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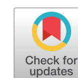

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ABSTRACT

The aim of this paper is to present the development and validation of a high-resolution full scan (HR-FS) electrospray ionization (ESI) liquid chromatography coupled to quadrupole Orbitrap mass spectrometer (LC/Q/Orbitrap MS) platform for the screening of prohibited substances in human urine according to World Antidoping Agency (WADA) requirements. The method was also validated for quantitative analysis of six endogenous steroids (epitestosterone, testosterone, 5α-dihydrotestosterone, dehydroepiandrosterone, androsterone and etiocholanolone) in their intact sulfates form. The sample preparation comprised a combination of a hydrolyzed urine liquid–liquid extraction and the dilute & shoot addition of original urine in the extracted aliquot. The HR-FS MS acquisition mode with Polarity Switching was applied in combination of the Quadrupole-Orbitrap mass filter. The HR-FS acquisition of analytical signal, for known and unknown small molecules, allows the inclusion of all analytes detectable with LC–MS for antidoping investigations to identify the use of known or novel prohibited substances and metabolites after electronic data files' reprocessing. The method has been validated to be fit-for-purpose for the antidoping analysis.

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1. Introduction

The World Antidoping Agency (WADA) is an international agency, which has the task to prevent and monitor any doping in sport. Sports movements and governments around the world collaborate with WADA to prevent, deter and define the rules with which such doping practices and duplicitous behavior of athletes using doping substances in sports can be challenged and fought on a united and global basis. The WADA prohibited List [1] includes among other substances pharmacological classes of drugs, either exogenous substances or endogenous substances administered exogenously, which define doping. The WADA antidoping control activity is based on the WADA accredited laboratories operating under the specifications of the WADA International Standard of Laboratories (ISL) [2]. In addition to these criteria, WADA publishes technical documents which provide analytical requirements to be fulfilled by laboratories. Examples are the Technical documents for Minimum required performance levels (MRPL) [3] which describes the main specifications for the analysis and detection of exogenous substances in human urine. Another example is the WADA Endogenous Anabolic Androgenic Steroids technical document TD2016EAAS [4] that provides the specifications for the analysis of the endogenous profile of small molecules present in urine, such as testosterone and testosterone-likes substances used for doping. The evaluation of the steroidal profile is done by the adoptive module of Urinary Athlete Biological Passport and a thorough review of the steroidal ABP has been published [5].

The doping control laboratories implement different analytical techniques in order to be able to detect a large variety of classes of prohibited substances. For that purpose, a Mass spectrometer (MS) coupled either with Gas Chromatography (GC/MS) or Liquid Chromatography (LC/MS), is the most standard technology used to ensure compliance with [1–4] in the analysis of urine samples. The use of GC/MS and LC/MS is complementary for the specifications [1–4]. The GC/MS is used as standard technique for small molecules, in particular for anabolic steroids, because these compounds provide a weak ionization yield when the atmospheric LC/MS interfaces like the electrospray ionization (ESI) is used [6].

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Several studies have been conducted by LC/MS concerning the screening for small molecules following the WADA's technical documents. The urine sample preparation can either be applied as direct urine LC/MS analysis using dilute &shoot (D&S) approach [7,8] or following urine extraction procedures such as [9] applied during the Olympics games in 2012 in the London Olympic Laboratory. The LC/MS systems are operated either in tandem MS/MS mode [7] or HR-FS acquisition that either with Quadrupole/Time-Of-Flight (LC/QTOF/MS) [10] or Orbitrap (LC/Q/Orbitrap/MS) [9] instruments.

The WADA prohibited List [1] includes prohibited pharmacological classes and the therein particular molecules are referred as examples, however it is open for other substances with a similar chemical structure or similar biological effect. Consequently, the screening procedure should be generic and comprehensive for all compounds detectable by the molecular profiling platform such as LC/MS; this should include the sample preparation and HR-FS MS acquisition of known and unknown molecules. Moreover, HR-FS MS acquisition and polarity switching for positive and negative ionized molecules are important parameters to be considered [11] to obtain comprehensive molecular profile detectable by an LC/MS system.

The limitations of the steroid profiles in anti-doping applications have been addressed for several years [5] and studies have been conducted to improve its effectiveness as a forensic biomarker tool to monitor and identify testosterone and analogs abuse [12]. In 1996, a study conducted by Dehennin et al. [13] proposed the introduction of epitestosterone sulfate in the athlete's steroid profile as a ratio of testosterone glucuronide to the total epitestosterone fractions. In another study, several additional endogenous steroid forms from those referred in [4] have been identified [14,15]. Untargeted metabolomics studies for the detection of steroid biomarkers have been performed as well [16–18]. Complementary to these studies [13], the sulfate fraction of endogenous steroid profile, in addition to the free and glucuronide fractions of [4], has been studied by several research groups in relation to the genotype for the UGT2B17 enzyme., where it is proposed that in case of exogenous testosterone intake, the ratio between androsterone glucuronide and epitestosterone glucuronide as a complement to testosterone and epitestosterone glucoronide ratio can be used especially in UGT2B17 *del/del* individuals. In addition, etiocholanolone sulfate was excreted at significantly higher levels in those individuals [19–22].

The detection and quantification of the intact conjugated endogenous and exogenous steroids by LC/MS has been studied and reviewed by several groups since '90s [23] and [24]. The quantification of glucuronidated and sulfated endogenous steroids by LC/MS has been reported earlier [25]. A similar approach has been followed in other studies for the analysis of intact conjugated steroids of endogenous and exogenous origin [26–28].

This article focuses on the application and validation of an LC/MS screening for small molecules using HR-FS acquisition with polarity switching. The method has been developed using an LC/Q/Orbitrap MS with a combination of D&S and a solvent extraction procedure for urine sample preparation. The method is intended to be generic in order to allow the detection of known designer drug urine metabolites [29] together with quantification of six intact endogenous sulfate steroids: epitestosterone (ES), testosterone (TS), 5α-dihydrotestosterone (5αDHTS), dehydroepiandrosterone (DHEAS), androsterone (AS) and etiocholanolone (ETIOS) using negative ionization mode, with possibility to detect unknown exogenous substances. This method is developed as a complementary for a previously published GC/QTOF/MS screening method [30]. Accordingly, the antidoping laboratories will have a completed GC and LC HR FS screening method with simultaneous measurement of steroid profile according to WADA Code specifications [4] and detection of intact sulfo-conjugated metabolites of Phase II metabolism.

2. Experimental

2.1. Material and methods

2.1.1. Reagents

β-Glucuronidase from *Escherichia Coli* (E.coli), for the enzymatic hydrolysis, was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Methanol (HPLC grade), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3) and acetonitrile were supplied by Sigma Aldrich (Darmstadt, Germany). Ethylacetate was supplied by Merck (Darmstadt, Germany). Formic acid (HCOOH) and 5 M ammonium formate ($HCOONH_4$) were from Agilent Technology.

2.1.2. Reference materials

The Reference Materials used in the current study were from Sigma Aldrich (Darmstadt, Germany), Steraloids (Newport, USA), LGC (Wesel, Germany), Toronto Research Chemicals TRC (Toronto, Canada), Cerilliant (Round Rock, USA), Lipomed (Arlesheim, Switzerland), Cayman (USA), NMI Australian Government National Measurement Institute (Pymble, Australia). Reference compounds that are not commercially available were provided by WADA Accredited Laboratories of Cologne (Germany), Ghent (Belgium), Rome (Italy), Athens (Greece), Barcelona (Spain) and Sydney (Australia), and the World Association of Antidoping Scientists (WAADS).

The deuterated internal standards of morphine 3-β-D-glycuronide-d3 and mefruside-d3 used for qualitative screening were obtained from TRC (Toronto, Canada). The deuterated internal standards used for quantitative screening were: 5α-Dihydrotestosterone Sulfate-d3 (5α-DHT-d3), Testosterone sulfate-d3 (TS-d3) and Androsterone sulfate-d4 (AS-d4) were all purchased from NMI (Pymble, Australia).

Stock standard solutions of the standard analytes were individually prepared in methanol. For validation purposes, working standard solution containing the standard analytes was prepared in methanol by subsequent dilutions of the stock solutions. The sulfo-conjugated steroids analytes were included in a different working solution. All solutions were stored at −20 °C in amber vials until use.

2.1.3. Sample preparation

To 5 mL urine, 1 mL phosphate buffer at pH 7 (Na_2HPO_4 0.8 M and NaH_2PO_4 0.4 M), 100 μL of β −Glucuronidase from *E. coli* and 50 μL of a methanolic solution of mefruside-d3 (10 $\mu g\,mL^{-1}$) morphine-3β-D-glucuronide-d3 (5 $\mu g\,mL^{-1}$) were used as internal standards (ISTDs). After addition of the internal standards to the sample, the mixture was incubated for 1.5 h at 50 °C. After hydrolysis, the pH was adjusted to 9–10 with a mixture of sodium hydrogen carbonate and sodium carbonate (10:1)(w/w). A liquid–liquid extraction with 5 mL of ethylacetate was performed using anhydrous sodium sulfate as salting-out agent. After centrifugation, the organic layer was separated from the aqueous phase by freezing the sample at −80 ° C. The organic phase was then acidified with 200 μL of 3 M acetic acid in ethylacetate and evaporated under nitrogen stream at 50 °C. The remaining residue was reconstituted with 200 μL of 80:20 LC mobile phase A/B (v/v) (see description of eluent at Section 2.1.4.1). 20 μL of the original urine were mixed with the reconstituted extract and 5 μL of the mixture were injected to the LC/MS system for analysis.

