Detection and characterization of urinary metabolites of boldione. Part I: phase I metabolites excreted free and conjugated with glucuronic acid or sulphate and released after alkaline treatment of the urine.

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Abstract

Boldione (1,4-androstadiene-3,17-dione) is included in the list of prohibited substances, issued by the World Antidoping Agency, in the group of exogenous anabolic steroids. Endogenous production of low concentrations of boldione has also been reported. The objective of the 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. In addition to boldione, eight metabolites were detected in the free fraction. Three of them were identified by comparison with standards: 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 to 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. In addition, four metabolites (M3, M4, M5, M6) increased their concentration in urine after treatment of the urine in alkaline conditions. Boldenone, epiboldenone, and hydroxylated metabolites of boldione, boldenone and 1-testosterone were detected as conjugates with sulphate. 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.

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 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-androtadien-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 hydrolysed in alkaline conditions [20-21]. This phase II metabolite has been recently identified as a conjugate with cysteine [22].

Limited data on boldione metabolism in humans has been published in the literature [6,16,23]. 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-testosteronre, 5β-androst-1-en-3α,17β-diol and (5β)-1-androstenedione. These metabolites were also previously identified as metabolites of boldenone [24]. Hydroxylated boldione, 5β-androst-1-en-6β-ol-3,17-dione and another unidentified metabolite appeared after solvolysis of the sulphate 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 [23]. 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 [24].

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. The developed methods for the detection of new metabolites have been developed and applied to samples from excretion studies. In the present paper, detection of metabolites excreted free, conjugated with glucuronic acid, with sulphate and metabolites released after alkaline treatment of the urines is described. In a second part of the study [25], the detection and characterization of metabolites of boldione conjugated with cysteine and N-acetylcisteine is described.

Experimental

Chemicals and reagents

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

Tert-butyl methyl ether (TBME, HPLC grade), ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade), potassium carbonate, sulphuric acid, 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

Sample preparation procedures were applied to extract different metabolic fractions from the urine matrix.

1. *Free fraction*

For the analysis of unconjugated fraction, 100 ng mL^{-1} of methyltestosterone (internal standard, ISTD) was added to urine samples (5 mL) and pH was adjusted by addition of 250 µL of 25% K_2CO_3 solution. Liquid-liquid extraction 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 ºC. The extracts were reconstituted with 100 µL of a mixture of deionized water:acetonitrile (50:50, v/v) and aliquots of 10 µL were analyzed by LC-MS/MS.

2. *Combined fraction (free and glucuronide conjugates)*

100 ng mL^{-1} of methyltestosterone (ISTD) 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 55ºC for 1h. After the sample reached the ambient temperature, 250 µL of 5% K_2CO_3 solution was 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 ºC. The extracts were reconstituted with 100 µL of a mixture of deionized water:acetonitrile (50:50, v/v) and aliquots of 10 µL were analyzed by LC-MS/MS.

3. *Sulphated fraction*

Sulphate conjugated metabolites were directly extracted from the urine samples using a liquid-liquid procedure described elsewhere [26]. 100 ng mL^{-1} of methyltestosterone

(ISTD) was added to urine samples (5 mL) and pH 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 100 µL of a mixture of deionized water:acetonitrile (90:10, v/v) and aliquots of 10 µL were analyzed by LC-MS/MS.

4. *Metabolites released after alkaline treatment*

Labile conjugated metabolites hydrolysed in alkaline conditions were extracted in conditions previously described [21].100 ng mL^{-1} of methyltestosterone (ISTD) was added to urine samples (5 mL) and were directly alkalinized by addition of 300 µL of KOH 6M (no previous enzymatic hydrolysis was performed). Incubation was perfo rmed during 15 minutes at 60 ºC. 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 100 µL of a mixture of deionized water:acetonitrile (50:50, 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) 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 1mM ammonium formate and 0.01% formic acid (solvent A) and methanol with 1mM 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 next paragraphs. The sample volume injected was always 10 µL.

The LC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation, Milford, MA) 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, 1,200 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 (Method 1, Table 1).

For the untargeted detection of sulphate 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) (Table 2).

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 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: 0-2h, 2-3h, 3-6h, 8-10h, 10-12h, 12-24h; spot urines at 26h, 30h, 32h, 36h, 38h, and 72h; and daily spot morning urines from days 5 to 15 after administration. Urine samples were stored at -20 ºC until analysis.

Results and discussion

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

Detection of boldione metabolites was accomplished by analysis by LC-MS/MS of urine samples obtained after administration of the drug and subjected to different sample preparation procedures to analyze the different metabolic fractions. MS/MS methods based on precursor ion scan of ions m/z 77, 91 and 105 in positive mode were applied to detect metabolites excreted in free fraction, in the combined fraction (free and glucuronide conjugates) and in the fraction of metabolites released after alkaline treatment. Precursor ion scan of ions m/z 77, 91 and 105 allow for the detection of compounds of steroidal nature [9-11]. After combining the information of all precursor ion scan methods, and contrasting the results between post- and pre-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 M5, androsta-1,4,6-triene-3,17-dione; metabolite M6, (5α)-1-androstenedione; and metabolite M8, (5α)-1-testosterone. For the rest of metabolites no standard was available.

