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High-throughput screening for various classes of doping agents using a new 'dilute-and-shoot' liquid chromatography-tandem mass spectrometry multi-target approach

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A new multi-target approach based on liquid chromatography – electrospray ionization tandem mass spectrometry (LC-(ESI)-MS/MS) is presented to screen for various classes of prohibited substances using direct injection of urine specimens. With a highly sensitive new generation hybrid mass spectrometer classic groups of drugs – for example, diuretics, beta2-agonists – stimulants and narcotics are detectable at concentration levels far below the required limits.

Additionally, more challenging and various new target compounds could be implemented. Model compounds of stimulant conjugates were studied to investigate a possible screening without complex sample preparation. As a main achievement, the integration of the plasma volume expanders dextran and hydroxyethyl starch (HES), commonly analyzed in time-consuming, stand-alone procedures, is accomplished. To screen for relatively new prohibited compounds, a common metabolite of the selective androgen receptor modulator (SARMs) andarine, a metabolite of growth hormone releasing peptide (GHRP-2), and 5-amino-4-imidazolecarboxyamide ribonucleoside (AICAR) are analyzed. Following a completely new approach, conjugates of di(2-ethylhexyl) phthalate (DEHP) metabolites are monitored to detect abnormally high levels of plasticizers indicating for illicit blood transfusion.

The assay was fully validated for qualitative purposes considering the parameters specificity, intra- (3.2–16.6%) and inter-day precision (0.4–19.9%) at low, medium and high concentration, robustness, limit of detection (1–70 ng/ml, dextran: 30 μg/ml, HES: 10 μg/ml) and ion suppression/enhancement effects.

The analyses of post-administration and routine doping control samples demonstrates the applicability of the method for sports drug testing. This straightforward and reliable approach accomplishes the combination of different screening procedures resulting in a high-throughput method that increases the efficiency of the labs daily work. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: sports drug testing; LC-MS/MS; dilute-and-shoot; high-throughput screening; plasma volume expanders; DEHP glucuronides

Introduction

In sports drug testing, the number of samples and doping agents constantly increases, thus multi-target approaches are required, combining high-throughput, simplified sample preparation, and a reliable detection for various classes of compounds. On the basis of modern and powerful analytical instruments consisting of liquid chromatographs coupled to sensitive mass spectrometers – for example, triple-quadrupole, time-of-flight (TOF) or Fourier transform (OrbiTrap) instruments – many new multi-target assays have been developed providing reliable detection of prohibited substances at the required limits. Currently, different screening procedures are utilized for the analysis of diuretics,[1–8] beta2-agonists,[6,7,9] stimulants,[4,6,7,10] narcotics,[6,7,11–13] and plasma volume expanders (PVE).[14–16] With the ongoing progress in the field of liquid chromatography and triple quadrupole mass spectrometry, new generation instruments have become accessible with the possibility of enhanced scan speed and scan-to-scan polarity switching. This achievement has allowed the development of new multi-target approaches that combine classic stand-alone screening procedures to detect many different categories of prohibited substances with versatile chemical structures. In contrast to TOF-based approaches, fast polarity switching allows the detection of acidic and basic compounds within a single run. Moreover, the high sensitivity of such instruments enables the identification of many prohibited substances without pre-concentration steps, resulting in very simple and fast 'dilute-and-shoot' methods. In sports drug testing, two assays have demonstrated its applicability for the combined detection of diuretics, stimulants, and narcotics using direct injection of urine specimens based on

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ultra-high performance liquid chromatography-tandem mass spectroscopy (UHPLC-MS/MS) and ultra-high performance liquid chromatography - quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS).[17,18]

Analyzing native urine specimens using direct injection provides a wide applicability to screen for many different compounds and their metabolites without time-consuming sample preparation steps. Using a high-sensitive next-generation triple-quadrupole mass spectrometer, a comprehensive multi-target approach was developed that, for the first time, enables a combined screening of diuretics, beta2-agonists, narcotics, stimulants, and their sulfo-conjugates and PVEs. One main achievement was the implementation of the PVEs dextran and HES, commonly analyzed in time-consuming stand-alone procedures.[14–16] Moreover, analyzing intact sulfo-conjugates of stimulating agents enables screening for numerous prohibited compounds and avoids complex sample preparation and hydrolysis steps. Additionally, the selective androgen receptor modulator (SARM) andarine (S-4), a main metabolite of the growth hormone releasing peptide (GHRP-2), namely D-Ala-D-(b-naphthyl)-Ala-Ala-OH (AA-3) and 5-amino-4-imidazolecarboxyamide ribonucleoside (AICAR) were implemented. As a brand new marker to indicate for illicit blood transfusion in sports drug testing, glucuroconjugated metabolites of di(2-ethylhexyl) phthalate (DEHP) were analyzed; DEHP is commonly used as a plasticizer in blood bags. The presented assay provides a powerful tool to cope with the increasing number of samples and prohibited substances and their metabolites in sports drug testing. It is emphasized that the combination of different screening methods reduces costs and speeds up sample turn-around time. Advantageously, only a small volume of urine in the μl range is needed, since the volume of urine in doping control is limited.

