Research article

Drug Testing and Analysis

Received: 29 June 2012 Revised: 14 September 2012 Accepted: 25 September 2012 Published online in Wiley Online Library: 23 October 2012

(www.drugtestinganalysis.com) DOI 10.1002/dta.1431

Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part II: Conjugates with cysteine and *N*-acetylcysteine

Oscar J. Pozo,[a]* Cristina Gómez,[a,b] Josep Marcos,[a,b] Jordi Segura[a,b] and Rosa Ventura[a,b]

The occurrence of boldione metabolites conjugated with cysteine and *N*-acetylcysteine in human urine was evaluated. Methods based on precursor ion scan of the protonated aminoacid (m/z 122 and m/z 164 for cysteine and *N*-acetylcysteine respectively) and neutral losses of the aminoacids (121 Da and 163 Da for cysteine and *N*-acetylcysteine respectively) were applied for the open detection of conjugates. Results for urine samples collected before and after boldione administration were compared. Using this approach, 24 metabolites (eleven conjugates with cysteine and thirteen conjugated with *N*-acetylcysteine) were detected. The metabolites were characterized by mass spectrometry and their potential structures were proposed based on this information. The structures of nine of these metabolites were confirmed by the synthesis of the conjugates. According to these results, a metabolic pathway for boldione involving this type of conjugation was presented. Up to our knowledge this is the first time that cysteine conjugates are presented for exogenous anabolic androgenic steroids and the first report of *N*-acetylcysteine conjugates for steroids. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: boldione; anabolic agents; metabolism; cysteine; *N*-acetylcysteine

Introduction

Anabolic androgenic steroids (AAS) are extensively metabolized[1–3] and studies on the metabolism are needed to elucidate the best markers for the detection of their misuse in sports. The best marker for the detection of a doping agent is not always the most abundant metabolite, but the metabolite excreted for the longest time after administration (so-called long-term metabolites) which offers the highest retrospectivity of the detection.[4]

Most of the metabolites used as markers of AAS misuse in the currently applied procedures in most anti-doping control laboratories have been identified using gas chromatography coupled to mass spectrometry (GC-MS).[1–3] However, GC-MS has some limitations for metabolic studies, being the need of derivatization of polar compounds and the need of hydrolysis of phase II metabolites the most important ones.

The occurrence of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) technology opened several alternatives for metabolic studies such as the use of precursor ion or neutral loss scan methods,[5–8] the greater sensitivity for some metabolites depending on the structure[9–11] and the possibility of the direct detection of phase II metabolites without a previous hydrolysis.[11–13]

Most important phase II metabolic reactions for AAS consist of conjugation with glucuronic or sulfuric acid.[1–3] Phase II metabolism is commonly studied using hydrolysis of the conjugates to phase I metabolites and identification of these phase I metabolites by GC-MS and/or LC-MS/MS. Hydrolysis with enzymes with β-glucuronidase activity has been mainly used for the study of metabolites conjugated with glucuronic acid,[1–3,14–18] whereas chemical hydrolysis is the most effective procedure to hydrolyze metabolites conjugated as sulfate.[16–19]

Recently, a new phase II metabolic pathway was reported for steroid hormones.[20] Cysteine (Cys) conjugates were found in human urine after testosterone administration. The occurrence of these metabolites was associated with the presence of an unreported three-steps metabolic biotransformation: 6,7-dehydrogenation as phase I metabolism followed by conjugation with glutathione and subsequent transformation in Cys conjugates. It was proven that both Cys and *N*-acetylcysteine (NAC) conjugates generated the polyunsaturated steroid after alkaline treatment. Therefore, they are behind the occurrence of recently reported testosterone metabolites appearing in urine after alkaline treatment.[21] The indirect detection of these metabolites has been found to be useful for the anti-doping control field since they can improve the detection of endogenous steroid misuse in some scenarios.[22,23]

Boldione (1,4-androstadien-3,17-dione) is a prohormone marketed as precursor of boldenone. During the last years, boldione metabolism was studied either by GC-MS and LC-MS/MS.[3,15,16] Recent studies on boldione metabolism in humans performed by our group[24] have shown that concentrations of some boldione metabolites increased after alkaline treatment of the samples, indicating the existence of labile phase II conjugates. Boldione and several of its

* *Correspondence to: Oscar J. Pozo, Bioanalysis and Analytical Services Research Group IMIM, Institut de Recerca Hospital del Mar, Doctor Aiguader, 88 08003 Barcelona, Spain. E-mail: opozo@imim.es*

a *Bioanalysis Research Group, IMIM, Hospital del Mar, Doctor Aiguader 88, 08003 Barcelona, Spain*

b *Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Doctor Aiguader 88, 08003 Barcelona, Spain*

metabolites[15,16,24] have a polyunsaturated carbonyl group in its structure and, therefore, they are potential candidates to be excreted as Cys or NAC conjugates. In fact, the presence of boldione in human urine collected after boldenone administration was reported after alkaline treatment of the sample.[14] These results indicated that additional phase II conjugates are produced for 1,4-androstadiene-3-keto steroids. Analogously to what have been found for testosterone, Cys conjugates and other conjugates formed in the mercapturic acid pathway are the main candidates to be the responsible for the occurrence of these metabolites.

The goal of this study was to evaluate the fraction of boldione metabolites conjugated with Cys and NAC. Several open screening methods based on precursor ion and neutral loss scans were applied for the detection of these conjugates. Metabolites were characterized using mass spectrometry and a potential structure was proposed. Synthesis of some of the metabolites was performed in order to ultimately confirm their structure.

Experimental

Chemical and reagents

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

Acetonitrile (ACN) and methanol (MeOH) (LC gradient grade), formic acid and ammonium formate (LC-MS grade), *tert*-butylmethyl ether (TBME), potassium hydroxide (analytical grade), hydrochloric acid fuming 37%, and cysteine (Cys) were purchased from Merck (Darmstadt, Germany). *N*-acetylcysteine (NAC) and sodium borohydride ($NaBH_4$) were obtained from Sigma-Aldrich (Steinheim, Germany).Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). The Sep-Pak® Vac RC (500 mg) C18 cartridges were purchased from Waters (Milford, MA, USA).

LC-MS/MS conditions

For the detection and characterization of the compounds, methods of LC-MS/MS were applied. The analyses were carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to a UPLC system, Acquity (Waters Associates, Milford, MA, USA) for the chromatographic separation. Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A capillary voltage of 3.0 kV was used in both positive and negative ionization mode. Cone voltages of 25 V and 20 V were selected for positive and negative ionization mode, respectively. The nitrogen desolvation temperature was set to 450 °C and the source temperature to 120 °C. The LC separation was performed using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm). The column temperature was set to 55 °C. Water and MeOH both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. Gradient elution and flow-rates used are described in each section.

