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Gas chromatographic quadrupole time-of-flight full scan high resolution mass spectrometric screening of human urine in antidoping analysis

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ABSTRACT

This paper presents the development and validation of a high-resolution full scan (FS) electron impact ionization (EI) gas chromatography coupled to quadrupole Time-of-Flight mass spectrometry (GC/QTOF) platform for screening anabolic androgenic steroids (AAS) in human urine samples. The World Antidoping Agency (WADA) enlists AAS as prohibited doping agents in sports, and our method has been developed to comply with the qualitative specifications of WADA to be applied for the detection of sports antidoping prohibited substances, mainly for AAS. The method also comprises of the quantitative analysis of the WADA's Athlete Biological Passport (ABP) endogenous steroidal parameters. The applied preparation of urine samples includes enzymatic hydrolysis for the cleavage of the Phase II glucuronide conjugates, generic liquid–liquid extraction and trimethylsilyl (TMS) derivatization steps. Tandem mass spectrometry (MS/MS) acquisition was applied on few selected ions to enhance the specificity and sensitivity of GC/TOF signal of few compounds. The full scan high resolution acquisition of analytical signal, for known and unknown TMS derivatives of AAS provides the antidoping system with a new analytical tool for the detection designer drugs and novel metabolites, which prolongs the AAS detection, after electronic data files' reprocessing. The current method is complementary to the respective liquid chromatography coupled to mass spectrometry (LC/MS) methodology widely used to detect prohibited molecules in sport, which cannot be efficiently ionized with atmospheric pressure ionization interface.

1. Introduction

Anabolic Androgenic Steroids (AAS) are the most frequently used class of prohibited substances by athletes [1,2] to boost their performance in sport activities. The detection of AAS in athletes' urine is a challenge for the doping control laboratories because of a) the low concentrations of the precursors and their metabolites, b) the low Minimum Required Performance Limits (MRPL) requested by the World Anti-Doping Agency (WADA) [3], c) the availability of designer steroids, which have similar activity and same or different chemical composition (formula) with known (not necessarily endogenous) steroids but different structures [4,5], d) the continuous discovery of new long-term metabolites of AAS that extend the retrospectivity of the consumption considerably (however the incorporation of these new metabolites in screening procedure is really helpful for the monitor of steroids abuse and has led in the past to numerous adverse analytical findings) [6–9], and e) the rumored use of "micro dosing", where athletes are doped with small doses which provide concentration in body fluids sampled for anti doping below the detection limit.

The doping control laboratories implement different analytical techniques in order to be able to detect a large variety of classes of prohibited substances. Mass spectrometry is the method of choice for the detection of the small molecules present in prohibited list of substances [1] combined either with gas chromatography (GC/MS) or with liquid chromatography (LC/MS). Due to their limited ionization efficiency, AASs are screened by GC/MS [10,11] and LC/MS are used for AASs, which can be efficiently ionized and hence selectively detected [12]. Only few free AASs are efficiently ionized, but there are numerous recent examples for the detection of intact Phase II metabolites of AASs by LC/MS analysis [13]. Regarding the LC/MS screening, WADA

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accredited laboratories use either LC triple quadrupole MS (LC/QQQ) [14,16] or LC High Resolution MS (LC/HRMS) − orbitrap or TOF − mass analyzers [15,17,18]. During the Olympic Games of London 2012 and Rio 2016, LC/HRMS orbitrap technology was used [18]. On the contrary, regarding the GC/MS screening, merely low resolution GC triple quadrupole technology (GC/QQQ) is used [10,11], while GC/HRMS has been proposed for special purposes as for the detection of Xenon [19].To the best of our knowledge, there is only one article regarding the use of full scan (FS) GC/HRMS technology as screening tool in the doping control field [20]. The main advantages of FS HRMS compared to triple quadrupole technology are the significantly reduced background noise originating from the urine matrix, the high resolving power and mass accuracy used as additional identification information and the capability to perform retesting of the samples by simply reprocessing the stored data files, whenever there is a special request for this such as when a new doping substance or its metabolite is discovered. The last feature (retesting) is gaining importance in sports drug testing, due to the impressive results that came out after the retesting of samples from the Beijing 2008 and the London 2012 Olympic Games some weeks before the Rio 2016 Olympic Games took place. While the percentage of positive cases coming from the original analysis of samples during the Games was far less than 1% (0.13% in Beijing 2008 [21], 0.16% in London 2012 [22]), the reanalysis of samples in 2016 of 1243 samples from Beijing 2008 and London 2012 Games that was reported previously negative, lead to additional identification of 98 positive cases, constituting an astonishing percentage of 8% [23].

In addition to screening for the exogenous compounds, GC/MS screening is used for the quantification of markers of the urinary WADA Athlete Biological Passport (ABP) Steroid Profile (SP) [24]. Currently, the SP consists of Testosterone (T), Epitestosterone (E), Androsterone (A), Etiocholanolone (Etio) 5α-androstan-3α,17β-diol (5αadiol), 5β-androstan-3α,17β-diol (5βadiol), as well as the ratios T/E, A/Etio, 5αadiol/5βadiol, A/T, 5αadiol/E. The analytical method used should be fit-for-purpose and allow covering the dynamic concentration range of listed compounds determined in both males and females. This means that the method should be able to quantify concentrations ranging from 2 ng/mL to more than 10 μg/mL in a single aliquot. WADA has set specific and strict requirements for the methods used for the quantification of the SP markers [24]. The implementation of GC/HRMS for quantification of the compounds listed in ABP-SP presents a challenge in terms of the dynamic range of currently available instruments.

In this paper, we described the use of high-resolution full scan gas chromatographic quadrupole Time-of-Flight mass spectrometry (GC/QTOF) to be used as screening platform for doping control purpose using FS and HRMS data. The method is validated for 73 analytes − mainly AAS but other categories of prohibited substances as well − at concentrations levels at or below the WADA MRPL [3]. Furthermore, this method is used for the quantification of the parameters of the SP to be included in the ABP [24]. To assess the performance of FS HRMS GC/QTOF approach, we present a comparison between GC/QQQ and GC/QTOF profiling of SP measured in the same sample set. The prospect and the feasibility of the implementation of FS obtained with HRMS as a routine screening method is discussed with aim to substitute the triple quadrupole method for doping control purpose.

