Research Article

Drug Testing and Analysis

Received: 8 June 2009 Revised: 11 July 2009 Accepted: 13 July 2009 Published online in Wiley Interscience: 21 September 2009

(www.drugtestinganalysis.com) DOI 10.1002/dta.50

Generic sample preparation and dual polarity liquid chromatography – time-of-flight mass spectrometry for high-throughput screening in doping analysis

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The requirements on initial testing in doping control are getting tighter regarding efficiency and speed while the scope of analytes is getting more diverse and, consequently, the need for high-throughput methods is apparent. In this study, a comprehensive screening method for doping agents in human urine is presented, based on solid phase extraction (SPE) and liquid chromatography–time-of-flight mass spectrometry (LCTOFMS). The method covers most of the compound groups in the list of prohibited substances by World Anti-Doping Agency (WADA). Mixed-mode SPE on two types of sorbent and the use of negative ionization mode besides the commonly used positive mode in electrospray ionization (ESI) allowed detection of acidic compounds, such as sulpho-conjugated metabolites. A run time of 8 minutes for each of the two ESI polarities was achieved. The method was validated regarding relative ionization efficiency, selectivity and signal to noise at the WADA's minimum required performance limit (MRPL) level, resulting in the acceptance of 197 compounds. A selection of 20 compounds was submitted for a more thorough validation, including extraction recovery, repeatability and linearity. Recovery and linearity (R^2) varied mainly between 83–115% and 0.78–0.99, respectively. Median values for repeatability at the MRPL and 10×MRPL levels were below 20%. A mean and median mass accuracy of 1.2 and 0.80 mDa, respectively, was achieved. The present method represents at the moment the widest coverage of low molecular weight prohibited substances for the screening in sports, providing an approach for further rationalisation of the analytical work-flow in the doping control laboratories. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: doping control; liquid chromatography-time-of-flight mass spectrometry (LC-TOFMS); high throughput; accurate mass; screening

Introduction

High throughput is the key word in today's doping analysis. While reporting time schedules are constantly tightened, the number of prohibited compounds on the list by World Anti-Doping Agency (WADA) is increasing.[1] Considering the limited sample volume available for testing, new analytical approaches are needed to respond to the challenge. Due to the requirements of selectivity and sensitivity the common approach for initial testing is based on the use of various mass spectrometric methods, applying predominantly gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) techniques. Comprehensive reviews of these strategies have been published elsewhere in detail.[2–4]

An optimal screening strategy would consist of a single analysis method for all analytes but, until now, the proposed methods have not been completely successful. To speed up initial testing and to rationalize the workflow in doping control laboratories, separate sample preparation steps have been combined and more compound groups have been included in analysis methods.[5–15] Most of the methods in doping control were based on LC-MS in the tandem mass spectrometric mode (MS/MS),[6,13,15] using fast LC with small dimension columns and small particle size to gain enhanced resolution, high peak capacity and high throughput. In food and veterinary analysis, fast multi-residue drug screening strategies have become the leading trend.[9,16–19]

Liquid-liquid extraction (LLE) with salting out is often preferred as one of the best generic sample preparation methods,[11,13,20,21] but it cannot be automated and its consumption of harmful solvents can be high. Comprehensive solid-phase extraction (SPE) methods have been applied occasionally and with success in doping screening analysis[9,22,23] and other fields of analytical toxicology.[24] The challenge of SPE is the high number of parameters that must be adjusted for optimum performance.

Several articles have recently been published on comprehensive doping screening, indicating the topicality of the subject. Our group presented a screening approach for 97 doping agents using mixed-mode (cation exchange + reversed phase) SPE, LC separation on an octadecyl reversed-phase column with conventional dimensions, electrospray ionization (ESI) in the positive mode and

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mass analysis by time-of-flight mass spectrometry (TOFMS), with a total analysis run time of 27 min.[22] Another group presented a screening method for 104 doping agents by combining rapid resolution LC-ESI-TOFMS and GC-TOFMS techniques and achieving an LC run time of 14 minutes.[7] Elsewhere, LC-ESI-MS/MS triple quadrupole instrumentation was applied in the dual polarity multiple reaction monitoring mode for the simultaneous detection of 72 doping agents within six different substance classes.[11] A column with conventional particle size was used and the total analysis run time was 19 minutes. The two latter papers relied on LLE as a sample preparation method applying two different extractions to cover both basic and acidic drugs. Very recently, a method was published relying on simple twofold dilution of urine samples prior to ultra-high-pressure (UP) LC-ESI-TOFMS screening.[25] The method included 103 compounds analysed in two consecutive 9 min runs of different MS polarity. In all of the studies mentioned, the compounds were analysed in their unconjugated form, originally free or after enzymatic hydrolysis of glucuronide conjugates, but the analysis of sulfo-conjugates was not discussed.

In our previous study we demonstrated the power of LC-TOFMS in comprehensive doping screening analysis.[22] However, the use of cation exchange-based mixed-mode SPE and positive ionization ESI excluded more polar and acidic compounds of interest, such as thiazide diuretics. Moreover, the LC run time was too long to meet the increasing productivity requirements. In this paper we extend the scope of LC-TOFMS screening by introducing a new generic extraction method followed by dual-polarity ESI in two separate runs. We also apply a column with a particle size smaller than 2 µm to achieve fast LC separation, meeting the requirements of high-throughput screening. The 207 tested doping agents of low molecular weight fall into the main drug groups prohibited by WADA: anabolic agents, β_2-adrenergic agonists, agents with anti-estrogenic activity, diuretics, compounds affecting oxygen transfer (efaproxiral), stimulants, narcotics, cannabinoids, glucocorticosteroids and β-blockers. The LC-TOFMS method is validated in terms of relative ionization efficiency and signal to noise (S/N) at the minimum required performance limit (MRPL) level, and for a selection of 20 compounds a more thorough validation is performed from a urine matrix, including extraction recovery, repeatability and linearity. In addition to the analysis of free unconjugated compounds and enzyme hydrolyzed glucuronide conjugates, examples of the analysis of intact sulfo-conjugated compounds are presented.

Experimental

Materials

17-Dihydroxyexemestane[26], *p*-hydroxymesocarb and its sulfo-conjugate[27] were chemically synthesized in-house and were kindly supplied by the Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki. Etamivan sulphate was enzymatically synthesized *in vitro* in-house as described earlier in reference[28]. The rest of the reference drugs and metabolites of pharmaceutical purity were purchased from various pharmaceutical suppliers.

Acetonitrile and methanol were purchased from Labscan (Poch Sa, Swinskiego, Poland). Ammonium formate was from Sigma (St Louis, MO, USA). Formic acid of UPLC/MS grade was obtained from LGC Promochem GmbH (Wesel, Germany). 2-Propanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). β-Glucuronidase *(E. coli)* K12 (80 U/mg at 25 °C) was from Roche (Mannheim, Germany). The other solvents and reagents were purchased from Merck (Darmstadt, Germany) and they were of high-performance liquid chromatography (HPLC) or analytical grade. Isolute IST HCX (130 mg) and HAX (130 mg) mixed-mode SPE cartridges were acquired from Biotage (Uppsala, Sweden). Dibenzepin and d_4-(9,11,12,12)-cortisol were selected as internal standards (IS) and diluted to a concentration of 100 and 1000 ng/mL in methanol respectively.

Urine samples used in this study were obtained from healthy volunteers. Spot urine samples from four male and four female volunteers were used to demonstrate the selectivity. In the validation process, pooled urine samples from two male and female volunteers (n = 4) were applied. The quality control samples were a part of the external quality assessment scheme of the doping control laboratory. The excretion urine sample of etamivan was obtained from a healthy male volunteer after oral administration of 40 mg of etamivan. The urine sample was collected 6 to 12 hours after the administration and was frozen for storage.

Sample preparation

The two-step SPE procedure is described in Figure 1. A 1-mL aliquot of human urine with 10 µL of IS solution and 375 µL 0.8 M sodium/potassium phosphate buffer, pH 7, was hydrolyzed with 15 µL of β-glucuronidase for 1 hour at 50 °C in a water bath. Analytes were first extracted with a HCX cartridge to collect basic and neutral analytes. Wash solutions from the HCX cartridge were further extracted with a HAX cartridge to extract acidic compounds. These two fractions were then combined and evaporated to dryness under a nitrogen stream at 45 °C. Dry residue was reconstituted with 150 µL of mobile phase (A : B; 90 : 10; v : v) and centrifuged for 10 minutes at 5700 *g* (8000 rpm). Finally, the supernatant was transferred into a sample vial for analysis.