2.1.4. Instrumentation

2.1.4.1. Chromatographic conditions. A Dionex UHPLC system (Thermo Scientific, Bremen, Germany) was used for the chromatographic separation. The system consisted of a vacuum degasser, a high-pressure binary pump, an autosampler with a temperature controlled sample tray set at 7 °C and a column oven set at 30 °C. Chromatographic separation was performed at 30 °C using a Zorbax Eclipse Plus C18 column (100 × 2.1 mm i.d., 1.8 μm particle size; Agilent Technologies). The mobile phase consisted of 5 mM ammonium formate in 0.02% formic acid (solvent A) and a mixture of acetonitrile/water (90:10 v/v) containing 5 mM ammonium formate and 0.01% formic acid (solvent B). A gradient elution program was employed at a constant flow rate of 0.2 mL min^{-1} with solvent B starting at 5% for 1 min, increasing to 32% in 2.5 mins and staying constant at 32% for 13 mins and then, set back to 100% within 8 mins, where the eluent composition was held for 2.5 min before returning to 5% within 1 min. The analysis run time was 28 min and the post-run equilibrium time was 4 min. The injection volume was 5 uL.

2.1.4.2. Mass spectrometric conditions. The mass spectrometer was a QExactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany) operated in the positive–negative polarity switching modes and equipped with a heated electrospray ionization (HESI) source. Source parameters were: sheath gas (nitrogen) flow rate, auxiliary gas (nitrogen) flow rate and sweep gas flow rate: 40, 10 and 1 arbitrary units respectively, capillary temperature: 300 °C, heater temperature: 30 °C, spray voltage: +4.0 kV (positive polarity) and −3.8 kV (negative polarity). The instrument operated in FS mode from *m/z* 100–1000 at 17,500 resolving power and duty cycle of 100 ms for both polarities and in MS/MS mode from *m/z* 100–1000 at 17,500 resolving power and duty cycle of 62 ms (product ion mode) for the analytes that showed matrix interfering peaks in the FS mode. The automatic gain control (AGC) was to 10^6. The mass calibration of the Orbitrap instrument was evaluated in both positive and negative modes daily and external calibration was performed prior to use following the manufacturer's calibration protocol.

2.1.5. Method validation

2.1.5.1. Qualitative method validation. The following validation parameters have been included in the experimental protocol and have been determined for all substances of interest. The identification capability is the ability of the method to detect the substance at the 50% of the Minimum Required Performance Levels (MRPL) concentration [3] in ten (10) different urine routine antidoping negative samples. In this study, 10 different urine matrices were spiked with the standard multicomponent solutions at concentrations equal to the 50% of MRPL or below (See Table 1). The specificity of the method was determined using the same experimental data obtained to assess the identification capability after checking the detection of substance of interest in the selected ion chromatographic time window obtained at specific *m/z* value. The absence of matrix interferences was evaluated by the analysis of the urine samples (blank sample) used also for the identification capability without being spiked. The Limit of detection (LOD) was determined as the lowest detectable concentration per substance with subsequent dilution cycles up to 1% of the concentration of the respective MRPL concentration. Sample carry over was evaluated by the detection of substances in blank urine samples injected after the injection of a urine sample spiked with the analytes at a concentration 20 times higher than the respective MRPL level. The target analyte recovery was determined for some selected analytes listed in Table 1 by comparing of the MS signal of the standards at 50% MRPL concentration spiked in urine sample prior sample preparation to the MS signal in urine sample spiked after sample preparation and prior evaporation step. Similarly, the matrix effects were determined for the same analytes by comparing the MS signal at the 50% MRPL concentration urine sample spiked after the extraction to the MS signal of the analytes spiked in the reconstitution solvent at equal concentrations. Matrix effects and extraction recovery were evaluated in 5 different urine matrices. The mass accuracy has been calculated in one urine sample used for identification capability experiment to assess eventual mass bias in the entire range of the full scan MS acquisition, for both ionization polarities.

2.1.5.2. Quantitative validation. For the endogenous steroids quantitative validation, the following validation parameters have been determined, in five experimental days. The linearity range of the calibration curves for each sulfate steroid in various concentrations has been evaluated. Seven points' calibration curves for quantification purposes were generated from spiking standards in steroid depleted urine samples. The depleted urine sample was prepared as follow: blank urine was collected from female children and was depleted from endogenous steroids using C18 SPE extraction. The linearity of the calibration curves were assessed in the concentration range of 1–200 ng mL^{-1} for TS, ES and 5αDHTS; 10–2000 ng mL^{-1} for AS and EtioS and DHEAS. The calibration curves were built from the peak area ratio of sulfo-conjugates steroids and TS-d3 for TS and ES, AS-d4 for AS EtioS and DHEAS and TS-d3 for 5αDHTS. The intermediate precision and the accuracy (% bias) for each sulfate steroid has been determined by the analysis of the spiked quality control (QC) samples at two concentration levels (low and high), which were prepared during 5 different experimental days and injected twice for each calibration curves. Both the intermediate precision and bias from the QC samples were used to estimate the combined Measurement Uncertainty (MU) for each steroid according to the following equation:

$$\text{Combined } MU = \sqrt{intermediate\,precision^2 + Bias^2}$$

3. Result and discussion

3.1. Method development and optimization

The method development was oriented to create a comprehensive analytical procedure to cover as many different small molecules as possible. Liquid-liquid extraction by ethylacetate as organic solvent was selected in order to achieve recovery of the sulfates phase II metabolites that remain unaffected after the enzymatic hydrolysis by the β-Glucuronidase from *E.Coli*. The extracted and reconstituted urine aliquot was enriched by 10% (v/v) original urine sample as part of D&S approach. The combined sample was analyzed by LC/MS. The D&S part of the analyzed sample is important for small molecules that are not extracted in the extraction process, such as melidonium, ethylglycuronide, FG4592, AICAR, finasteride carboxylic acid metabolite and ritalinic acid. The use of the ethylacetate as extraction solvent and the D&S contribution of the analyzed sample do not contaminate the LC/MS system and do not change the maintenance schedule of the LC/MS system compared with an LC/MS system used to analyze extracted aliquots only. Moreover, the liquid chromatographic column was replaced after approximately 1200 urine sample injections.

One of the main aspects during method development was to optimize the liquid chromatographic separation of AS to ETIOS. To achieve this goal in the current instrumental configuration and settings, several isocratic sections as part of the overall eluent gradient program, were tested to achieve the liquid chromatographic method described in the experimental part 2.1.4.1.

The MS acquisition was performed using an Orbitrap mass analyzer in order to maximize the advantages from simultaneous

Table 1
Theoretical and experimental data used for qualitative validation of 304 analytes.

No.	Compound	Spiked Conc. (ng mL^{-1})	RT[g] (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD[a] (ng mL^{-1})	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
	Diuretics and Masking Agents									
1	Acetazolamide	100	5.1	[M−H]−	220.9809	[M+H]+	222.9954	2		
2	Althiazide	25	13.9	[M−H]−	381.9762	[M+NH4]+	401.0168	5		
3	Amiloride	100	5.0	[M−H]−	230.0552	[M+H]+	228.0406	2		
4	Asozemide	50	15.3	[M−H]−	369.0001			1	−19.3 (7.2)	82.5 (4.8)
5	Bendroflumethiazide	100	21.4	[M−H]−	420.0305	[M+NH4]+	439.0711	2		
6	Benzthiazide	100	19.4	[M−H]−	429.9762	[M+H]+	431.9908	2		
7	Brinzolamide	50	6.45	[M+H]+	384.0716			2.5	−29.5 (8.3)	93.3 (6.3)
8	Bumetanide	100	21.9	[M+H]+	365.1166	[M−H]−	363.1020	2		
9	Buthiazide	100	15.1	[M−H]−	352.0198	[M+NH4]+	371.0604	2		
10	Canrenone	100	22.8	[M+H]+	341.2111			2		
11	Chlorothiazide	100	5.7	[M−H]−	293.9415			2		
12	Chlorthalidone	100	7.6	[M−H]−	337.0055	[M+H]+	339.0201	2		
13	Clopamide	100	9.0	[M+HCOO]−	390.0896	[M+H]+	346.0987	2		
14	Conivaptan	50	19.2	[M+H]+	499.2129			1	−20 (21)	91.6 (6.5)
15	Cyclopenthiazide	100	21.5	[M−H]−	378.0355			2	−25.1 (4.8)	28.3 (9.3)
16	Cyclothiazide	100	20.5	[M−H]-	388.0198			10	−21.5 (3.8)	26.8 (9.4)
17	Dichlorphenamide	100	7.8	[M−H]−	302.9073			2		
18	Dorzolamide	100	5.3	[M+H]+	325.0345	[M−H]−	323.0199	2	−48 (12)	84 (14)
19	Epitizide	100	16.5	[M−H]−	423.9480			2	−2.5 (7.6)	35.1 (8.8)
20	Eplerenone	50	14.5	[M+H]+	415.2115			1	+5 (18)	101 (12)
21	Ethacrynic acid	100	20.9	[M−H]−	301.0040	[M+H]+	303.0185	2		
22	Furosemide	100	11.8	[M−H]−	329.0004			2		
23	Hydrochlorothiazide	100	6.0	[M−H]−	295.9570	[M+NH4]+	314.9978	2		
24	Hydroflumethiazide	100	7.0	[M−H]−	329.9836			1		
25	Lixivaptan	50	24.2	[M−H]−	472.1234			5		
26	Mebutizide	50	22.0	[M−H]−	380.0511			5		
27	Methazolamide	50	6.2	[M+H]+	237.0111			1	+5 (15)	42.2 (7.4)
28	Methylchlorthiazide	25	11.0	[M−H]−	357.9495	[M+NH4]+	376.9901	2		
29	Metolazone	100	12.8	[M+H]+	366.0674	[M−H]−	364.0528	2		
30	Indapamide	100	16.4	[M+H]+	366.0674	[M−H]−	364.0528	2		
31	Mozavaptan	50	9.6	[M+H]+	428.2333			1	−12 (12)	92.8 (5.6)
32	Piretanide	100	21.0	[M+H]+	363.1006	[M−H]−	361.0864	2		
33	Polythiazide	100	21.4	[M−H]−	437.9636	[M+NH4]+	457.0042	2		
34	Probenecid	25	21.7	[M−H]−	284.0962			1		
35	Quinethazone	50	6.3	[M+H]+	290.0361			1	+23.2 (7.2)	95.2 (5.1)
36	Relcovaptan	50	22.1	[M+H]+	637.1285			1	+21.5 (7.1)	96.1 (4.6)
37	Thiazide Artifact ACB[e]	100	5.4	[M−H]−	283.9572			2		
38	Thiazide Artifact ATFB[f]	100	6.7	[M−H]−	317.9835			2		
39	Tolvaptan	100	23.0	[M+H]+	449.1626	[M−H]−	447.1481	2	−44.2 (6.8)	94 (10)
40	Torasemide	100	8.65	[M+H]+	349.1330	[M−H]−	347.1183	2	−45.8 (9.9)	78.1 (6.3)
41	Triamterene	100	6.0	[M+H]+	254.1147			2		
42	Trichlormethiazide	25	9.9	[M−H]−	377.8949	[M−H]−[c]	379.892	1		
43	Xipamide	20	21.6	[M−H]−	353.0368	[M+H]+	355.0510	1		
	Anabolic Agents									
44	16β-Hydroxyprostanazol	2.5	11.5	[M+H]+	329.2224			1.25		
45	3-Hydroxyprostanazol	2.5	15.2	[M+H]+	329.2224			0.6		
46	4-Hydroxyprostanazol	2.5	16.9	[M+H]+	329.2224			0.6		
47	6β-Hydroxyfluoxymesterone	2.5	7.6	[M−H]−	397.2032			0.25		
48	Boldenone	2.5	19.7	MSMS (+)	287.2 >121.0650			0.25		
49	Methyldienolone	2.5	20.5	MSMS (+)	287.2 >135.0806			0.6	−63.2 (6.4)	86.7 (6.8)
50	Boldione	2.5	21.2	[M+H]+	285.1849			0.25		
51	Bolasterone	2.5	22.7	[M+H]+	317.2475			0.25		
52	Calusterone	2.5	23.0	[M+H]+	317.2475			0.25		