After analysis of the combined fraction, which includes compounds excreted in free form and compounds excreted as glucuronoconjugates, five additional metabolites not previously detected 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 metabolites are the main boldenone metabolites in humans and it has been described that they are mainly excreted as glucuronoconjugates [24]. Boldenone and metabolite M9 were ionized by formation of the $[M+H]^+$ ion. However, for metabolites M10, M11 and M12 the ion $[M+H-H_20]^+$ was formed instead of the ion $[M+H]^+$, indicating that the conjugated 3-keto function is not present in their structures [9] (Table 3).

Structures of some of the metabolites have been confirmed by comparison of the data obtained in excretion study samples with pure standards of the metabolites. For metabolites not 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 metabolites. For confirmation of these structures, the use of pure standards will be mandatory. The structures of the metabolites detected are depicted in Figure 1 and 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 metabolites available as standards are presented. Spectra of boldione and boldenone are governed by a product ion at m/z 121, formed by cleavage in the B ring and characteristic of 1,4-diene-3-keto structure [27,28]. CID mass spectra of androsta-1,4,6-triene-3,17-dione (M5) is dominated by the ion at m/z 147, characteristic of steroids with a double bond in position 6. 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), an additional loss of water (102Da, m/z 185 for M6, and m/z 187 for M8 and M9) [29,30].

Metabolites M2 and M3

The MM of metabolites M1 and M2 is 302 Da. This indicates for the M1 a double hydroxylation and for the M2 a reduction and subsequent hydroxylation , with respect to boldione. Identical product ion mass spectra were obtained for both compounds suggesting that they are isomers (Figure 3). The presence of ion at m/z 121 as the base peak, confirms the existence of a 1,4-diene-3-keto structure, as for boldione and boldenone. Therefore, the reduction has to occur in the 17-keto group to form a 17-hydroxy-metabolite.

The mass spectrometric data does not indicate the position of the additional hydroxyl group. However, taking into consideration the normal metabolic pathways for anabolic steroids [4], hydroxylation in C16 is one of the most feasible possibilities (Figure 1).

Metabolite M4

The MM of metabolite M4 is 300 Da, indicating an hydroxylation with respect to boldione. In addition to [M+H]+ ion, another ion at m/z 283 ($[M+H-H_2O]^+$ was observed in the MS spectra. In the CID spectrum of $[M+H]^+$ ion (Figure 3) , the product ion at m/z 121 is not the base peak, indicating that one of the hydroxyl groups is incorporated in the A or B rings. 6β-hydroxylation is one of the common metabolic transformations of compounds of steroidal nature [4], and a 6β-hydroxy-metabolite (androst-1,4-diene-6β-ol-3,17-dione) was identified as boldenone metabolite [24]. Therefore, the most feasible structure for metabolite M4 is androst-1,4-diene-6β-ol-3,17-dione (Figure 1).

Metabolite M1

The MM of metabolite M1 is 316 Da, suggesting the addition of two hydroxyl groups. As for metabolite M4, ion at m/z 121 is not the base ion of the MS/MS spectrum (Figure 3), indicating that one of the hydroxyl groups is incorporated in the A or B rings, probably in C6. The position of the second hydroxyl group could not be proposed based on mass spectrometric data available (Figure 1). However, as indicated for metabolites M2 and M3, taking into consideration the normal metabolic pathways for anabolic steroids [4], hydroxylation in C16 is one of the most feasible possibilities (Figure 1). Therefore, androst-1,4-diene-6β,16-diol-3,17-dione is the proposed structure for metabolite M1.

Metabolites M6 and M7

Metabolites M6 and M7 have a similar mass spectrum indicating 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 [14] as boldione metabolite. Both metabolites were identified by Schanzer et al [21] 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 $[M+H-H_20]^+$ 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 a MM of 288 Da, indicating two reductions with respect 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).

Metabolite M12 has a MM of 290 Da, indicating an additional reduction with respect to metabolites M10 and M11. Additional reduction of the 17-keto group is proposed (Figure 1).

Metabolites M10-M12 were described by other authors either after administration of boldione [16] and also after administration of boldenone [24].

2. Metabolites released after alkaline treatment

It was seen that four of the metabolites detected in the free fraction (M3, M4, M5, M6) 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 hydrolyzed in alkaline conditions are conjugates with cysteine or N-acetylcysteine [22]. 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 performed, metabolites M3, M4, M5 and M6 bear in their structure a polyunsaturated carbonyl group (Figure 1). So, they have the adequate structure for conjugation with glutathione. Therefore, according to our results, metabolites M3, M4, M5 and M6 are excreted in free form and also as conjugates with cysteine or N-acetylcisteine. Characterization of these conjugates is described in the second part of our study on boldione metabolism [25].

3. Metabolites excreted as conjugates with sulphate

For the detection of metabolites conjugated with sulphate, a precursor ion scan method of ion at m/z 97 in negative mode was applied. Ion at m/z 97 is the hydrogensulphate anion, and it is common to all steroid sulphates in negative mode [12,31]. After combining the information between pre and post-administration samples, seven sulphated metabolites were considered as potential boldione metabolites (Table 4). Boldenone sulphate was identified by comparison with a pure standard. Additionally, epiboldenone (M13) sulphate was identified using analytical data of a previous study of our group dealing with boldenone metabolism [12]. Boldenone and epiboldenone sulphates have been recently described as minor metabolites of boldenone in humans [12].

