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Detection of boldenone and its major metabolites by liquid chromatography—tandem mass spectrometry in urine samples

F. Buiarelli [a], G.P. Cartoni [a,*], F. Coccioli [a], L. Giannetti [b], M. Merolle [a], B. Neri [b], A. Terracciano [a]

[a] *Università di Roma "La Sapienza", Dipartimento di Chimica P.le A. Moro, 5-00185 Rome, Italy*
[b] *Istituto Zooprofilattico Sperimentale Regioni Lazio e Toscana-Roma, Via Appia Nuova, 1411-00178 Rome, Italy*

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Abstract

Boldenone is an androgenic anabolic steroid (AAS) intensively used for growth promoting purposes in animals destined for meat production and as a performance enhancer in athletics. Therefore its use is officially banned either in animals intended for consumption or in humans. Because most anabolic steroids are completely metabolized and usually no parent steroid is excreted, metabolite identification is crucial to detect the illegal use of anabolic steroids either in humans or in livestock.

The aim of this work is the investigation of 17β-boldenone and its main metabolites, 17β-sulphate, 17β-glucuronide, 5β-androst-1-en-17β-ol-3-one and 17α-boldenone, in human and bovine urine developing a multiresidue analysis.

After solid phase extraction of urine samples, detection is carried out by high performance liquid chromatography–tandem mass spectrometry in multiple reaction monitoring. The average recovery for all the investigated compounds is above 70%. The developed method is very easy, quick and highly specific. Linearity, precision, decision limit and detection capability were also evaluated.

Keywords: Liquid chromatography–tandem mass spectrometry; Urine; Boldenone metabolites; Conjugated boldenone

1. Introduction

17β-Boldenone (androsta-1,4-dien-17β-ol-3-one) is a synthetic androgenic steroid hormone with anabolic properties, first synthesized in 1956 by dehydrogenation of the male hormone testosterone with selenium dioxide [1]; its structure is highly similar to testosterone with dehydrogenation at the C-1,2 position.

As other anabolic steroids, its illegal use to increase body mass and enhance physical conditioning it is widely demonstrated.

17β-Boldenone is included in the 2005 list of banned substances in sports of the World Anti-Doping Agency (WADA) [2], and at the same time its illegal administration for growth promotion purposes in animals destined for meat production (cattle fattening) is coming particularly intensive. The forensic chemistry of natural or/and synthetic steroids as illegal abuse substances requests the development of analytical methods able to identify both the parent drug and its metabolites, to demonstrate its effective administration. The AASs and their metabolites are excreted in urine together with a large variety of compounds like salts, endogenous steroids, and metabolites of medicine if any is taken, the concentration of which, like that of the anabolic agents in the urine sample, can vary a lot, from a few $\mu g\,mL^{-1}$ to $pg\,mL^{-1}$.

Biotrasformation reactions of drugs are generally divided into two classes. In phase I reactions, polar functional groups are introduced into a drug molecule via typical oxidation, reduction and hydrolysis. In phase II reactions, the drug and/or its phase I metabolites are conjugated with endogenous species such as glucuronic acid, sulphate, amino acids, biliary acids and so on, forming soluble conjugate products which are readily excreted from the body via urine or faeces. Conju-

* Corresponding author. Tel.: +39 06 49913713; fax: +39 06 490631.
E-mail address: giampaolo.cartoni@uniroma1.it (G.P. Cartoni).

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gation with glucuronic and sulphate acid is a common route of metabolism for steroids and may make up to 90% of the excreted metabolites. They are therefore an important class of compounds for drug screening in sports [3]. The human metabolism of boldenone was investigated in 1971 [4]. 17β-Boldenone has been reported to be excreted in human urine as free and conjugate. Its main metabolites are 5β-androst-1-en-17β-ol-3-one, 5β-androst-1-en-3α-ol-17-one and 5β-androst-1-en-3α,17β-diol [5], while 17α-boldenone is considered to be the main metabolite in equine and cattle urine [6].

However, enforcement of the ban on the use of boldenone on bovines is complicated and under debate since 1996 [7]. Common methods utilized in the determination of steroids and steroids conjugates involve extraction of the bulk of compounds by Amberlite XAD-2 or silica C-18 liquid solid extraction (LSE), followed by group fractionation on surface-modified Sephadex. These methods are general and separate neutral steroids, glucuronides, monosulphates and disulphates but with low selectivity within these groups [8]. Residue analysis of boldenone and its metabolites in urine, faeces or hair samples is usually performed by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) with electron impact ionization (EI), after hydrolysis, extraction, purification and derivatization [9,10]. In fact, due to their low volatility and thermal instability, drug conjugates are not directly amenable to GC–MS, so these compounds are commonly enzymatically hydrolyzed or by other means, such as hydrochloric acid hydrolysis with aqueous media, for further characterization. Because the steroid conjugates dominate the urinary profile, it would be to great advantage to directly determine the intact steroid conjugates, thereby eliminating the time-consuming and potentially inaccurate hydrolysis procedures [11]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) can be a robust, easy and sensitive alternative to GC–MS [12]. In this work, we present a LC–MS/MS method, with electrospray ionization (ESI), for the multiresidue detection and quantification of 17β-boldenone, 17α-boldenone, 5β-androst-1-en-17β-ol-3-one, 17β-boldenone sulphate and 17β-boldenone glucuronide in urine samples. Fig. 1 shows the chemical structure of the analytes investigated. A liquid–solid extraction technique using cartridges, packed with a polymeric sorbent, is used to isolate the target analytes from urine samples, bypassing the hydrolysis step. It is shown that the method is effective for the extraction and quantification of traces of 17β-boldenone and its metabolites in urine samples. Urine samples were collected from stated untreated calves, from stated untreated human volunteers and from a human volunteer after oral ingestion of 17β-boldenone and analysed, in order to assess whether boldenone residues are from endogenous or exogenous origin, and to evaluate the role of conjugation in monitoring boldenone abuse.

2. Experimental

2.1. Chemicals and reagents

Methanol (HPLC grade) was obtained from Carlo Erba (Milan, Italy); formic acid and ammonium formate were obtained from Fluka (Sigma–Aldrich, Milan, Italy). All reagents were of analytical grade. Ammonium formate 6 mM was prepared dissolving 378.4 mg of ammonium formate in 1000 mL of water. Ultrapure water was produced with a Pure Lab^{TM} system (USF Elga, Ransbach-Baumbach, Germany).

17β-Boldenone (β-Bold) 5β-androst-1en-17β-ol-3one (5β-Androst) and 17β-boldenone sulphate (β-BoldSulf) Na salt was obtained from Steraloids (Newport, RI, USA).