Liquid chromatography

An Agilent 1200 (Agilent Technologies, Waldbronn, Germany) series rapid resolution LC system with a micro vacuum degasser, autosampler, binary pump and column oven was used for chromatography. Zorbax Eclipse Plus rapid resolution HT C18 column 50 × 2.1 mm (1.8 µm) from Agilent with in-line frit was used in gradient mode at 40 °C. The mobile phase consisted of 2.5 mM ammonium formate/0.1% formic acid (A) and 2.5 mM ammonium formate/0.1% formic acid in 90% acetonitrile (B). The flow rate was 0.4 mL/min. The proportion of B was held at 10% for one minute and then linearly increased to 40% in 2 min, to 70% in 1 min, to 90% in 2 min and held at 90% for 0.5 min and then back to 10% in 0.5 min. The column equilibration pre-run time (10% of B) was 1 min and the analysis cycle time was 8 minutes. The injection volume was 3 µL. HyStar version 3.2 by Bruker Daltonics (Bremen, Germany) was used to control the LC instrument.

Mass spectrometry

The TOF mass spectrometer was a Broker Daltonics micrOTOF, equipped with an orthogonal ESI ion source. Ionization was performed in both positive and negative modes. Ionization parameters were optimized with direct injection of a set of compounds with different chemical properties covering the whole chromatographic separation time scale at a concentration of 1 µg/mL for each compound by an external syringe (KD Scientific syringe pump, MA, USA). Two separate runs were performed

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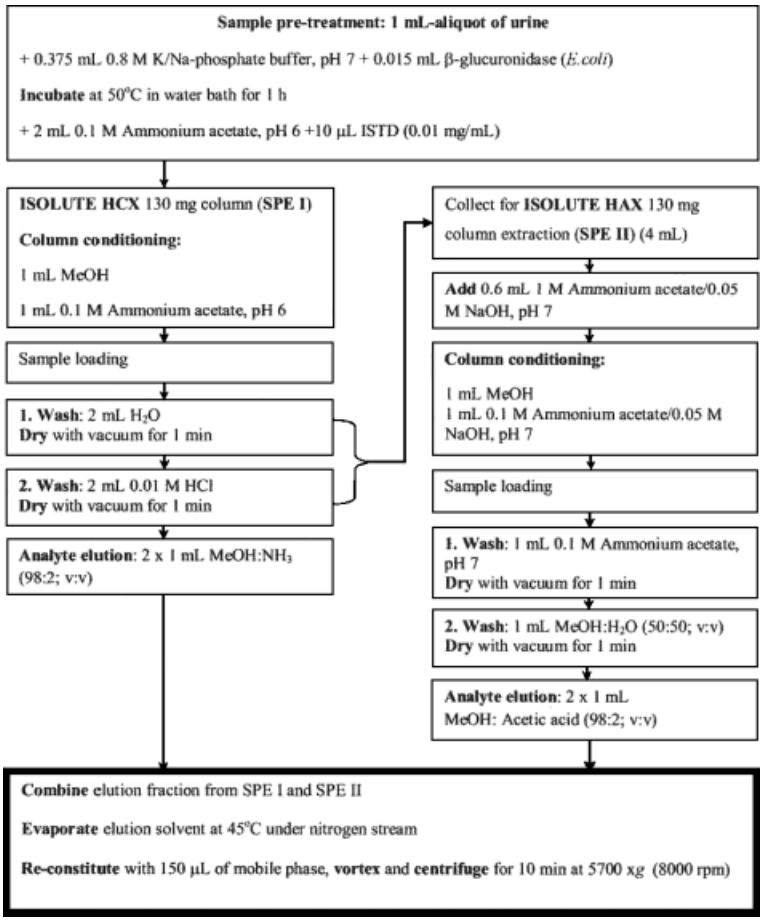

Figure 1. Generic extraction procedure involving acidic and basic solid phase extraction (SPE).

to cover both polarities because of the hardware features of TOFMS. The following compounds were selected for positive ionization optimization: amphetamine, bunolol, buprenorphine, canrenone, clenbuterol, mefruside, oxandrolone, phenylephrine and ritalinic acid. Parameters in negative ionization mode were optimized with the following compounds: 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (metabolite of cannabis, THC-COOH), acetazolamide, chlorothiazide, dexamethasone, etacrynic acid, mersalyl acid, modafinil and *p*-hydroxymesocarb sulphate. The nebulizer and dry gas flows (nitrogen) were 1.6 bar and 8.0 L/min respectively. The drying temperature was 200 °C. For the positive mode the applied capillary voltage was 4500 V, the capillary exit was 85.0 V and skimmer 1 was set to 35.0 V. The transfer time of the ions from hexapole to orthogonal acceleration was 40 µs and 5 µs was applied for pre pulse storage. Hexapole radio frequency was 45.0 Vpp. The corresponding values for negative mode were 3400 V, −97.6 V, −45.7 V, 35.0 µs, 10.0 µs and 135.7 V. The main optimized parameters were capillary exit, skimmer 1 and hexapole RF which affected the *m/z* values transmitted to the ion optics. The criterion for optimization was to obtain good intensity and resolution for the test compounds. Mass spectral data were collected within the range of *m/z* 50–600. On an average, resolution for *m/z* 296 (dibenzepin) was 11 000. TOFMS was operated with micrOTOF control version 3.2 (build 23) by Bruker Daltonics.

Daily external calibration of TOFMS for positive and negative ionization modes was performed with sodium formate solution containing 10 mM sodium hydroxide in 2-propanol/0.2% formic acid (1 : 1, *v*/*v*) by syringe injection. The calibration was performed in the quadratic and high precision calibration mode covering the whole mass range with a minimum of seven sodium formate clusters (positive: Na(NaCOOH)n, negative: $HCOO(NaCOOH)_n$, n = 1–8). An automated internal post-run mass scale calibration of individual samples was based on an injection of the calibrant at the beginning and at the end of each run, the latter being for manual verification of calibration stability.

Relative ionization efficiencies (relative to dexamethasone) were determined for all the studied compounds in positive and negative ionization modes. Solutions of each studied compound and dexamethasone (reference compound) were prepared separately at a concentration of 500 ng/mL into 1 mL of mobile phase (A : B; 90 : 10; v : v). An Agilent 1200 LC autosampler was used for injection and sample flow was introduced directly to the ESI ion source. Measurement time was 1.5 minutes per sample with a 0.5 min washout time. Injection volume was 20 µL. The relative intensities of $[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$ and $[M+HCOO]^-$ were calculated.

Data evaluation

LC-TOFMS acquisition data were processed with TargetAnalysis version 1.1 (Build 192) and DataAnalysis macro (version 3.4) by Bruker Daltonics, similar to our previous study.[22] The two-level search criteria for target masses included

1) mass tolerance (1. < 8 ppm, 2. < 15 ppm)

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2) retention time window (1. < 0.2 min, 2. < 0.3 min)
3) isotopic pattern match SigmaFit™ (SigmaFit) (1. < 0.03, 2. < 0.05).

SigmaFit (by Bruker Daltonics) is an exact numerical comparison of theoretical and measured isotopic patterns. It provided an additional identification parameter for accurate mass measurement. An entry fulfilling the first 'level' was reported as positively identified, but if any of the three parameters were between the two levels, an entry was probably identified. Compounds without retention time were tentatively identified. In the report listing the two first levels were highlighted with different colours. Extracted ion chromatograms (EIC) of the expected $[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$ or $[M+HCOO]^-$ ions of each compound were generally created by the application macro with a 3 mDa window.

The in-house database was created as described in Kolmonen *et al*.[22] Mixtures of five to seven compounds in mobile phase (A : B, 90 : 10, v : v) were used, in which the concentration for each compound was 5 µg/mL. The in-house database for positive ionization comprised 195 entries for the studied prohibited substances and their possible adducts. For negative ionization, the database included 67 entries. Some compounds were included in both databases due to their ionization characteristics. The database contained retention time (RT), molecular formula of the compounds and theoretical monoisotopic masses, which were calculated with the Bruker Simulate Isotopic Pattern tool based on their molecular formula. Due to matrix interference, individual screening parameters for SigmaFit and mass accuracy were applied for some of the compounds.