Product ion scan spectra in negative mode of steroid sulphates are characterized by the presence of only one ion at m/z 97, no other ions that could help on structural elucidation are formed [12]. For this reason, only tentative structures could be proposed for the other five metabolites, taking into consideration the molecular masses of the peaks detected, as indicated in Table 4. Thus, one hydroxylated metabolite of boldione (M4), epiboldenone (M13), two hydroxylated metabolites of boldenone (M2 and M3) and one hydroxylated metabolite of 1-testosterone (M14) are excreted as a sulphate conjugates. The formation of hydroxylated metabolites of boldione excreted in the sulphated fraction was also described by Uralets and Gillette [16].

4. Phase I metabolites of boldione

In Figure 1, a complete profile 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, with cysteime or N-acetylcisteine, or with sulphate.

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

Reduction in Δ^4 of boldione resuts 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 [24], only the 5β-isomer (M9) is produced. Hydroxylation of 1-testosterone results in the formation of metabolite M14, only excreted as conjugate with sulphate.

Reduction of boldione in Δ^4 and 3-keto forms metabolites M10, M11 and M12, only excreted as conjugates with glucuronic acid. Finally, 6,7-dehydrogenation results in the formation of androsta-1,4,6-triene-3,17-dione.

Some of the metabolites (M3,M4, M5 and M6) are excreted as conjugates with cysteine or N-acetylcysteine [25].

5. **Detection times**

An SRM method was developed to monitor all metabolites in urine samples (Table 2). The method was found to be selective for all the selected analytes as no interferences were found in any of three blank samples analyzed. Urine samples collected up to 15 days after the administration of an oral dose of boldione to a healthy volunteer were analyzed using the SRM method to evaluate the excretion profiles of the metabolites and their detection times.

Detection times of each metabolite are listed in Tables 3 and 4. Some of the metabolites were detected only for few hours after administration (M2, M12). Others were detected up to 72h after administration (M6, M8 in the free fraction; boldenone, M9 and M11 excreted as glucuronoconjugates; M3, M5 and M6, excreted as conjugates with cysteine or N-acetylcysteine; and boldenone, epiboldenone and hydroxy-boldenone sulphates), and the longest detection was obtained with metabolite M4. This metabolite was detected in the free fraction up to 38 h after administration, and in samples treated in alkaline conditions it was detected up to 5 days (Table 3). Therefore, metabolite M4 is mainly excreted as a conjugate with cysteine or N-acetylcysteine [25]. An example of the detection of metabolite M4 is given in Figure 4, where chromatograms of the free fraction and the fraction obtained after alkaline treatment in pre-administration samples and samples obtained 2 and 5 days after administration are presented. A peak of metabolite 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 [24]. In our excretion study, these metabolites were detected up to 72h after administration. Therefore, the use of metabolite M4 as a marker of boldione use can significantly increase the retrospectivity of the detection.

Conclusion

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 $\Delta^4$and 3-keto function (metabolites M10 to M12). From all these metabolites, six were not previously described for boldione.

In addition, metabolites M3 to M6 increased their concentration after alkaline treatment of the urine indicating that they are also excreted as conjugates with cysteine or N-acetylcysteine. These conjugates were not described before for boldione. Finally, seven metabolites were excreted as sulphate 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 our excretion study, the longest restrospectivity was obtained for metabolite M4 excreted as conjugate with cysteine or N-acetylcysteine , which could be detected after 5 days of boldione administration.

Acknowledgements

Financial support received from World Antidoping Agency (research grants 09A5DP and 09A20RV) is gratefully acknowledged.

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Tables

Table 1. Precursor ion scan methods.

	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

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

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	4,5	+	317	121	30	25
M2, M3	3,4/3,7	+	303	121	30	25
M4	4,1	+	301	121	30	20
			283	97	30	25
Androsta-1,4,6-triene-3,17-dione (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 sulphate	4,9	-	365	350	60	30
epiboldenone (M13) sulphate	5,4	-	365	350	60	30
M4 sulphate	2,6/3,1	-	379	97	60	30
M2,M3 sulphates	3,8/4,3	-	381	80	60	30
M14 sulphate	5,5	-	383	97	60	30

Table 3. Potential boldione metabolites detected 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 open detection of metabolites.

Fraction	Metabolite	MM (Da)	$[M+H]^+$	RT (min)	Detection time
A	M1	316	317	7,1	24h
	M2	302	303	5,9	10h
	M3	302	303	6,1	38h
	M4	300	301	4,1	38h
	Androsta-1,4,6-triene-3,17-dione (M5)	282	283	9,3	38h
	Boldione	284	285	9,6	38h
	(5α)-1-androstenedione (M6)	286	287	12,5	72h
	(5β)-1-androstenedione (M7)	286	287	12,8	38h
	(5α)-1-testosterone (M8)	288	289	14,0	72h
B	Boldenone	286	287	10,4	72h
	(5β)-1-testosterone (M9)	288	289	14,7	72h
	M10	288	271*	16,8	38h
	M11	288	271*	18,1	72h
	M12	290	273*	18,9	8h
C	M3	302	303	6,1	72h
	M4	300	301	4,1	Day 5
	M5	282	283	9,3	72h
	M6	286	287	12,5	72h

*For metabolites M10 to M12, ion $[M+H-H_2O]^+$ was formed instead of $[M+H]^+$

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

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

Figures

Figure 1. Proposed metabolic profile regarding phase I metabolites for boldione.