Urine treatment

For the direct detection of the Cys and NAC conjugated steroids, urine samples (5 ml) were passed through a C18 column, previously conditioned with 2 ml of MeOH and 2 ml of H_2O. The column was then washed with 2 ml of H_2O and, finally, the analytes were eluted with 2 ml of MeOH. The methanolic extracts were evaporated under nitrogen stream in a bath at 50 °C. Finally, the residue was dissolved in 150 μl of a mixture of H_2O:ACN (9:1,*v/v*) and a volume of 10 μl was directly injected into the system.

Open screening method for the detection of cysteine and N-acetylcysteine conjugates

Open screening methods based on neutral loss and precursor ion scan modes (Table 1) were applied to samples collected before and after boldione administration. A gradient program was used at a flow-rate of 300 μl/min; the percentage of organic solvent was linearly changed as follows: 0 min, 10%; 1 min, 10%; 20 min, 60%; 21 min, 90%; 22 min, 90%; 22.5 min, 10%; 25 min, 10%.

General procedure for the synthesis of cystein-*S*-yl- and *N*-acetylcystein-*S*-yl-adducts of the steroids

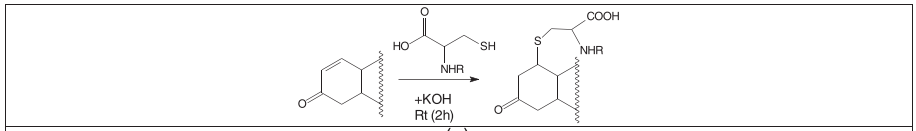
The general scheme of the synthesis is shown in Figure 1a. Each steroid (1 equiv) is dissolved in 3 volumes of MeOH and is added with 1 volume of cysteine or *N*-acetylcysteine (100 equiv) dissolved in 6 M KOH. The mixture is left under vigorous stirring for 2 h. After that, the organic solvent is removed under vacuum at 27 °C and the remaining aqueous solution is neutralized with 6 M HCl, filtered and purified.

General procedure for the reduction of cystein-*S*-yl- and *N*-acetylcystein-*S*-yl-adducts of the steroids

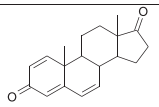
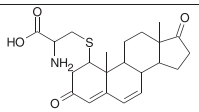
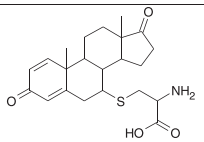
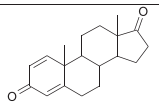
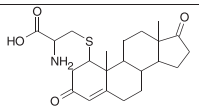
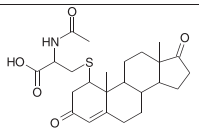
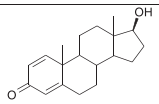
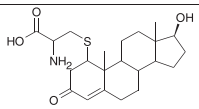
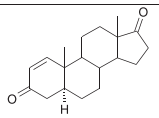
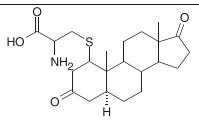
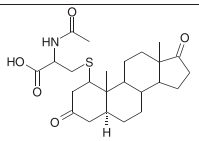
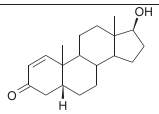
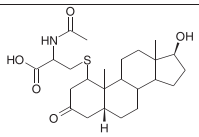
Reduction was performed by adding 100 mg of $NaBH_4$ to a solution of standard in 6 ml of methanol and 1 ml of water. After the mixture had been stirred at room temperature for 5 h,

Table 1. Neutral loss and precursor ion scan methods applied for the open screening detection of cysteine and *N*-acetylcysteine conjugates

Mode	Cone (V)	Selected loss (Da)	Selected product ion (m/z)	Collision energy (eV)	Conjugate
Neutral loss	20	121	-	20	Cysteine
	20	163	-	20	*N*-acetylcysteine
Precursor ion scan	20	-	122	10	Cysteine
	20	-	164	10	*N*-acetylcysteine



(a)

AAS	Structure	R	Metabolite	Metabolite code
ATD		H		Cys 1
		H		Cys 2
Boldione		H		Cys3
		$COCH_3$		NAC 1/ NAC 12
Boldenone		H		Cys 5
1-AD		H		Cys 11
		$COCH_3$		NAC 2
Bmet		$COCH_3$		NAC5

(b)

Figure 1. (a) General scheme for the synthesis of the conjugates. R = H, cysteine; R = $COCH_3$, *N*-acetylcysteine (b) Structures of the chemicals and the metabolites synthesized in this study.

a few drops of acetic acid were added and it was evaporated to dryness.

Mass spectrometry characterization of synthesized compounds

For the characterization of the compounds conjugated with Cys and NAC, synthesized conjugates were dissolved in a mixture of H_2O:ACN (9:1,*v/v*) and 10 µl were injected into the system. Full-scan methods acquired in both positive and negative ionization modes were applied. The *m/z* range was selected from 200 to 500 in order to acquire the potential adducts and in-source fragments. After that, the collision-induced dissociation behaviour of the target analytes was evaluated at different collision energies (from 10 to 40 eV). A gradient program was used at a flow-rate of 300 µl/min; the percentage of organic solvent was linearly changed as follows: 0 min, 10%; 1 min, 10%; 20 min, 60%; 21 min, 90%; 22 min, 90%; 22.5 min, 10%; 25 min, 10%.

Confirmation of the identity of the metabolites

Comparison between the analytes and the reference materials is necessary in order to unequivocally confirm the structure of the target compound. For this purpose, several transitions were selected for every target analyte in the SRM method (Table 2). The ratios between the different transitions were evaluated for each target compound. Finally, the ion ratios obtained for the target compound in urine and the corresponding reference standard were compared.

Samples

Urine samples obtained after administration of 200 mg of boldione to one healthy volunteer (male, Caucasian, 26 years old, 65 Kg) were studied. The clinical protocol was approved by the Local Ethical committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). Samples collected before and 3 h after boldione administration were used in this study.

Table 2. SRM method used for the confirmation of the presence of synthesized compounds

Metabolite code	Cone Voltage (V)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
Cys1/Cys2	20	404	97	30
			147	30
			283	20
Cys3	20	406	121	30
			147	20
			285	10
Cys5	20	408	121	30
			135	20
			287	20
Cys11	20	408	122	20
			185	30
			287	20
NAC1/ NAC12	20	448	121	30
			147	20
			285	10
NAC2	20	450	69	30
			121	30
			122	30
			164	10
			185	30
			203	30
			287	10
NAC5	20	452	122	30
			164	20
			187	30
			289	20

Results and discussion

Open detection of cysteine and *N*-acetylcysteine conjugates

Cys and NAC conjugates share a common behaviour in CID in positive ionization mode. At low collision energy they can loss either the neutral aminoacid moiety generating the $[M+H]^+$ of the free steroid or the neutral steroid producing the $[M+H]^+$ of the aminoacid.[20] Therefore, both precursor ion scan and neutral loss scan approaches are theoretically applicable for the open detection of Cys and NAC. Potential metabolites were selected by comparing the chromatograms acquired for samples collected before and after boldione administration.