Validation procedure

All 207 compounds were validated with respect to ionization efficiencies, the MRPL level (four replicates), S/N ratios and selectivity. A more comprehensive validation of the method was performed for 20 selected compounds, including analytes with diverse physico-chemical properties covering both ESI polarities. The list of the selected compounds with their chemical structures is presented in Figure 2. The full validation consisted of extraction recovery, intra-day (five replicates) and inter-day (five days) repeatability at the MRPL and 10×MRPL levels, selectivity and linearity. The measurement of extraction recoveries was performed by spiking compounds at the MRPL or 10×MRPL level before and after SPE extraction. Absolute peak areas were normalized to the peak area of the IS (dibenzepin or d_4-cortisol) at both polarities. The selectivity was demonstrated with blank urine samples from healthy volunteers (female and male, n = 4 for each). Linearity was measured at five concentration levels corresponding to MRPL, 5×MRPL, 10×MRPL, 25×MRPL and 50×MRPL. Ion suppression in positive and negative ion modes was studied by injecting an extracted pooled urine blank sample to a direct inlet flow of dexamethasone (1 µg/mL, 0.3 mL/min) and the potential changes in the intensity of dexamethasone were examined.[29] The performance of

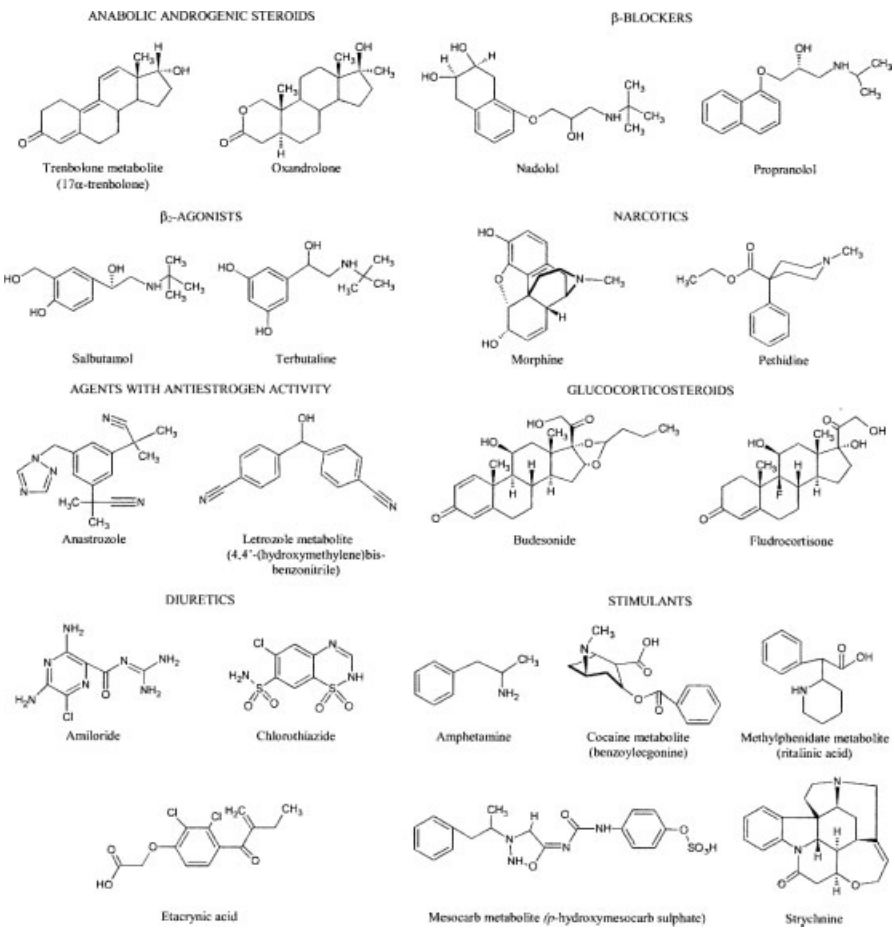

Figure 2. Structures of the selected compounds for extensive method validation.

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the method was evaluated with five external quality control samples.

Results and Discussion

Sample preparation

The extraction method was performed in two steps (Figure 1): after enzymatic hydrolysis of glucuronide-conjugated compounds with β-glucuronidase *(E. coli)* urine samples were extracted in mixed-mode strong cation exchange/C8 SPE columns (HCX). Wash solutions were collected for extraction of acidic compounds in mixed-mode strong anion exchange/C8 SPE columns (HAX). The basic and neutral compounds were eluted from HCX columns in basic conditions and acidic compounds from HAX columns in acidic conditions and the extracts were combined resulting in a single sample for LC-TOFMS analysis. The method was developed based on the following test compounds: amphetamine, bunolol, buprenorphine, canrenone, clenbuterol, mefruside, oxandrolone, phenylephrine and ritalinic acid.

In the development and optimization of sample preparation, only the SPE technique was considered, as it is amenable to automation. The initial aim was to develop a single SPE extraction method using silica or polymer-based sorbents, and various SPE extraction columns were tested: SampliQ OPT (Agilent), Sep-Pak C18, Oasis HLB (Waters Corporation, Milford, MA, USA). However, despite optimization, the extraction recoveries for test compounds were mainly as low as 30%–50%. Hence it became apparent that two SPE cartridges with an opposite mixed-mode retention mechanism were needed to expand the analyte selection. In the final procedure, the established HCX extraction method[22] with minor modifications was completed by HAX extraction to recover acidic compounds too. The extraction recoveries of the test compounds, except phenylephrine, obtained with the final procedure, were over 75%. Phenylephrine had the lowest recovery, 46%, yet it was much more than obtained with other cartridges in which the recoveries were less than 10%.

The developed SPE sample preparation method proved to be sufficiently generic, since analytes in their free form, glucuronide conjugates (after enzymatic hydrolysis) and even intact sulfo-conjugates could be analysed. Many compounds with a phenylalkylamine structure, such as etilefrine and etamivan, are largely excreted as sulfo-conjugates in urine.[30] As the enzyme used did not possess sulfatase activity, sulfo-conjugates were detected intact, as demonstrated by the validation of p-hydroxymesocarb sulphate and by the detection of etamivan sulphate synthesized enzymatically *in vitro* in-house.[28] The presence of etamivan sulphate was verified with a urine sample from an excretion study involving a healthy volunteer (Figure 3).

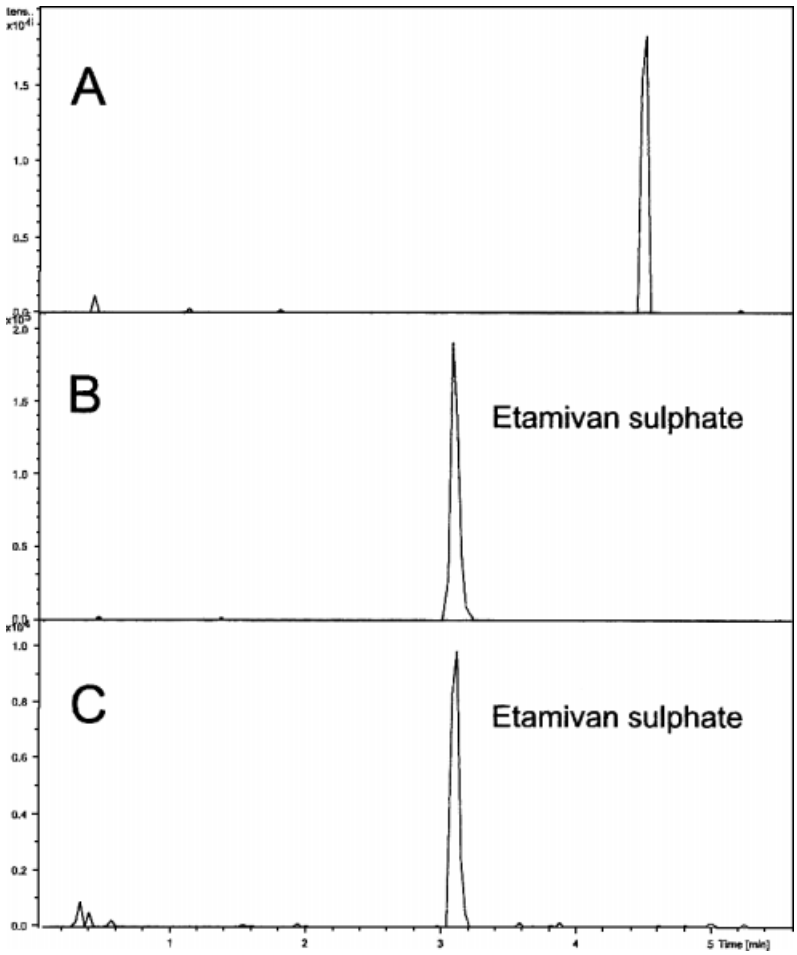

Figure 3. Detection of intact etamivan sulphate in urine. Extracted ion chromatograms (EIC) for etamivan sulphate $[M-H]^-$ *m/z* 302.07038 (A) blank urine sample, (B) urine after administration of etamivan, and (C) in-house *in vitro* enzymatically synthesized etamivan sulphate.