Table 1 (*Continued*)

No.	Compound	Spiked Conc. (ng mL^{-1})	RT[g] (min)	Main Ion	Ion Mass (*m*/*z*)	Secondary Ion	Ion Mass (*m*/*z*)	LOD[a] (ng mL^{-1})	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
53	9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (Fluoxymesterone MT)	2.5	23.8	[M+H]+	319.2068	[M+HCOO]−	363.1977			
54	Fluoxymesterone	2.5	15.4	[M+H]+	337.2173	[M+HCOO]−	381.2083	0.5		
55	11α-Hydroxymethyltestosterone (Formebolone MT)	2.5	11.1	[M+H]+	319.2268			0.5		
56	2-hydroxymethyl-11α,17β-dihydroxy-17α-methylandrost-1,4-diene-3-one (Formebolone MT)	2.5	8.3	MSMS (+)	347.2 >147.0804 347.2 > 281.1898			0.25		
57	Gestrinone	2.5	22.3	[M+H]+	309.1849			0.05		
58	16β-Hydroxystanozolol	1	16.0	[M+H]+	345.2537			1		
59	4β-Hydroxystanozolol	1	15.1	[M+H]+	345.2537			0.5		
60	3′-Hydroxystanozolol	1	14.2	[M+H]+	345.2537			1		
61	18-nor-17β-hydroxymethyl,17α-methylandrost-1,4,13-trien-3-one (Methandienone long term-MT)	1	21.9	MSMS (+)	299.2 >147.0804 299.2 > 269.1894			0.1	−71.2 (9.4)	98 (15)
62	Methyltrienolone	2.5	20.4	MSMS (+)	285.2 >227.1430 285.2 > 159.0804			0.1	−67.1 (7.9)	90.3 (4.8)
63	Oxandrolone “night watch” MTs	*	22.1/22.7	MSMS (+)	305.2 >275.2006 305.2 > 257.1900					
64	Stanozolol	1	22.3	[M+H]+	329.2584			1		
65	THG	2.5	23.3	[M+H]+	313.2162			0.25		
66	Trenbolone	2.5	17.2	[M+H]+	271.1693			0.05		
67	17-Epitrenbolone	2.5	19.8	[M+H]+	271.1693			0.05		
68	Mesterolone sulfate MT	*	16.9	[M−H]−	383.1898					
69	Mesterolone hydroxy sulfate MT	*	8.5	[M−H]−	399.1847					
70	Ractopamine	10	6.2	[M+H]+	302.1751	[M−H]−	300.1605	0.5		
71	LGD4033	1	23.7	[M+HCOO]−	383.0836			0.1	−28.4 (4.9)	89.6 (5.4)
72	S1	2.5	23.9	[M−H]−	401.0766			0.1		
73	S4 (Andarine)	2.5	21.9	[M−H]−	440.1075	[M+H]+	442.1219	0.05	−13.1 (5.4)	92.9 (9.1)
74	O-dephenylandarine (Andarine MT)	2.5	14.9	[M−H]−	307.0547			0.025		
75	S9	2.5	24.4	[M−H]−	417.0473			0.1		
76	S22 (Ostarine)	2.5	23.0	[M−H]−	388.0915	[M+NH4]+	407.1320	0.05	−14 (11)	88 (13)
77	O-dephenylostarine (Ostarine MT)	2.5	11.5	[M−H]−	287.0647			0.025		
78	Zilpaterol	2.5	4.7	[M+H]+	217.0972			0.25		
	BETA-2 Agonists									
79	Bambuterol	10	7.1	[M+H]+	368.2182			0.2		
80	Brombuterol	5	7.2	[M+H]+	366.9838			0.1	−15 (13)	88.7 (7.1)
81	Cimaterol	5	4.8	[M+H]+	220.1444	[M+H−H_2O]+	202.1338	0.1	+13 (13)	85.4 (4.1)
82	Cimbuterol	5	5.4	[M+H]+	234.1601					
83	Clenpenetrol	5	7.5	[M+H]+	291.1025			0.1	−23 (17)	86.2 (6.3)
84	Clenproperol	5	6.3	[M+H]+	263.0712			0.1	−25 (16)	87.1 (3.8)
85	Fenoterol	10	5.5	[M+H]+	304.1543					
86	Fenoterol Artifact		5.7	[M+H]+	316.1543					
87	Formoterol	20	6.6	[M+H]+	345.1809	[M−H]−	343.1663	0.4		
88	Higenamine	10	5.3	[M+H]+	272.1272			0.5		
89	Coclaurine	10	5.8	[M+H]+	286.1438			0.5		
90	Indacaterol	5	12.2	[M+H]+	393.2173			0.5	−35 (19)	77 (11)
91	Mabuterol	5	7.7	[M+H]+	311.1133			0.1	−21 (11)	87.8 (5.2)

Table 1 (*Continued*)

No.	Compound	Spiked Conc. (ng mL^{-1})	RT[g] (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD[a] (ng mL^{-1})	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
92	Mapenterol	5	9.0	[M+H]+	325.1289			1.0	−30 (16)	85.5 (8.1)
93	Olodaterol	5	7.0	[M+H]+	387.1914			0.1	−24 (17)	88.3 (5.8)
94	Pirbuterol	5	4.6	[M+H]+	241.1547			2.0		
95	Procaterol	5	5.1	[M+H]+	291.1703			0.5		
96	Reproterol Artifact	5	5.3	[M+H]+	402.1772			0.5		
97	Ritodrine	10	5.7	[M+H]+	288.1594			0.5		
98	Salbutamol	100	4.7	[M+H]+	240.1594	[M−H]−	238.1449	1.0		
99	Salmeterol	10	20.8	[M+H]+	416.2795	[M−H]−	414.2650	0.2		
100	Terbutaline	10	4.7	[M+H]+	226.1438	[M−H]−	224.1292	0.2		
101	Tulobuterol	5	6.6	[M+H]+	228.1147			0.1	−24 (14)	86.1 (4.3)
102	Vilanterol	5	18.6	[M+H]+	486.1809			0.1	−31 (16)	82 (13)
	HORMONE AND METABOLIC MODULATORS									
103	6α-Hydroxytestosterone (6-OXO MT)	10	8.2	[M+H]+	305.2111			0.5	−50 (12)	88.7 (4.2)
104	Anastrozole	10	16.0	[M+H]+	294.1713			0.2		
105	Androsta-1,4,6-triene-3,17-dione (ATD)	8	21.0	[M+H]+	283.16930			0.2		
106	17β-Hydroxyandrost-1,4,6-triene-3-one (ATD MT)	10	18.3	[M+H]+	285.1849			0.2		91.5 (6.0)
107	Clomiphene 4-hydroxy MT	*	21.7	[M+H]+	422.1881					
108	Clomiphene-hydroxy-methoxy MT	*	21.8/22.1	[M+H]+	452.1987					
109	Clomiphene-N-desethyl-hydroxy MT	*	21.3	[M+H]+	394.1568					
110	Exemestane	10	22.5	[M+H]+	297.1849			1		
111	17β-Hydroxyexemestane (Exemestane MT)	10	21.9	[M+H]+	299.2006	MSMS 299.2 >135.0804		0.2	−62 (7.4)	91.9 (5.6)
112	GW1516	10	24.5	[M−H]−	452.0607					
113	GW1516 sulfoxide		21.8	[M−H]−	468.0557					
114	Bis(4-cyanophenyl)methanol (Letrozole MT)	10	17.9	[M−H]−	233.0720	[M+HCOO]-	279.0775	1		
115	Melidonium	50	1.15	MSMS 147.11 > 58.0659				12.5		
116	Raloxifene	10	11.5	[M+H]+	474.1734			1		
117	Tamoxifene 3-hydroxy-4-methoxy	10	22.2	[M+H]+	418.2377			1		
118	Testolactone	10	12.6	[M+H]+	301.1798			0.2		
119	Tetrahydrotestolactone	*	21.4	[M+H]+	305.2111					
120	Toremifene	10	23.1	[M+H]+	406.1932					
121	Toremifene-carboxy metabolite Tamoxifene-carboxy metabolite	*	13.8	[M+H]+	402.2064					
122	Trimetazidine	10	5.6	[M+H]+	267.1703			0.4	−43 (18)	68 (11)
123	AICAR	4700	1.5	[M+H]+	259.1037	[M−H]-	257.0891			
	STIMULANTS									
124	1-Benzylpiperazine	50	5.1	[M+H]+	177.1386			1		