2. Experimental

2.1. Material and methods

2.1.1. Reagents

Sodium hydrogen carbonate and diethyl ether were supplied by Merck (Darmstadt, Germany). Methanol (HPLC grade), 2-Propanethiol, di-potassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), ammonium iodide (NH_4I), sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) were supplied by Sigma Aldrich (Darmstadt, Germany). β-Glucuronidase from *Escherichia Coli* (E.coli) was supplied by Roche (Mannheim, Germany). MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was supplied by Chemische Fabrik Karl Bucher (Waldstetten, Germany). Perfluorotributylamine (PFTBA) from Agilent.

2.1.2. Reference materials

The following internal standards (ISTD) were purchased from LGC (Wesel, Germany): etiocholanolone-D5 (d5 Etio), androsterone glucuronide-D4 (d4A Glu), testosterone-D3 (d3T), epitestosterone-D3 (d3E), 5β-androstane-3α-17β-diol-D5 (D5-5βAdiol). The remaining reference materials of the study were purchased from LGC (Wesel, Germany), TRC (Toronto, Canada), Sigma Aldrich (Darmstadt, Germany), Steraloids (Newport, USA), and Cerilliant (Round Rock, USA). Stock standard solutions of the analytes were individually prepared in methanol. For validation purposes, working standard solution containing the analytes was prepared in methanol by subsequent dilutions of the stock solutions. All solutions were stored at −20 °C in amber vials. The steroid profile analytes were included in a different working solution.

Urine samples from excretion study of dehydrochloromethyltestosterone (oral turinabol), Desoxymethyltestosterone (Madol), Oxymetholone, Mathandienone, Oxandrolone were donated by the Doping Control Laboratory of Athens, Greece or provided by the World Association of Antidoping Scientists (WAADS).

2.1.3. Sample preparation

Two and a half (2.5) mL of urine aliquot is hydrolyzed by 50 μL of beta-glucuronidase enzyme from *E. Coli* and incubated for 90 min in 50 °C after the addition of 25 μL of ISTD mixture (d3T, d3E, d4A Glu, d5 Etio, d5-5βAdiol) and 1 mL pH 7 phosphate buffer that was prepared by adding 169.8 g of $K_2HPO_4{\cdot}3H_2O$ and 54 g KH_2PO_4 in 1 L of water. After hydrolysis, the urine is buffered by $NaHCO_3$: Na_2CO_3 (10:1) and extracted at pH 9–10 by 5 mL diethyl ether. The sample is centrifuged at 3000 rpm for 12 min and the organic phase is separated from the aqueous phase in frozen conditions at −80 °C and evaporated under nitrogen flow at 50 °C. The residue was TMS derivatized by adding 50 μL of derivatization reagent MSTFA/NH_4I/2-Propanthiol (1000:4:8) and was incubated in 100 °C for 60 min.

2.1.4. Instrumentation

2.1.4.1. GC/QTOF. The GC/MS system used in the current study is an Agilent GC 7890 coupled with an Agilent 7200 QTOF MS (G3850-64101) equipped with 5% Phenyl polysilphenylene-siloxane capillary column (30 m length, 0.25 mm ID, 0.1 μm film thickness, SGE BP X5) and back flush system. The quadrupole device prior the TOF MS analyzer provides the capability of applying MS/MS experiments. Helium was used as carrier gas with a constant flow set at 1.1 mL/min. Two microliters were injected in split mode of 20:1. The injection port and the interface temperatures were set at 280 °C. Initial oven temperature was 160 °C, ramped at 10 °C/min to 200 °C, then ramped at 2 °C/min to 220 °C, ramped at 6 °C/min to 292 °C, 50 °C/min up to 310 °C and held for 3 min, total run time 29.36 min. Two (2) GHz extended dynamic range (EDR) acquisition mode was used for TOF data acquisition. The acquisition rate was 5 spectra per sec, 200 msec per spectrum, number of transients per spectrum was 2718. The used GC/MS has the capacity of acquiring MS data in high-resolution FS mode with a mass accuracy < 5 ppm mass error in EI mode depending on the concentration of the analytes. The MS range (80–670 *m/z*) is capable of covering MS acquisition of all small molecules analyzed by the GC/QTOF. To correct for an eventual shift in *m/z*, a mass calibration procedure was introduced in the analysis sequence after every three aliquot injections. The instrument calibrator was Perfluorotributylamine (PFTBA, Agilent).

2.1.4.2. GC/QQQ. Agilent GC 7890 coupled with an Agilent 7000C QQQ MS is the routine GC/MS screening system of Antidoping Lab Qatar and it is equipped with an Agilent 7693 auto sampler with 10 μL syringe, split/splitless system and the same SGE BPX5 column that was used in the GC/QTOF system described in the previous section. The same oven temperature program presented in 2.1.4.1 was followed. Injection volume was 2 μL in a split ratio of 1:10. Helium was used as carrier gas at 1.1 mL/min flow for GC separation and EI at 70 eV was used for compound ionization. Helium was used also as a quench gas at a flow of 2.25 mL/min and nitrogen as a collision gas at a flow of 1.5 mL/min. The data acquisition was performed in multiple reaction monitoring (MRM) acquisition mode with a collision energy ranging between 5 and 35 eV.

2.1.5. Method validation

2.1.5.1. Qualitative method validation. In order to demonstrate the suitability of the FS HRMS method a validation process was carried out, where the guidelines of the WADA International Standard for Laboratories (ISL) [25] were followed. For that purpose, urine samples were collected by anonymous consented donors. Analysis of 10 different blank urine samples spiked with the reference material solution mixture of AAS at a concentration level of 50% of MRPL [3] was performed for the evaluation of Limit of Detection (LOD) and Identification Capability (IC) validation parameters. The chromatographic Signal to Noise (S/N) ratio of higher than 3 was used as detection criterion. The specificity of the developed method was evaluated by analyzing 10 different blank urine samples to demonstrate the absence of any interfering peaks at the retention times of the analytes of interest. The specific *m/z* and retention times were used to identify particular AAS analytes. The test for carryover was performed by analyzing a negative urine sample after the injection of the same urine sample spiked with the reference material solution mixture of AAS substances at a concentration level of 10 times the MRPL. The criterion to detect AAS compounds was used as assess the presence of carryover. The extraction recovery was characterized with percentage expressing the difference between the sample spiked with reference material solution mixture of AAS before the extraction and a sample spiked at the end of the extraction procedure with respect of peak height at a concentration level of 50% of MRPL.