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Table 1. Chromatographic and ionization parameters for all included doping agents at the minimum required performance limit (MRPL) level. For compounds marked with bold the data are for the concentration level of 10×MRPL

Compound		Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
ANABOLIC AGENTS												
1	Allyltrenbolone	$C_{21}H_{26}O_2$	311.20056				10	1.45	705	7.4	ni	+
2	Boldenone	$C_{19}H_{26}O_2$	287.20056				10	1.31	34	6.6	ni	+
3	**Fluoxymesterone metabolite (9α-fluoro-17α-methyl-androst-4-ene-3α, 6β,11β,17β-tetrol)**	$C_{20}H_{29}FO_3$	337.21735				10	0.75	1576	0.18	ni	+
4	Formebolone metabolite (2-hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one)	$C_{25}H_{30}O_4$	347.22169				10	0.94	4535	1.1	ni	+
5	**Furazabol**	$C_{20}H_{30}N_2O_2$		348.26455			10	nd	nd	0.15	ni	+
6	Gestrinone	$C_{21}H_{24}O_2$	309.18491				10	1.40	127	1.0	ni	+
7	**4-Chlorodehydromethyltestosterone metabolite (6β-hydroxy-4-chlorodehydromethyltestosterone)**	$C_{20}H_{27}ClO_2$	335.17723				10	nd	nd	0.030	ni	+
8	Methandienone metabolite (6β-hydroxymethandienone)	$C_{20}H_{28}O_2$	301.21621				2	1.38	393	2.9	ni	+
9	**Methandienone metabolite (17-epimethandienone)**	$C_{21}H_{29}O$	299.23694				2	nd	nd	0.27	ni	+
10	Methyltrienolone	$C_{19}H_{24}O_2$	285.18491				10	1.32	466	1.16	ni	+
11	Oxandrolone	$C_{19}H_{30}O_3$		324.25332			10	1.32	9367	5.0	ni	+
12	Oxandrolone metabolite (17-epioxandrolone)	$C_{19}H_{30}O_3$		324.25332			10	1.44	2943	2.4	ni	+
13	**Stanozolol metabolite (3′-hydroxystanozolol)**	$C_{21}H_{32}N_2O_2$	345.25366		343.23910		2	nd	nd	0.66	3.7	−
14	Stanozolol metabolite (16β-hydroxystanozolol)	$C_{21}H_{32}N_2O_2$	345.25366				2	0.96	176	3.8	ni	+
15	Tetrahydrogestrinone (THG)	$C_{21}H_{28}O_2$	313.21621				10	1.49	36	1.7	ni	+
16	Trenbolone metabolite (17α-trenbolone)	$C_{18}H_{22}O_2$	271.16926				10	1.31	500	5.3	ni	+
17	Zeranol	$C_{18}H_{26}O_5$	323.18530		321.17075		10	1.26	234	0.22	12	−
	AGENTS WITH ANTI-ESTROGENIC ACTIVITY											
18	Aminoglutethimide	$C_{13}H_{16}N_2O_2$	233.12845				50	0.44	nd	4.2	ni	+
19	Anastrozole	$C_{17}H_{19}N_5$	294.17132		292.15677		50	1.26	609	10	7.4	+
20	Clomiphene	$C_{26}H_{28}ClNO$	406.19322				50	1.43	55 084	0.019	ni	+
21	Cyclofenil	$C_{23}H_{24}O_4$	365.17434				50	nd	nd	ni	ni	
22	Exemestane	$C_{20}H_{24}O_2$	297.18491				50	1.42	226	3.0	ni	+

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Table 1. (*Continued*)

	Compound	Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
23	Exemestane metabolite (17-dihydroexemestane)	$C_{20}H_{26}O_2$	299.20056				50	1.38	930	4.1	ni	+
24	Letrozole	$C_{17}H_{11}N_5$	286.10872		284.09417		50	1.25	26 631	1.2	55	–
25	Letrozole metabolite (4, 4′-(hydroxymethylene)bis-benzonitrile)	$C_{15}H_{10}N_2O$	235.08659		233.07204		50	1.26	1967	ni	11	–
26	Raloxifene	$C_{28}H_{27}NO_4S$	474.17336		472.15880		50	1.15	425	1.4	2.2	±
27	Tamoxifen	$C_{26}H_{29}NO$	372.23219				50	1.46	168	0.12	ni	+
28	Toremifene	$C_{26}H_{28}ClNO$	406.19322				50	1.43	1556	0.34	ni	+
	β_2-AGONISTS											
29	Bambuterol	$C_{18}H_{29}N_3O_5$	368.21800				500	0.99	314	16	ni	+
30	Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.08690				2	0.89	1392	0.033	ni	+
31	Fenoterol	$C_{17}H_{21}NO_4$	304.15434		302.13978		100	0.29	720	4.4	11	–
32	Formoterol	$C_{19}H_{24}N_2O_4$	345.18088		343.16633		100	0.90	71	2.4	5.5	±
33	Isoetharine	$C_{13}H_{21}NO_3$	240.15942		238.14487		100	0.18	40 510	8.4	6.2	+
34	Orciprenaline	$C_{11}H_{17}NO_3$	212.12812		210.11357		100	0.13	403	7.3	2.7	+
35	**Rimiterol**	$C_{12}H_{17}NO_3$	224.12812		222.11357		100	0.13	nd	4.3	3.7	±
36	Ritodrine	$C_{17}H_{21}NO_3$	288.15942		286.14487		100	0.35	8123	13	11	±[b]
37	Salbutamol	$C_{13}H_{21}NO_3$	240.15942		238.14487		100	0.16	692	23	10	+
38	Salmeterol	$C_{25}H_{37}NO_4$	416.27954		414.26498		100	1.27	200	1.5	4.8	–
39	Terbutaline	$C_{12}H_{19}NO_3$	226.14377		224.12922		100	0.16	125	6.2	2.0	+
	β-BLOCKERS											
40	Acebutolol	$C_{18}H_{28}N_2O_4$	337.21218		335.19763		500	0.88	690	15	8.3	+
41	Alprenolol	$C_{15}H_{23}NO_2$	250.18016				500	1.10	356	29	ni	+
42	Atenolol	$C_{14}H_{22}N_2O_3$	267.17032				500	0.16	627	17	ni	+
43	Befunolol	$C_{16}H_{21}NO_4$	292.15434				500	0.91	323	12	ni	+
44	Betaxolol	$C_{18}H_{29}NO_3$	308.22202				500	1.12	219	21	ni	+
45	Bevantolol	$C_{20}H_{27}NO_4$	346.20129				500	1.14	166	12	ni	+
46	Bisoprolol	$C_{18}H_{31}NO_4$	326.23259				500	1.05	222	14	ni	+
47	Bufetolol	$C_{18}H_{29}NO_4$	324.21694				500	1.04	109	14	ni	+
48	Bufuralol	$C_{16}H_{23}NO_2$	262.18016				500	1.14	280	20	ni	+
49	Bunitrolol	$C_{14}H_{20}N_2O_2$	249.15975				500	0.87	362	21	ni	+
50	Bunolol	$C_{17}H_{25}NO_3$	292.19072				500	0.92	503	32	ni	+
51	Bupranolol	$C_{14}H_{22}NO_2Cl$	272.14118				500	1.11	2463	15	ni	+
52	Carteolol	$C_{16}H_{24}N_2O_3$	293.18597				500	0.44	1289	15	ni	+
53	Carvedilol	$C_{24}H_{26}N_2O_4$	407.19653		405.18198		500	1.21	189	41	68	±
54	Celiprolol	$C_{20}H_{33}N_3O_4$	380.25438		378.23983		500	0.98	172	21	13	+

Table 1. (*Continued*)

	Compound	Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
55	Esmolol	$C_{16}H_{25}NO_4$	296.18564				500	0.95	35	16	ni	+
56	Indenolol	$C_{15}H_{21}NO_2$	248.16451				500	1.03	262	22	ni	+
57	Labetalol	$C_{19}H_{24}N_2O_3$	329.18597		327.17142		500	1.03	23 766	11	128	–
58	Mepindolol	$C_{15}H_{22}N_2O_2$	263.17540				500	0.81	141	22	ni	+
59	Metipranolol	$C_{17}H_{27}NO_4$	310.20129				500	1.08	1189	11	ni	+
60	Metoprolol	$C_{15}H_{25}NO_3$	268.19072				500	0.89	346	29	ni	+
61	Nadolol	$C_{17}H_{27}NO_4$	310.20129				500	0.60	828	17	ni	+
62	Oxprenolol	$C_{15}H_{23}NO_3$	266.17507				500	1.00	144	23	ni	+
63	Penbutolol	$C_{18}H_{29}NO_2$	292.22711				500	1.27	2189	19	ni	+
64	Pindolol	$C_{14}H_{20}N_2O_2$	249.15975				500	0.48	266	33	ni	+
65	Practolol	$C_{14}H_{22}N_2O_3$	267.17032				500	0.20	68	19	ni	+
66	Propafenone	$C_{21}H_{27}NO_3$	342.20637				500	1.24	130	14	ni	+
67	Propranolol	$C_{16}H_{21}NO_2$	260.16451				500	1.08	210	26	ni	+
68	Sotalol	$C_{12}H_{20}N_2O_3S$	273.12674		271.11219		500	0.18	47	14	10	±[b]
69	Timolol	$C_{13}H_{24}N_4O_3S$	317.16419				500	0.86	117	22	ni	+
70	Toliprolol	$C_{13}H_{21}NO_2$	224.16451				500	0.94	1080	24	ni	+
	CANNABINOIDS											
71	Cannabis metabolite (cannabidiol)	$C_{21}H_{30}O_2$	315.23186				15	1.23	4923	0.021	ni	+
72	Cannabis metabolite (11-nor-9-carboxy-Δ9-tetrahydrocannabinol, THC-COOH)	$C_{21}H_{28}O_4$	345.20604		343.19148		15	1.71	40	0.017	0.35	+
	DIURETICS											
73	**Acetazolamide**	$C_4H_6N_4O_3S_2$	222.99541		220.98086		250	0.26	nd	0.11	2.0	–
74	Amiloride	$C_6H_8N_7OCl$	230.05516				250	0.18	194	0.26	ni	+
75	Bendroflumethiazide	$C_{15}H_{14}F_3N_3O_4S_2$			420.03051		250	1.32	3251	ni	18	–
76	Benzthiazide	$C_{15}H_{14}ClN_3O_4S_3$			429.97622		250	1.27	1056	ni	26	–
77	Bumetanide	$C_{17}H_{20}N_2O_5S$	365.11657		363.10202		250	1.38	1212	0.64	18	–
78	Canrenone	$C_{22}H_{28}O_3$	341.21112				250	1.43	852	1.4	ni	+
79	Chlorothiazide	$C_7H_6ClN_3O_4S_2$			293.94155		250	0.30	4998	ni	121	–
80	Chlorthalidone	$C_{14}H_{14}ClN_3O_4S$	356.04663				250	0.98	326	0.24	ni	+
81	Clopamide	$C_{14}H_{20}ClN_3O_3S$	346.09867		344.08411		250	1.10	8458	3.6	18	–
82	Cyclothiazide	$C_{14}H_{16}ClN_3O_4S_2$			388.01980		250	1.29	1377	ni	9.2	–
83	Dichlorphenamide	$C_6H_6Cl_2N_2O_4S_2$	304.92188		302.90733		250	0.91	1081	0.04	22	–
84	Etacrynic acid	$C_{13}H_{12}Cl_2O_4$	303.01854		301.00399		250	1.40	3959	0.31	3.1	–
85	Furosemide	$C_{12}H_{11}ClN_2O_5S$	331.01500		329.00044		250	1.20	1663	0.06	8.5	–
86	Hydrochlorothiazide	$C_7H_8ClN_3O_4S_2$			295.95720		250	0.36	242	ni	11	–