Table 1 (*Continued*)

No.	Compound	Spiked Conc. (ng mL^{-1})	RT[g] (min)	Main Ion	Ion Mass (*m*/*z*)	Secondary Ion	Ion Mass (*m*/*z*)	LOD[a] (ng mL^{-1})	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
125	1-(3-Chloro)phenyl-Piperazine	25	6.8	[M+H]+	197.0840			1.25	−61.9 (7.7)	87 (15)
126	2Amino-NEthyl-Phenylbutane	25	6.7	[M+H]+	178.1590			1.25	−57 (12)	91.2 (5.0)
127	4-Ethylephedrine	50	6.8	[M+H]+	194.1539	[M+H−H_2O]+	176.1434	2.5	−38.6 (9.5)	88.8 (5.5)
128	4-Methylhexanamine	50	5.9	[M+H]+	116.1434			1		
129	Tuaminoheptane	50	6.2	[M+H]+	116.1434			1		
130	1.4-Dimethylpentylamine	50	6.0	[M+H]+	116.1434					
131	1.3-Dimethylbutylamine	50	5.1	[M+H]+	102.1277			1	+11 (26)	65 (12)
132	6-Hydroxybromantane	10	23.9	[M+H]+	322.0801	[M+H]+[d]	324.0781	1		
133	Adrafinil	50	9.0	$C_{13}H_{10}$	167.0855	[M−H]−	288.0700	1		
134	Amiphenazole	50	5.1	[M+H]+	192.0590			5		
135	Amphepramone	50	6.0	[M+H]+	206.1539			1		
136	Amphetamine	50	5.6	[M+H]+	136.1121	[M+H−NH_3]+	119.0855	1		
137	β-Methylphenethylamine	25	5.7	[M+H]+	136.1121	[M+H−NH_3]+	119.0855	1.25	−42.0 (9.2)	79.6 (4.5)
138	Benfluorex	50	20.8	[M+H]+	352.1519			1		
139	Benzoylecgonine	50	6.0	[M+H]+	290.1387			20		
140	Benzphetamine	50	9.4	[M+H]+	240.1747			1		
141	Carphedone	50	6.8		219.1128		174.0913	1		
142	Cathine	50	5.0	[M+H−H_2O]+	134.0964	[M+H]+	152.1070	1		
143	Cathinone	25	5.1	[M+H]+	150.0913			6.25		
144	Clobenzorex	50	10.8	[M+H]+	260.1201			1		
145	Cropropamide	50	10.4	[M+H−$C_2H_5NH_2$]+	196.1332	[M+H]+	241.1911	1		
146	Crotethamide	50	7.9	[M+H−$C_2H_5NH_2$]+	182.1176	[M+H]+	227.1754	1		
147	Cyclazodone	50	8.2	[M+H]+	215.0826		217.0972	1		
148	DiMethylamphetamine	50	6.0	[M+H]+	164.1434			1		
149	Ethylamphatemine	50	6.2	[M+H]+	164.1434			1		
150	Mephentermine	50	6.2	[M+H]+	164.1434			1		
151	Dobutamine	50	6.2	[M+H]+	302.1751	[M−H]−	300.1605	5		
152	Ecgonine methylester	10	1.3	[M+H]+	200.1281			1		
153	Ephedrine/Pseudoephedrine	50	5.3	[M+H]+	166.1226	[M+H−H_2O]+	148.1121	1		
154	Etafedrine	50	5.8	[M+H]+	194.1539			1		
155	Ethamivan	50	8.1	[M+H]+	224.1281			1		
156	Etilefrine	50	3.1	[M+H]+	182.1176			20		
157	Etilefrine-Sulfate	*	3.0	[M−H]−	260.0598	[M+H]+	262.0744			
158	Famprofazone	50	23.5	[M+H]+	378.2540	[2M+H]+	755.5007	1		
159	Fenbutrazate	50	23.3	[M+H]+	368.2220			1		
160	Fencamfamine	50	8.1	[M+H]+	216.1747			1		
161	Fencamine	50	6.3	[M+H]+	385.2346	[M−H]−	383.2201	1		
162	Fenethylline	50	6.6	[M+H]+	342.1925			1		
163	Fenfluramine	50	8.6	[M+H]+	232.1308			1		
164	Fenproporex	50	6.1	[M+H]+	189.1386			1		
165	Flephedrone	50	5.6	[M+H]+	182.0976			1	−38 (12)	42 (16)
166	Furfenorex	50	7.6	[M+H]+	230.1539			1		
167	HMA	50	4.8	[M+H]+	182.1176	[M+H−NH_3]+	165.0910	10	−35 (15)	55 (10)
168	HMMA	50	5.0	[M+H]+	196.1332	[M−CH_3NH]+	165.0910	5	−18 (12)	71.7 (9.0)
169	Heptaminol	50	4.1	[M+H]+	146.1539	[M+H−H_2O]+	128.1434	1		
170	Isometheptene	50	6.6	[M+H]+	142.1590			1		
171	MDA	50	5.8	[M+H−NH3]+	163.0754	[$C_8H_6O_2$]+	135.0440	1		
172	MDMA	50	6.0	[M+H]+	194.1176	[M−CH_3NH]+	163.0754	1		
173	Mefenorex	50	7.4	[M+H]+	212.1201			0.2		
174	Mephedrone	10	6.1	[M+H]+	178.1226	[M+H−H_2O]+	160.1121	1		
175	Methedrone	25	6.0	[M+H]+	194.1176			1.25	−42.4 (8.0)	78.9 (8.1)
176	Methoxyphenamine	50	6.3	[M+H]+	180.1383	[M−CH_3NH]+	149.0961	1		

Table 1 (*Continued*)

No.	Compound	Spiked Conc. (ng mL^{-1})	RT[g] (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD[a] (ng mL^{-1})	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
177	Methylephedrine	50	5.5	[M+H]+	180.1383	[M+H−H_2O]+	162.1277	1		
178	Methylphenitate	50	6.7	[M+H]+	234.1489			1		
179	Modafinil	50	9.7	$C_{13}H_{10}$	167.0855			1		
180	Modafinil carboxylate MT	50	8.0	$C_{13}H_{10}$	167.0855			5	−46 (10)	3.1 (0.8)
181	Morazone	50	7.4	[M+H]+	378.2176			1	−11 (13)	77.6 (4.0)
182	N,N-dimethylphenethylamine	25	5.5	[M+H]+	150.1277			1.25	−44 (13)	77 (13)
183	Nikethamide	50	6.4	[M+H]+	179.1179			1		
184	Norfenefrine-Sulfate	*	1.5	[M−H]−	232.0285	[M+H]+	234.0431			
185	Norfenfluramine	10	7.4	[M+H]+	204.0995			0.25		
186	Octopamine	500	1.3	[M+H−H_2O]+	136.0757			50		
187	Octopamine_sulfate	*	1.3	[M−H]−	232.0285	[M+H]+	234.0431			
188	Oxilofrine	50	2.0	[M+H]+	182.1176		164.1070	5		
189	Oxilofrine_sulfate	*	2.0	[M+H]+	262.0757	[M−H]−	260.0598			
190	PEA	500	5.0	[M+H]+	122.0969				79.4 (9.9)	62.2 (6.4)
191	PVP	50	7.0	[M+H]+	232.1696			2.5	60 (10)	94 (10)
192	Pemoline	50	6.1	[M+H]+	177.0659			1		
193	Pentetrazol	50	6.0	[M+H]+	139.0978			1		
194	Phendimetrazine	10	5.8	[M+H]+	192.1383			0.2		
195	Phenmetrazine	50	5.8	[M+H]+	178.1226			1		
196	Pholedrine	50	4.3	[M+H]+	166.1226	[M−CH_3NH]+	135.0804	1		
197	Prenylamine	50	22.0	[M+H]+	330.2216			1		
198	Prolintane	50	8.4	[M+H]+	218.1903			1		
199	Propylhexedrine	50	7.3	[M+H]+	156.1747			1		
200	Ritalinic acid	50	5.9	[M+H]+	220.1332			12.5	96.6 (6.9)	2.1 (0.3)
201	D-methamphetamine	50	5.7	[M+H]+	150.1277			1		
202	Phenpromethamine	50	5.8	[M+H]+	150.1277			1		
203	Ortetamine	50	6.2	[M+H]+	150.1277			1		
204	Phentermine	50	6.0	[M+H]+	150.1277			1		
205	p-Methylamphetamine	50	6.4	[M+H]+	150.1277			1		
206	Selegiline	50	6.5	[M+H]+	188.1434			1		
207	Selegiline desmethyl MT	50	6.6	[M+H]+	174.1280					
208	Sibutramine	50	20.9	[M+H]+	280.1827			1		
209	Sibutramine desmethyl MT	50	20.4	[M+H]+	266.1670			2.5	40.9 (8.8)	89.9 (9.6)
210	Strychnine	50	6.0	[M+H]+	335.1754			1		
211	Sydnocarb	50	22.6	[M+H]+	323.1502			1		
212	p-Hydroxysydnocarb	10	16.4	[M+H]+	339.14520			0.5	+34.0 (8.8)	93.3 (5.5)
213	p-Hydroxyamphetamine	50	3.9	[M+H]+	152.1077	[M+H−NH_3]+	135.0802	5		
	NARCOTICS									
214	3-Methylfentanyl	1	12.3	[M+H]+	351.2431			0.1		
215	6-Acetylmorphine	25	5.7	[M+H]+	328.1543			0.5		
216	Buprenorphine	2.5	12.1	[M+H]+	468.3108			0.05		
217	Norbuprenorphine	2.5	7.6	[M+H]+	414.2638			0.25		
218	Methadone	5	19.8	[M+H]+	310.2165			0.1		
219	EDDP (Methadone MT)	25	13.2	[M+H]+	278.1903			0.5	−43.3 (5.4)	85.5 (8.7)
220	Fentanyl	1	9.9	[M+H]+	337.2274			0.1		
221	Norfentanyl	1	6.2	[M+H]+	233.1648			0.1		
222	Hydromorphone	25	4.8	[M+H]+	286.1438			1		
223	Morphine	25	3.4	[M+H]+	286.1438			5		
224	Oxycodone	25	5.7	[M+H]+	316.1543			0.5		
225	Oxymorphone	25	4.3	[M+H]+	302.1378	[M+Na]+	324.1206	0.5		
226	Pentazocine	25	8.2	[M+H]+	286.2165			0.5		
227	Pethidine	25	7.3	[M+H]+	248.1645			0.5		
228	Racemoramide	25	19.8	[M+H]+	393.2537			0.5		