2.1.5.2. Quantitative validation of the ABP SP. Six points' calibration curves were made by spiking steroid stripped urine; blank urines were collected from female children and were stripped from endogenous steroids after C18 SPE extraction and collection of the urine eluent. Calibration curves were analyzed in each day when validation was performed (total analysis time was 5 days) in the concentration range of interest per endogenous steroid present in SP of ABP. The calibration curves were established over the concentration ranges of 2–400 ng/mL for T and E; 100–8000 ng/mL for A and Etio; and 4–800 ng/mL for 5αadiol and 5βadiol. The calibration curves were built from the peak height ratio of steroids and the above referred standard reference mixture containing deuterated endogenous steroids. The assessment of concentration accuracy of the SP method was performed using two levels of spiked Quality Control (QC) samples, which were prepared and injected twice for each calibration curve. The accuracy of the method was estimated by calculating the (%) relative bias of the experimental concentration with respect of the theoretical concentration in the QC samples. The intermediate precision was determined from the data of the QC sample collected during the 5 different experimental days. Both the intermediate precision and bias from the QC samples were used to estimate the combined Measurement Uncertainty for each steroid. At the Antidoping Lab Qatar, the Agilent 7000C GC/QQQ is used as a routine antidoping screening GC/MS instrument for small molecules, similar to already published methods [10,11]. For a period of three months, approximately 700 samples analyzed routinely with GC/QQQ in our laboratory were reanalyzed with the HRMS GC/QTOF for both qualitative and quantitative analytes.

3. Result and discussion

3.1. Method organization

The sample preparation as described in 2.1.3. based on the generic liquid–liquid extraction with diethyl ether at pH 9-10 and desalting step, which approach is commonly used to extract doping substances from urine matrix. Before the extraction step, the steroids deconjugation of the Phase II glucuronide conjugates was performed by enzymatic hydrolysis using the β-glucuronidase from *E. coli*, as indicated in [24]. The final step of the sample preparation was TMS derivatization of the extracts. Trimethylsylilation was performed by MSTFA/ammonium iodide/propanethiol mixture. Under these conditions, both the hydroxyl and the keto steroidal groups are derivatized [26]. A slow temperature gradient was applied in order to better separate and reduce matrix interferences spreading in a longer period of time in the background matrix and to achieve baseline chromatographic separation of isomers such as androsterone-di-TMS to etiocholanolone-di-TMS and 5α-androstane-3α, 17β-diol-di-TMS to 5β-androstane-3α, 17β-diol-di-TMS. Apart from some exceptions, the analytes were detected in fortified urine samples at a concentration of 50% of MRPL; i.e. in 2.5 ng/mL for most of the AAS in FS MS mode. The FS MS acquisition mode, in combination with the slow chromatographic temperature gradient program, allows potentially the detection of unlimited number of new analytes in the repertoire of the screening method without the need to modify the chromatographic conditions and revalidations of the existing method's substances.

Results evaluation comprised tracing the target analytes extracted ion chromatograms. The creation of extracted ion chromatograms *m/z* windows for proper evaluation of the MRPL and elimination of matrix interferences that may reveal minor chromatographic peaks of the prohibited substances is of utmost importance. The extraction of ion chromatograms were performed by the instrument' software (Agilent Mass Hunter Quantitative Analysis for QTOF version B.07.01). The optimized conditions for the evaluation of extracted ion chromatograms comprised the per-compound compilation [27] at a mass accuracy for each substance of ± 20 ppm. Other mass accuracies for the generation of the extracted ion chromatogram were tested between 20 and 100 ppm. We have found that the peak of interest is lost in extracted window lower than 20 ppm and increasing level of matrix interference was observed at 100 ppm.

3.2. Qualitative validation results

Table 1 shows the qualitative validation data for LOD, IC and recovery rates of AAS substances of the current method. Apart from a few exceptions, e.g. 1-testosterone metabolite, which has interference at low concentration and 13β, 17α-diethyl-3α,17β-dihydroxy-5α gonane, the LOD of 50% WADA MRPL of 2.5 ng/mL were achieved for the AASs listed in Table 1. Similarly, FS HRMS approach enables specific detection most of the AAS with low LOD. Other prohibited substances than AAS such as stimulants, narcotics, b2-agonists, diuretics, beta-blockers, and other AASs, analyzed by LC/MS, were not included in the current study and therefore not listed in Table 1. Several metabolites are not available as synthesized reference materials, but they are available in excretion urines. For these substances, the LOD and IC cannot be applied and only the specificity in blank urine samples is considered.

Fig. 1 shows the typical mass errors of the representative AAS' screening diagnostic ions over the entire mass range at a concentration

Table 1
GC/QTOF analytes (Substance, Derivative, RT, Theoretical *m/z* of diagnostic ions, Recovery%, LOD).

