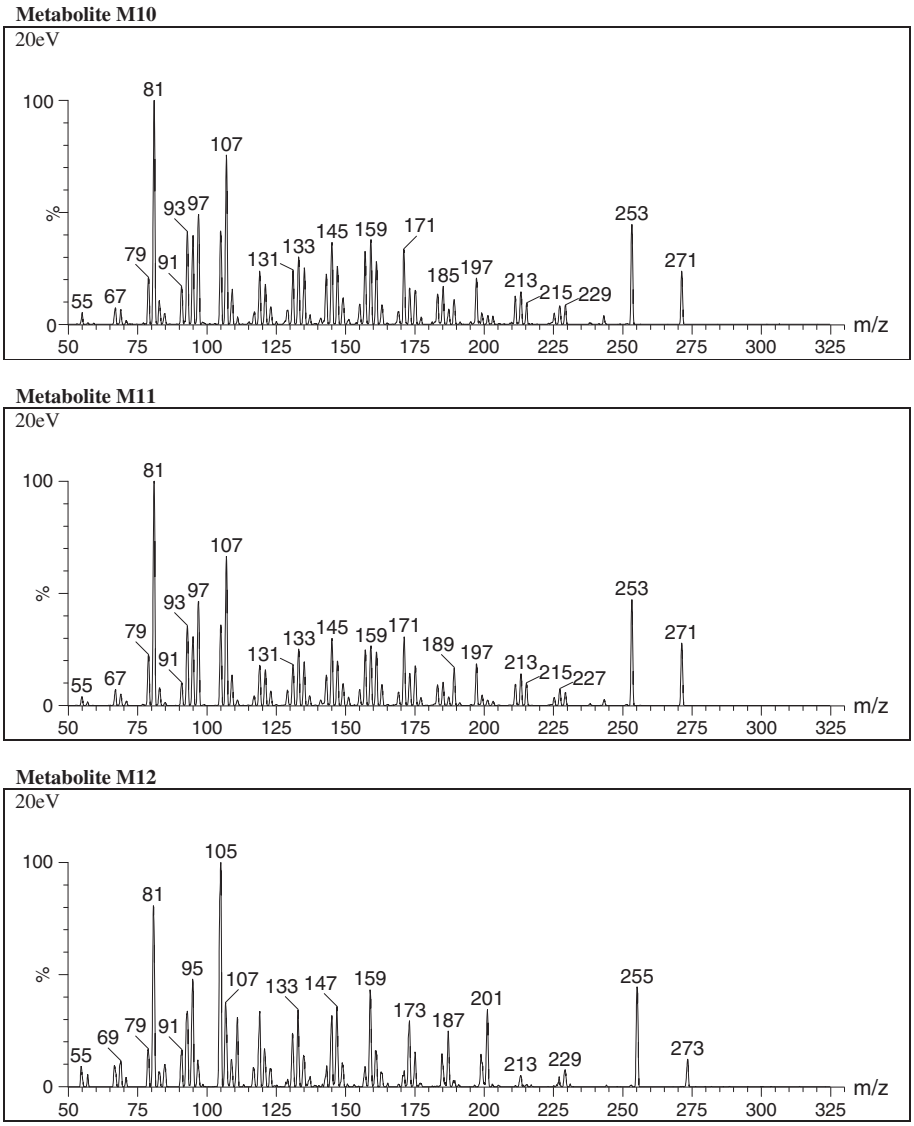

Figure 3. (Continued)

Table 4. Potential boldione metabolites excreted as sulfates. Retention times (RT) were obtained with the gradient described for the detection of sulfates

Metabolite	MM (Da)	$[M-H]^-$	RT (min)	Detection time
Boldenone sulfate	366	365	4,9	72 h
Epiboldenone (M13) sulfate	366	365	5,4	72 h
M4 sulfates	380	379	2,6	24 h
	380	379	3,1	26 h
M2, M3 sulfates	382	381	3,8	38 h
	382	381	4,3	72 h
M14 sulfate	384	383	5,5	36 h

analytical method. For these reasons, it may happen that some metabolites show lower detection times than the proposed precursor, as occurred for metabolites M1 and M4. Metabolite M1 is proposed to be produced from metabolite M4, however the detection time of metabolite M1 is substantially lower than for metabolite M4 (Table 3) due to its lower abundance and/or its poorer sensitivity.