Table 1 (*Continued*)

No.	Compound	Spiked Conc. ($ng\,mL^{-1}$)	RT[g] (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD[a] ($ng\,mL^{-1}$)	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
229	Sufentanyl	1	15.7	[M+H]+	387.2101			0.02		
	CANNABINOIDS									
230	Carboxy-Δ9-tetrahydrocannabinol	75	25.0	[M−H]−	343.1915	[M+HCOO]−	389.1970			
231	JWH-250	0.5	25.8	[M+H]+	336.1958			0.1		
232	JHW-250 5-hydroxypentyl MT	0.5	22.7	[M+H]+	352.1902			0.1	−44.6 (9.0)	87.8 (5.1)
233	JWH-200	0.5	22.4	[M+H]+	385.1918			0.05		
234	JWH-122	0.5	27.5	[M+H]+	356.2009			0.2		
235	JWH-73 N-(4-hydroxbutyl) MT	0.5	23.0	[M+H]+	344.1645			0.05		
236	JWH-73 N-butanoic acid MT	0.5	22.9	[M+H]+	358.1438	[M−H]−	356.1292	0.05		
237	JWH-18 N-(4-hydroxypentyl) MT	0.5	23.5	[M+H]+	358.1802			0.2		
238	JWH-18 pentanoic acid MT	0.5	23.2	[M+H]+	372.1594			0.05		
	GLUCOCORTICOIDS									
239	Beclomethasone	15	16.9	[M+H]+	409.1776	[M+HCOO]−	453.1686	1.5		
240	Betamethasone	15	14.4	[M+H]+	393.2072	[M+HCOO]−	437.1981	0.3		
241	Dexamethasone	15	14.9	[M+H]+	393.2072	[M+HCOO]−	437.1981	0.3		
242	6β-Hydroxybudesonide	15	12.5	[M+HCOO]−	491.2287	[M+H]+	447.2377	0.3	−45 (12)	90 (11)
243	Budesonide	15	22.2	[M+H]+	431.2428	[M+HCOO]−	475.2337	0.3		
244	Ciclesonide	15	27.7	[M+H]+	541.3160			4		
245	Clobetasol propionate	15	24.0	[M+H]+	467.1995			1	−65.5 (5.0)	88.5 (7.1)
246	Deflazacort 6βhydroxy desacetyl MT	15	6.3	[M+H]+	416.2068	[M+HCOO]−	460.1970	1.5	−65.1 (7.0)	80 (11)
247	Deflazacort desacetyl MT	15	10.4	[M+H]+	400.2118	[M+HCOO]−	444.2028	0.3		
248	Desonide	15	17.8	[M+HCOO]−	461.2184	[M+H]+	417.2272	0.3		
249	Fludrocortisone	15	10.5	[M+H]+	381.2072	[M+HCOO]−	425.1981	0.3		
250	Flumethasone	15	15.3	[M+HCOO]−	455.1887	[M+H]+	411.1978	0.3		
251	Fluocortolone	15	20.5	[M+H]+	377.2123	[M+HCOO]−	421.2032	1.5		
252	Fluorometholone	15	20.8	[M+H]+	377.2123	[M+HCOO]−	421.2032	0.75	−27 (11)	90.9 (3.4)
253	Fluprednisolone	15	10.0	[M+H]+	379.1915	[M+HCOO]−	423.1825	0.3		
254	Fluticasone 17β-carboxy MT	15	15.8	[M+H]+	397.1821	[M−H]−	395.1676	1.5		
255	Fluticasone prop. 17β-carboxy MT	15	21.9	[M+H]+	453.2083	[M−H]−	451.1938	0.3	−60.1 (9.4)	55.0 (5.4)
256	Methylprednisolone	15	13.3	[M+H]+	375.2166	[M+HCOO]−	419.2075	1.5		
257	Prednisolone	15	9.8	[M+H]+	361.2009	[M+HCOO]−	405.1919	1.5		
258	Prednisone	15	10.1	[M+H]+	359.1853	[M+HCOO]−	403.1762	1.5		
259	Triamcinolone acetonide	15	18.2	[M+H]+	435.2177	[M+HCOO]−	479.2087	0.3		
260	Flunisolide	15	18.9	[M+H]+	435.2177	[M+HCOO]−	479.2087	0.3		
261	Triamcinolone	15	6.8	[M+H]+	395.1864	[M+HCOO]−	439.1774	0.3		
	BETA-BLOCKERS									
262	Acebutolol	50	6.4	[M+H]+	337.2122	[M−H]−	335.19760	0.5		
263	Alprenolol	50	9.3	[M+H]+	250.1802			0.5		
264	Atenolol	50	4.9	[M+H]+	267.1703			0.5		
265	Befunolol	25	6.6	[M+H]+	292.1543			0.5	−3.2 (8.6)	91.1 (4.0)
266	Betaxolol	50	9.6	[M+H]+	308.2220			0.5		
267	Bisoprolol	50	7.9	[M+H]+	326.2326			0.5		
268	Bufarolol	25	10.5	[M+H]+	262.1802			0.5	−14 (11)	86.9 (3.9)
269	Bupranolol	25	9.5	[M+H]+	272.1412			0.5	−13 (12)	86.0 (5.1)
270	Carteolol	50	5.6	[M+H]+	293.1860			0.5		
271	Carvedilol	50	15.5	[M+H]+	407.1965	[M−H]−	405.1821	0.5		
272	Celiprolol	50	7.0	[M+H]+	380.2544	[M−H]−	378.2399	0.5		
273	Esmolol	25	7.0	[M+H]+	296.1856			1.25	−40 (10)	89.4 (8.1)
274	Labetalol	50	7.9	[M+H]+	329.1860	[M−H]−	327.1715	0.5		
275	Levobunolol	50	6.7	[M+H]+	292.1907			0.5		
276	Metipranolol	25	8.7	[M+H]+	310.2013			1		
277	Metoprolol	50	6.6	[M+H]+	268.1907			0.5		

Table 1 (*Continued*)

No.	Compound	Spiked Conc. (ng mL^{-1})	RT[g] (min)	Main Ion	Ion Mass (m/z)	Secondary Ion	Ion Mass (m/z)	LOD[a] (ng mL^{-1})	% Matrix Effect[a,b] (SD)	% Extraction Recovery[a,b] (SD)
278	Nadolol	50	5.7	[M+H]+	310.2013	[M+HCOO]−	354.1921	0.5		
279	Nebivolol	25	20.2	[M+H]+	406.1824			0.5	−35 (15)	77.8 (8.8)
280	Oxprenolol	50	7.5	[M+H]+	266.1751			0.5		
281	Penbutolol	25	20.5	[M+H]+	292.2271			0.5	−25.5 (7.7)	80.1 (6.2)
282	Pindolol	50	6.0	[M+H]+	249.1598			0.5		
283	Propranolol	50	9.0	[M+H]+	260.1645			0.5		
284	Sotalol	50	4.9	[M+H]+	273.1267	[M−H]−	271.1122	0.5		
285	Timolol	50	6.3	[M+H]+	317.1642			0.5		
	CONFOUNDING FACTORS									
286	Ethyl glucuronide	5000	1.1	MSMS 221.07 >75.0070		[M−H]−	221.0660			
287	Finasteride carboxy-MT	*	10.3	[M+H]+	403.2591	[M−H]−	401.2446			
288	Fluconazole	50	6.7	[M+H]+	307.1110			1.0	−20 (12)	94.4 (5.1)
289	Miconazole	50	23.8	[M+H]+	414.9924			1.0		
290	Ketoconazole	50	20.3	[M+H]+	531.1560			2.5	−63 (14)	82.1 (8.3)
	OTHER SUBSTANCES									
291	Roxadustat (FG4592)	1	23.1	[M+H]+	353.1132			0.25	−17 (10)	54.2 (9.1)
292	Ibutamoren	1	16.3	[M+H]+	529.2479			0.2		
293	Efaproxiral	10	22.0	[M−H]−	340.1553	[M+H]+	342.1700	0.2		
294	2-Hydroxyflutamide	*	19.6	[M−H]−	291.0588					
295	Hydrocodone	5	5.9	[M+H]+	300.1594					
296	Codeine	5	5.4	[M+H]+	300.1594			0.1		
297	Pipradrol	50	7.7	[M+H]+	268.1696	[M+H−H_2O]+	250.1590	1		
298	Telmisartan	25	22.7	[M+H]+	515.2442			0.5	−54 (10)	86 (13)
299	Tramadol	25	6.7	[M+H]+	264.1958			0.5		
300	Bupropion	25	7.8	[M+H]+	240.1150			1		
301	Caffeine		5.7	[M+H]+	195.0877					
302	Mitraginine	25	13.4	[M+H]+	399.2278			0.5	−16 (10)	79.5 (8.3)
303	Tapentadol	25	6.8	[M+H]+	222.1852			0.5	−17.3 (7.5)	89.1 (3.2)
304	Norephedrine	10	4.8	[M+H]+	134.0964	[M+H−H_2O]+	152.1070	0.2		

*Compound available from excretion urine reference material.

[a] LOD, Matrix Effects and Extraction Recovery, were estimated according to the main ion intensities.

[b] Matrix Effects and Extraction Recovery, were evaluated for a limited number of selective analytes.

[c] Isotope of ^{37}Cl.

[d] Isotope of ^{81}Br.

[e] 4-Amino-6-chloro-1,3-benzenedisulfonamide.

[f] 4-Amino-6(trifluoromethyl) benzene-1,3-disulfonamide.

[g] RT referred to retention time.