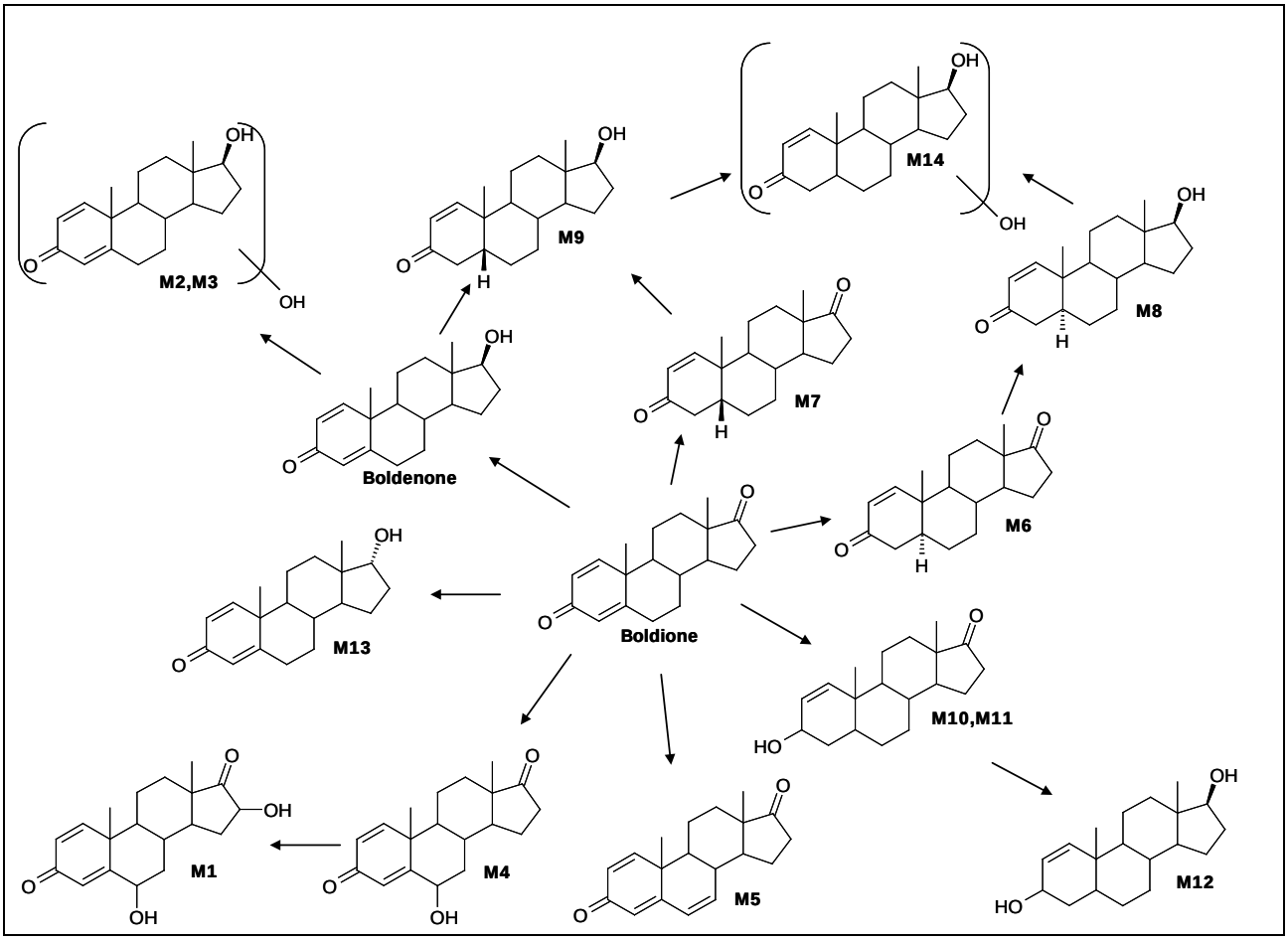
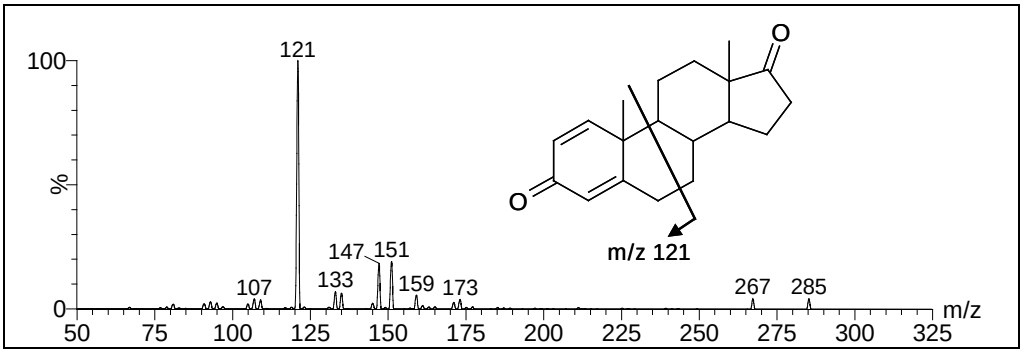
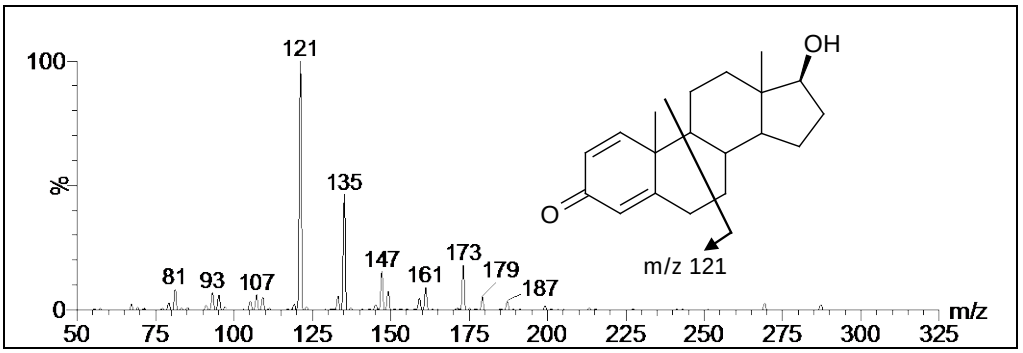