Detection times commonly ranged from a few hours after administration (e.g. M2 excreted free and M12 obtained after hydrolysis with β-glucuronidase) to 72 h after administration (M6, M8, in the free fraction; boldenone, M9, and M11 after hydrolysis with β-glucuronidase; boldenone, epiboldenone and hydroxy-boldenone sulfates). In general, metabolites released after alkaline treatment presented longer retrospectivity. Among them M4 detected by this approach had the longest detection time and it could be detected up to 5 days (Table 3). The detectability of M4 after alkaline treatment was more than 3 fold longer than its detection as unconjugated. Therefore, metabolite M4 is mainly excreted as a conjugate with cysteine or *N*-acetylcysteine.[24] An example of the detection of metabolite M4 is given in Figure 4, chromatograms of the free fraction and the fraction obtained after alkaline treatment in pre-administration samples and

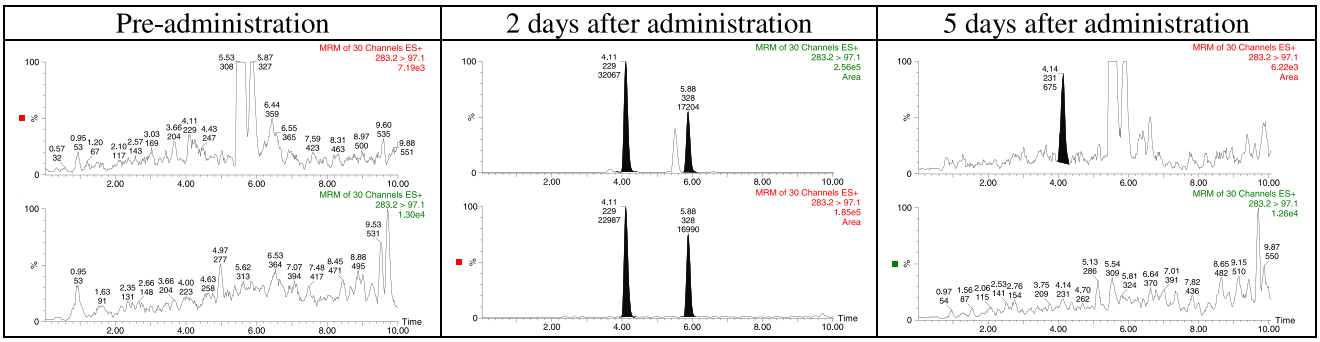

Figure 4. SRM chromatograms of the transition *m/z* 283 to *m/z* 97 characteristic of metabolites M4 (RT 4.1 min) and M5 (RT 4.9 min) in the free fraction (bottom) and in the alkaline fraction (top) in samples collected before administration and 2 and 5 days after boldione administration.

samples obtained 2 and 5 days after administration are presented. A peak for M4 is detected in the sample collected 5 days after administration treated in alkaline conditions.

Boldione is normally detected by monitoring the main boldenone metabolites (boldenone and metabolite M9) excreted in the glucuronoconjugated fraction.[23] In our excretion study, these metabolites were detected up to 72 h after administration. Therefore, the use of metabolite M4 detected after alkaline treatment of the sample can significantly increase the retrospectivity of the detection of boldione misuse.

Conclusions

Boldione metabolites have been studied in different metabolic fractions. In addition to boldione, eight metabolites were detected in the free fraction resulting from hydroxylations (metabolites M1 to M4), 6,7-dehydrogenation (metabolite M5), and reduction in Δ^4 (metabolites M6 and M7) and further reduction of the 17-keto group (metabolites M8 and M9). Five metabolites were detected only as glucuronoconjugates: boldenone and its main metabolite (metabolite M9) and three metabolites resulting from reduction in Δ^4 and 3-keto function (metabolites M10 to M12). Six of these metabolites were not previously described for boldione.