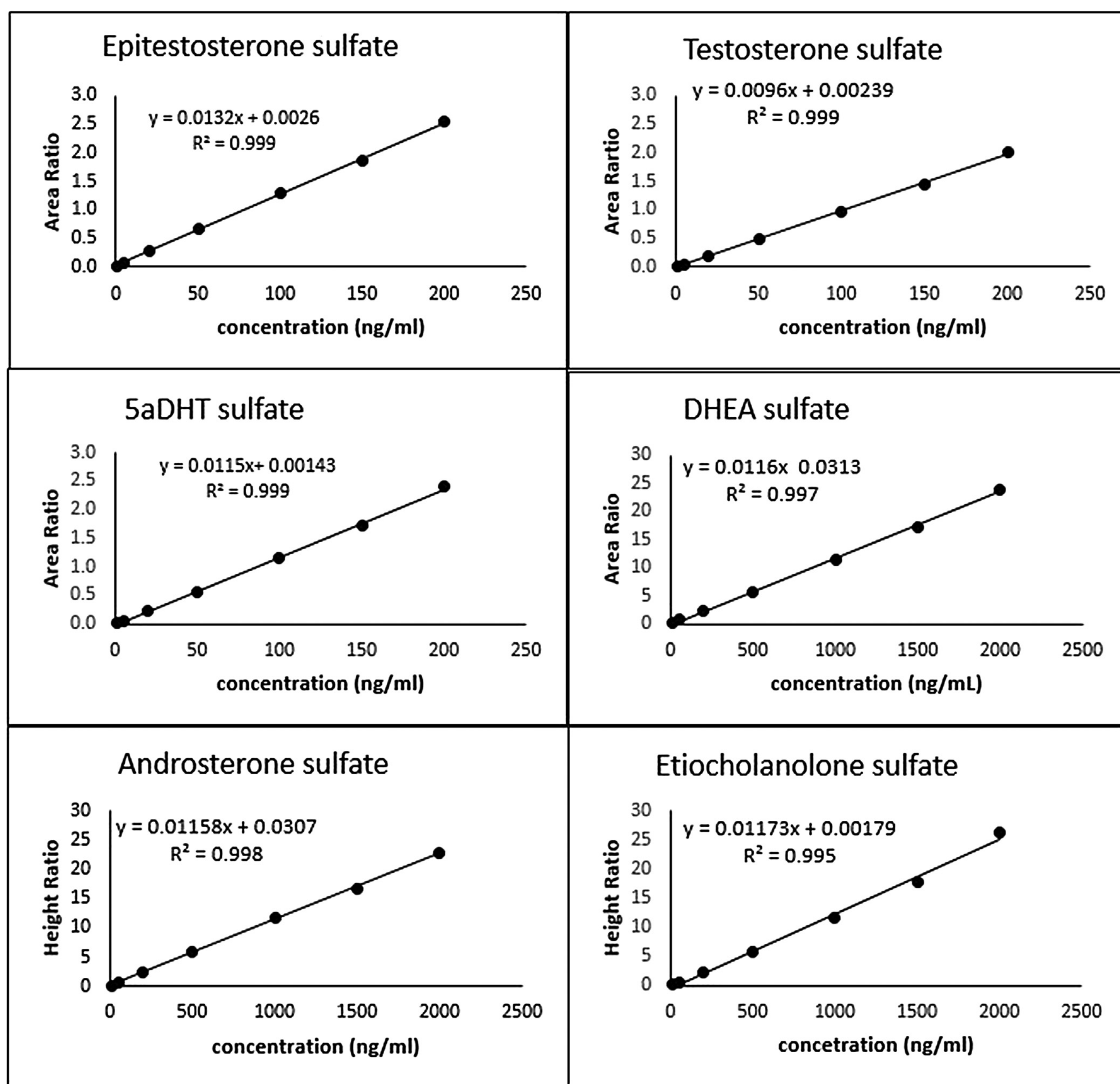

Fig. 1. Calibration curves of ES, TS, 5A-DHTS, DHEAS, AS and ETIOS.

Table 2
Summary of the data used for quantitative validation of endogenous sulfate steroids.

Compound name	RT[a] (min)	Ion (*m/z*)	QC level (ng/ml)		bias%		Intermediate precision (%)		MU%	
			QC1	QC2	QC1	QC2	QC1	QC2	QC1	QC2
TS	10.1	367.1585	2.5	100	17.5	14.0	3.8	4.1	17.9	14.5
ES	10.9	367.1585	2.5	100	14.1	5.7	3.2	3.4	14.4	6.6
5αDHTS	13.2	369.1741	2.5	100	2.8	7.5	2.3	0.3	3.6	7.5
DHEAS	12.7	367.1585	25	1000	9.9	2.4	2.4	2.2	10.1	3.2
ANDROS	16.3	369.1741	25	1000	17.9	15.7	1.6	1.7	18	15.8
ETIOS	17.2	369.1741	25	1000	10.3	4.8	3.0	1.2	10.7	4.9
TS-d3	10.1	370.1773	Internal standards							
AS-d4	16.2	373.1992								
5αDHTS −d3	13.0	372.1929								

[a] RT referred to retention time.

conditions of polarity switching, full scan in high-resolution mode and tandem MS acquisition, which provide the best performance for the selected substance classes that should be analyzed in complex sample with important matrix interferences. The MS resolution of the acquisition method defined as full width at half maximum (FWHM) at 200 *m/z* was of 70,000 and resulted in chromatographic peaks with 4 acquisition points for each polarity mode. Under those conditions, the introduction of simultaneous

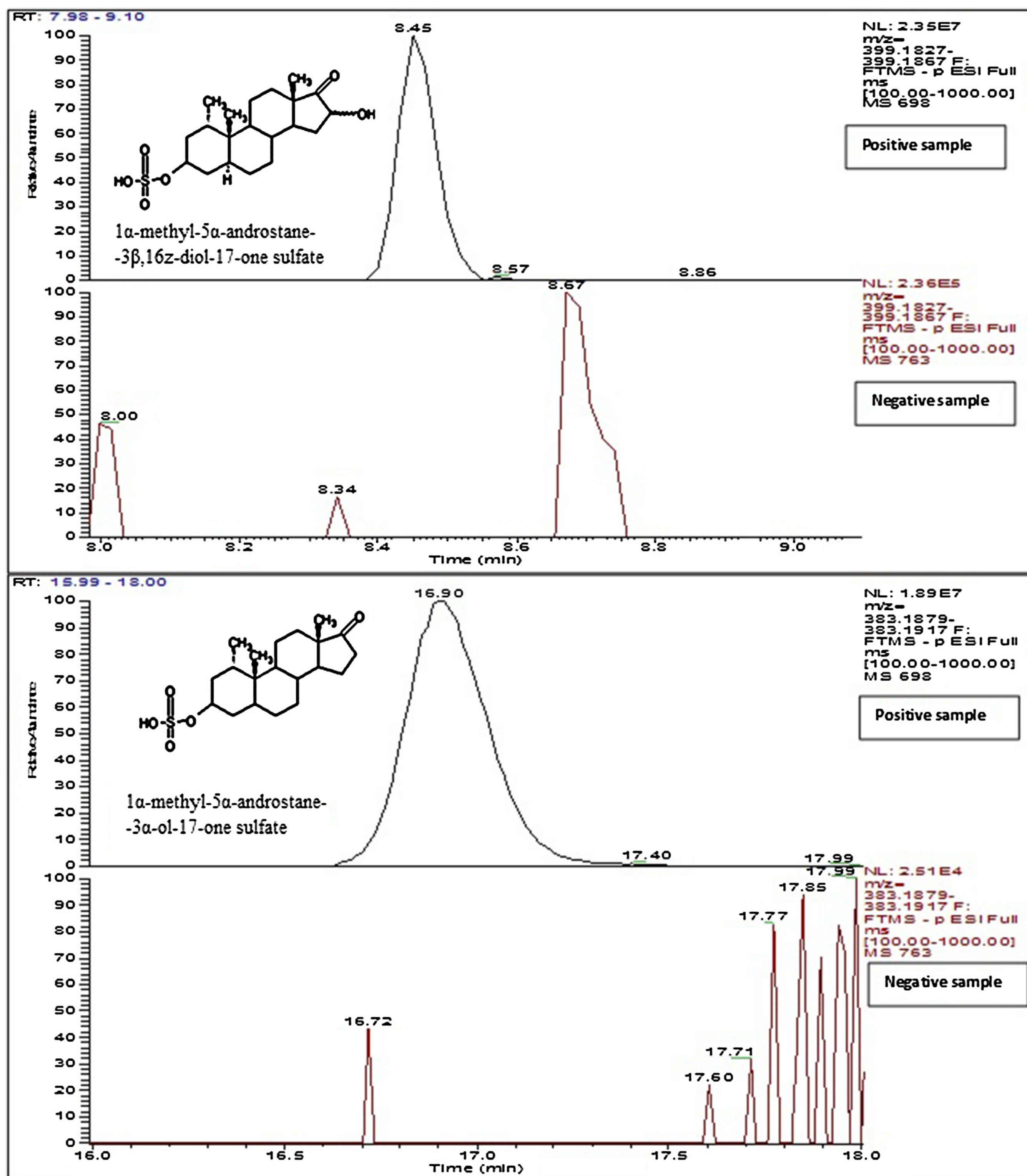

Fig. 2. Extracted ion chromatograms at ±5 ppm mass window of two mesterolone sulfate metabolites in a urine sample suspected for illegal use of mesterolone anabolic steroid and in a negative urine sample.

tandem MS acquisition would create long duty cycle leading to low sensitivity due to low number of sampling point of a chromatographic peak. In the current application, however the FWHM was set to 17,500, which resulted in 8–9 acquisition points for an average chromatographic peaks in polarity switching mode, while addition of the acquisition up to three MS/MS resulted in 5–6 acquisition points per chromatographic peak. Respectively, a mass window of ±5 ppm was applied for extraction of the ion chromatograms for all analytes of interest, except for stimulants and narcotics that are eluted at an early retention time where window of the extracted ion chromatogram window was set at ±10 ppm.