Based on this common MS behaviour of Cys conjugates, two different analytical approaches were developed for the open detection of Cys conjugates (Table 1): (1) a neutral loss method of 121 Da (neutral loss of Cys) and (2) a precursor ion scan method for *m/z* 122 ($[M+H]^+$ for Cys). The acquisition of the neutral loss of 121 Da allowed for the detection of seven peaks (Cys1, Cys2, Cys3, Cys4, Cys5, Cys8 and Cys9) which were not present in the pre-administration sample (Figures 2a and 2e). These peaks were selected as potential boldione metabolites conjugated with Cys. The application of the second approach (precursor ion scan of *m/z* 122) allowed for the detection of eleven peaks (Cys3, Cys4, Cys5, Cys6, Cys7, Cys8, Cys9, NAC2, NAC3, NAC4, NAC5 and the peak marked as asterisk in Figure 2f) which were not present in the blank sample 122 (Figures 2b and 2f). Among them, and based on the *m/z* of the precursor, six metabolites could be associated with Cys metabolites whereas four metabolites (NAC2, NAC3, NAC4, and NAC5) could be assigned to NAC conjugates. One peak (marked with an asterisk in Figure 2f) corresponding to boldenone-glucuronide was also observed by the use of the precursor ion scan method.

In order to confirm the presence of less abundant isobaric metabolites, extracted ion chromatograms (XIC) for the detected $[M+H]^+$ were evaluated. One additional metabolite (Cys10) eluting at 15.5 min was observed when extracting the m/z 410 (Table 3). Overall, ten metabolites conjugated with Cys were detected by both developed methods.

Similarly to the detection of Cys conjugates, two analytical approaches for the detection of NAC conjugates were developed. Using the neutral loss of 163 Da (neutral loss of NAC), five peaks were detected in the post-administration urine which were not present in the basal one (Figures 2c and 2g). The method based on the precursor ion scan of *m/z* 164 allowed for the detection of nine peaks absent in the basal sample (Figures 2d and 2h). Three of them (NAC3, NAC4, and NAC5) were also detected by the neutral loss of 163 Da whereas NAC2, NAC3, NAC4 and NAC5 were also detected by the precursor ion scan of *m/z* 122. The presence of additional isobaric metabolites was evaluated by studying the XIC of the detected $[M+H]^+$. Using this strategy, two additional metabolites (NAC12 and NAC13 at *m/z* 448 and 466 respectively) were detected (Table 3).

In summary, ten potential boldione metabolites conjugated with Cys and 13 conjugated with NAC could be detected using the developed open screening strategies. In order to propose a feasible structure for the detected metabolites, their mass spectrometric behaviour was studied. In some cases, the structure could be confirmed by the synthesis of the metabolites. The MS behaviour of synthesized material was also used as guide for the proposal of metabolites structure.

Characterization and confirmation of the detected metabolites conjugated with cysteine

Cys1 and Cys2

Cys1 and Cys2 exhibited an *m/z* of 404 as $[M+H]^+$ (Table 3) which corresponds to 2 Da less than the Cys conjugate expected for boldione. Therefore, the addition of an extra double bond to boldione and the subsequent conjugation with Cys is the most feasible explanation for the occurrence of this metabolite. The product ion scan of both compounds at low collision energy exhibited the abundant neutral loss of 121 Da characteristic of Cys conjugates[20] (Table 4). At higher collision energies, the product ion spectra of both metabolites were similar to the reported for 1,4,6-androstatriene-3,17-dione (ATD).[24] Therefore, Cys conjugates of ATD were postulated as structures for Cys1 and Cys2.

Both C1 and C7 are potential sites for the conjugation of ATD with Cys. The synthesis of the Cys conjugates of ATD revealed the occurrence of two peaks at the same retention times of the metabolites found in urine. These two compounds were confirmed to be the metabolites present in the post-administration urine by the acquisition of a SRM method (Figure 1b, Table 5).

Cys3

Cys3 exhibited the $[M+H]^+$ expected for the Cys conjugate of boldione (*m/z* 406, Table 3). Besides the neutral loss of 121 Da obtained at low collision energy, the product ion spectra