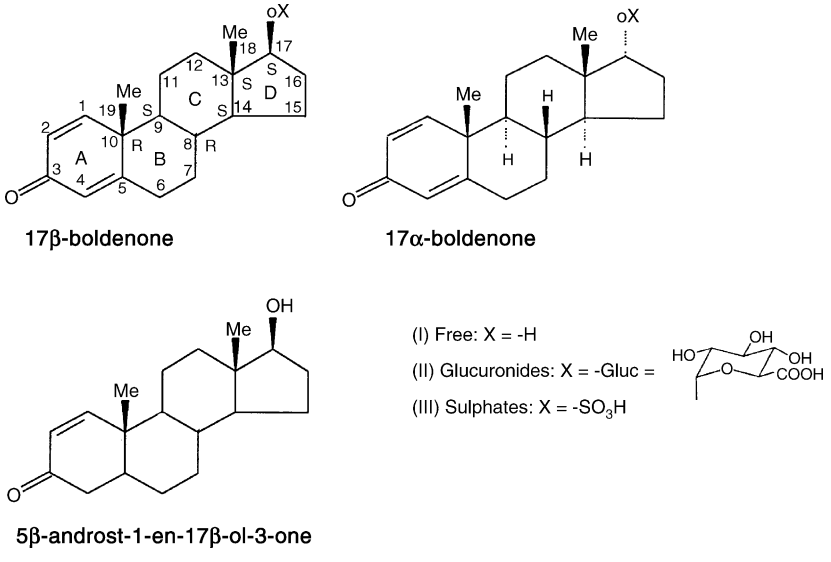

Fig. 1. Chemical structure of the analytes investigated.

Table 1
Optimized electrical parameters for MS and MS/MS analysis

Compound	Parameters				Experiment
	IS (kV)	OR (V)	RNG (V)	RO_2 (V)	
Glucuronides/sulphates	−5.0	−45	−250	55	1
Free	+5.5	30	100	−35	2

17α-Boldenone (α-Bold), 17β-boldenone glucuronide (β-BoldGluc) K salt, the deuterated d_3-17β-boldenone (d_3Bold), the deuterated epitestosterone glucuronide (d_3ETGluc) K salt trihydrated and 16,16,17-d_3-testosterone sulphate (d_3TSulf) Na salt, were obtained from NARL (Pymble, NSW 2073, Australia). Liquid–solid extraction cartridges used, were Strata X (Phenomenex, Torrance, CA, USA), packed with 60 mg 3 mL^{-1} of polymeric sorbent (mixed polymeric and reversed phase), obtained from Chemtek Analytica (Bologna, Italy). Stock standard solutions (1 mg mL^{-1}) in methanol were prepared monthly and stored at −20 °C in the dark. Working solutions were daily prepared by appropriate dilution by H_2O–MeOH 1:1 (v/v). Authentic analytical standard mixtures of the compounds were prepared from 1 mg mL^{-1} stock solutions and had solvent compositions that were close to, or equal to, the mobile phase composition.

2.1.1. Urine samples

Bovine urine samples were collected from calves younger than 1-year-old. Blank human urine samples were collected casually during the day from volunteers of different ages (from 25 to 75 years old) and at different times of the day. Urine samples from the volunteer, who ingested a single dose of 70 mg (1 mg kg^{-1} body weight) of boldenone, were collected systematically for 5 days. Samples were stored in the dark, at −20 °C until assay.

2.2. Instrumentation

LC analysis was carried out by a Shimadzu two-pump system LC-10 AD (Shimadzu, Kyoto, Japan) at a flow rate of 150 μL min^{-1} of CH_3OH-ammonium formate (6 mM) 60:40, previously degassed (sparging with He), under an isocratic elution. The eluent from the column was directly transferred to the ion-spray interface with no post-column split.

The used LC column, Nucleosil C_{18} (Shandon, UK, 250 mm × 2.1 mm i.d. × 5 μm) equipped with a guard column Phenomenex C_{18} (40 mm × 2.1 mm i.d.), was slurry packed in our laboratory [13]. The LC system was equipped with an injector Rheodyne 8125, with a sample loop of 5 μL.

The mass spectrometer PE-SCIEX API 365 (Perkin-Elmer Sciex Instruments, Foster City, CA, USA) equipped with a TurboIon Spray interface, was used to perform MS and MS/MS analyses. Acquisition parameters were optimized in ion spray mode by direct continuous pump infusion of standard working solutions of the analytes (10 ng μL^{-1}in MeOH–ammonium formate 6 mM 1:1 (v/v)) at a flow rate of 10 μL min^{-1} in the ion source of the mass spectrometer.

The atmospheric pressure ionization (API) source voltage was set in negative ionization mode for glucuronides and sulphates (experiment 1), and in positive mode for free steroids (experiment 2), respectively. Air was used as nebulizing gas, nitrogen as curtain gas and as collisional gas. Curtain and collision gases were set at 10, 6 and 5 (arbitrary units) for the nebulizer. The collisional energy was adjusted by the variation of the voltage difference between the high pressure entrance quadrupole (Q_0 = 10 V) and the collisional cell quadrupole RO_2; it was optimized to achieve the highest sensitivity for each analyte. The vaporizer was set at 350 °C. Table 1 shows the optimized parameters of the mass spectrometer. Data acquisitions were performed preliminarily on the pure standard compounds in full scan (mass range 50–500 Da) using the first quadrupole to choose an abundant precursor ion. MS/MS product ion scans were then

Table 2
Precursor ions, fragments and LC retention times for steroids analysed in MRM ion spray

	Polarity	Precursor *m/z*	Products *m/z*	t_R (min)
Sulphates				
17-β-Boldenone sulphate (β-BoldSulf) (MW = 366)	–	365 $(M-H)^-$	350, 177, 97	7.78
d_3-Testosterone sulphate (d_3TestSulf) (MW = 371)	–	370 $(M-H)^-$	370, 97	9.23
Glucuronides				
17-β-Boldenone glucuronide (β-BoldGluc) (MW = 462)	–	461 $(M-H)^-$	85, 75, 113	7.45
d_3-Epitestosterone glucuronide (d_3ETGluc) (MW = 467)	–	466 $(M-H)^-$	113, 290, 85	10.82
Free				
17-α-Boldenone (α-Bold) (MW = 286)	+	287 $(M+H)^+$	121, 135, 173	27.45
17-β-Boldenone (β-Bold) (MW = 286)	+	287 $(M+H)^+$	121, 135, 173	23.15
d_3-17-β-Boldenone (d_3Bold) (MW = 289)	+	290 $(M+H)^+$	121, 138	
5β-Androst-1EN-17βOL-3ONE (5β-Androst) (MW = 288)	+	289 $(M+H)$+	187, 121	41.69