Figure 2. Product ion mass spectra of $[M+H]^+$ ion of boldione, boldenone, androsta-1,4,6-triene-3,17-dione (M5), (5α)-1-androstenedione (M6), (5α)-1-testosterone,(M8) and (5β)-1-testosterone (M9), available as standards..

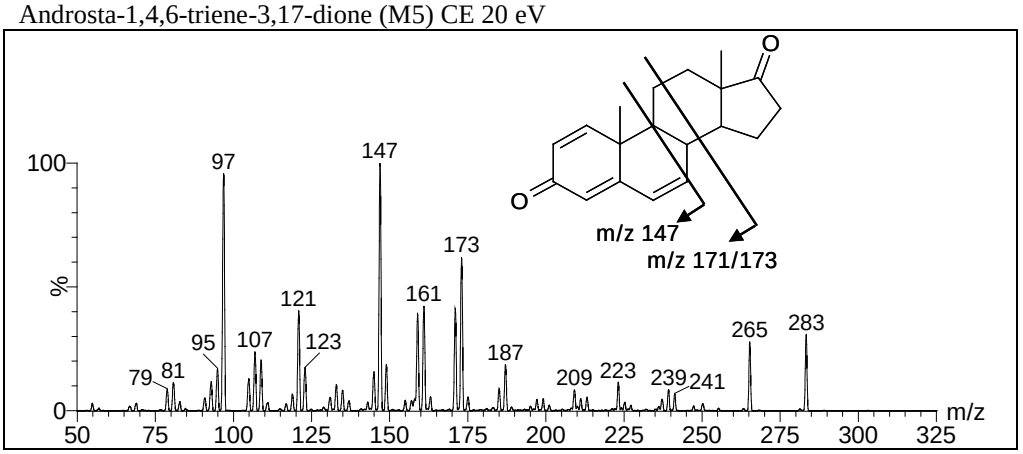
Boldione CE 20 eV



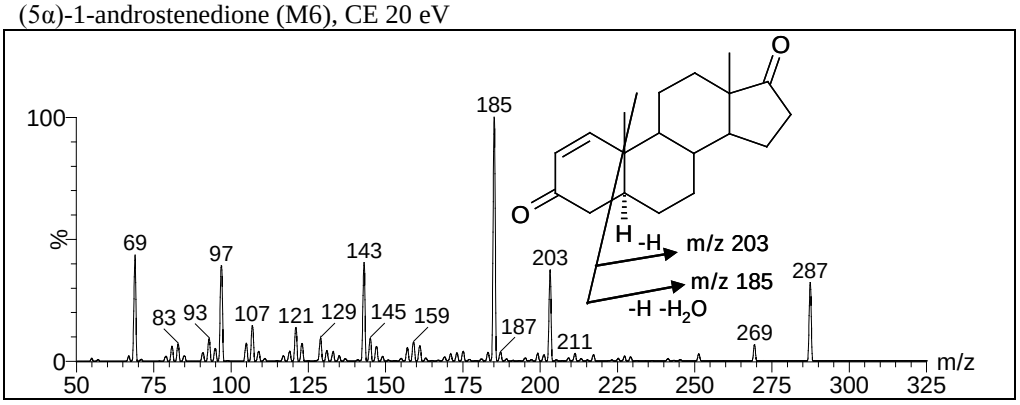
Boldenone CE 20 eV



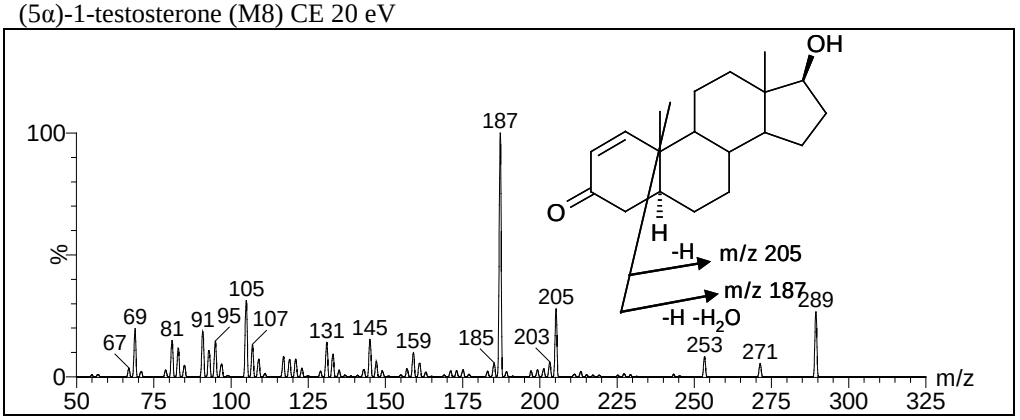
Androsta-1,4,6-triene-3,17-dione (M5) CE 20 eV