In addition, metabolites M2, M3, M4, M5, M7, and M9 increased their concentration after alkaline treatment of the urine suggesting that they are also excreted as conjugates with cysteine or N-acetylcysteine. These conjugates were not described before for AAS. Finally, seven metabolites were excreted as sulfate conjugates and only one was described previously. In summary, the use of LC-MS/MS has allowed the identification of new phase I and phase II metabolites of boldione. In the present excretion study, the longest restrospectivity was obtained for metabolite M4 excreted as conjugate with cysteine or *N*-acetylcysteine, which could be detected 5 days after a boldione administration.

The endogenous presence of boldione has been reported at very low concentrations.[19–21] Additionally, boldione is normally screened by the detection of boldenone and its main metabolite, M9,[6] and these compounds have been found also to be endogenous.[12,23] In our case, boldione was detected in all samples collected before administration only after alkaline treatment and none of the rest of the metabolites was detected in those samples. However, to select the most adequate marker for the detection of boldione misuse, endogenous production of these metabolites should be checked in a larger number of samples including those with endogenous concentrations of boldenone and its main metabolite.

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References

[1] F. Hartgens, H. Kuipers. Effects of androgenic-anabolic steroids in athletes. *Sport Med* **2004**, *34*, 513.
[2] WADA 2012 prohibited list. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2012/WADA_Prohibited_List_2012_EN.pdf June **2012**.
[3] WADA 2010 laboratory statistics. Available at: http://www.wada-ama.org/Documents/Resources/Statistics/Laboratory_Statistics/WADA_2010_Laboratory_Statistics_Report.pdf June **2012**.
[4] W. Schänzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **1996**, *42*, 1001.
[5] W. Schänzer, M Donike. Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal. Chim. Acta* **1993**, *275*, 23.
[6] P. van Eenoo, F.T. Delbeke. Metabolism and excretion of anabolic steroids in doping control - New steroids and new insights. *J. Steroid Biochem. Mol. Biol.* **2006**, *101*, 161.
[7] O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Recent developments in MS for small molecules: application to human doping control analysis. *Bioanalysis* **2012**, *4*, 197.
[8] B.G. Wolthers, G.P.B. Kraan. Clinical applications of gas chromatography and gas chromatography-mass spectrometry of steroids. *J. Chromatogr. A* **1999**, *843*, 247.
[9] O.J. Pozo, P. Van Eenoo, K. Deventer, F.T. Delbeke. Detection and characterization of anabolic steroids in doping analysis by LC-MS. *TrAC, Trends Anal. Chem.* **2008**, *27*, 657.
[10] O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke. Detection and characterization of a new metabolite of 17α-methyltestosterone. *Anal. Chem.* **2008**, *80*, 1709.
[11] O.J. Pozo, R. Ventura, N. Monfort, J. Segura, F.T. Delbeke. Evaluation of different scan methods for the urinary detection of corticosteroid metabolites by liquid chromatography tandem mass spectrometry. *J. Mass Spectrom.* **2009**, *44*, 929.
[12] C. Gomez, O.J. Pozo, H. Geyer, J. Marcos, M. Thevis, W. Schänzer, *et al.* New potential markers for the detection of boldenone misuse. *J. Steroid Biochem. Mol. Biol.* **2012**, *132*, 239.
[13] F. Badoud, E. Grata, J. Boccard, D. Guillarme, J.L. Veuthey, S. Rudaz, *et al.* Quantification of glucuronidated and sulfated steroids in human urine by ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* **2011**, *400*, 503.
[14] D.J. Borts, L.D. Bowers. Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry. *J. Mass Spectrom.* **2000**, *35*, 50.
[15] L. Hintikka, T. Kuuranne, A. Leinonen, M. Thevis, W. Schänzer, J. Halket, *et al.* Liquid chromatographic-mass spectrometric analysis

of glucuronide-conjugated anabolic steroid metabolites: method validation and interlaboratory comparison. *J. Mass Spectrom.* **2008**, *43*, 965.