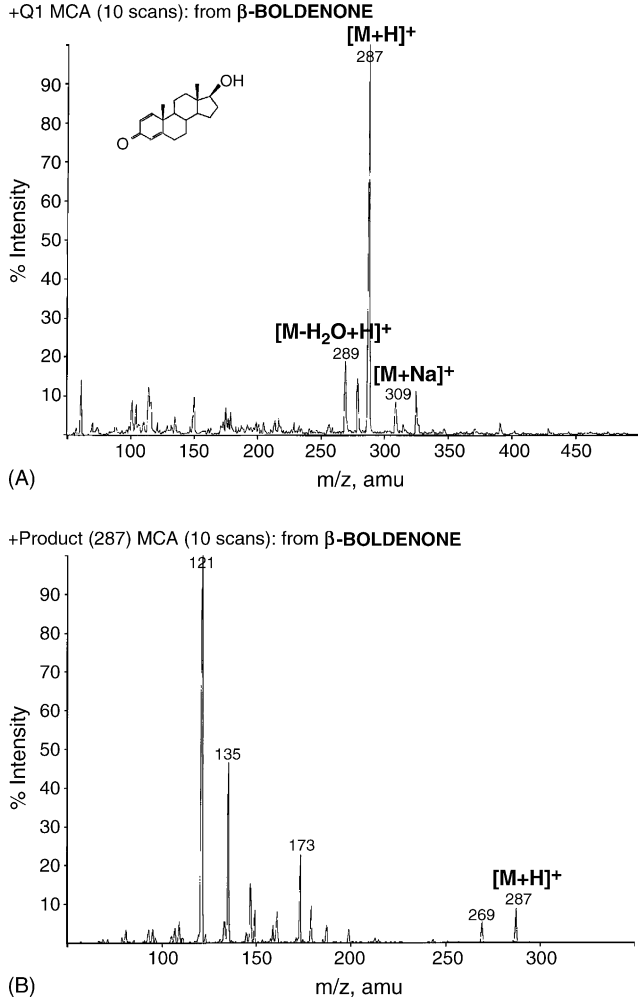

Fig. 2. (A) Full scan API MS spectrum, in positive ionization, of β-boldenone standard. (B) Full scan API MS/MS spectrum of β-boldenone standard. Precursor ion *m/z* 287. MS and MS/MS conditions as in Table 1. The spectra were obtained by infusion (10 μL min^{-1}) of standard solution 10 ng μL^{-1} in MeOH–ammonium formate 6 mM.

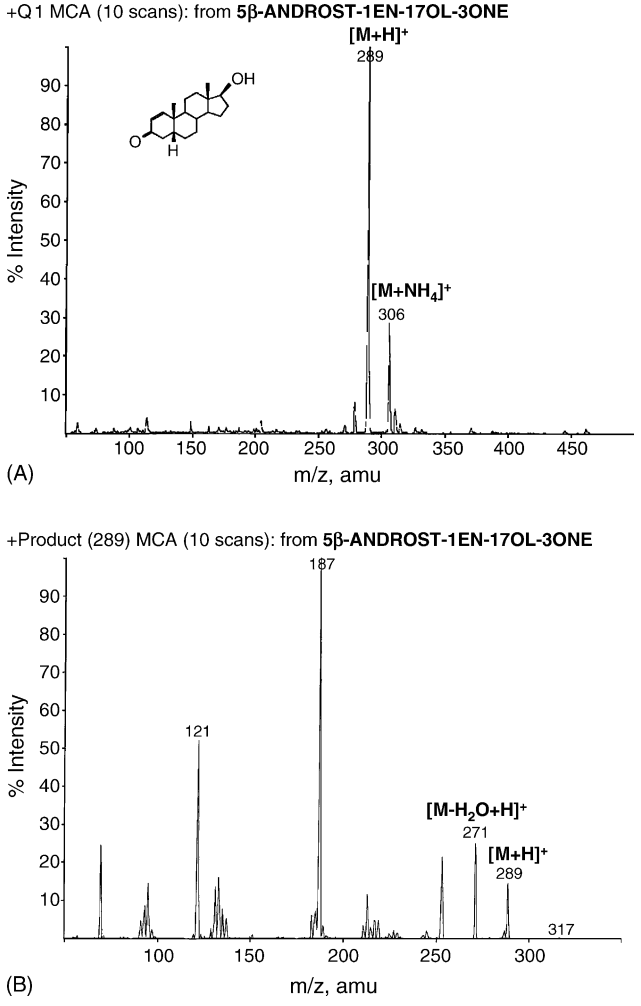

Fig. 3. (A) Full scan API MS spectrum, in positive ionization, of 5β-androst-1en-17ol-3one standard. (B) Full scan API MS/MS spectrum of 5β-androst-1en-17ol-3one standard. Precursor ion *m/z* 289. MS and MS/MS conditions as in Table 1. The spectra were obtained by infusion as in Fig. 2.

recorded from *m/z* 50–500 Da. Finally all analyses, both on standards and on samples, were carried out by LC–MS/MS in MRM mode, monitoring two diagnostic product ions from each chosen precursor ion to obtain high specificity and sensitivity.

2.3. Sample preparation

A solid phase extraction (SPE) protocol, optimized in our laboratory in a previous work [14], has been followed for the extraction of the investigated compounds from urine samples.

Fifty microliters of an internal standard (ISTD) mixture of d_3ETGluc (10 ng μL^{-1}), d_3TSulf (2 ng μL^{-1}) and d_3Bold (2 ng μL^{-1}) were added to 5 mL of urine sample, afterwards adjusted to pH 3.5 with few droplets of formic acid. The sample was loaded on a Strata × SPE column, linked to a vacuum system, previously conditioned with 3 mL of methanol and washed with 6 mL of 2 mM aqueous formic acid (pH 3.5). After a washing step with 3 mL of 2 mM aqueous formic acid–MeOH 95:5, steroids were eluted from the cartridge by 3 mL of MeOH and directly collected in a vial. The eluate was evaporated to dryness under nitrogen stream at 45 °C.

Table 3
Acquisition scheme

Acquisition period	Polarity	Time (min)	Experiment	Detected compounds
[I]	–	0–16.50	1	Glucuronides sulphates
[II]	+	16.50–45	2	Free