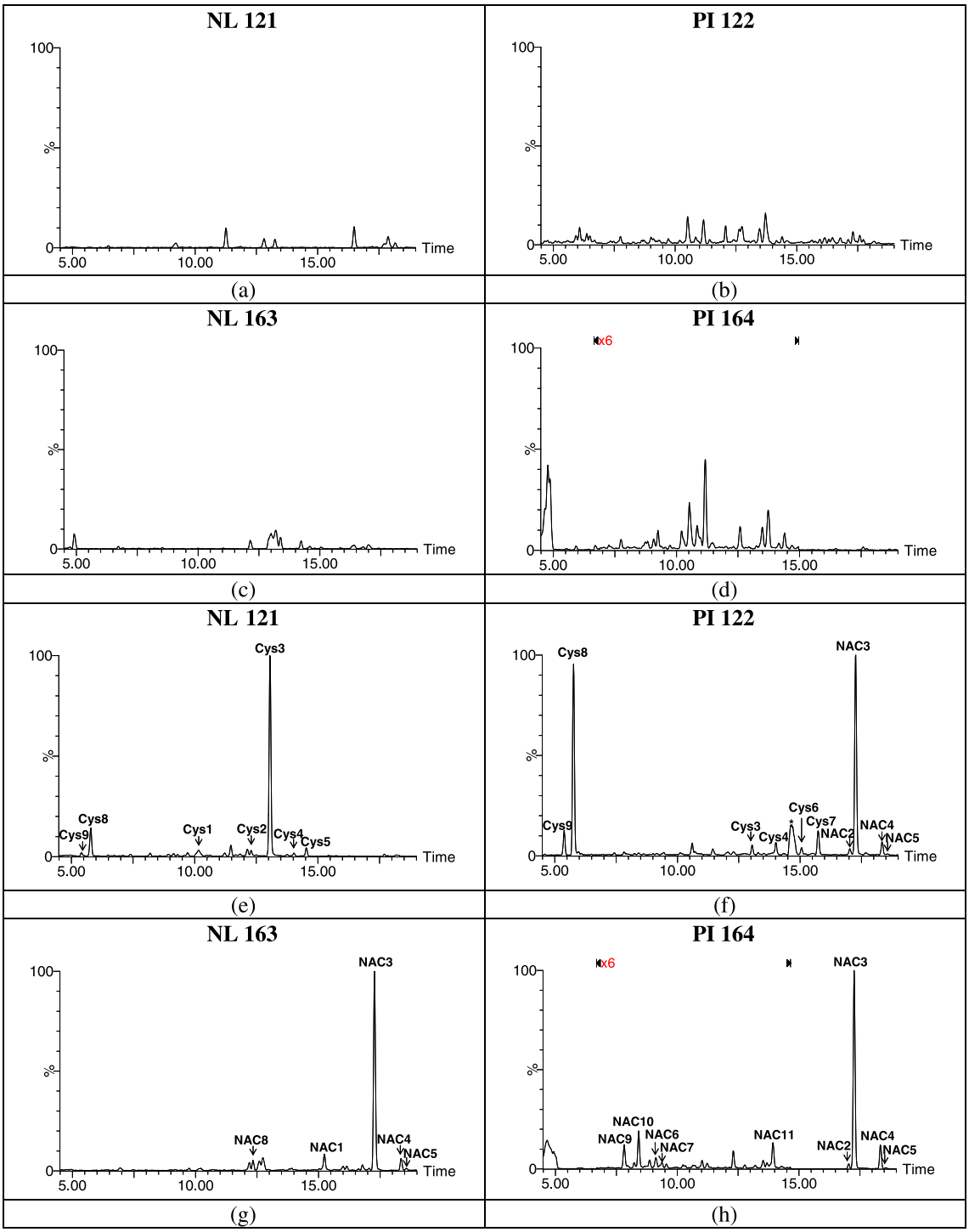


Figure 2. Open screening detection of cysteine and *N*-acetylcysteine conjugates in a urine sample collected before boldione administration (a)–(d) and 4 h after boldione administration (e-h) using: Neutral loss of 121 Da (a, e), Precursor ion scan scan of *m/z* 122 (b,f), Neutral loss of 163 Da (c,g) and Precursor ion scan of *m/z* 164 (d,h).

at 30 eV showed an abundant peak at *m/z* 121 (Table 4), characteristic of 1,4-dien-3-keto steroids[25,26] and base peak in the boldione spectrum.[24] Therefore, Cys conjugate of boldione can be postulated as the structure for Cys3. This fact was confirmed after comparing the SRM results of the post-administration sample with those obtained for the synthesized material (Figure 1b, Table 5).

Cys4, Cys5 (and Cys11)

Both Cys4 and Cys5 shared the m/z 408 as $[M+H]^+$ (Table 3). This *m/z* is consistent with a reduction in the boldione structure followed by conjugation with Cys. Reduction of the keto moiety in C17 and the double bond Δ^4 were described as phase I metabolites for boldione generating boldenone and 1-androstendione, respectively.[3,16,24]

Table 3. Boldione metabolites detected by open screening methods

Metabolite	RT	$[M+H]^+$	PI 122	NL 121	PI 164	NL 163
Cys1	10.1	404	✗	✓	✗	✗
Cys2	12.3	404	✗	✓	✗	✗
Cys3	13.1	406	✓	✓	✗	✗
Cys4	14.0	408	✓	✓	✗	✗
Cys5	14.5	408	✗	✓	✗	✗
Cys6	15.1	410	✓	✗	✗	✗
Cys7	15.8	410	✓	✗	✗	✗
Cys8	5.8	422	✓	✓	✗	✗
Cys9	5.4	424	✓	✓	✗	✗
Cys10	15.5	410	✗*	✗	✗	✗
NAC1	15.3	448	✗	✗	✗	✓
NAC2	17	450	✓	✗	✓	✗*
NAC3	17.3	450	✓	✗	✓	✓
NAC4	18.3	452	✓	✗	✓	✗
NAC5	18.6	452	✗	✗	✓	✓
NAC6	9.16	464	✗	✗	✓	✗*
NAC7	9.4	464	✗	✗	✓	✗
NAC8	12.4	464	✗	✗	✗	✓
NAC9	7.8	466	✗*	✗	✓	✗*
NAC10	8.4	466	✗*	✗	✓	✗*
NAC11	13.9	466	✗	✗	✓	✗*
NAC12	15.0	448	✗	✗	✗	✗*
NAC13	13.3	466	✗	✗	✗	✗*

* Only detectable after XIC.

The product ion scan of Cys4 at low collision energy shows besides the neutral loss of 121 Da and the ion at *m/z* 122 corresponding to the $[M+H]^+$ of the Cys (Table 4). This last ion was not observed in Cys1, Cys2, and Cys3 probably because the large conjugation of the carbonyl group produced after the neutral loss of Cys makes this pathway more abundant. This fact suggests the presence of a steroid with low conjugation in C3 and therefore reduction of the Δ^4 is the most feasible structure for Cys4. The presence of several specific ions such as *m/z* 69, or losses of 84 Da and 102 Da supports this assignment.[5,24,25,27,28] The synthesis of the Cys conjugate of (5α)-1-androstenedione showed similar fragmentation pathway but with different abundances and eluting at different retention time. Therefore, the (5β)-1-androstenedione conjugated with Cys can be postulated as structure for Cys4.

Although the synthesis of the Cys conjugate of (5α)-1-androstenedione confirmed that it was not the structure for Cys4, the SRM method developed for its confirmation allowed for its detection in the post administration sample. It was present after administration of boldione although at extremely low concentrations which hampered its detection by open screening methods. (5α)-1-androstenedione conjugated with Cys was confirmed by retention time and ion ratios and was labeled as Cys11 (Table 5).

The product ion spectrum of Cys5 at 30 eV exhibited an abundant peak at *m/z* 121 (Table 4) confirming the presence of a 1,4-androstadien-3-keto moiety.[24–26] Therefore, the Cys conjugate of boldenone was postulated as structure for Cys5. This structure was confirmed by the synthesis of the conjugated (Figure 1b, Table 5).

Cys6, Cys7 and Cys10

Three metabolites with $[M+H]^+$ at *m/z* 410 were detected (Cys6, Cys7 and Cys10, Table 3). This *m/z* implied two reductions in the boldione structure and the subsequent conjugation with Cys. Since Cys is postulated to be conjugated to unsaturated carbonyl groups, and based on the metabolism described for boldione and boldenone,[5,14,24] a reduction of both the keto moiety in C17 and the double bond Δ^4 before conjugation with Cys was postulated as metabolic pathway. However, the synthesis of the Cys conjugates of both (5α)-1-testosterone and (5β)-1-testosterone revealed that this hypothesis was not acceptable since different product ion spectra and retention times were obtained.

Contrarily to the rest of the Cys metabolites observed, the product ion spectra of Cys6, Cys7 and Cys10 at low collision energy was not dominated by the expected ion at $[M\text{-}121\ Da]^+$. Instead of this neutral loss, an ion at *m/z* 271, corresponding to the loss of Cys and water, was the most abundant ion in these conditions (Table 4). Besides this loss of Cys and water, an additional molecule of water was removed in all three metabolites generating the ion at *m/z* 253. The loss of the maximum number of water molecules in a steroid structure is associated with low conjugated AAS.[25] For these reasons, a reduction of the carbonyl group in C3 from Cys4 and/or Cys11 was postulated for Cys6, Cys7, and Cys10 (Figure 3).

The treatment of the synthesized Cys11 with $NaBH_4$ produced a totally reduced derivative (reduced at C3 and C17). This synthesized compound showed a similar fragmentation pathway that Cys6, Cys7 and Cys10, i.e. instead of the loss of Cys, it exhibited a neutral loss of Cys and water followed by an extra loss of water. Although the synthesis of these metabolites is still necessary, this fact supports the assignment of their structure as the shown in Figure 3.