[16] V.P. Uralets, P.A. Gilette, New anabolic steroids available as nutritional supplements: 5-alpha-androtan-3,17-diol, 1,4-androstandien-3,17-dione and 5-alpha-androst-1-en-17-ol-3-one, in *Recent advances in Doping analysis (10)* Proceedings of the 20th Cologne Workshop on Dope Analysis *Sport und Buch Strauss*, (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck). Cologne, Germany, **2002**, 73.

[17] M.A. Popot, S. Boyer, L. Menaut, P. García, Y. Bonnaire, D. Lesage. Boldenone, testosterone and 1,4-androstadiene-3,17-dione determination in faeces from horses, untreated and after administration of androsta-1,4-diene-3,17-dione (boldione). *Biomed. Chromatogr.* **2008**, *22*, 662.

[18] B. Destrez, E. Bichon, L. Rambaud, F. Courant, F. Monteau, G. Pinel, *et al*. Criteria to distinguish between natural situations and illegal use of boldenone, boldenone esters and boldione in cattle 2. Direct measurement of 17beta-boldenone sulpho-conjugate in calf urine by liquid chromatography–high resolution and tandem mass spectrometry. *Steroids* **2009**, *74*, 803.

[19] K. Verheyden, H. Noppe, L. Vanhaecke, K. Wille, J. Vanden Bussche, K. Bekaert, *et al*. Excretion of endogenous boldione in human urine: influence of phytosterol consumption. *J. Steroid Biochem. Mol. Biol.* **2009**, *117*, 8.

[20] O.J. Pozo, J. Marcos, R. Ventura, A. Fabregat, J. Segura. Testosterone metabolism revisited: discovery of new metabolites. *Anal Bional. Chem.* **2010**, *398*, 1759.

[21] A. Fabregat, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Alternative markers for the long-term detection of oral testosterone misuse. *Steroids* **2011**, *76*, 1367.

[22] Y. Kim, M. Jun, W. Lee. Characterization of boldione and its metabolites in human urine by liquid chromatography/electrospray ionization mass spectrometry and gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 9.

[23] W. Schänzer, M. Donike. Metabolism of boldenone in man: gas chromatographic/mass spectrometric identification of urinary excreted metabolites and determination of excretion rates. *Biol. Mass Spectrom.* **1992**, *21*, 3.

[24] O.J. Pozo, C. Gómez, J. Marcos, J. Segura, R. Ventura. Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part II: conjugates with cysteine and N-acetylcysteine. *Drug Test. Anal.* **2012**, *4*, 786.

[25] R. Ventura, M. Roig, N. Monfort, P. Sáez, R. Bergés, J. Segura. High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. *Eur. J. Mass Spectrom.* **2008**, *14*, 191.

[26] O.J. Pozo, P. Van Eenoo, K. Deventer, S. Grimalt, J.V. Sancho, F. Hernandez, *et al*. Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 4009.

[27] M. Thevis, H. Geyer, U. Mareck, W. Schänzer. Screening for unknown synthetic steroids in human urine by liquid chromatography-tandem mass spectrometry. *J. Mass Spectrom.* **2005**, *40*, 955.

[28] M. Thevis, U. Bommerich, G. Opfermann, W. Schänzer. Characterization of chemically modified steroids for doping control purposes by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2005**, *40*, 494.

[29] K. Verheyden, B. Le Bizec, D. Courtheyn, V. Mortier, M. Vandewiele, W. Gillis, *et al*. Mass spectrometric detection of and similarities between 1-androgens. *Anal. Chim. Acta* **2007**, *586*, 57.

[30] L. Yi, J. Dratter, C. Wang, J.A. Tunge, H. Desaire. Identification of sulfation sites of metabolites and prediction of the compounds' biological effects. *Anal. Bioanal. Chem.* **2006**, *386*, 666.