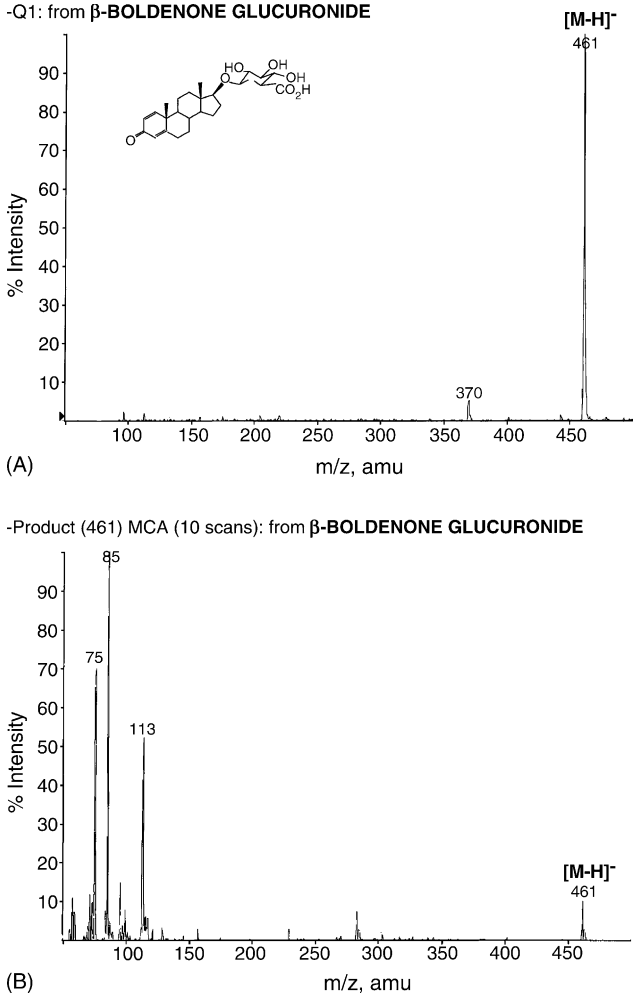

Fig. 4. (A) Full scan API MS spectrum, in negative ionization, of β-boldenone glucuronide standard. (B) Full scan API MS/MS spectrum of β-boldenone glucuronide standard. Precursor ion *m/z* 461. MS and MS/MS conditions as in Table 1. The spectra were obtained by infusion as in Fig. 2.

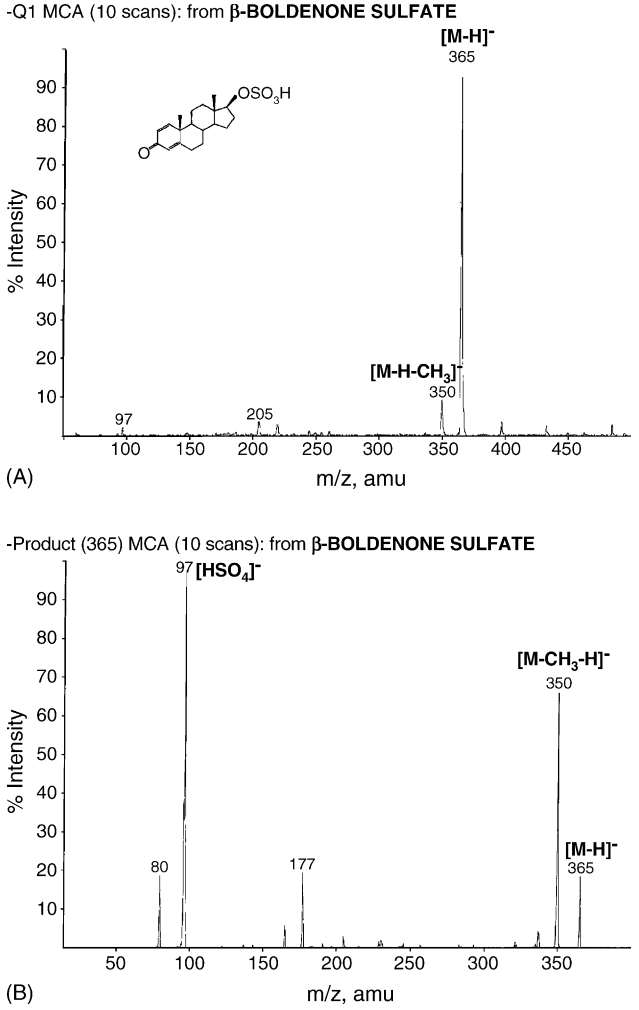

Fig. 5. (A) Full scan API MS spectrum, in negative ionization, of β-boldenone sulphate standard. (B) Full scan API MS/MS spectrum of β-boldenone sulphate standard. Precursor ion *m/z* 365. MS and MS/MS conditions as in Table 1. The spectra were obtained by infusion as in Fig. 2.

The dried residue was dissolved in 50 μL of MeOH–water 1:1 (v/v) and aliquots of solution (5 μL) analysed by LC–MS/MS system. All urine samples were prepared in double aliquots.

2.4. Recovery

The extraction recovery of the method was tested on a pool of human blank urine samples, spiked with the standards of the interested compounds, adding 50 μL of the ISTD mixture after the extraction process. For the determination of the recovery the samples were analysed by LC–MS/MS in MRM mode and compared with calibration standard solutions (analytes + ISTD dissolved directly in MeOH–ammonium formate 6 mM 1:1 (v/v)). Two aliquots of both spiked samples and standard solutions (with the same final concentration) were run in triplicate (six injections for each concentration level). Standard graphs were prepared daily by plotting peak area ratios of analytes to IS versus analyte concentration. The curves were employed to evaluate the extraction efficiency of the analytes. Spiked urine samples were prepared at different known analyte concentrations (1, 10, 50 ng mL^{-1}, corresponding to a final concentration in MeOH, respectively, 0.1, 1, 5 ng μL^{-1}).

2.5. Calibration and quantitation procedure

Matrix calibration curves were performed by LC–MS/MS analyses (MRM mode) of extracts of blank urine samples spiked with mixtures of the interested analytes, at different known concentrations (1, 5, 10, 25, 50 ng mL^{-1}) and the ISTDs. Samples were prepared in duplicate and injected in triplicate into the LC–MS/MS. Graphs were calculated by least squares linear fitting of the peak area ratio analyte/ISTD (using the most abundant ions) versus analyte nominal concentration. Graphs were used to interpolate concentrations of these analytes both in the fortified and in real samples.

Table 4
Detection limit (CCα) and detection capability (CCβ) values for the investigated analytes

Compounds	Detection limit ($ng\,mL^{-1}$)	Detection capability ($ng\,mL^{-1}$)
β-BoldGluc	0.40	0.55
β-BoldSulf	0.75	0.99
β-Bold	0.52	0.70
α-Bold	0.70	0.93
5β-Androst	0.42	0.56

2.6. Linearity, precision, accuracy, specificity, decision limit and detection capability

Calibration curves were used to evaluate linearity of the method. Urine fortified samples, at different known concentrations (1, 5, 10, 25, 50 $ng\,mL^{-1}$), were prepared to ensure satisfactory results in terms of precision and accuracy. Precision was calculated by the relative standard deviation (R.S.D.) for repeated measurements, and accuracy by assessing the agreement between the measured and nominal concentrations. According to Commission Decision 2002/657/EC [15], specificity of the procedure, decision limit (CCα) and detection capability (CCβ) were evaluated. Specificity was estimated by analysing 20 independent blank urine samples from different calves and humans. Decision limit (CCα) was calculated using 20 independent blank urine samples to calculate the signal to noise ratio at the retention time of the analyte. Three times the signal to noise ratio was used as decision limit. Decision capability (CCβ) was calculated fortifying 20 blank urine samples at the decision limit. The value of (CCα) plus 1.64 times the standard deviation of reproducibility of the measured content equals (CCβ).