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Table 1. (*Continued*)

Compound		Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
87	Indapamide	$C_{16}H_{16}N_3O_3ClS$	366.06737		364.05281		250	1.26	4118	2.2	22	–
88	Mefruside	$C_{13}H_{19}ClN_2O_5S_2$	383.04967		381.03511		250	1.29	3538	0.64	16	–
89	Mersalyl acid	$C_{13}H_{16}HgNO_6$					250			ni	ni	
90	Metolazone	$C_{16}H_{16}N_3O_3ClS$	366.06737		364.05281		250	1.20	821	1.8	15	–
91	Probenecid	$C_{13}H_{19}NO_4S$	286.11076		284.09620		250	1.40	438	1.5	35	–
92	Spironolactone	$C_{24}H_{32}O_4S$	417.20941				250	1.43	392	0.012	ni	+
93	Torasemide	$C_{16}H_{20}N_4O_3S$	349.13289		347.11834		250	1.10	1141	3.2	17	–
94	Triamterene	$C_{12}H_{11}N_7$	254.11487				250	0.67	63	52	ni	+
95	Trichlormethiazide	$C_8H_8Cl_3N_3O_4S_2$			379.89195		250	1.07	183	ni	6.7	–
96	Xipamide	$C_{15}H_{15}ClN_2O_4S$	355.05138		353.03683		250	1.36	964	0.53	65	–
	GLUCOCORTICOSTEROIDS											
97	Beclomethasone	$C_{22}H_{29}ClO_5$	409.17763		407.16308		30	1.27	5307	1.1	2.0	±
98	Betamethasone	$C_{22}H_{29}FO_5$	393.20718		391.19263		30	1.24	101	1.0	1.0	±
99	Budesonide	$C_{25}H_{34}O_6$	431.24282			475.23265	30	1.40	211	1.4	5.4	±
100	Ciclesonide	$C_{32}H_{44}O_7$	541.31598		539.30143		30	0.99	1776	0.05	0.086	–
101	Deoxycorticosterone	$C_{21}H_{30}O_3$	331.22677		329.21222		30	1.38	1538	7.3	0.072	+
102	Desonide	$C_{24}H_{32}O_6$	417.22717			461.21699	30	1.29	188	3.2	8.4	±
103	Dexamethasone	$C_{22}H_{29}FO_5$	393.20718		391.19263		30	1.25	96	1.0	1.0	+
104	Fludrocortisone	$C_{21}H_{29}FO_5$	381.20718			425.19701	30	1.16	220	1.5	20	±
105	Flumethasone	$C_{22}H_{28}F_2O_5$	411.19776			455.18759	30	1.25	113	1.6	20	±
106	Flunisolide	$C_{24}H_{31}FO_6$	435.21774			479.20757	30	1.29	2345	2.6	14	±
107	Methylprednisolone	$C_{22}H_{30}O_5$	375.21660			419.20643	30	1.22	737	1.1	10	±
108	Prednisolone	$C_{21}H_{28}O_5$	361.20095			405.19078	30	1.15	216	1.4	17	±
109	Prednisone	$C_{21}H_{26}O_5$	359.18530		357.17075		30	1.15	451	1.4	5.1	±
110	Tibolone	$C_{21}H_{28}O_2$	313.21621				30	1.40	169	0.16	ni	+
111	Triamcinolone	$C_{21}H_{27}FO_6$	395.18644		393.17189		30	1.03	168	0.58	6.7	–
112	Triamcinolone acetonide	$C_{24}H_{31}FO_6$	435.21774			479.20867	30	1.08	187	2.1	9.9	–
	ENHANCEMENT OF OXYGEN TRANSFER											
113	Efaproxiral (RSR 13)	$C_{20}H_{23}NO_4$	342.16999		340.15543		250	1.40	1372	9.4	64	–
	NARCOTICS											
114	Buprenorphine	$C_{29}H_{41}NO_4$	468.31084				10	1.2	18 510	2.1	ni	+
115	Buprenorphine metabolite (norbuprenorphine)	$C_{25}H_{35}NO_4$	414.26389				200	1.0	15 819	0.9	ni	+
116	Codeine	$C_{18}H_{21}NO_3$	300.15942				200	0.29	1528	1.6	ni	+
117	Dextromoramide	$C_{25}H_{32}N_2O_2$	393.25366				200	1.3	1269	11	ni	+
118	Ethylmorphine	$C_{19}H_{23}NO_3$	314.17507				200	0.65	480	11	ni	+
119	Fentanyl	$C_{22}H_{28}N_2O$	337.22744				200	1.1	1959	27	ni	+

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Table 1. (*Continued*)

	Compound	Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
120	Fentanyl metabolite (norfentanyl)	$C_{14}H_{20}N_2O$	233.16484				200	0.79	1655	26	ni	+
121	Heroin	$C_{21}H_{23}NO_5$	370.16490				200	0.93	4122	0.5	ni	+
122	Heroin metabolite (6-monoacetylmorphine)	$C_{19}H_{21}NO_4$	328.15434				200	0.38	112	7.0	ni	+
123	Hydromorphone	$C_{17}H_{19}NO_3$	286.14377				200	0.16	128	12	ni	+
124	Methadone	$C_{21}H_{27}NO$	310.21654				200	1.3	921	30	ni	+
125	Methadone metabolite (2-ethyldiene-1,5-dimethyl-3,3-diphenylpyrrolidine, EDDP)	$C_{20}H_{23}N$	278.19033				200	1.2	6406	23	ni	+
126	3-Methylfentanyl	$C_{23}H_{30}N_2O$	351.24309				200	1.2	1450	12	ni	+
127	Morphine	$C_{17}H_{19}NO_3$	286.14377				200	0.14	624	17	ni	+
128	Oxycodone	$C_{18}H_{21}NO_4$	316.15434				200	0.36	5690	11	ni	+
129	Oxycodone metabolite (noroxycodone)	$C_{17}H_{19}NO_4$	302.13869				200	0.34	4842	7.6	ni	+
130	Oxymorphone	$C_{17}H_{19}NO_4$	302.13869		300.12413		200	0.15	17 252	855	34	+
131	Pentazocine	$C_{19}H_{27}NO$	286.21654				200	1.0	47 491	29	ni	+
132	Pethidine	$C_{15}H_{21}NO_2$	248.16451				200	0.96	219	30	ni	+
133	Pethidine metabolite (norpethidine)	$C_{14}H_{19}NO_2$	234.14886				200	0.96	1254	20	ni	+
134	Pholcodine	$C_{23}H_{30}N_2O_4$	399.22783				200	0.10	213	4.0	ni	+
	STIMULANTS											
135	Amfepramone	$C_{13}H_{19}NO$	206.15394				500	0.49	285	4.2	ni	+
136	2-Aminoheptane	$C_7H_{17}N$	116.14338				500	0.68	nd	24	ni	+
137	Amiphenazole	$C_9H_9N_3S$	192.05899				500	1.1	7704	0.012	ni	+
138	Amphetamine	$C_9H_{13}N$	136.11208				500	0.34	315	3.5	ni	+
139	Amphetaminil	$C_{17}H_{18}N_2$	251.15428				500	nd	nd	ni	ni	
140	Benzphetamine	$C_{17}H_{21}N$	240.17468				500	1.1	342	21	ni	+
141	Brucine	$C_{23}H_{26}N_2O_4$	395.19654				500	0.78	229	5.2	ni	+
142	Caffeine	$C_8H_{10}N_4O_2$	195.08765				500	0.37	549	5.6	ni	+
143	Carphedon	$C_{12}H_{14}N_2O_2$	219.11280		217.09825		500	0.88	358	5.4	0.029	+
144	Cathine	$C_9H_{13}NO$	152.10699				500	0.21	78	5.4	ni	+
145	Cathinone	$C_9H_{11}NO$	150.09134				500	0.22	13	7.9	ni	+
146	4-bromo-2,5-dimethoxyphenethylamine (2C-B)	$C_{10}H_{14}NO_2Br$	260.02807				500	0.95	2486	3.6	ni	+
147	Chlorophentermine	$C_{10}H_{14}ClN$	184.08875				500	0.93	133	4.5	ni	+
148	Chlorprenaline	$C_{11}H_{16}ClNO$	214.09932				500	0.75	577	24	ni	+
149	Clobenzorex	$C_{16}H_{18}ClN$	260.12005				500	1.1	3466	18	ni	+
150	Cocaine	$C_{17}H_{21}NO_4$	304.15434				500	0.95	262	21	ni	+
151	Cocaine metabolite (benzoylecgonine)	$C_{16}H_{19}NO_4$	290.13869				500	0.77	236	11	ni	+
152	Cropropamide	$C_{13}H_{24}N_2O_2$	241.19105				500	1.2	1641	82	ni	+