3.2. Validation results

3.2.1. Qualitative validation results

The identification capability, specificity, matrix interferences, LOD, recovery and matrix effects results for the validated target

substances are shown in Table 1. Substances ionizable by ESI with difficulties to meet the validation specifications were analysed by the GC/MS screening, like aminoglutethimide, 4-androstene-3,6,17-trione, HU-210 and norfenephrine. Table 1 summarizes the results of qualitative validation and represents all classes of 304 prohibited substances that were detected with score 10/10 in the different urine matrices tested at concentrations equal to or below 50% of the MRPL. Our approach was able to detect even substances showing ion suppression more than 70% or extraction recovery less than 20%. For most of the substances the LOD are in the level of 10% MRPL or lower. Carry over between injections was not observed at injected concentration of 20 times higher than the MRPL levels [3].

3.2.2. Quantitative validation results

The validation data related to the sulfate endogenous steroids are summarized as follow: the representative calibration curves of the 6 steroids are presented in Fig. 1; precision, accuracy expressed as% of bias and combined measurement uncertainties of the steroids are shown in Table 2; calibration curves obtained following the protocol described in section 2.1.5.2 were calculated from the peak area ratio of sulfo-conjugates steroids and the deuterated standards except for AS and EtioS, calibration curves for AS and EtioS were calculated from the height ratio of the analytes due to the low chromatographic resolution and considerable overlap of the chromatographic peaks of AS and EtioS at high concentration level. The validation results demonstrated high analytical performance with respect of linearity, accuracy (2.4–17%), intermediate precision (1.2–4.1%), and combined measurement uncertainties (3.2–18%) over the range of 1–200 ng mL^{-1} for TS, ES and 5αDHTS and over the range 10–2000 ng mL^{-1} for AS, EtioS and DHEAS. Weighted linear regression was applied to calculate the calibration curves due the wide concentration range of the analytes, which resulted in larger biases at low concentration levels when non-weighted linear regression was used. The analytical results described in this study can be extended to other exogenous and endogenous steroids.

3.3. Application to real samples

The current method had been applied as screening method to official antidoping samples, among others, reported as Adverse Analytical Findings (AAF, positive case) based on the GC/MS technology detection of AAS abuse. The examples presented herein do not represent an exhaustive study of the sulfate metabolites of the respective AAS, but the detection of sulfate metabolites based on matching the *m/z* of the sulfate conjugate metabolites published in literature. The first example concerns with a sample contained a metabolite (1α-methyl-5α-androstane-3α-ol-17-one) of the anabolic steroid of mesterolone at the concentration of 3 ng mL^{-1}, without the presence of mesterolone parent compound. Our LC/MS method was applied to this sample in order to test and corroborate the results obtained using standard GC/MS approach. Two mesterolone sulfate metabolites with parent ion *m/z* of 383.1898 and 399.1847 Da, and the non-sulfate metabolite detected by GC/MS with *m/z* of 488 > 433 were detected in full scan LC/MS data. Fig. 2 shows the extracted ion chromatogram for mesterolone sulfate metabolites in the sample presented together with blank urine sample. These two mesterolone sulfate metabolites are considered to be markers of mesterolone abuse [31]. The second example is related to a positive case reported for the AAS nandrolone based on the GC/MS detection of the metabolites 19-Norandrosterone and 19-Noretiocholanolone from the free and glucuronide urine fraction at a concentration of approximately 50 ng mL^{-1} for 19-Norandrosterone. The current LC/MS method analysis revealed two peaks of *m/z* 355.1585 attributed to 19-Norandrosterone sulfate and 19-Noretiocholanolone sulfate not present in the LC/MS of negative samples [27] (Fig. 3). In a third example, a sample reported for boldenone detected by GC/MS at a concentration of the level of 500 ng mL^{-1} analyzed in parallel by the LC/MS method in MS/MS mode showed the peak *m/z* 365.1428 > 350.1188 for boldenone sulfate and epiboldenone sulfate [26] (Fig. 4).

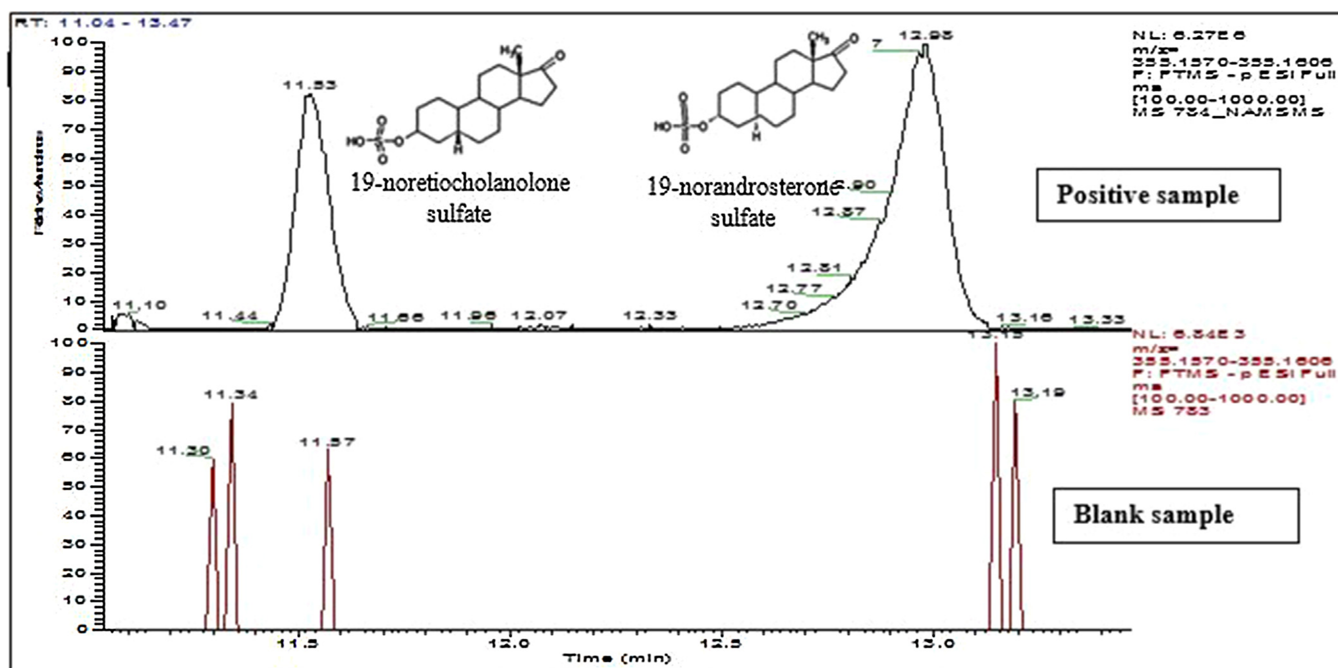

Fig. 3. Extracted ion chromatograms at ±5 ppm mass window of 19-nornadrosterone sulfate and 19-noretiocholanolone sulfate in a urine sample suspected for use of nandrolone anabolic steroid and a negative sample.

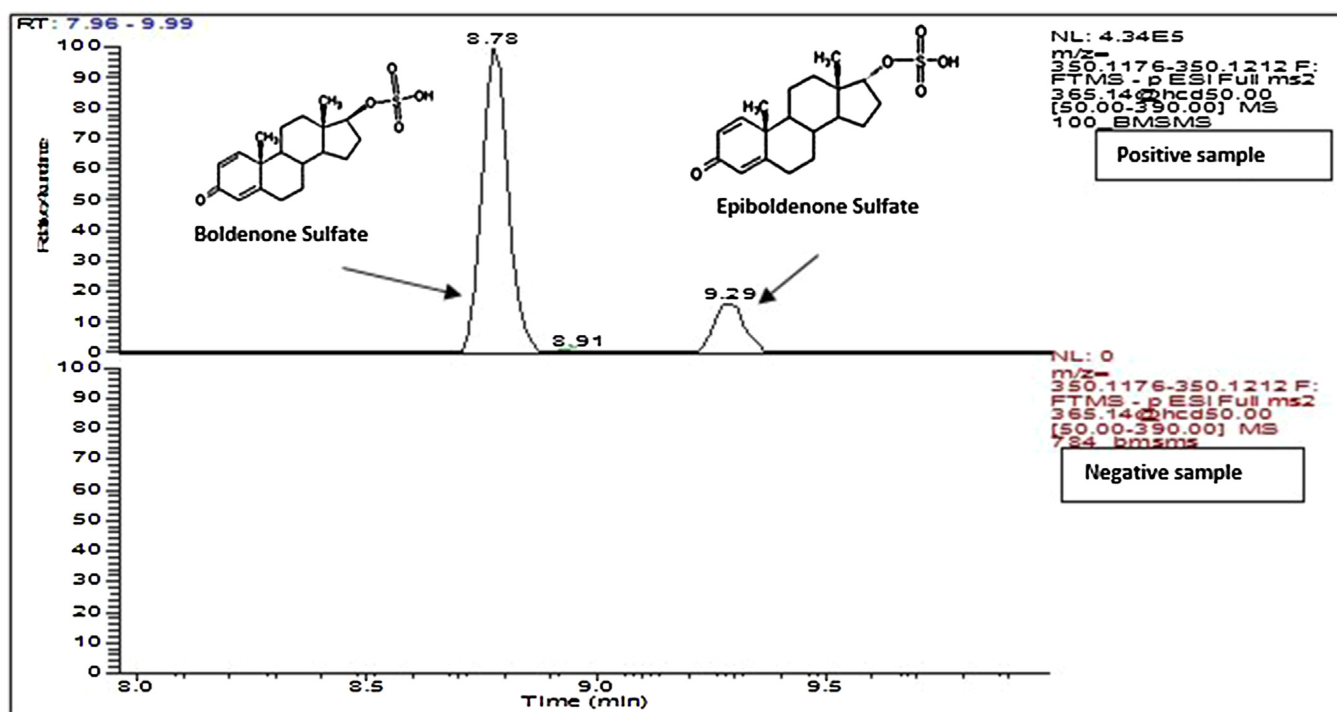

Fig. 4. Extracted ion chromatograms at ±5 ppm mass window of 19-nornadrosterone sulfate and 19-noretiocholanolone sulfate in a urine sample suspected for use of nandrolone anabolic steroid and a negative sample.