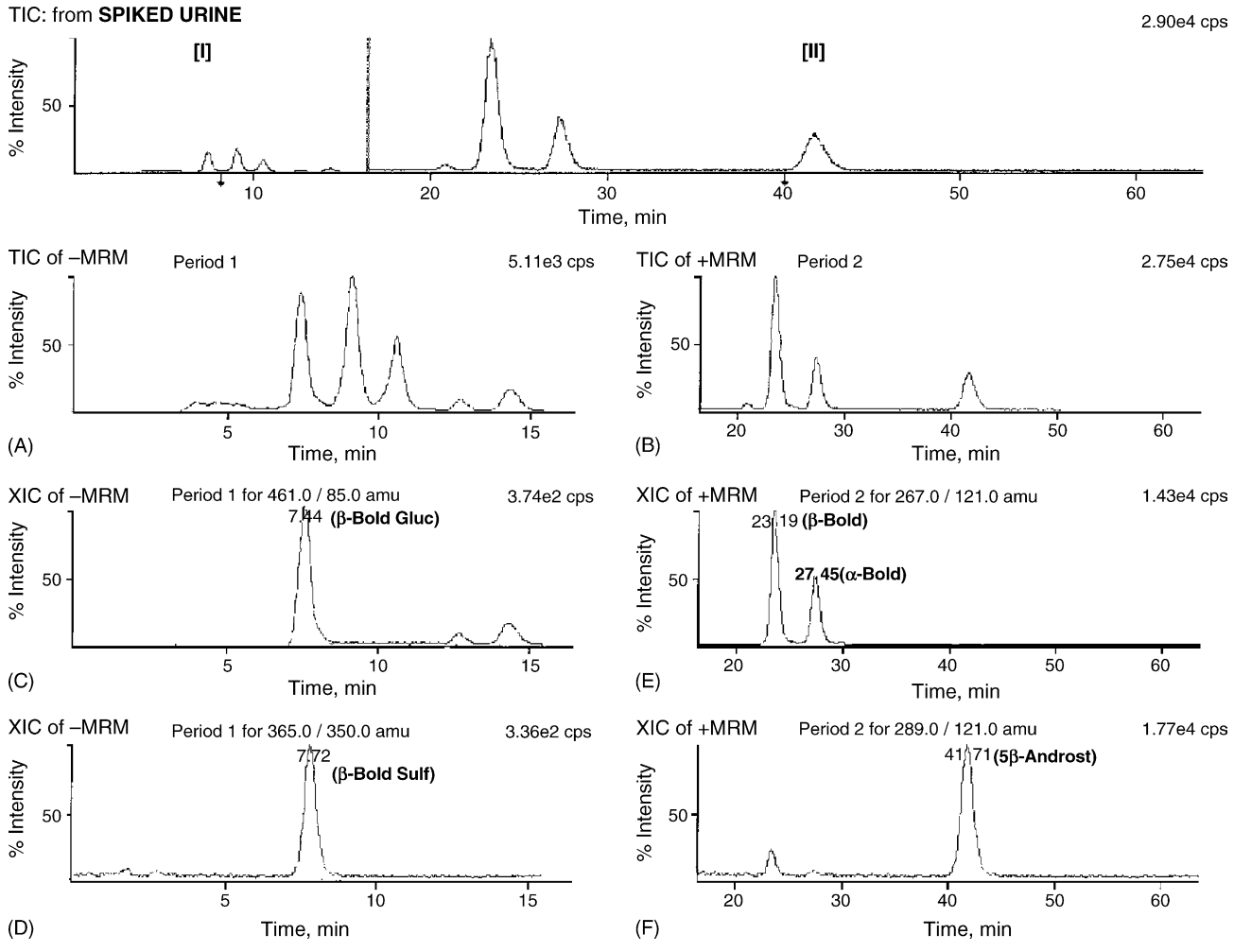

Fig. 6. TIC in MRM mode of a standard mixture of the target analytes added to bovine blank urine (5 $ng\,\mu L^{-1}$ for β-BoldSulf; 2.5 $ng\,\mu L^{-1}$ for the others). Letters [I] and [II] refer to the two different periods of data acquisition. Acquisition time [I] = 0–16.5 min, [II] = 16.5–45 min. (A) TIC in MRM mode the first experiment 1 (negative ionization) in acquisition period [I]; (B) TIC in MRM mode the second experiment 2 (positive ionization) in acquisition period [II]; elution condition as in the text, experimental conditions as in Tables 1 and 2. (C)–(F) XICs (extracted ion currents) from Fig. 6 of the most intense transitions: respectively *m/z* 461–85 for β-boldenone glucuronide, *m/z* 365–350 for β-boldenone sulphate, *m/z* 287–121for α- and β-boldenone and *m/z* 289–121 for 5β-androst-1en-17ol-3one.