Cys8 and Cys9

Cys8 and Cys9 exhibited $[M+H]^+$ at *m/z* 422 and *m/z* 424, respectively (Table 3). Therefore, they imply a hydroxylation. Both product ions exhibited the neutral loss of Cys and the ion at *m/z* 122. Additionally, both showed an abundant additional loss of water (*m/z* 283 and *m/z* 285 for Cys8, and Cys9, respectively) which can be explained by the hydroxylation of the metabolite (Table 4).

The molecular weight established for Cys8 (421 Da) is in agreement with a hydroxylation of boldione. The absence in the product ion spectrum at high collision energy of the ion at *m/z* 121, characteristic of 1,4-diene-3-keto AAS suggested that the hydroxyl group is in the B ring. A hydroxyl group in C6 can be neutrally removed as a water molecule in CID generating a double bond in Δ^6. Thus, the hydroxylation in C6 (i.e. 6-hydroxy-boldione) would explain that the ions observed for Cys8 at 30 eV (*m/z* 97 and *m/z* 147) also appeared in Cys1 and Cys2 which have a double bond in Δ^6 (Table 4). 6-Hydroxy-boldione was also identified in the free fraction of boldione.[3,24]

Regarding Cys9, its molecular weight (423 Da) suggested a hydroxylation either from boldenone or from 1-androstenedione. The product ion spectra acquired at 30 eV showed an abundant ion at *m/z* 121 (Table 4) indicating the presence of a 1,4-diene-3-keto moiety in the free steroid. Therefore, a Cys conjugate of a hydroxy-boldenone was the most feasible

Table 4. Main ions observed in the product ion spectra at different collision energies for the detected boldione metabolites conjugated with *N*-acetylcysteine

Metabolite	$[M+H]^+$	Collision energy (eV)	Main ions (*m/z*) observed in the product ion scan (in brackets relative abundance)
Cys1	404	10	404 (100); 283 (60)
		20	265 (100); 283 (80); 173 (50)
		30	147 (100); 97 (50); 173 (50); 105 (30)
Cys2	404	10	283 (100)
		20	283 (100)
		30	97 (90); 147(100); 123 (90)
Cys3	406	10	285 (100)
		20	285 (100); 267 (25); 151 (30); 147 (40); 121 (60)
		30	151 (20); 147 (25); 121 (100)
Cys4	408	10	122 (100); 287 (40); 408 (70)
		20	122 (100); 287 (80); 69 (30)
		30	69 (100); 122 (100); 287 (60)
Cys5	408	10	287 (100)
		20	287 (100); 121 (40); 135 (60); 269 (50)
		30	121 (100); 135 (70);143 (30); 173 (30)
Cys6	410	10	410 (100); 271 (70); 392 (40)
		20	271 (100); 253 (40)
		30	97 (100); 147 (100);161 (80); 253 (80); 81 (70); 107 (50); 133 (50); 171 (50); 271 (50)
Cys7	410	10	410 (100); 271 (60); 122 (40)
		20	271 (100); 253 (40); 122 (40)
		30	253 (100); 97 (90); 81 (70); 147 (70); 175 (70); 271 (60)
Cys8	422	10	301 (100); 283 (70); 122 (50)
		20	283 (100); 122 (70); 187(60); 265 (50); 301 (40); 97 (30)
		30	97 (100);147 (50); 187 (60); 265 (40)
Cys9	424	10	303 (100); 285 (80); 122 (70)
		20	285 (100); 303 (40); 267(50); 121(80)
		30	121 (100); 133 (60); 147 (60); 159 (40); 171 (60); 285 (50)
Cys10	410	10	410 (100); 271 (60)
		20	271 (100); 253 (60); 122 (50); 177 (50)
		30	253 (100); 303 (70); 97 (70); 163 (50)
NAC1	448	10	448 (100); 285 (95)
		20	285 (100); 147 (50); 121 (40); 151 (40)
		30	121 (100); 147 (40); 151(30)
NAC2	450	10	164 (100)
		20	164 (100); 122 (50); 287 (50)
		30	122 (100); 164 (40), 203 (25), 185 (20)
NAC3	450	10	164 (100); 287 (30)
		20	164 (100); 122 (50); 287 (70)
		30	122 (100); 69 (80); 164 (30), 185 (25)
NAC4	452	10	434 (100); 164 (50); 271 (30)
		20	164 (100); 271 (90); 253 (70); 81 (40); 130 (40)
		30	122 (100); 253 (60); 81 (50); 130 (50); 107 (40)
NAC5	452	10	289 (100); 452 (70); 164 (60)
		20	289 (100); 271 (95); 164 (90), 205 (20), 187 (20)
		30	143 (100); 121 (70); 122 (70)
NAC6	464	10	464 (100); 164 (90); 283 (60); 302 (50)
		20	283 (100); 187 (80); 164 (60); 97 (45); 147 (45); 265 (40); 301 (40)
		30	283 (100); 121 (90); 147 (50);187 (40); 265 (60)
NAC7	464	*n.d.*	
NAC8	464	10	302 (100); 301 (80); 464 (30)
		20	302 (100); 301 (50); 137 (60); 151 (50); 284 (30)
		30	137 (100); 147 (25)
NAC9	466	10	164 (100); 466 (25)
		20	164 (100); 122 (50); 303 (30)
		30	122 (100); 147 (50); 164 (40)

(continues)

Table 4. *(Continued)*

Metabolite	$[M+H]^+$	Collision energy (eV)	Main ions (*m/z*) observed in the product ion scan (in brackets relative abundance)
NAC10	466	10	164 (100); 466 (50); 303 (30)
		20	164 (100); 122 (80); 285 (25)
		30	122 (100); 164 (40)
NAC11	466	10	466 (100); 164 (50); 285 (40);303 (20)
		20	164 (100); 122 (50); 267 (50); 303 (50)
		30	122 (100); 159 (40); 285 (40)
NAC12	448	*n.d.*	
NAC13	466	*n.d.*	

n.d. Not detected in product ion scan

Table 5. Confirmation of the presence of synthesized metabolites in urine after boldione administration