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Table 1. (*Continued*)

Compound		Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
153	Crotetamide	$C_{12}H_{22}N_2O_2$	227.17540				500	1.0	1728	3.5	ni	+
154	Cyclazodone	$C_{12}H_{12}N_2O_2$	217.09715		215.08260		500	1.0	3291	6.6	3.8	+
155	Dimethylamphetamine	$C_{11}H_{17}N$	164.14338				500	0.95	137	19	ni	+
156	Dimethyltryptamine	$C_{12}H_{16}N_2$	189.13863				500	1.6	608	33	ni	+
157	Doxapram	$C_{24}H_{30}N_2O_2$	379.23801				500	1.0	1762	9.5	ni	+
158	Ephedrine	$C_{10}H_{15}NO$	166.12264				500	0.25	295	22	ni	+
159	Etafedrine	$C_{12}H_{19}NO$	194.15394				500	0.38	186 418	29	ni	+
160	Etamivan	$C_{12}H_{17}NO_3$	224.12812				500	1.0	53	18	ni	+
161	Ethylamphetamine	$C_{11}H_{17}N$	164.14338				500	0.59	93	28	ni	+
162	Etilefrine	$C_{10}H_{15}NO_2$	182.11756				500	0.14	2606	14	ni	+
163	Famprofazone	$C_{24}H_{31}N_3O$	378.25399				500	1.3	10 438	10	ni	+
164	Fencamfamin	$C_{15}H_{21}N$	216.17468				500	1.0	182	25	ni	+
165	Fenetylline	$C_{18}H_{23}N_5O_2$	342.19245				500	0.92	1046	11	ni	+
166	Fenfluramine	$C_{12}H_{16}NF_3$	232.13076				500	1.1	79 772	38	ni	+
167	Fenproporex	$C_{12}H_{16}N_2$	189.13863				500	0.49	562	18	ni	+
168	Heptaminol	$C_8H_{19}NO$	146.15394				500	0.16	224	15	ni	+
169	*p*-Hydroxyamphetamine	$C_9H_{13}NO$	152.10699				500	0.15	856	3.9	ni	+
170	Isometheptene	$C_9H_{19}N$	142.15903				500	0.84	98	11	ni	+
171	3,4-Methylenedioxy-α-ethyl-*N*-methylphenethylamine (MBDB)	$C_{12}H_{17}NO_2$	208.13321				500	0.81	402	26	ni	+
172	3,4-Methylenedioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	180.10191				500	0.38	8	13	ni	+
173	3,4-Methylenedioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	194.11756				500	0.45	261 085	25	ni	+
174	Mefenorex	$C_{12}H_{18}ClN$	212.12005				500	0.95	103	21	ni	+
175	Mephedrone	$C_{11}H_{15}NO$	178.12264				500	0.62	217	22	ni	+
176	Mephentermine	$C_{11}H_{17}N$	164.14338				500	0.65	148	19	ni	+
177	Mesocarb	$C_{18}H_{18}N_4O_2$	323.15025				500	1.4	815	11	ni	+
178	Mesocarb metabolite (*p*-hydroxymesocarb)	$C_{18}H_{18}N_4O_3$	339.14517				500	1.2	994	12	ni	+
179	Mesocarb metabolite (*p*-hydroxymesocarb sulphate)	$C_{18}H_{18}N_4O_6S$	419.10198		417.08743		500	1.1	242	0.21	2.4	±
180	Methamphetamine	$C_{10}H_{15}N$	150.12773				500	1.1	106	0.047	ni	+
181	Methoxyphenamine	$C_{11}H_{17}NO$	180.13829				500	0.75	47 114	29	ni	+
182	*p*-Methylamphetamine	$C_{10}H_{15}N$	150.12773				500	0.79	10 321	14	ni	+
183	Methylephedrine	$C_{11}H_{17}NO$	180.13829				500	0.29	9112	35	ni	+
184	Methylphenidate	$C_{14}H_{19}NO_2$	234.14886				500	0.89	233	40	ni	+
185	Methylphenidate metabolite (ritalinic acid)	$C_{13}H_{17}NO_2$	220.13321				500	0.67	97	2.0	ni	+
186	Modafinil metabolite (modafinil acid)	$C_{15}H_{15}NO_2S_2$	275.07364				500	nd	nd	ni	ni	+

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Table 1. (*Continued*)

Compound		Molecular formula	Target ions and their theoretical monoisotopic masses				MRPL ng/mL	RRT	S/N	Relative Ionisation[a]		ESI Polarity
			$[M+H]^+$	$[M+NH_4]^+$	$[M-H]^-$	$[M+HCOO]^-$				Positive	Negative	
187	Nikethamide	$C_{10}H_{14}N_2O$	179.11789				500	1.6	1638	6722	ni	+
188	Octopamine	$C_8H_{11}NO_2$	154.08626				500	0.11	83	0.69	ni	+
189	Ortetamine	$C_{10}H_{15}N$	150.12773				500	0.70	136	13	ni	+
190	Oxilofrine	$C_{10}H_{15}NO_2$	182.11756				500	0.11	10722	11	ni	+
191	Pemoline	$C_9H_8N_2O_2$	177.06585				500	0.48	5355	2.8	ni	+
192	Pentetrazol	$C_6H_{10}N_4$	139.09782				500	0.40	2696	48	ni	+
193	Phendimetrazine	$C_{12}H_{17}NO$	192.13829				500	0.38	445	25	ni	+
194	Phenmetrazine	$C_{11}H_{15}NO$	178.12264				500	0.38	13344	25	ni	+
195	Phentermine	$C_{10}H_{15}N$	150.12773				500	0.53	108	9.5	ni	+
196	**Phenylephrine**	$C_9H_{13}NO_2$	168.10191				500	0.12	86	9.4	ni	+
197	Phenylpropanolamine	$C_9H_{13}NO$	152.10699				500	0.20	356	6.9	ni	+
198	Picrotin	$C_{15}H_{21}NO_7$	328.13908				500	0.91	4980	1.0	ni	+
199	Pipradlol	$C_{18}H_{21}NO$	268.16959				500	1.0	944	22	ni	+
200	Prolintane	$C_{15}H_{23}N$	218.19033				500	1.0	2506	30	ni	+
201	Propylhexedrine	$C_{10}H_{21}N$	156.17468				500	0.96	89	27	ni	+
202	Pseudoephedrine	$C_{10}H_{15}NO$	166.12264				500	0.26	104	22	ni	+
203	Pyrovalerone	$C_{16}H_{23}NO$	246.18524				500	1.1	5444	13	ni	+
204	Selegiline	$C_{13}H_{17}N$	188.14338				500	0.76	587	32	ni	+
205	Selegiline metabolite (*N*-desmethylselegiline)	$C_{12}H_{15}N$	174.12773				500	0.69	98	39	ni	+
206	Sibutramine metabolite (*N*-desmethylsibutramine)	$C_{16}H_{24}ClN$	266.16700				500	1.3	160	15	ni	+
207	Strychnine	$C_{21}H_{22}N_2O_2$	335.17540				200	0.64	1947	12	ni	+

Notes:
[a] relative to dexamethasone
[b] validation in negative ionization mode
nd = not detected
ni = no ionization at optimized conditions
± compounds are included in both positive and negative databases

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Liquid chromatography – time-of-flight mass spectrometry

Another aim of the study was to transfer the existing LC method for doping screening[22] to a rapid resolution column (50 × 2.1 mm, 1.8 μm), maintaining the resolution. The analysis run time of 8 min was optimized with test compounds with respect to the total number of the analytes and the matrix background. Centrifugation of the samples prior to analysis was essential to avoid column blockage.