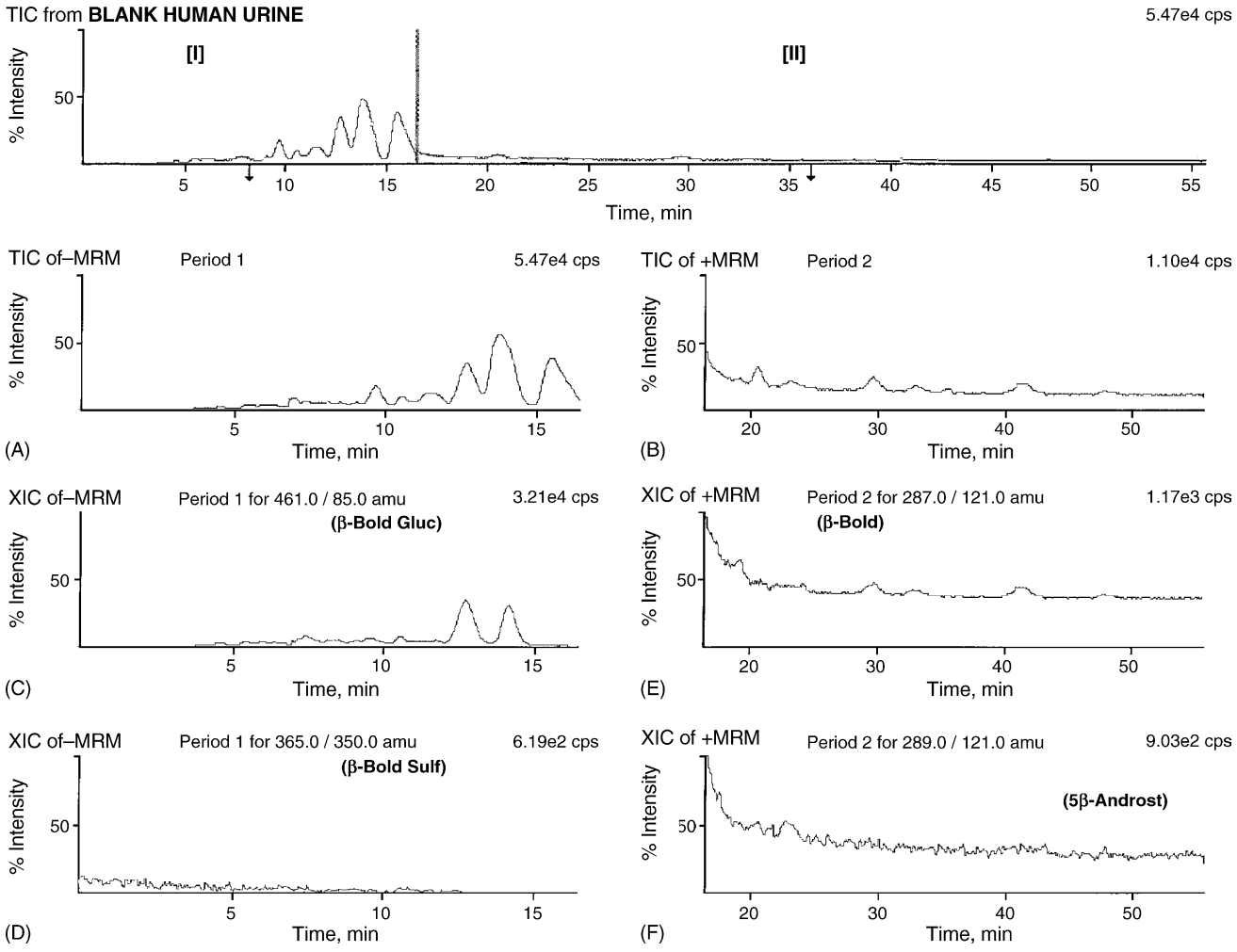

Fig. 7. TIC in MRM mode of a human blank urine sample. Letters [I] and [II] as in Fig. 6. (A) TIC in MRM mode the first experiment 1 (negative ionization) in acquisition period [I]; (B) TIC in MRM mode the second experiment 2 (positive ionization) in acquisition period [II]; elution condition as in the text, experimental conditions as in Tables 1 and 2. (C)–(F) XICs (extracted ion currents) from Fig. 7 of the most intense transitions as in Fig. 6.

3. Results and discussion

3.1. LC–MS and LC–MS/MS analysis

First acquisitions by mass spectrometer were made on quadrupole 1 (Q_1) in full scan mode using standard solutions of β-Bold and β-BoldSulf, α-Bold, 5β-Androst, β-BoldGluc, d_3Bold, d_3ETGluc and d_3TSulf in continuous infusion. The development of the MRM-LC–MS/MS method usually first requires experiments carried out by infusion-MS on standard solutions to determine suitable API parameters for the best sensitivity and S/N ratio, as well as to determine the molecular related ions. Acquisitions with heated nebulizer (HN)-APCI source were also tested, but no advantages were observed. Table 2 summarizes the precursor and the main product ions obtained in MS/MS for the analytes examined. For each class of compounds (free, sulphates, glucuronides) a deuterated internal standard was acquired. MS spectra and MS/MS spectra of free steroids were acquired in positive ionization. The spectrum is dominated by protonated molecular ion $[M+H]^+$, which easily provides the molecular weight. Fig. 2A shows the API mass spectrum of β-Bold. The presence of the peak at m/z 269 $[M-H_2O+H]^+$ and of the adduct at m/z 309 $[M+Na]^+$ is observed. Fig. 3A shows the MS spectrum of 5β-Androst, were the base peak is the pseudo-molecular ion at m/z 289 $[M+H]^+$, but the presence of the adduct $[M+NH_4]^+$ is also relevant. The precursor ions of the investigated compounds were pseudo-molecular ions $[M+H]^+$. The MS/MS spectra of free steroids show common product ions at m/z 121 (Figs. 2B and 3B). Other relevant fragments, at m/z 135 for α-Bold and β-Bold (Fig. 2B) and at m/z 187 for 5β-Androst (Fig. 3B), were observed. Glucuronides and sulphates were analysed both in positive and in negative ionization. Because of their acidic feature, glucuronides and sulphates are more efficiently ionized in negative mode and easily deprotonated in liquid phase. The precursor ion was generally $[M-H]^-$. No significative product ions were observed in the mass spectra, due to the extremely mild API conditions. Fig. 4A and B shows the MS and MS/MS spectra of β-BoldGluc, respectively. The product ions m/z 85 and 113, common to other