4. Conclusion

The presented LC/MS screening method was developed and validated for the analysis of endogenous and exogenous substances, with aim to expands the analytical repertoire of small molecules to the detection of sulfate Phase II metabolites and allow the quantitative determination of endogenous sulfate steroids in their intact form. This becomes very important, because the sulfoconjugated steroid profile in this project can be analyzed in all samples without any further analysis and without creating any interferences to glucuroconjugated steroid profile required by WADA [4]. The use of the combined ethylacetate liquid–liquid extraction of hydrolyzed urine and the D&S urine addition allow to extract both sulfate derivatives and intact steroids and drugs. In future study, we aim to assess the method for analysis of large number of steroid sulfate metabolites. This expansion is important due to fact that several studies [20,22,27] show that sulfate metabolites can be detected much later after steroid abuse due to the longer half-life of sulfate metabolites compared to non-conjugated forms. Besides the advantage of the detection time of sulfoconjugated metabolites of exogenous steroids, they are eliminated in greater quantities in comparison to their glucuronide analogues, resulting in better retrospective detection capabilities [32].

The high-resolution full scan LC/MS data with the polarity switching MS acquisition mode provides the fingerprint of all compounds detected by an LC/MS(/MS) system with several advantages over targeted analysis of compounds. These include the high MS specificity without reduction in the sensitivity of detection, the ability to identify all compounds, which can be detected by an LC/MS system in positive and negative ionization modes, the ability to reprocess the collected data retrospectively, to search for new metabolites, unknown prohibited designer drugs, and to obtain information on the overall metabolomic state of a person, which may reflect illegal use of performance enhancer compounds.

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References

[1] World Anti-doping Agency, World Anti-doping Agency (WADA) Prohibited List, 2017, https://www.wada-ama.org/sites/default/files/resources/files/2016–09-29_wada_prohibited_list_2017_eng_final.pdf (Accessed 12 August 2017).
[2] World Anti-Doping Agency, World Anti-Doping Agency (WADA) International Standard for Laboratories (ISL 2016), 2016, https.//www.wada-ama.org/sites/default/files/resources/files/isl_june_2016.pdf (Accessed 6 August 2017).
[3] World Anti-Doping Agency, World Anti-doping Agency (WADA) TD2017MRPL, 2017, https.//www.wada-ama.org/sites/default/files/resources/files/wada-td2017mrpl-en_0. pdf (Accessed 31 August 2017).
[4] World Anti-Doping Agency, Endogenous Anabolic Androgenic Steroids: Measurement and Reporting. TDAAS, 2016, ver. 1.0. https://www.wada-ama.org/en/resources/science-medicine/td2016-eaas (Accessed 15 August 2017).
[5] T. Kuuranne, M. Saugy, N. Baume, Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling, Br. J. Sports Med. 48 (2014) 848–855.
[6] O.J. Pozo, P. Van Eenoo, K. Deventer, F.T. Delbeke, Ionization of anabolic steroids by adduct formation in liquid chromatograph electrospray mass spectrometry, J. Mass Spectrom. 42 (2007) 497–516.
[7] K. Deventer, O.J. Pozo, A.G. Verstraete, P. Van Eenoo, Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology, Trends Anal. Chem. 55 (2014) 1–13.
[8] C. Görgens, S. Guddat, A. Thomas, P. Wachsmuth, A. Orlovius, G. Sigmund, M. Thevis, W. Schänzer, Simplifying and expanding analytical capabilities for various classes of doping agents by means of direct urine injection high performance liquid chromatography high resolution/high accuracy mass spectrometry, J. Pharm. Biomed. Anal. 131 (2016) 482–496.

[9] A. Musenga, D. A.Cowan, Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control, J. Chromatogr. A 1288 (2013) 82–95.
[10] A. Vonaparti, E. Lyris, Y.S. Angelis, I. Panderi, M. Koupparis, A. Tsantili-KakouLidou, R.J.B. Peters, M.W.F. Nielen, C. GeorgakopouLos, Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometry, Rapid Comm. Mass Spectrom. 24 (2010) 595–1609.
[11] T. Friedmann, U. Flenker, C. Georgakopoulos, M. Alsayrafi, P. Sottas, S. Williams, R. Gill, Evolving concepts and techniques for anti-doping, Bioanalysis 4 (13) (2012) 1667–1680.
[12] D.W. Andersen, K. Linnet, Screening for anabolic steroids in urine of forensic cases using fully automated solid phase extraction and LC–MS-MS, J. Anal. Toxicol. 38 (2014) 637–644.
[13] L. Dehennin, P. Lafarge, Ph. Dailly, D. Bailloux, J.P. Lafarge, Combined profile of androgen glucuro- and sulfoconjugates in postcompetition urine of sportsmen: a simple screening procedure using gas chromatography-mass spectrometry, J. Chromatogr. B 687 (1996) 85–91.
[14] P.Van Renterghem, P. Van Eenoo, P. Sottas, M. Saugy, F. Delbeke, Delbeke pilot study on subject-based comprehensive steroid profiling:novel biomarkers to detect testosterone misuse in sports, Clin. Endocrinol. (Oxf.) 75 (2011) 134–140.
[15] O.J. Pozo, J. Marcos, R. Ventura, A. Fabregat, J. Segura, Testosterone metabolism revisited: discovery of new metabolites, Anal. Bioanal. Chem. 398 (2010) 1759–1770.
[16] J. Boccard, F. Badoud, E. Grata, S. Ouertani, M. Hanafi, G. Mazerolles, P. Lanteí ri, J. Veuthey, M. Saugy, S. Rudaz, A steroidomic approach for biomarkers discovery in doping control, Forensic Sci. Int. 213 (2011) 85–94.
[17] M. Raro, M. Ibaín˜ez, R. Gil, A. Fabregat, E. Tudela, K. Deventer, R. Ventura, J. Segura, J. Marcos, A. Kotronoulas, J. Joglar, M. Farreí, S. Yang, Y. Xing, P. Van Eenoo, E. Pitarch, F. Hernaíndez, J. Sancho, Oí.J. Pozo, Untargeted metabolomics in doping control: detection of new markers of testosterone misuse by ultrahigh performance liquid chromatography coupled to high-resolution mass spectrometry, Anal. Chem. 87 (2015) 8373–8380.
[18] A. Palermo, F. Botrè, X. de la Torre, N. Zamboni, Non-targeted LC-MS based metabolomics analysis of the urinary steroidal profile, Anal. Chim. Acta 964 (2017) 112–122.
[19] F. Badoud, J. Boccard, C. Schweizer, F. Pralong, M. Saugy, N. Baume, Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight massspectrometry, J. Steroid Biochem. Mol. Biol. 138 (2013) 222–235.
[20] J. Schulze1, M. Johansson, J. Thörngren, M. Garle, A. Rane, L. Ekström, SULT2A1 gene copy number variation is associated with urinary excretion rate of steroid sulfates, Front. Endocrinol. (Lausanne) 4 (2013), 88.
[21] T. Piper, W. Schänzer, M. Thevis, Genotype-dependent metabolism of exogenous testosterone –new biomarkers result in prolonged detectability, Drug Test. Anal. 8 (2016) 1163–1173.
[22] J. Schulze, J. Thö rngren, M. Garle, L. Ekström, A. Rane, Androgen sulfation in healthy UDP-Glucuronosyl transferase 2B17 enzyme-deficient men, J. Clin. Endocrinol. Metab. 96 (11) (2011) 3440–3447.
[23] D. Borts, L. Bowers, Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry, J. Mass Spectrom. 35 (2000) 50–61.
[24] C. Gomez, A. Fabregat, Ó. Pozo, J. Marcos, J. Segura, R. Ventura, Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism, Trends Anal. Chem. 53 (2014) 106–116.
[25] F. Badoud, E. Grata, J. Boccard, D. Guillarme, J. Veuthey, S. Rudaz, M. Saugy, Quantification of glucuronidated and sulfated steroids in human urine by ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry, Anal. Bioanal. Chem. 400 (2011) 503–516.
[26] E. Tudela, K. Deventer, L. Geldof, P. Van Eenoo, Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography-high resolution mass spectrometry, Drug Test. Anal. 7 (2015) 95–108.
[27] S. Rzeppa, G. Heinrich, P. Hemmersbach, Analysis of anabolic androgenic steroids as sulfate conjugates using high performance liquid chromatography coupled to tandem mass spectrometry, Drug Test. Anal. 7 (2015) 1030–1039.
[28] G. Balcells, O. Pozo, A. Esquivel, A. Kotronoulas, J. Joglar, J. Segura, R. Ventura, Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1389 (2015) 65–75.
[29] W. Abushareeda, A. Fragkaki, A. Vonaparti, Y. Angelis, M. Tsivou, K. Saad, S. Kraiem, E. Lyris, M. Alsayrafi, C. GeorgakopouLos, Advances in the detection of designer steroids in anti-doping, Bioanalysis 6 (6) (2014) 881–896.
[30] W. Abushareeda, E. Lyris, S. Kraiem, A.A. Wahaibi, S. Alyazidi, N. Dbes, A. Lommen, M. Nielen, P.L. Horvatovich, M. Alsayrafi, C. Georgakopoulos, Gas chromatographic quadrupole time-of-flight full scan high resolution mass spectrometric screening of human urine in antidoping analysis, J. Chromatogr. B 1063 (2017) 74–83.
[31] P. Kiousi, Y.S. Angelis, A.G. Fragkaki, W. Abushareeda, M. Alsayrafi, C. Georgakopoulos, E. Lyris, Markers of mesterolone abuse in sulfate fraction for doping control in human urine, J. Mass Spectrom. 50 (2015) 1409–1419.
[32] A.T. Cawley, R. Kazlauskas, G.J. Trout, A.V. George, Determination of urinary steroid sulfate metabolites using ion paired extraction, J. Chromatogr. B 825 (2005) 1–10.