Name	Derivative	RT (min)	Ions (m/z)	Recovery%	LOD, (ng/ml)	Detection in 10 urine aliquots
1-5α-androstenedione	di-OTMS	15.08	430.2718, 415.2483,	82	2.5	
18-normetenol	OTMS	8.7	358.2686, 253.1951, 216.1873	87	2.5	10
1-androstene 3 β,17 β diol	di-OTMS	14.49	405.2640	117	2.5	9
1-Testosterone	di-OTMS	15.45	432.2874, 194.1121	97	2.5	10
3αOH-Tibolone	di-OTMS	16.31	443.2796	NA	NA	excretion urine
3 βOH-Tibolone	di-OTMS	15.19	443.2769, 353.2295	94	2.5	10
17α-methyl-5α-androstane-3α,17β-diol	di-OTMS	15.69	270.2342, 450.3344	130	1	10
17α-methyl-5β-androstane-3α,17β-diol	di-OTMS	15.87	270.2342, 450.3344	94	1	10
5α-androstan-3α,17β-diol	di-OTMS	14.21	256.2186, 241.1951	NA	4 (as LOQ[a])	10
5β-androstan-3α,17β-diol	di-OTMS	14.55	256.2186, 241.1951	NA	4 (as LOQ)	10
6-OXO androstenedione	tri-OTMS	18.34	501.2671, 516.2906	123	2.5	10
Androsterone msms	di-OTMS	13.58	434.3031 − > 419.2796	NA	100 (as LOQ)	10
Bolasterone metabolite (7α,17α-dimethyl 5β-androstane 3α,17β-diol)	di-OTMS	17.11	374.2999, 269.2264	99	2.5	9
Bolasterone PC	di-OTMS	18.33	460.3187, 445.2953	97	2.5	10
Boldenone metabolite (5β-androst-1-ene 17β-ol-3-one)	di-OTMS	12.43	194.1121	99	2.5	10
Calusterone met (7β,17α-dimethyl-5β-androstane-3α,17β-diol)	di-OTMS	16.8	284.2499, 374.2999, 269.2264	106	2.5	10
Calusterone PC	di-OTMS	18.56	460.3187, 445.2953	NA	2.5	9
Clenbuterol ms/ms	OTMS, NTMS	6.15	335.0690 − > 300.1001 335.0690- > 227.0525	66	0.1	10
Clostebol met (4-chloroandrost-4-en-3α-ol-17-one)	di-OTMS	17.49	466.2485, 468.2587	110	2.5	10
Cyclofenil m1	tri-TMS	20.24	422.2092	97	10	10
D3-epitestosterone.	di-OTMS	15.86	435.3063 > 420.2828	NA	NA	ISTD
D3-testosterone	di-OTMS	16.67	435.3063 > 420.2828	NA	NA	ISTD
Danazol m1(Ethisterone)	tri-TMS	18.64	456.2874, 441.2640	103	2.5	9
desoxymethyltestosterone I(17α-methyl-5α-androstan-2ξ,3α,16ξ,17β-tetrol)	tetra-OTMS	21.00	626.4033	NA	NA	excretion urine
desoxymethyltestosterone II(17α-methyl-5α-androstan-2ξ,3α, 17β-triol)	tri-OTMS	15.15	523.3454	NA	NA	excretion urine
desoxymethyltestosterone M1 (17α-methyl-5α-androstan-2α,3α,17β-triol)	tri-OTMS	17.95	523.3454	NA	NA	excretion urine
desoxymethyltestosterone M2LT(18-nor17,17-dimethyl-5α-androst-13-en-2ξ,3α-diol)	di-OTMS	10.85	448.3187	NA	NA	excretion urine
Drostanolone PC	di-OTMS	16.75	448.3187	99	2.5	10
Drostanolone met (Drostanolone 3ol 17one)	di-OTMS	14.26	448.3187	106	2.5	10
Epimetendiol	di-OTMS	12.55	358.2686, 448.3187	103	1	10
Epitestosterone	di-OTMS	15.99	432.2874 > 209.1356 432.2874 > 417.2640,	NA	2(as LOQ)	10
ethylestranol		12.4	270.2342	80	2.5	10
etiocholanolone msms	di-OTMS	14.11	434.3031 > 419.2796	NA	100 (as LOQ)	10
Fluxymesterone met(9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one)	di-OTMS	15.35	462.2780, 447.2545,	106	2.5	10
Formebolone met(Dealdehyde-formebolone)	tri-TMS	19.14	534.3375	97	2.5	10
Formestane	tri-OTMS	19.25	518.3062, 503.2828	95	20	10
Furazabol	OTMS	21.54	387.2462, 402.2697	94	2.5	10
furazanol met(16β-hydroxyfurazabol)	di-OTMS	24.35	490.3042, 218.1153	54	2.5	10
Letrozol met	$C_{17}H_{11}N_5$	9.91	217.0760, 291.0917	112	6.25	9
MDA	di-NTMS	5	188.1285	81	20	10
mesterolone met(1α-methyl-5α-androstane-3α-ol-17-one)	di-OTMS	15.47	448.3187, 235.1513	89	2.5	10
Mesterolone PC	di-OTMS	16.3	433.2953, 448.3187	101	2.5	10
Methasterone met(2α,17α-dimethyl-5α-androstane-3A,17β-diol)	di-OTMS	16.24	449.3266, 374.2999	118	2.5	10
Methasterone PC	di-OTMS	18.3	462.3344, 419.2796, 332.2530, 372.2843	105	2.5	10
Methenolone	di-OTMS	17.24	446.3031, 431.2796, 195.1200, 208.1278	100	2.5	9
Methenolone met (3α-hydroxy-1-methylen-5α-androstan-17-one)	di-OTMS	15.06	446.3031, 431.2796	97	2.5	10
Methyl-1-testosterone	di-OTMS	17.24	446.3031, 431.2796, 194.1121, 356.2530	100	2.5	9
mibolerone	di-OTMS	17.78	446.3031, 431.2796, 301.1982	89	2.5	10
Androsterone mono-TMS	mono-OTMS	13.46	272.2135	NA	NA	10
19-norandrosterone	di-OTMS	11.73	405.2640, 315.2139, 420.2874	116	1	9
19-Noretiocholanolone	di-OTMS	13.13	405.2640, 315.2139, 420.2874	116	2.5	10
Norclostebol	di-OTMS	20.15	452.2328, 417.2640	73	2.5	10
Norethandrolone m1 (17α-ethyl-5α-estrane-3α,17β-diol)	di-OTMS	16.65	241.1951, 331.2452	97	2.5	10
Norethandrolone m2(17α-ethyl-5β-estrane-3α,17β-diol)	di-OTMS	17.52	241.1951, 331.2452	107	2.5	10
Norfenefrine	di-OTMS, di-	4.82	174.1129, 426.2131	2	50	10

(continued on next page)