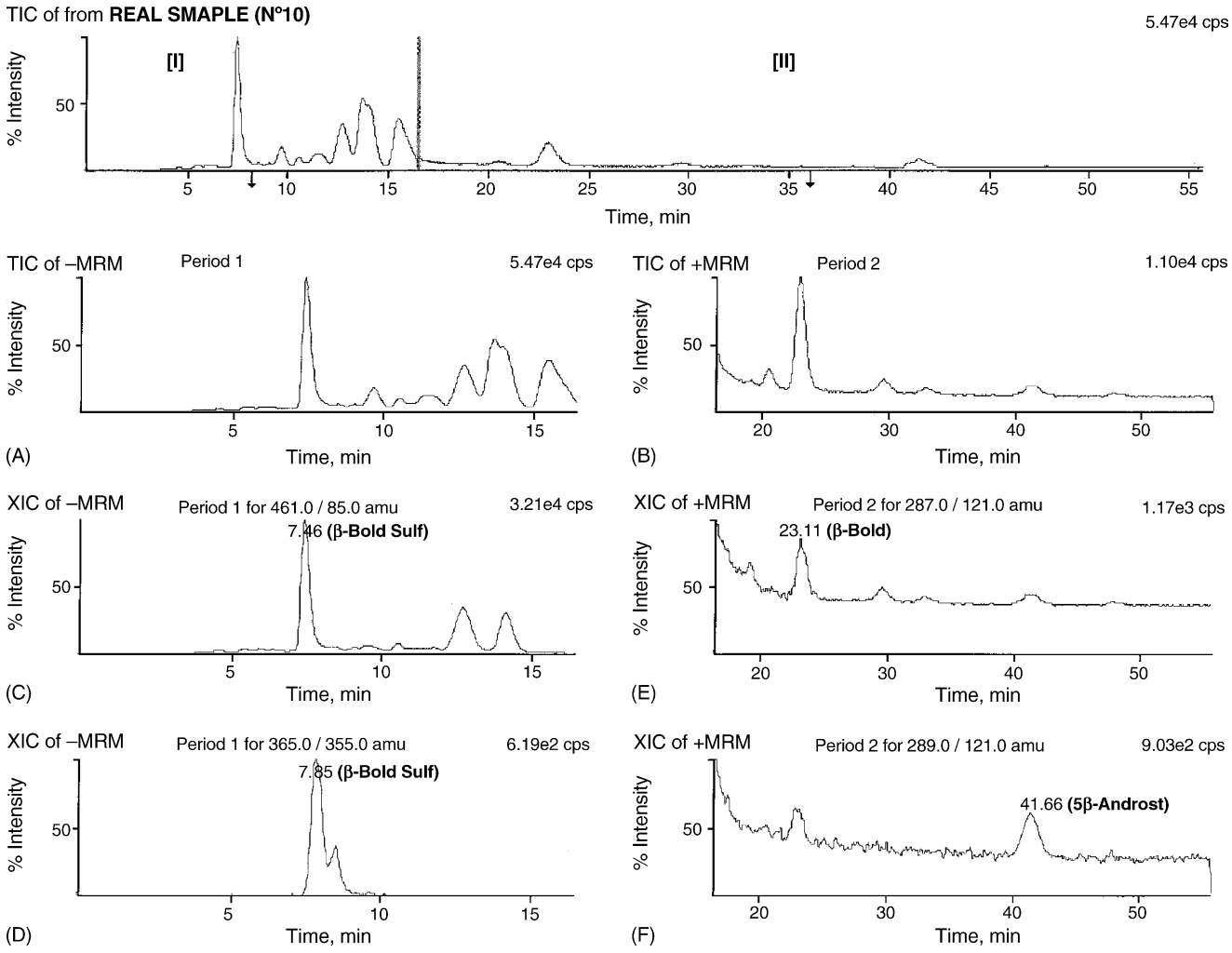

Fig. 8. TIC in MRM mode of a human real sample (29 h after administration). Letters [I] and [II] as in Fig. 6. (A) TIC in MRM mode the first experiment 1 (negative ionization) in acquisition period [I]; (B) TIC in MRM mode the second experiment 2 (positive ionization) in acquisition period [II]; elution condition as in the text, experimental conditions as in Tables 1 and 2. (C)–(F) XICs (extracted ion currents) from Fig. 8 of the most intense transitions as in Fig. 6.

glucuronide compounds, are indicative for the detection of glucuronides, but not diagnostic for unambiguous identification of steroid structure. In fact these product are due to glucuronic moiety [14]. Fig. 5A and B shows the MS and MS/MS spectra of β-BoldSulf. The MS/MS spectrum shows the presence of the ion *m/z* 97 $[HSO_4]^-$, typical fragment of sulphate steroidal compounds, while the fragment at *m/z* 350 derives from the loss of a methyl group $[M - CH_3{-}H]^-$, as previously studied [3]. For all the compounds, electrical parameters for MS/MS experiments were chosen to reduce the intensity of the precursor ion to about 15% (see Table 1).

The LC–MS/MS experiments were performed following the transitions of the two most intense ions (underlined in Table 2) of each analysed compound. Table 3 shows a multiresidue chromatographic analysis performed in total run time of 45 min and divided into two periods. Negative ionization of glucuronides and sulphates was performed from 0 to 16.50 min (first period [I]) with parameters experiment 1 of Table 1; positive ionization of free steroids was performed from 16.50 to 45 min (second period [II]) with parameters of experiment 2 of Table 1.

Fig. 6 shows the total ion chromatogram (TIC) in MRM mode of a standard mixture (5 ng μL^{-1} for β-BoldSulf; 2.5 ng μL^{-1} for the others) of the target analytes added to a blank bovine urine matrix. In particular Fig. 6A shows the TIC of the experiment 1, relative to glucuronides and sulphates, in the first acquisition period [I]. Fig. 6C and D shows the extracted ion currents (XICs) of the most intense transitions of β-BoldGluc and β-BoldSulf respectively. Fig. 6B shows the TIC of the experiment 2, relative to free steroids, in the second acquisition period [II], while Fig. 6E and F the XICs of the most intense transitions of them. All investigated compounds result to be well separated and detected with a good response factor. Figs. 7 and 8 show two human urine sample chromatograms, before and after (29 h) the ingestion of β-Bold: the presence of boldenone and its metabolites is well evident. All human and bovine blank urine samples

Table 5
Recovery values, intra and interday precision for 17β-boldenone and its main metabolites by HPLC-MRM-MS/MS

Analytes	Spike level (ng mL^{-1})	Concentration found (ng mL^{-1})	Accuracy (%)	Intraday precision (R.S.D.%) (n = 6)	Interday precision (R.S.D.%) (n = 18)	Recovery (%) (n = 6)
β-Bold	1.0	0.76	24	7.89	9.64	76
	5.00	4.05	19	5.68	8.09	
	10.00	7.99	20	4.01	7.80	80
	25.00	17.52	30	7.24	7.97	
	50.00	36.11	28	9.05	8.52	72
α-Bold	1.00	0.71	29	7.04	9.54	71
	5.00	3.90	22	4.36	7.11	
	10.00	8.32	17	5.65	7.91	83
	25.00	17.25	31	8.11	10.60	
	50.00	36.12	28	10.02	11.84	72
5β-Androst	1.00	0.71	29	7.04	9.62	71
	5.00	3.62	28	6.80	8.45	
	10.00	7.89	21	7.01	8.78	79
	25.00	20.02	20	8.20	10.63	
	50.00	36.51	27	7.56	9.05	73
β-BoldSulf	1.00	0.72	28	5.56	7.82	72
	5.00	3.91	22	6.91	8.65	
	10.00	9.02	10	4.88	7.43	90
	25.00	19.49	22	8.26	11.43	
	50.00	39.47	21	8.56	10.88	79
β-BoldGluc	1.00	0.74	26	6.76	8.90	75
	5.00	4.05	19	7.91	10.42	
	10.00	8.27	17	7.50	9.87	83
	25.00	19.75	21	7.90	9.34	
	50.00	40.51	19	8.81	10.67	81

analysed show no interfering peaks at the retention times of β-Bold and its metabolites.

3.2. Quantitation results

Five calibration levels in double aliquots, were used to build the calibration curves; 5 μL of extracts were injected three times each. The relative calibration graphs are given respectively by the equations:

- β-BoldGluc: $y = (0.0260 \pm 0.0004)x - (0.011 \pm 0.005)$, $r^2 = 0.999$;
- β-BoldSulf: $y = (0.259 \pm 0.009)x + (0.126 \pm 0.103)$, $r^2 = 0.998$;
- β-Bold: $y = (0.693 \pm 0.012)x + (0.224 \pm 0.154)$, $r^2 = 0.999$;
- α-Bold: $y = (0.430 \pm 0.013)x + (0.170 \pm 0.162)$, $r^2 = 0.998$;
- 5β-Androst: $y = (0.093 \pm 0.001)x - (0.023 \pm 0.018)$, $r^2 = 0.999$.