		Synthetic		Sample	
Metabolite	Transition	Relative abundance (%)	RT (min)	Relative abundance (%)	RT (min)
Cys1	404 > 97	36 %	10.23	35.7 %	10.20
	404 > 147	100 %		100 %	
	404 > 283	57 %		54 %	
Cys2	404 > 97	30 %	12.45	27 %	12.42
	404 > 147	29 %		27 %	
	404 > 283	100 %		100 %	
Cys3	406 > 121	67.3 %	13.05	80 %	13.08
	406 > 147	26 %		28.9 %	
	406 > 285	100 %		100 %	
Cys5	408 > 121	100 %	14.53	100 %	14.56
	408 > 135	62.8 %		67.7 %	
	408 > 287	35 %		27.8 %	
Cys11	408 > 122	100 %	15.04	100 %	15.00
	408 > 185	26.8 %		21.1 %	
	408 > 287	34.2 %		35.9 %	
NAC1	448 > 121	100 %	15.28	100 %	15.25
	448 > 147	42 %		41 %	
	448 > 285	83 %		81 %	
NAC2	450 > 69	1.8	17.08	2.6	17.05
	450 > 121	0.8		1.1	
	450 > 122	39.5		40..9	
	450 > 164	100 %		100 %	
	450 > 185	5.8		6.1	
	450 > 203	2.8		3.6	
	450 > 287	3..7		3.9	
NAC5	452 > 122	65.2 %	18.61	68.3 %	18.58
	452 > 164	100 %		100 %	
	452 > 187	30.1 %		27.9 %	
	452 > 289	68.5 %		74.8 %	
NAC12	448 > 121	100 %	15.03	100 %	15.00
	448 > 147	32 %		31 %	
	448 > 285	87 %		69 %	

candidate for Cys9. The abundant *m/z* 121 also suggested that the hydroxyl group is distant from the A ring. Based on the common metabolism of AAS,[1–3] 16-hydroxy-boldenone which has also been identified in the free fraction of boldione[24] was selected as potential structure for Cys9 (Figure 3).

Therefore, Cys conjugates of 6-hydroxy-boldione and 16-hydroxy-boldenone can be postulated for Cys8 and Cys9 respectively. However, due to the absence of reference materials for these steroids, their structures could not be confirmed at this point.

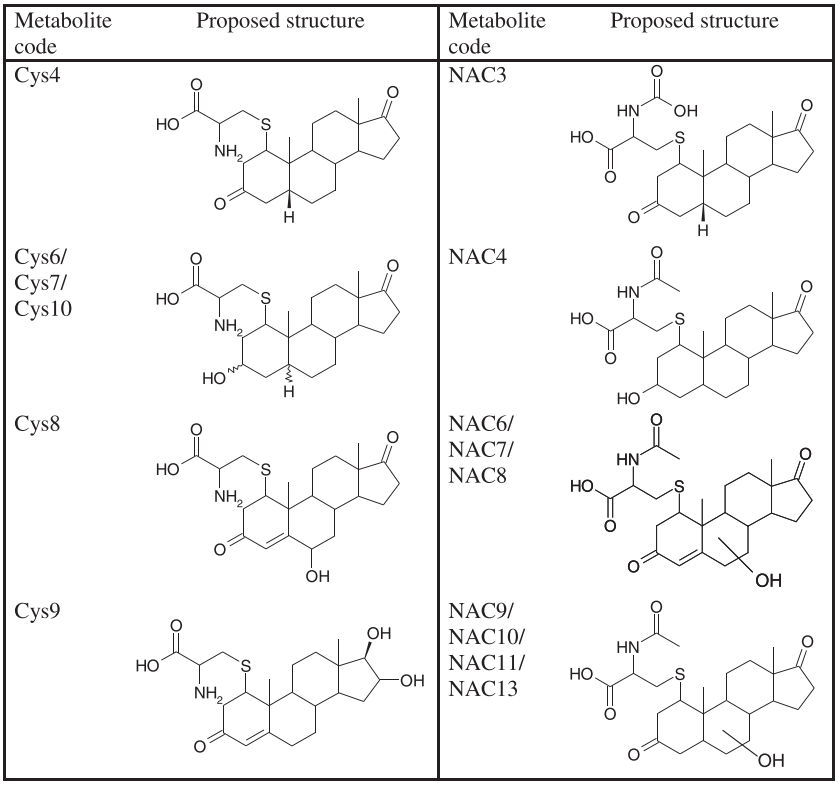


Figure 3. Proposed structures for non-synthesized metabolites.

Characterization and confirmation of the detected metabolites conjugated with *N*-Acetylcysteine

NAC1 and NAC12

NAC1 showed a $[M+H]^+$ at *m/z* 448 corresponding to the NAC conjugate of boldione. The product ion scan at low collision energy showed an abundant loss of 163 Da corresponding to the neutral loss of the NAC forming the free boldione. At 30 eV the characteristic ion at *m/z* 121 dominated the spectrum. The confirmation of boldione conjugated with NAC as structure for NAC1 was performed by the comparison with the reference synthesized material (Table 5).

Surprisingly, the synthesis of this conjugate revealed the presence of two isobaric compounds. Both sharing similar product ion spectra, but eluting at slightly different retention times (15.3 for NAC1 and 15.0 for the isobaric compound). When confirming the presence of NAC1 in the urine sample, the isobaric compound at smaller concentrations was also present, showing that it is also a boldione metabolite (NAC12). The structure of NAC12 is not obvious, as it can be produced either by the addition of the NAC at C4 or at the β-side of C1. Additional experiments are needed in order to confirm the structure of NAC12.

NAC2 and NAC3

The molecular weight of NAC2 and NAC3 (449 Da) suggest a reduction in the boldione structure and the subsequent conjugation with NAC. As stated in the case of Cys4 and Cys5, either the carbonyl group at C17 or the Δ^4 are the most feasible locations for this reduction to take place. Besides the ions corresponding to the neutral loss of NAC and those at *m/z* 164 and 122, the product ion spectra of NAC2 and NAC3 exhibited ions at *m/z* 69, 185 and 203 (Table 4). This fact together with the absence of the ion at *m/z* 121 allowed for the establishment of the Δ^4 as the reduction site. NAC2 and NAC3 could be due to the NAC conjugation of the 5α and 5β isomers of 1-androstendione.

Since the required materials are commercially available, the NAC conjugated for (5α)1-androstendione was synthesized. It was found to be coincident with NAC2 when using the SRM confirmatory method (Table 5). All the selected transitions were also found for NAC3 although with different ion ratios suggesting that NAC3 is the 5β isomer of 1-androstendione conjugated with NAC (Figure 3).

NAC4 and NAC5

Similarly to Cys6, Cys7, and Cys10, the $[M+H]^+$ observed for NAC4 and NAC5 (*m/z* 452, Table 3) suggested the occurrence of two reductions. The product ion of NAC5 at low collision energy exhibited the common loss of 163 Da and the ions at *m/z* 164. At higher collision energies, the occurrence of the ion at *m/z* 187, *m/z* 205 and *m/z* 69 (Table 4) suggested the presence of a 1-ene-3-keto moiety in the free AAS.[5,25,27,28] NAC conjugates of 5α and 5β isomers of 1-testosterone were synthesized. NAC5 share the ion ratios and the retention time with the 5β isomer (Figure 4). Therefore, it was concluded that NAC conjugate of 5β-1-testosterone was the structure of NAC5.