Table 1 *(continued)*

Name	Derivative	RT (min)	Ions (m/z)	Recovery%	LOD, (ng/ml)	Detection in 10 urine aliquots
	NTMS					
OT M3(4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androstan-3α-ol)	di-OTMS	18.5	379.2218, 343.2452, 253.1982	NA	NA	excretion urine
OT EPIM3(4-chloro-18-nor-17 α-hydroxymethyl,17β-methyl-5 β-androstan-3α-ol)	di-OTMS	17.5	379.2218, 343.2452, 253.1982	NA	NA	excretion urine
OT M4(4-chloro-18- nor-17β-hydroxymethyl,17α-methylandrost-4-en-3α-ol)	di-OTMS	18.4	377.2062, 287.1561	NA	NA	excretion urine
OT EPIM4(4-chloro-18-nor-17 α- hydroxymethyl,17β-methylandrost-4-en-3α-ol)	di-OTMS	17.4	377.2062, 287.1561	NA	NA	excretion urine
OT II(4-chloro- 3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one)	tetra-OTMS	21.3	656.3330	NA	NA	excretion urine
Oxabolone PC	tri-OTMS	18.67	506.3062	102	2.5	10
Oxymesterone	tri-OTMS	20.81	534.3375, 519.3141, 389.2327	84	2.5	10
stenbolone	di-OTMS	16.23	446.3031, 208.1278	106	2.5	10
Testosterone	di-OTMS	16.83	417.2640, 432.2874	NA	2(as LOQ)	10
Oxandrolone NW 1 (17α-hydroxymethyl-oxandrolone)	Mono-OTMS	18.4	273.1849	NA	NA	excretion urine
Oxandrolone NW 2(17β-hydroxymethyl-oxandrolone)	Mono-OTMS	18.6	273.1849	NA	NA	excretion urine
THCCOOH	di-OTMS	18.68	488.2773, 473.2538, 371.2401	80	5	10
5α-Zearalanol	tri-OTMS	19.3	433.2225	83	2.5	10
5β-Zearalanol	tri-OTMS	19.5	433.2225	97	2.5	10
Oxymethelone M1(18-nor-2ξ,17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol)	tri-OTMS	19.90	447.3109, 357.2608	NA	NA	excretion urine
Oxymethelone M2 LT(18-nor-17β-hydroxymethyl-17α-methyl-2ξ −methyl-5α-androst-13-en-3α-one)	di-OTMS	17.20	357.2608	NA	NA	excretion urine

[a] LOQ: limit of quantification for the endogenous steroids.

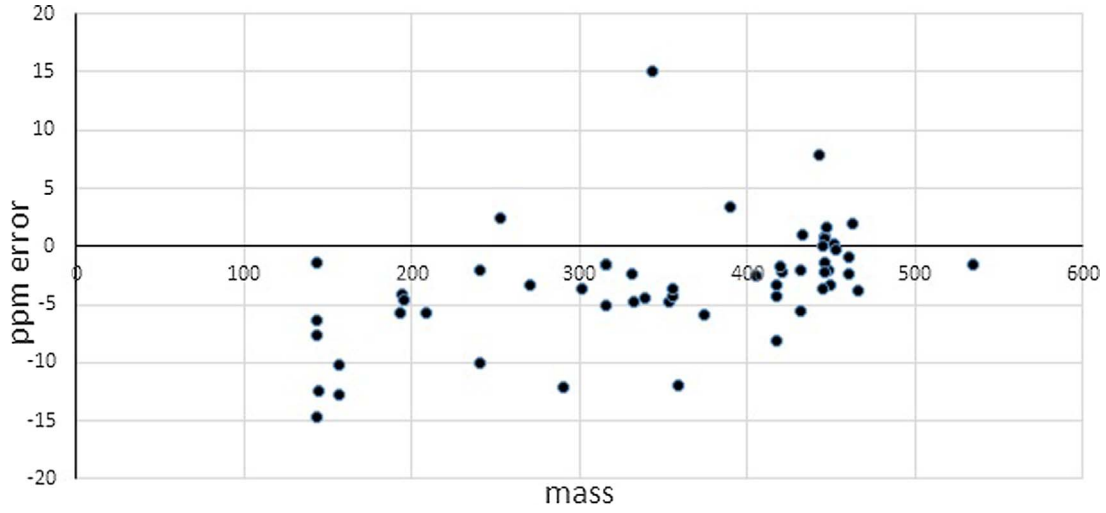

Fig. 1. Mass errors of representative AAS' diagnostic ions over the entire mass range.

level of 2.5 ng/mL in urine matrix and FS acquisition mode. At all examples in Fig. 1, 70% of the mass errors were lower than 5 ppm, 18% between 5 and 10 ppm and 12% of mass error were higher than 10 ppm. The mass errors were considered sufficient for the conditions of the AASs spiked concentrations, urine matrices and FS acquisition. The acquisition rate presented in 2.1.4.1. provided 30–50 data points which is sufficient to identify and quantify analytes peak in the acquired FS GC/MS data. The mass accuracy and the quantitative analysis were also influenced by the instrument's dynamic concentration range. More specifically, substances injected at concentrations greater than 1000 ng/mL resulted in saturation of the MS detector as indicated by the instrument's software. The signals of Etio and A above the 3rd highest calibration concentration of 1000 ng/mL showed sign of detector saturation. This problem was overcome by the use of the MSMS of EI GC/MS ions instead of FS acquisition and using ion transition of *m/z* 434.3031–419.2796 for Etio and A. On the other hand, in the lower concentration range, the detection of the beta2-agonist clenbuterol at 50% MRPL of 100 pg/mL cannot be achieved by FS mode, but only in MS/MS mode (Table 1, Fig. 2). The acquisition for T and E was also performed in MS/MS mode to improve detection at 2 ng/mL concentration level and to differentiate from the close eluting 11-βOH-etiocholanolone.