Good linearity for all the analytes was observed over the concentration range examined. Values of decision limit (CCα) and detection capability (CCβ), calculated as

Table 6
Excretion values of β-boldenone and its main metabolites

Sampling (h)	β-BoldGluc		β-BoldSulf		β-Bold		5β-Androst	
	ng mL^{-1}	R.S.D.	ng mL^{-1}	R.S.D.	ng mL^{-1}	R.S.D.	ng mL^{-1}	R.S.D.
0	n.d.		n.d.		n.d.		n.d.	
2	6.48×10^4	8.58	1.15	9.05	6.40	7.38	n.d.	
5	13.8×10^4	7.67	15.5	8.72	30.3	8.06	1.74	6.88
15	7.04×10^4	7.58	10.3	9.60	22.0	8.36	2.50	6.96
19	2.84×10^4	8.32	6.99	8.86	10.9	9.14	2.81	7.43
22	2.70×10^3	9.03	5.00	8.45	1.03	8.96	14.5	6.99
29	1.19×10^3	8.76	3.79	9.20	n.d.		8.98	7.77
39	6.20×10^2	7.21	2.23	7.68	n.d.		9.89	8.06
63	1.60×10^2	8.54	0.98	8.25	n.d.		5.21	7.35
87	1.51	9.76	n.d.		n.d.		1.46	7.75
111	n.d.		n.d.		n.d.		n.d.	

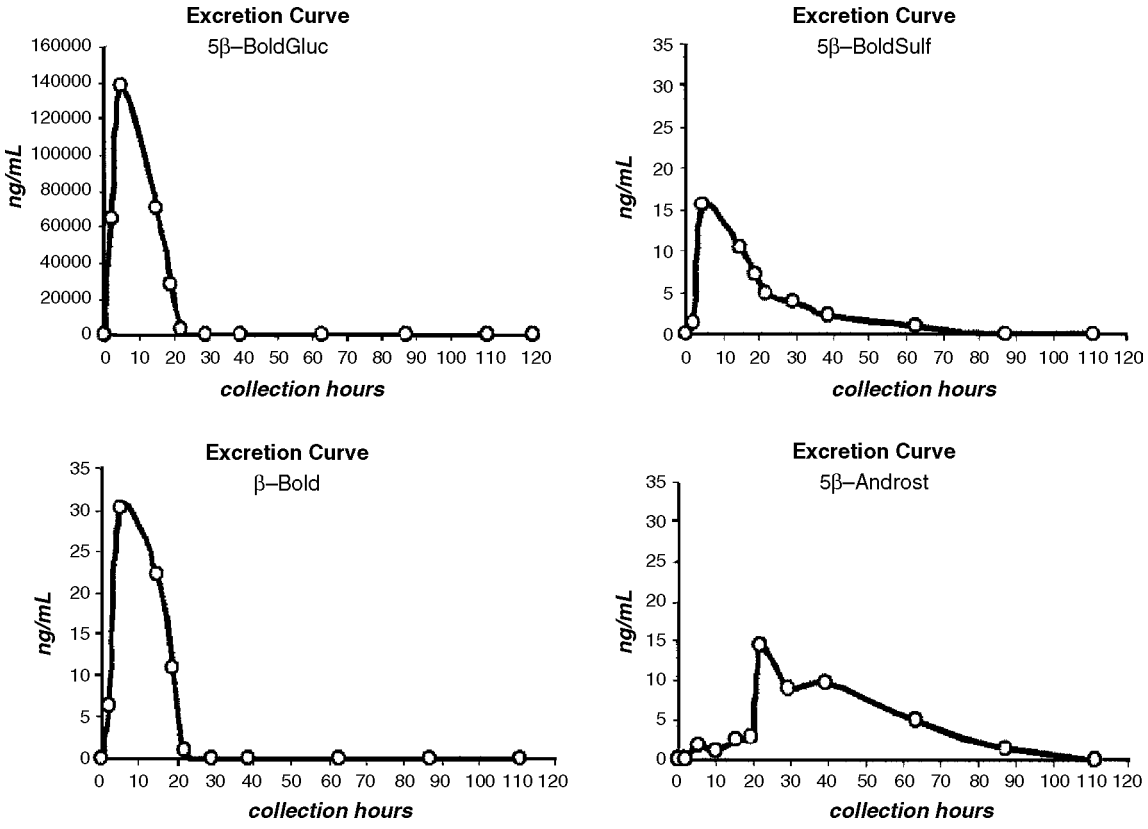

Fig. 9. Excretion curves of β-boldenone and its main metabolites in human urine of the volunteer, who referred the ingestion of a single dose of 70 mg (1 mg kg^{-1} body weight) of β-boldenone.

described above, are reported in Table 4. Calibration curves were used to evaluate both intraday (repeatability) and interday (intermediate) precision. Precision data were evaluated injecting spiked samples six times each at different concentration levels over three different days. Fresh extracts were processed from a urine sample containing the metabolites studied. Table 5 summarizes the intra and interday precision of the analytical procedure and the recovery results in urine. The intraday precision ($n = 6$) ranged from 4 to 10%, the interday precision ($n = 18$) from 7 to 12%. Average recovery ($n = 6$) was above 70% for each analyte. The procedure was accurate for all the concentration tested, as shown in Table 5, and ranged from 5 to 15%.

3.3. Application to real samples

This method was applied to human urine. Samples were prepared in duplicate and injected in triplicate into the LC–MS/MS. Human urinary excretion of boldenone metabolites after oral ingestion with a single dose of 70 mg of boldenone, was monitored. Blank urine samples were collected for 3 days before the administration of boldenone; no metabolite was detected. After boldenone ingestion, a different tendency in excretion between metabolites was observed, as shown in Table 6. Conjugated glucuronide was the main metabolite of boldenone in humans, whose excretion was very high; the highest value was measured around 19 h after administration. To analyse this metabolite, samples were diluted with blank urine sample up to 10,000 times before the extraction. The excretion values of the other analytes were of the same magnitude order. Fig. 9 shows the excretion profile of the investigated analytes.

β-BoldSulf excretion reaches a maximum at 5 h from administration, as well as for β-Bold and β-BoldGluc, while 5β-Androst concentration starts to increase substantially after 19 h, whereas the trends of the others are decreasing. At the fifth day after the treatment the concentration of all metabolites falls below the detection limit (CCα) values. Free α-boldenone was not detected in humans.

4. Conclusion

A multiresidue liquid chromatographic–tandem mass spectrometric method has been developed for direct analysis of free and conjugated β-boldenone and its main metabolites, in urine samples. Sample preparation is easy and fast to perform because it does not require particular analytical treatments. The extracts are directly analysed by reversed phase LC–MS/MS in MRM mode, in two different acquisition periods, by different ionization modes. Moreover, other relevant advantages of the present method over GC–MS procedures include the elimination of hydrolysis and derivatization steps

prior to the chromatographic separation. This method allows to determine the ratios between conjugated and free fractions, thus reducing the risk of false positive or misleading results.

It is particularly valuable for investigating the excretion profile of β-boldenone and its metabolites into biological fluids. It can result particularly helpful for such laboratories involved in official routine analysis for monitoring the illegal use of anabolic steroids.

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