(5α)-1-androstenedione (M6), CE 20 eV



(5α)-1-testosterone (M8) CE 20 eV



(5β)-1-testosterone (M9) CE 20 eV

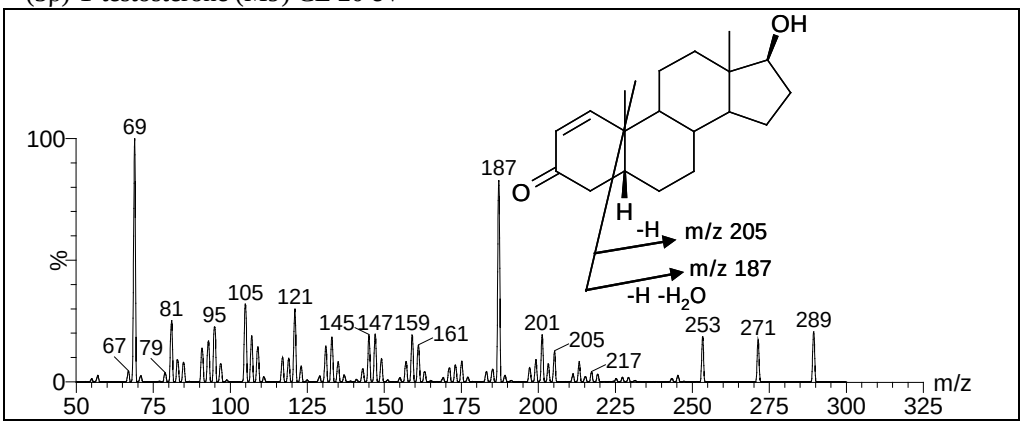
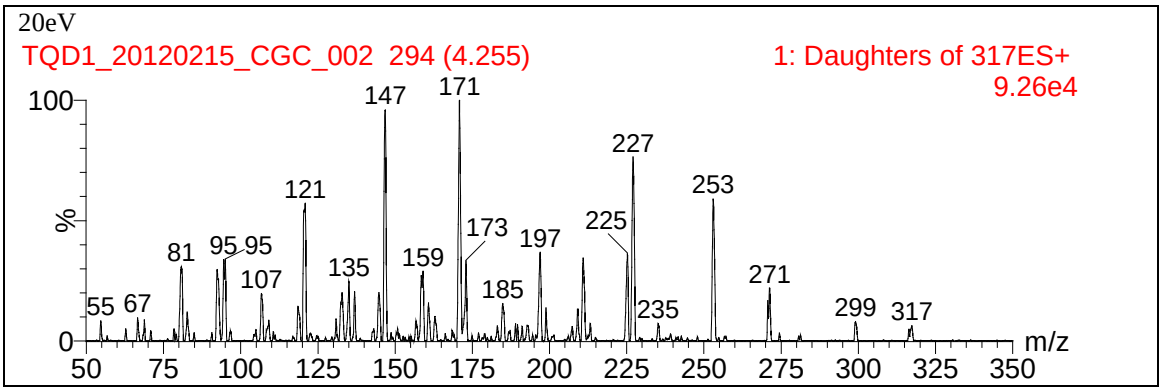
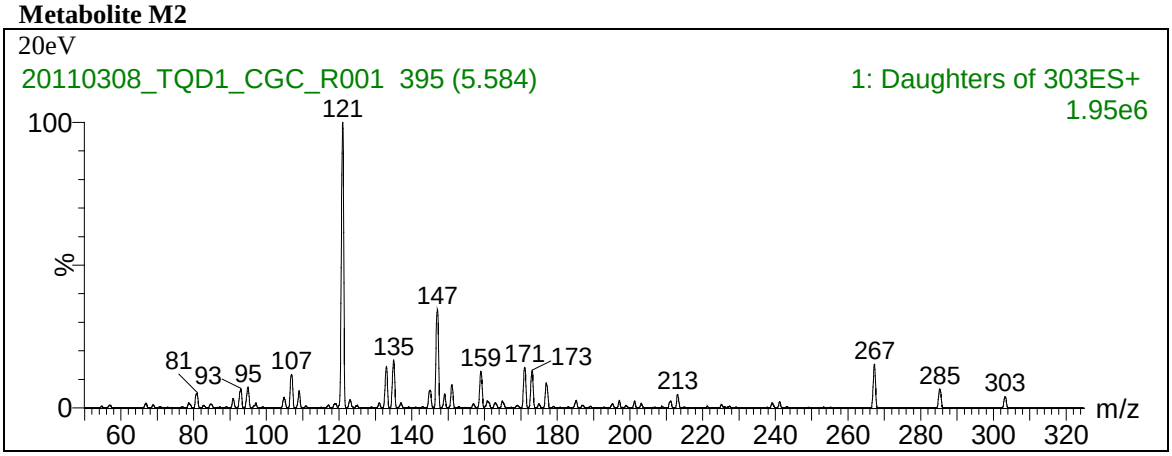


Figure 3. Product ion mass spectra of $[M+H]^+$ ion of metabolites M1, M2, M3, M4, M7, M10, M11 and M12.