3.3. Quantitative validation results

The GC/QTOF method was validated also for the quantitative

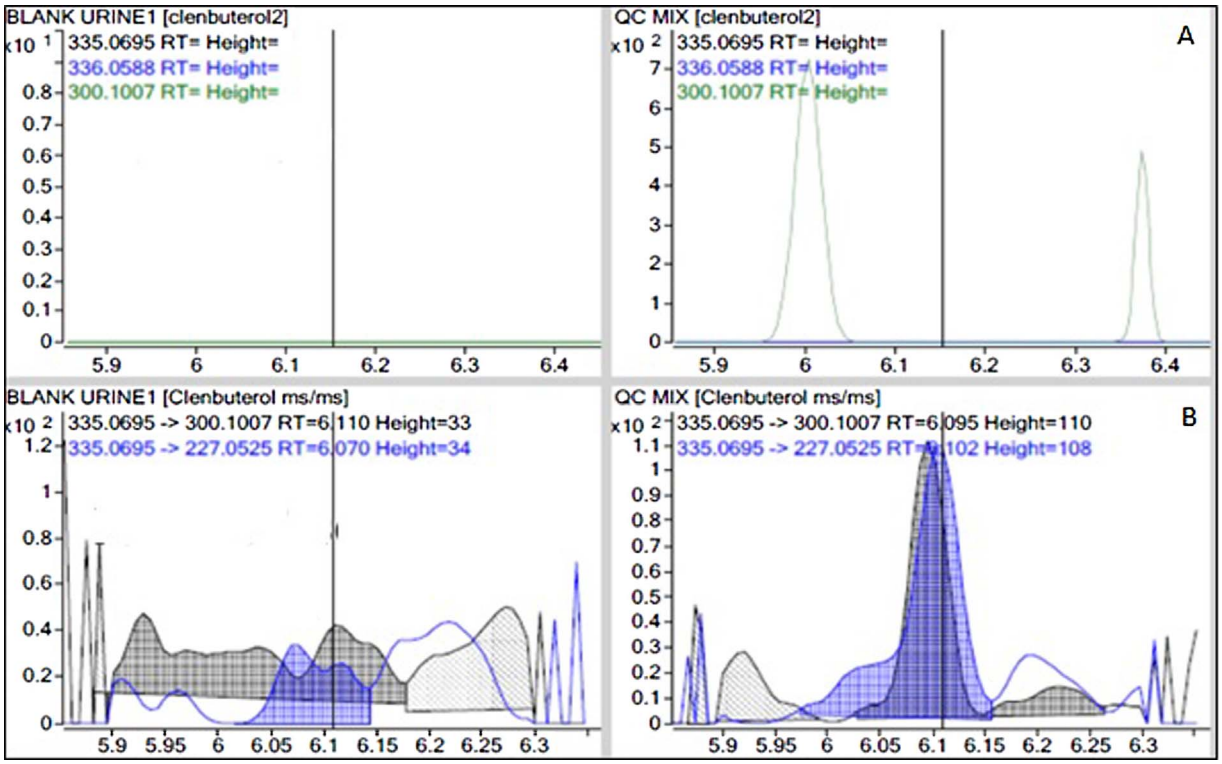

Fig. 2. Clenbuterol detection at 0.1 ng/mL. (A) blank and spiked urine in full scan mode. (B) blank and spiked urine in MS/MS mode.

Table 2
Concentrations levels used for the construction of the calibration curves.

Compound name	Calibration range (ng/ml)	r2 of one day	Slope of one day	Intercept of one day	Level[a] (ng/ml)	Intermediate precision (%)	Biases (%)	MU (%)[b]
Androsterone (A)	100–8000	0.999	0.00160	−0.18370	400	6.2	8.2	10.4
					2000	4.4	5.4	6.9
Etiocholanolone (ETIO)	100–8000	0.999	0.00140	−0.06240	400	5.2	7.5	9.2
					2000	2.8	5.7	6.3
5α-androstandiol(5αadiol)	4–800	0.995	0.01520	−0.26740	40	13.5	15.1	20.2
					200	8.7	8.9	12.5
5β-androstandiol(5βadiol)	4–800	0.993	0.00030	−0.00470	40	9.5	11.5	14.8
					200	5.9	7.5	9.5
Testosterone (T)	2–400	0.996	0.00030	−0.00250	20	7.9	11.5	14
					100	11.4	8.6	14.3
Epitestostrone (E)	2–400	0.995	0.00020	−0.00220	20	7.4	17.8	19
					100	9.8	9.8	13.8

[a] Referred to the level of QCs used.
[b] combined $MU = \sqrt{intermediateprecision^2 + Bias^2}$.

analysis of the six steroids of the steroidal ABP according to the approach described in [24]. Table 2 shows the validation results for the six endogenous steroids characterized by linearity range, correlation coefficient, slope and intercept, bias, intermediate precision and combined uncertainty. The combined uncertainty for the determination of A, Etio, 5αadiol, 5βadiol, T and E, as estimated during method validation in order to fulfill the requirements described in [24]. The Limit of Quantitation (LOQ) is considered the lowest concentration in the calibration curve for each steroid [24].

3.4. Analytical performance comparison GC/QQQ vs GC/QTOF

For a period of 3 months in 2016, approximately 700 aliquots selected from ongoing routine analysis of antidoping samples for screening and confirmatory purpose were analyzed with GC/QTOF in parallel to the routine GC/QQQ instrument. During analysis period of 3 months, the GC part of the GC/MS required only maintenance, which consisted of replacement of the liner and septum and cutting the beginning of the column. The criteria of the MS maintenance initiation,