Liquid chromatography mobile phase composition with and without ammonium formate was tested and for certain compounds the presence of ammonium formate was essential due to retention (e.g. β-blockers) or adduct formation (e.g. oxandrolone).

The analytical column proved to be robust and conducted 1200 analyses without any loss in chromatographic performance. The retention times of the analytes were repeatable: at the MRPL level, the median RSD% of relative RT was 0.32, allowing the use of a RT window of ±0.2 min (Table 2). An isocratic part at the beginning of the gradient (1 min) was required to achieve sufficient retention for hydrophilic compounds, for example phenylephrine. Peak shapes were generally good, and in case of co-eluting peaks, the compounds could be identified with accurate mass and SigmaFit.

Both the positive and negative ionization mode was applied to cover the wide variety of doping agents. Especially the number of diuretics, representing an extremely heterogeneous group of compounds, was significantly increased along with the use of negative polarity, as shown also in several earlier studies.[6,11,13,20,23,31–34] For those compounds that were detectable in both modes, the use of dual polarity provided an additional confirmation of identification. As matrix interference is typically less in negative ion mode, it offers an alternative for those compounds that suffer from a high background in positive polarity (such as glucocorticosteroids).

Ionization efficiencies relative to dexamethasone, which is ionized in both polarities, were recorded for each analyte in both polarities to establish the database of the most prominent target ions (Table 1). In the positive ion mode, 175 analytes were detected mainly as protonated molecule $[M+H]^+$, but with certain analytes, such as oxandrolone and 17-epioxandrolone, the most abundant ion was ammonium adduct $[M+NH_4]^+$, as suggested earlier.[35] The observations were analogous in the negative polarity (29 analytes), as most analytes were detected as deprotonated molecule $[M-H]^-$ and some compounds, such as triamcinolone acetonide, yielded a formate adduct $[M+HCOO]^-$. Spironolactone had a characteristic fragment of *m/z* 341.2112 under optimized analysis conditions. In general anabolic agents exhibited low ionization efficiencies (< 1) and some of them could not be detected at the MRPL level. On the other hand, clomiphene also had a low ionization efficiency (0.019), but had an S/N > 50 000 at the MRPL level, obviously because of the optimal chromatographic retention resulting in a lower matrix interference and a higher MRPL level. For glucocorticosteroids, the ionization efficiencies were similar to those of dexamethasone and hence they were detectable at the MRPL level. β-Blockers had high ionization efficiencies and were clearly detected at the MRPL level. The highest ionization efficiency of all compounds was observed with nikethamide (> 6700).

Due to TOFMS hardware features two separate runs were required to cover both polarities but, still, a total analysis time of 16 minutes was achieved. This is competitive with the recent studies reporting total run times of 14 min[7], 19 min[11] and 2 × 9 min.[25]

Method validation

The LC-TOFMS method was validated with 207 analytes in replication experiments for relative ionization efficiency and S/N at the MRPL level (Table 1). Here a 10×MRPL level was applied to 3′-hydroxystanozolol, 6β-hydroxy-4-chlorodehydromethyltestosterone, 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol (fluoxymesterone metabolite), 17-epimetandienone, acetazolamide, furazabol, phenylephrine and rimiterol. From the whole set, only 10 compounds could not be detected either at MRPL or 10×MRPL with the following detection problems: insufficient ionization (6β-hydroxy-4-chlorodehydromethyltestosterone, 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol, amphetaminil and furazabol), non-optimal LC conditions and/or matrix interference (3′-hydroxystanozolol, 17 – epimetandienone, acetazolamide, aminoglutethimide and rimiterol), evaporation during sample preparation (2-aminoheptane) or in-source fragmentation (modafinil acid metabolite).

The LC-TOFMS method was further validated from urine matrix at the MRPL level with a selection of 20 compounds falling into eight groups of prohibited substances (Table 2). The validation procedure consisted of the determination of extraction recovery, linearity ranging from MRPL to 50×MRPL, intraday and interday repeatability at the MRPL and 10×MRPL levels, selectivity and ion suppression. Generally, the extraction recovery was above 80% but chlorothiazide and etacrynic acid exhibited exceptionally low recoveries of 10% and 15%, respectively. Nevertheless, these compounds could be detected with S/N ratios over 3000 at the MRPL level. Linearity of the method was satisfactory with correlation coefficients (R^2) ranging from 0.78 to 0.99. However, linearity was poor for benzoylecgonine ($R^2 = 0.68$) probably due to the degradation of its ester structure under basic conditions during sample preparation. Median values for intraday repeatability at the MRPL and 10×MRPL levels were 11% and 6%, respectively, and for interday repeatability 19% and 16% respectively.

Accurate mass measurement was generally performed using a 3 mDa window. In the validation study from urine matrix, mean and median mass accuracy was 1.2 and 0.80 mDa, respectively, and the mean and median values for SigmaFit were 0.045 and 0.027, respectively (Table 2). For a few compounds, however, mass errors and SigmaFit values were higher than those set in the search criteria; thus individual search criteria were applied in the database. This behaviour was due to interference from the urine background and reflected the need for an even higher chromatographic and mass resolution.

The selectivity of the method was demonstrated with drug-free urine samples (n = 8). No interfering compounds were detected at the retention times of the analytes as shown in Figure 4 and no false positive entries were reported from the database search. Ion suppression was most intensive between 0.3–0.5 min (maximum of −87%) but only a few analytes, morphine, salbutamol and phenylephrine, eluted in this range. For the first two compounds, S/N ratios were still good (> 600) but phenylephrine could be detected only at the 10×MRPL level. However, the effect of ion suppression on threshold compounds, such as morphine and salbutamol, may be overcome by using deuterated analogs. Based on total ion chromatograms, matrix interference was heaviest around 1 min and between 2.5 and 5 min.

The suitability of the present screening method was demonstrated with external quality control samples, and the results

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Table 2. Method validation results for selected compounds in urine

Compound	Extraction recovery[a]	conc.	RRT RSD%	Mass error [mDa]	SigmaFit	Repeatability (RSD%) of peak areas[a] intraday (n = 5)	Repeatability (RSD%) of peak areas[a] interday (n = 5)	Linearity correlation coefficient R^2	Linear range [ng/mL]
Oxandrolone	100	MRPL	0.30	0.60	0.014	5	15	0.85	10–500
		10×MRPL				3	9		
Trenbolone metabolite (17α-trenbolone)	105	MRPL	0.12	−1.8	0.170[b]	21	25	0.99	10–250
		10×MRPL				3	8		
Salbutamol	105	MRPL	3.2	0.40	0.006	15	13	0.99	100–2500
		10×MRPL				8	5		
Terbutaline	93	MRPL	3.1	0.78	0.020	16	19	0.98	100–5000
		10×MRPL				5	18		
Anastrozole	95	MRPL	0.00	−0.42	0.028	18	19	0.98	50–2500
		10×MRPL				1	16		
Letrozole metabolite (4, 4′-(hydroxymethylene)bis-benzonitrile)	92	MRPL	0.21	1.8	0.027	9	23	0.92	50–2500
		10×MRPL				17	37		
Amiloride	87	MRPL	0.80	0.82	0.044	9	15	0.93	250–12 500
		10×MRPL				7	13		
Chlorothiazide	10	MRPL	0.00	−1.2	0.049	21	20	0.94	250–12 500
		10×MRPL				10	11		
Etacrynic acid	15	MRPL	0.68	−2.9	0.052[b]	9	21	0.79	250–6250
		10×MRPL				19	24		
Amphetamine	95	MRPL	0.68	−0.48	0.002	14	3	0.97	500–25 000
		10×MRPL				8	16		
Cocaine metabolite (benzoylecgonine)	86	MRPL	0.00	−0.86	0.014	10	22	0.68	500–12 500
		10×MRPL				6	5		
Mesocarb metabolite (*p*-hydroxymesocarb sulphate)	90	MRPL	0.14	−3.1[b]	0.060[b]	30	19	0.93	500–12 500
		10×MRPL				12	19		
Methylphenidate metabolite (ritalinic acid)	86	MRPL	0.54	0.98	0.011	8	23	0.92	500–25 000
		10×MRPL				2	19		
Strychnine	89	MRPL	0.53	0.48	0.030	11	10	0.94	500–12 500
		10×MRPL				6	14		
Morphine	95	MRPL	0.0	−1.1	0.026	14	14	0.96	200–5000
		10×MRPL				6	20		
Pethidine	100	MRPL	0.60	0.38	0.003	10	13	0.96	200–10 000
		10×MRPL				19	10		
Budesonide	115	MRPL	0.25	−0.26	0.036	7	41	0.88	30–1500
		10×MRPL				4	25		
Fludrocortisone	83	MRPL	0.34	4.9[b]	0.280[b]	13	17	0.92	30–750
		10×MRPL				8	27		
Nadolol	89	MRPL	2.6	−0.40	0.014	5	17	0.95	500–25 000
		10×MRPL				6	22		
Propranolol	93	MRPL	0.26	−0.02	0.016	6	13	0.78	500–25 000
		10×MRPL				6	11		
	Average	MRPL	0.72	1.2	0.045	13	20		
		10×MRPL				8	16		
	Median	MRPL	0.32	0.80	0.027	11	19		
		10×MRPL				6	16		