Both ion ratios and retention times of the synthesized compounds were not in agreement with the observed in the post-administration urine for NAC4. The product ion spectra at low collision energy of NAC4 did not exhibited the common loss of 163 Da. Instead, an ion at *m/z* 271 corresponding to the loss of

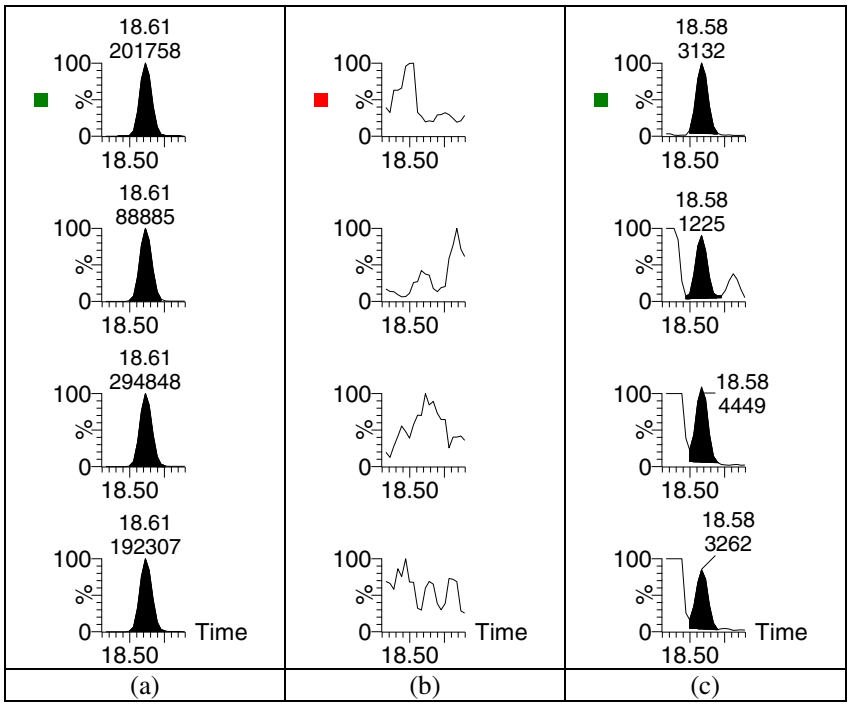


Figure 4. Confirmation of the presence of NAC5 in urine after boldione administration. SRM chromatograms for (a) synthesized reference material, (b) urine collected before boldione administration and (c) urine collected 4 h after boldione administration.

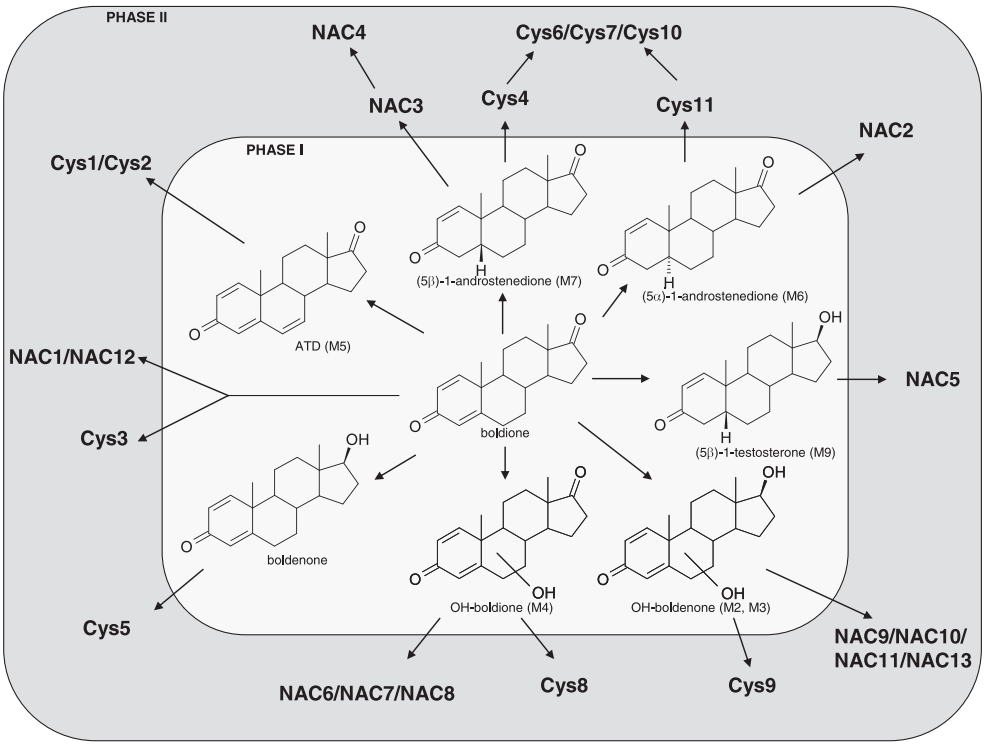


Figure 5. Metabolic pathway for boldione involving Cys and NAC conjugation.

NAC and water was observed. A similar situation was observed with Cys6, Cys7 and Cys10. As stated above, a reduction of the carbonyl group at C3 from either NAC3 or NAC2 can be the cause of this behavior (Figure 3). The complete reduction of NAC2 provided a compound with similar mass spectrometric behavior supporting this assignment.

NAC6, NAC7 and NAC8

Three metabolites (NAC6, NAC7 and NAC8) with a molecular weight corresponding to the NAC conjugated of a hydroxyl-boldione metabolite (463 Da) were detected. Among them, the sensitivity obtained for NAC7 did not suffice for the acquisition of

an adequate product ion spectra and therefore its structure could not be postulated.

The product ion spectra at low collision energy of NAC6 and NAC8 exhibited the common neutral loss of 163 Da and the ion at m/z 164 (Table 4). When increasing the collision energy, NAC6 exhibited an abundant ion at *m/z* 121 suggesting the presence of an intact 1,4-diene-3-keto structure. Therefore, it can be postulated that the hydroxyl group is located far away from the A ring. C16 is the most feasible position for the hydroxylation. In the product ion spectrum of NAC8 at higher collision energy, ions at *m/z* 137 and 147 were detected. This fact implies a modification in the A ring of the steroid and therefore, a C6 hydroxylation seems to be the most feasible metabolic transformation before conjugation with NAC (Figure 3). Obviously, the synthesis of these compounds is needed in order to ultimately confirm their structures.

NAC9, NAC10, NAC11, and NAC13

NAC9, NAC10, NAC11, and NAC13 have a $[M+H]^+$ corresponding to a reduction and a hydroxylation previous to the conjugation with NAC. The product ion spectra of these compounds only exhibited the ions at *m/z* 164 and *m/z* 122 and the neutral losses of NAC and NAC and water as major ions. This fact can be related to a reduction of the Δ^4 which would prevent for the generation of more specific ions needed for the postulation of a feasible structure.