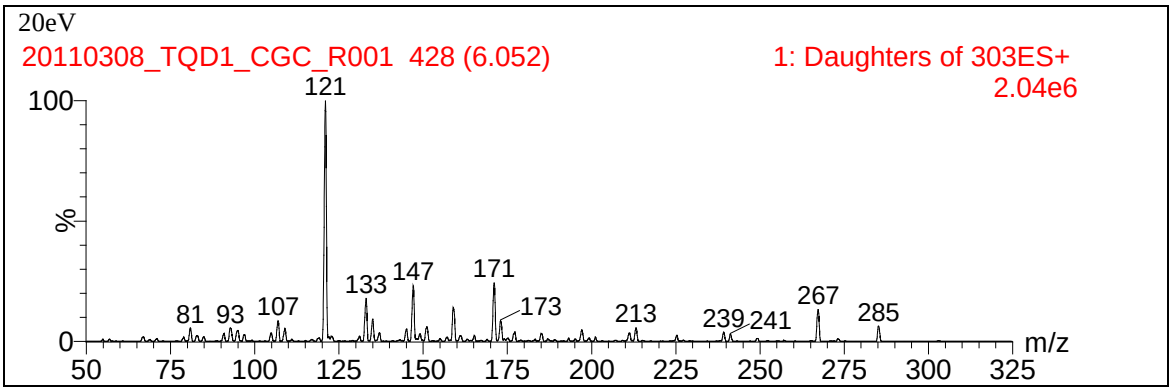
Metabolite M1



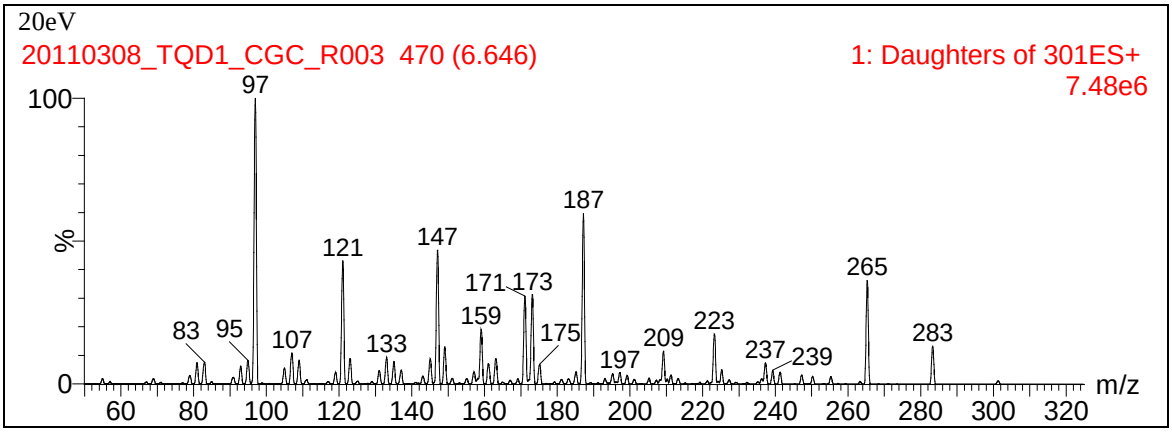
Metabolite M2



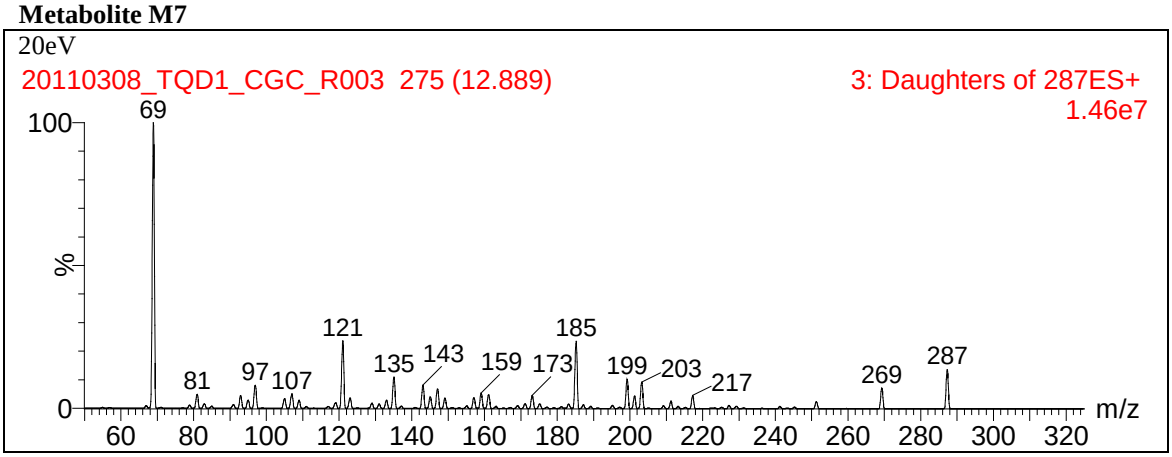
Metabolite M3



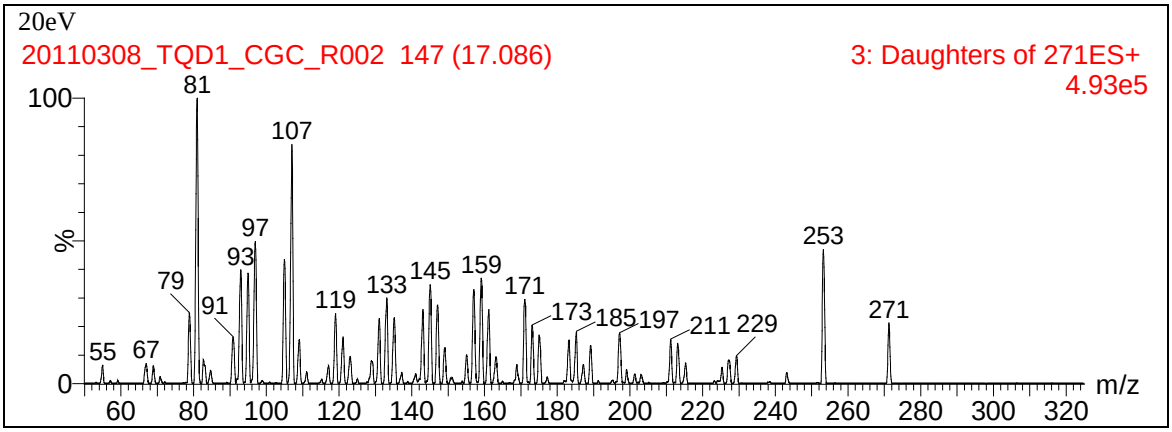
Metabolite M4



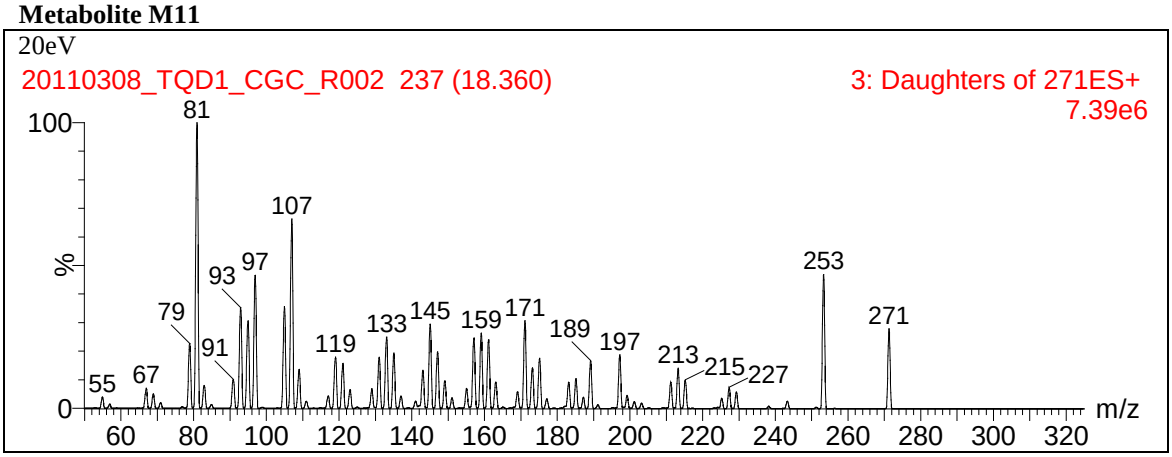
Metabolite M7



Metabolite M10



Metabolite M11



Metabolite M12