[a] relative to ISTD
[b] outside primary search criteria; individual search parameters included in the database

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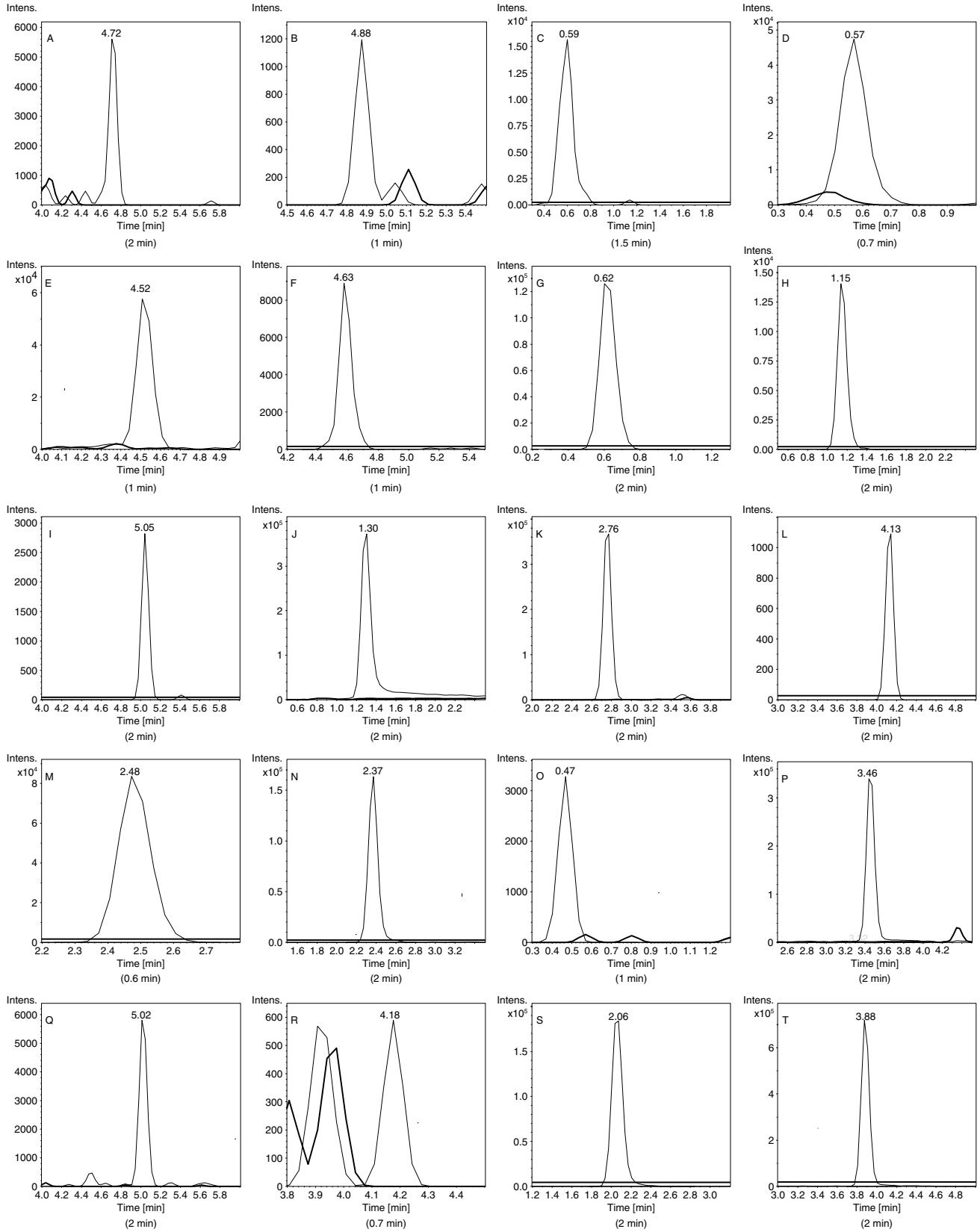

Figure 4. Extracted ion chromatograms (EIC) of the validation compounds at the minimum required performance limit (MRPL) with a mass window of ±3 mDa. Bold lines represent the urine blank sample and the number in parenthesis identifies the window width. A = Trenbolone metabolite (17α-trenbolone) $[M+H]^+$ B = Oxandrolone $[M+NH_4]^+$ C = Salbutamol $[M+H]^+$ D = Terbutaline $[M+H]^+$ E = Anastrozole $[M+H]^+$ F = Letrozole metabolite (4,4′-(hydroxymethylene)bis-benzonitrile) $[M-H]^-$ G = Amiloride $[M+H]^+$ H = Chlorothiazide $[M-H]^-$ I = Etacrynic acid $[M-H]^-$ J = Amphetamine $[M+H]^+$ K = Cocaine metabolite (benzoylecgonine) $[M+H]^+$ L = Mesocarb metabolite (*p*-hydroxymesocarb sulphate) $[M+H]^+$ M = Methylphenidate metabolite (ritalinic acid) $[M+H]^+$ N = Strychnine $[M+H]^+$ O = Morphine $[M+H]^+$ P = Pethidine $[M+H]^+$ Q = Budesonide $[M+H]^+$ R = Fludrocortisone $[M+H]^+$ S = Nadolol $[M+H]^+$ T = Propranolol $[M+H]^+$.

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Table 3. The findings from external quality control samples by the present LC-TOFMS method

Sample number	Content of the quality control sample (concentration [ng/mL])	Findings	Mass Error [mDa]	SigmaFit	ΔRT [min]
1	Blank	None			
2	Zeranol (36)	None			
3	Tetrahydrogestrinone (19)	Tetrahydrogestrinone	0.50	0.030	0.01
	Indapamide (337)	Indapamide	0.50	0.040	0.00
4	*p*-Hydroxymesocarb (712)	*p*-Hydroxymesocarb	0.70	0.011	0.00
5	Salbutamol (1293)	Salbutamol	−1.30	0.015	−0.01

are listed in Table 3. The findings, excluding the anabolic agent zeranol, were congruent with the results of the prevailing doping analysis methods. Detection of the low zeranol concentration was interfered by co-eluting matrix compounds resulting in poor mass accuracy (> 4 mDa).

The present study feature a much more extensive variety of doping agents than was reported in the recently published papers,[7,11,25] but still allows detection of most compounds with good mass accuracy. In addition, from the vast LC-TOFMS data even non-target compounds can be recovered, if a new enquiry is made afterwards. The stability of high mass accuracy in TOFMS over a wide dynamic range is critical in routine screening analysis. However, in several LC- TOFMS drug-screening methods[7,14,17,36] a wide mass window, typically 10 mDa, was used in the analysis of biological material while mass accuracy was on average above 5 ppm (2 mDa), the limit of accurate mass measurement. Interestingly, a window as wide as 50 mDa was required in the recent 'dilute and shoot' doping screening applying UPLC hybrid quadrupole TOFMS.[25] In pesticide screening methods, however, the reported mass accuracies met the requirements better.[16,19,37] The mass accuracy performance obtained clearly follows from the whole analytical procedure: the complexity of matrix, the number and concentration of target analytes, selectivity of sample preparation and chromatography and, finally, the performance of the TOFMS analyser and software. It is evident that high mass accuracy is a prerequisite for a reliable screening analysis and a legible report layout.

Conclusions

The present method for a wide range of small molecules, namely anabolic agents, β_2-agonists, hormone antagonists and modulators, diuretics, enhancers of oxygen transfer, stimulants, narcotics, cannabinoids, glucocorticosteroids and β-blockers is a major step towards a 'universal' doping screening method. By featuring generic sample preparation and accurate mass measurement in a narrow 3 mDa window, the present LC- TOFMS method is amenable to high-throughput doping screening, even for intact sulfo-conjugates in urine. Two consecutive analysis runs of different polarity expand the selection of analytes and improve reliability. The high S/N ratios obtained emphasize the possibility of detecting drug concentrations well below MRPL levels. Adding new compounds to the target database is easy, even if reference material is not available, since mere knowledge of the molecular formula is sufficient for preliminary identification. The non-selective sample preparation and fast chromatography used set high demands for the TOF mass analyser. A better performance was, however, obtained here in terms of mass accuracy than generally reported for LC-TOFMS methods in comparable doping screening methods. The wide scope of the present method is clearly helpful in rationalizing doping screening, but other methods are still required for the appropriate detection of anabolic agents and corticosteroids, as well as in peptide and protein analysis.

Acknowledgments

We wish to acknowledge Tina Suominen and Teija Moisander for their valuable technical assistance.

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