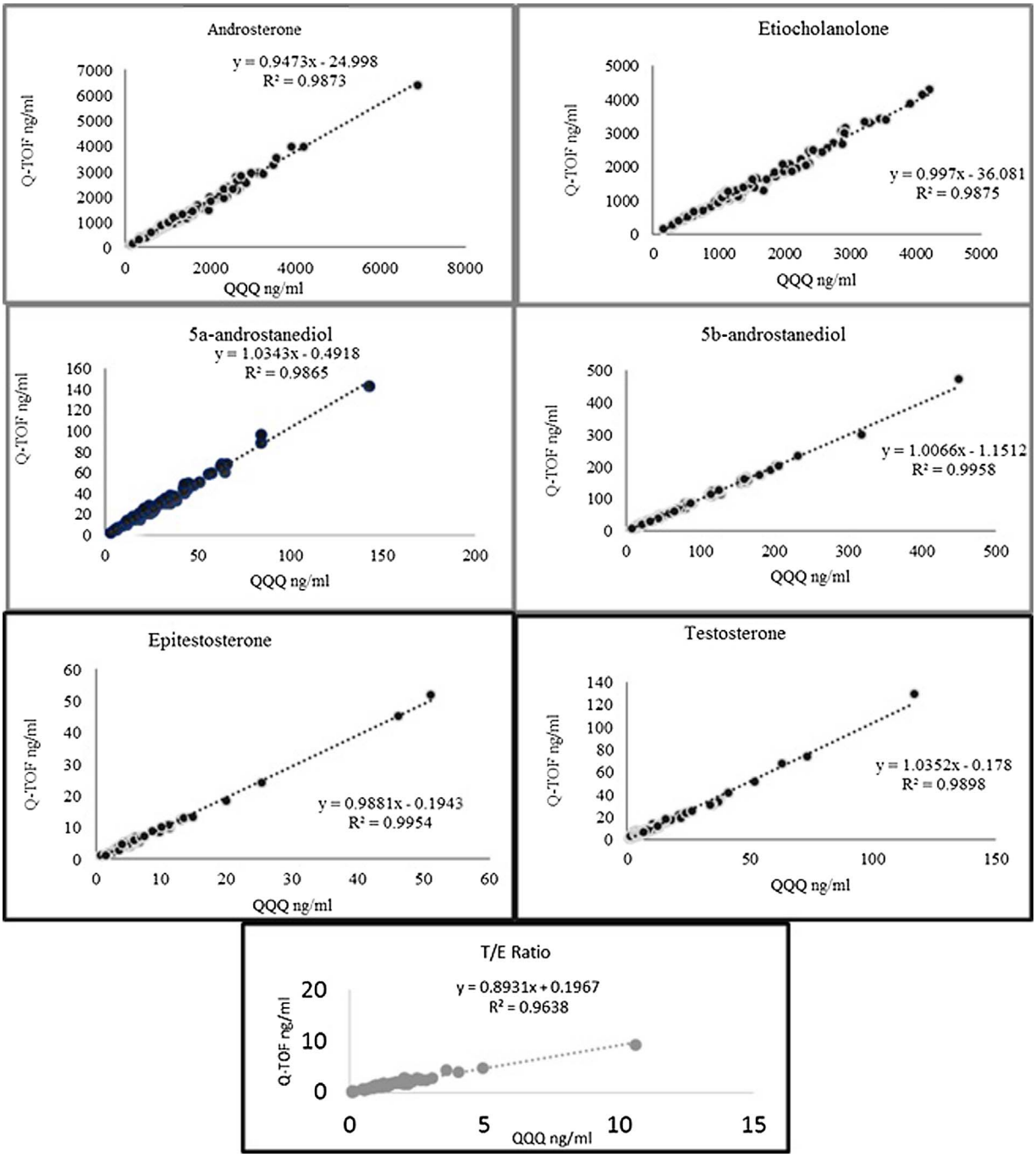

Fig. 3. Correlation of SP measured with GC/QQQ and GC/QTOF characterized by correlation coefficients.

except those used to accept in-house quality control samples, is the mass accuracy and the stability of the mass calibration within sequence of multiple aliquots analysis. The, mass accuracy in the system suitability mass calibration algorithm were kept below 3 ppm error for the PFTBA ions. Similarly, calibration of TOF mass axis ran within the analytical sequence every 3 samples injections, resulted in PFTBA mass errors at the level of 5 ppm, which were corrected down to 1 ppm level. The lock mass correction of the mass calibration within the acquisition requires the simultaneous infusion of the calibrant PFTBA during sample analysis. This option was not used in order to avoid detector saturation and subsequent reduction of the instrument's dynamic range.

The analytical performance of both GC/QQQ and GC/QTOF in terms of analytes qualitative detection was comparable; i.e. all findings as analytes detected in GC/QQQ were also detected in GC/QTOF in samples used for screening, confirmation and proficiency testing samples. In relation to the quantitative analysis of the six steroids of the

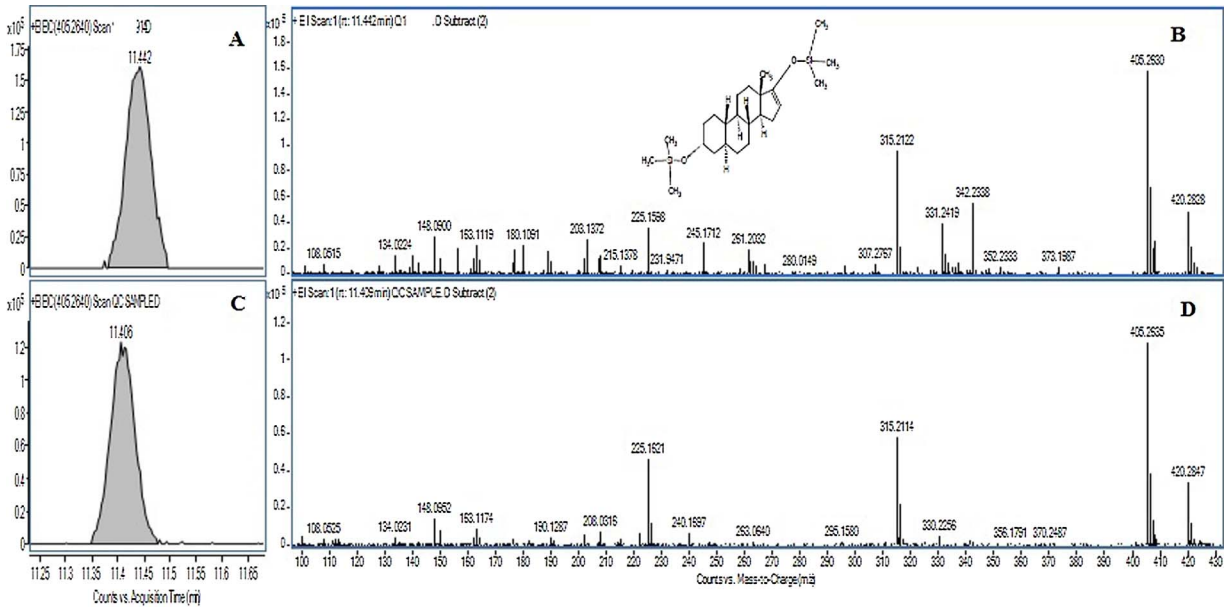

Fig. 4. Analysis of real sample of 19NA: (A) extracted ion chromatogram at 20 ppm mass window,of 19NA in an original urine sample estimated at 3.9 ng/mL. (B) full scan spectrum 19NA in an original sample. (C) extracted ion chromatogram at 20 ppm mass window, of 19NA in a positive control at 5 ng/mL. (D) full scan spectrum 19NA in a positive control.

Table 3
Mass accuracies for the main ions for 19NA in the original samples and positive control.

	Real sample		QC sample	
Theoretical mass	Experimental mass	Mass error (ppm)	Experimental mass	Mass error (ppm)
420.2874	420.2828	−10.9	420.2874	0
405.2640	405.2630	−2.5	405.2635	−1.2
315.2139	315.2122	−7.0	315.2114	−9.5

(All errors are negative, showing systematic negative bias).