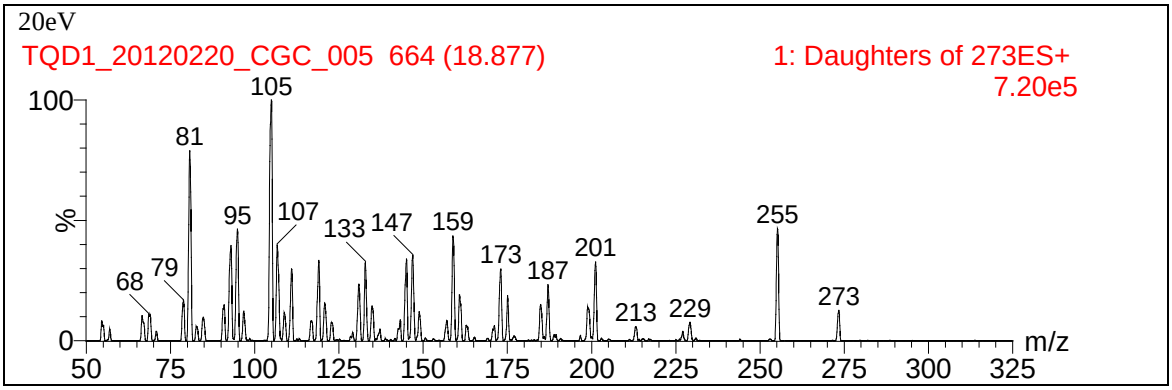


Figure 4. SRM Chromatograms of the transition m/z 283 to m/z 97 characteristic of metabolites M4 (RT 4.1 min) and M5 (RT 4.9 min) in the free fraction (bottom) and in the alkaline fraction (top) in samples collected before administration and 2 and 5 days after boldione administration.