Cysteine and *N*–acetylcysteine metabolic pathways for boldione

Based on the results presented, the metabolic pathway involving Cys and NAC conjugation is presented for boldione (Figure 5). Since conjugation with either Cys or NAC is postulated to be produced from a Michael's addition between the aminoacid and a conjugated double bond of the steroid,[20] phase I metabolites can be extrapolated from the conjugated detected in urine. Thus, several known phase I metabolites were involved in the formation of the detected conjugates. All of them (4,5-hydrogenation, 6,7-dehydrogenation, C17-reduction and hydroxylations in C16 and C6) have been described for boldione.[3,15,16,24]

6,7-Dehydrogenation was also described for testosterone[20] and some Δ^6 metabolites have been found for other steroids.[1,29] The conjugation of these phase I metabolites either with Cys or with NAC provided most of the metabolites detected in this study.

According to our results, Cys and NAC conjugates can suffer additional metabolism. This fact would explain the occurrence of metabolites Cys6, Cys7, Cys10, and NAC4. Phase I metabolites obtained after reduction of the Δ^4 seem to be conjugated either with Cys or with NAC producing Cys4/Cys11 and NAC2/NAC3, respectively. The subsequent reduction of the carbonyl function in C3 would produce Cys6, Cys7, Cys10, and NAC4. This biotransformation seems to be the most likely pathway since the addition of a Cys/NAC group in C1 would need the presence of a carbonyl group at C3.

Conclusions

The presence of Cys and NAC conjugates in human urine after boldione administration has been confirmed. The neutral loss and precursor ion scan methods developed allowed for the detection of 24 boldione metabolites. Eleven of them were conjugated with Cys and thirteen were conjugated with NAC. All metabolites were characterized by LC-MS/MS and nine of them were confirmed by the synthesis of the reference material.

Similarly to several metabolites reported for testosterone,[20] Cys conjugates were mainly obtained from these phase I metabolites with a conjugated keto moiety. However, whereas NAC metabolites were not found for testosterone, it has been demonstrated that this biotransformation is an important metabolic pathway for boldione. Up to our knowledge, this is the first report of NAC conjugation for steroid metabolism. It is expected that this metabolic pathway is common to other anabolic agents.

Phase I metabolites observed in this study (Figure 5) were already reported for boldione and/or boldenone suggesting that conjugation with Cys/NAC is a phase II metabolism which complements conjugation as sulfate and glucuronide. Alkalization of the sample in order to release Cys/NAC conjugates after a retro-Michael's reaction seems to be the most sensitive treatment for the detection of these conjugates.[20] Thus, the alkaline release of the conjugate for metabolites Cys3, NAC1, and NAC12 will result in the detection of boldione, whereas alkaline treatment of Cys5 will form boldenone. In the same way, alkaline release of the conjugates Cys9/NAC9/NAC10/NAC11/NAC13, Cys8/NAC6/NAC/7NAC8, Cys1/Cys2, Cys4/NAC3, and NAC5 will result in the metabolites M2/M3, M4, M5, M7, and M9 respectively reported in the first part of this study.[24] The hydrolysis of Cys11/NAC2 will produce the metabolite M6. However the amount of this metabolite was not found to be increased after alkaline treatment. This can be due to the low concentrations of this metabolite excreted as Cys11/NAC2 compared with the amount excreted free.

The occurrence of conjugates with Cys and NAC in the metabolism of AAS opens new alternatives for the detection and confirmation of the AAS misuse. Therefore, their inclusion in screening methods can be useful for the doping control field.

Acknowledgements

This work was supported by grant from Instituto de Salud Carlos III FEDER, (CP10/00576). Financial support of WADA (09A5DP and 11A22OP) and grant by the Generalitat de Catalunya (2009GR00492 to the research team) are also acknowledged. The help of Andreu Fabregat is acknowledged.

References

[1] W. Schänzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **1996**, *42*, 1001.
[2] 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.
[3] P. Van Eenoo, F.T. Delbeke. Metabolism and excretion of anabolic steroids in doping control - New steroids and new insights. *J. Steroid Biochem.* **2006**, *101*, 161.
[4] 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.
[5] 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.
[6] O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke. Efficient approach for the comprehensive detection of unknown anabolic steroids and metabolites in human urine by liquid chromatography - Electrospray-tandem mass spectrometry. *Anal. Chem.* **2008**, *80*, 1709.

[7] 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.
[8] O.J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, S. Grimalt, J.V. Sancho, *et al*. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids* **2009**, *74*, 837.
[9] D.H. Catlin, M.H. Sekera, B.D. Ahrens, B. Starcevic, Y.C. Chang, C.K. Hatton. Tetrahydrogestrinone: discovery, synthesis, and detection in urine. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1245.
[10] A. Leinonen, T. Kuuranne, T. Kotiaho, R. Kostiainen. Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids* **2004**, *69*, 101.
[11] 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.* **2012**, *132*, 239.
[12] 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.
[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] 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.
[15] 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.
[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 W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (Eds.) *Recent Advances in Doping Analysis* (10). Sport und Buch Strauss, Cologne, Germany, **2002**, pp.73.
[17] M.K. Parr, G. Fuβhöller, N. Schlörer, G. Opfermann, T. Piper, G. Rodchenkov, *et al*. Metabolism of androsta-1,4,6-triene-3,17-dione and detection by gas chromatography/mass spectrometry in doping control. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 207.
[18] Y.L. Tseng, C.Y. Sun, F.H. Kuo. Detection and quantification of glucuro- and sulfoconjugated metabolites in human urine following oral administration of xenobiotic 19-norsteroids. *Steroids* **2006**, *71*, 817.
[19] A.T. Cawley, R. Kazlauskas, G.J. Trout, A.V. George. Determination of urinary steroid sulfate metabolites using ion paired extraction. *J. Chromatogr. B* **2005**, *825*, 1.
[20] O.J. Pozo, A. Fabregat, A. Kotronoulas, J. Marcos, J. Joglar, I. Alfonso, *et al*. Testostetrone metabolites released alter Basic treatment. Where do they come from? (2012) Presented in the Manfred Donike Workshop, 30th Cologne Workshop on Dope Analysis, Cologne, Germany, **2012**.
[21] O.J. Pozo, J. Marcos, R. Ventura, A. Fabregat, J. Segura. Testosterone metabolism revisited: discovery of new metabolites. *Anal. Bioanal. Chem.* **2010**, *398*, 1759.
[22] 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.
[23] A. Fabregat, O.J. Pozo, P. Van Renterghem, P. Van Eenoo, J. Marcos, J. Segura, R. Ventura. Detection of dihydrotestosterone gel, oral dehydroepiandrosterone, and testosterone gel misuse through the quantification of testosterone metabolites released after alkaline treatment. *Drug Test. Anal.* **2011**, *3*, 828.
[24] C. Gomez, O.J. Pozo, A. Fabregat, J. Marcos, K. Deventer, P. van Eenoo, *et al*. Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part I: Phase I metabolites excreted free, as glucuronide and sulfate conjugates, and released after alkaline treatment of the urine. *Drug Test. Anal.* **2012**, *4*, 775.
[25] 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.
[26] 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.
[27] 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.
[28] 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.
[29] O.J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, W. Van Thuyne, M.K. Parr, *et al*. *Drug Metab. Dispos.* **2009**, *37*, 2153.