ABP-SP, in Fig. 3, the correlation graphs of the GC/QTOF estimated quantitative steroid profile versus the respective quantitative profile obtained with the accredited GC/QQQ are presented. For T and E the LOQ in GC/QQQ was 1 ng/mL, while in GC/QTOF, it was 2 ng/mL. All correlation coefficients shown in Fig. 3 were above 0.94 showing high agreement between the two screening platforms.

3.5. Application of GC/QTOF to real samples and verification of reference materials

Fig. 4 shows the ion chromatogram of a real sample of the AAS nandrolone main metabolite 19-norandrosterone di-TMS estimated at 3.9 ng/mL is presented together with the respective full scan spectrum, to be compared with a Quality Control Positive sample spiked at 5 ng/mL. The mass accuracies of the most characteristics ions in the real sample and in the Quality Control Positive spiked samples are presented in Table 3. Similarly, Fig. 5 shows the extracted ion chromatograms of newly discovered long-term metabolites of the AAS dehydrochloromethyltestosterone [6] M3 and epi-M3 in a sample obtained from an excretion study sample provided by WAADS. The mass accuracies of the main characteristics ions of M3 from this sample are presented in Table 4. This procedure is useful to verify the identity of non-commercially available reference materials, where the certificate of analysis does not exist.

4. Conclusions

The development of a new GC/QTOF method for the screening and confirmatory GC/MS analysis of small molecules not subjected in LC/MS analysis for the WADA antidoping system is presented. The presented method was validated at LOD of 50% MRPL for the majority of the target analytes in FS acquisition mode. Our data shows that the SP profile obtained with high-resolution GC/QTOF meet the WADA specifications [24]. The quality of the FS fragment mass spectra obtained for representative AAS was proven by the achieved mass accuracy of the main characteristic ions at the level of 15 ppm mass window. The specificity of the method for the studied AAS is similar to the routine method used at ADLQ based on tandem MS in a GC/QQQ instrument. This study demonstrated the robustness in terms of manufacturer and in-house ADLQ maintenance and instrument failures of the GC/QTOF instrument in a typical routine analysis workload where more than 700 samples were analyzed in a period of 3 months. The acquisition of FS data in the same MS cycle, together with possible tandem MS acquisitions, permits the retrospective analysis of the acquired GC/TOF data of sample analyzed for official antidoping purpose to detect other non-targeted substances. These non-targeted substances could be illegal designer drugs or AAS long-term metabolites that prolong AAS detection abuse in the human urine samples, and detection with our approach is possible even for analytes, which were not known at the time of the data acquisition.

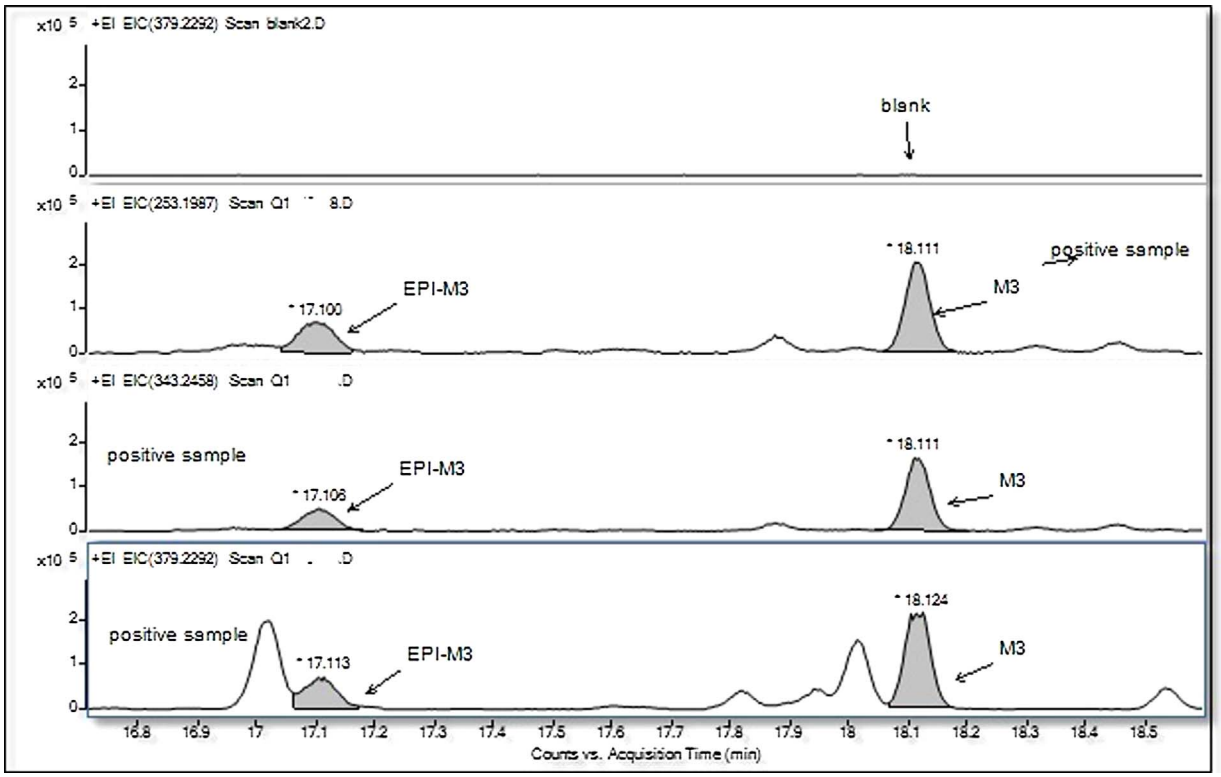

Fig. 5. Extracted ion chromatograms at 20 ppm mass window, of newly discovered long-term metabolites of the AAS dehydrochloromethyltestosterone M3 and epi-M3 in a real case and in a blank urine samples.

Table 4
Mass accuracies for the main ions of dehydrochloromethyltestosterone m3.

Theoretical mass	Experimental mass	Mass error(ppm)
379.2218	379.2292	19.5
343.2452	343.2504	15.1
253.1982	253.1988	2

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