Experimental

Chemicals and reagents

AICAR, amiloride, bumetanide, chlorothiazide, chlorthalidone, dextran, ephedrine, ethacrynic acid, hydrochlorothiazide, indapamide, norephedrine, pseudoephedrine, salbutamol, salmeterol were obtained from Sigma (Deisendorf, Germany).

The reference materials of althiazide, cyclopenthiazide, cyclothiazide, epithiazide, ethiazide, hydroflumethiazide and meticrane were provided from Simon Biddle from HFL Sport Science, Fordham, UK.

Andarine (S-4), GHRP-2 metabolite, $^{13}C_5$-AICAR, ethamivan sulfate, p-hydroxyamphetamine sulfate and p-hydroxymethamphetamine sulfate were synthesized in-house as reference materials and the dephenylated aglycon of andarine was synthesized *in vitro*.[19,20]

Buthiazide and azosemide were purchased from Boehringer (Mannheim, Germany), methadone, piretanide and furosemide from Hoechst AG (Frankfurt, Germany), bemethiazide and hydroxyethyl starch (Infukoll, 6%) from Schwarz-Pharma (Monheim, Germany).

Acetazolamide was acquired from Lancaster Synthesis Ltd. (Morecambe, UK), bendroflumethiazide from ICI Pharma GmbH (Plankstadt, Germany), benzoylecgonine from the Institute of Forensic Medicine of University of Cologne (Cologne, Germany), benzthiazide from Knoll AG (Ludwigshafen, Germany), chlorazanile from Heumann Pharma GmbH (Nürnberg, Germany), clopamide from Sandoz Pharmaceuticals GmbH (Nürnberg, Germany), clorexolone from Rhone-Poulenc Pharma (Norderstedt, Germany), diclofenamide from Dr. Mann Pharma (Berlin, Germany), fentanyl from Janssen-Cilag (Neuss, Germany), formoterol from Novartis Pharma GmbH (Nürnberg, Germany), mefruside from Bayer AG (Leverkusen, Germany), methylclothiazide from Abbott Laboratories Ltd. (Queensborough, England), metolazone from Searle GmbH (München, Germany), muzolimine from Zyma GmbH (München, Germany), norpseudoephedrine from European Directorate for the Quality of Medicines & HealthCare (Council of Europe, Strasbourg, France), oxycodone from LGC Standards GmbH (Wesel, Germany), piretanide from Cassella-Riedel Pharma GmbH (Frankfurt, Germany), polythiazide from Pfizer Deutschland GmbH (Berlin, Germany), probenecid form MSD Sharp & Dohme GmbH (Haar, Germany), ritalinic acid from Isotec Inc. (Miamisburg, Ohio, USA), trichlormethiazide from Merck (Darmstadt, Germany), xipamide from Beiersdorf AG (Hamburg, Germany). All reference materials were of analytical purity.

Glacial acetic acid and ammonium acetate were purchased from Sigma (Steinheim, Germany). All reagents were of analytical grade. Acetonitrile (LC-MS grade) was supplied by VWR International GmbH (Darmstadt, Germany). Standard solutions and other aqueous solutions were prepared using deionized water (Sartorius Stedim Biotech S. A., Aubagne, France).

LC-MS/MS analysis

Chromatographic separation of target analytes was achieved on an Agilent 1100 Series HPLC system equipped with a Nucleodur C_{18} Pyramid analytical column (2 x 50 mm, 3 μm particle size; Macherey-Nagel, Düren, Germany) connected to a Phenomenex Gemini C_6-phenyl (4 x 2.0 mm) pre-column. The mobile phase consisted of 5 mM ammonium acetate buffer containing 0.1% glacial acetic acid (pH = 3.5, mobile phase A) and acetonitrile (mobile phase B). A linear gradient at a flowrate of 0.35 ml/min was employed starting at 0% B, increasing to 90% B within 4.5 min and re-equilibrating (0.5 ml/min) at 0% B for 6.25 min. The overall runtime was 10.75 min injection-to-injection duration. The injection volume used was 5 μl.

Tandem mass spectrometry was carried out using a hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex 5500 QTrap; Darmstadt, Germany) controlled by Analyst Software 1.5 (AB Sciex). Fast polarity switching (50 msec) electrospray ionization was used with the following conditions: ionspray voltage +5500 V (positive) and -4500 V (negative), ion source temperature 450 °C, nitrogen was used as curtain, nebuliser, and auxiliary gas. The analytes and the IS (mefruside) were detected utilizing multiple reaction monitoring (MRM) of diagnostic ion transitions at dwell times of 10 msec. For optimisation of the orifice potential and the collision energy solutions of pure reference compounds for each analyte were directly injected using a 1-ml syringe at flow rate of 10 μl/min. Nitrogen was used as collision gas (3.5×10^{-3} Pa) delivered from a nitrogen generator (CMC Instruments, Eschborn, Germany). Target ion transition of MRM experiments are listed in Table 1.

Preparation of reference solutions

A stock standard solution was prepared of each individual compound at a concentration of 1 mg/ml in acetonitrile or methanol and stored at -20 °C, except of dextran and hydroxyethyl starch (HES) that were prepared in water and stored at 4 °C. The reference working solution mix of the compounds was prepared at the concentration level of 10 μg/ml in methanol.

Table 1. Chromatographic and mass spectrometric parameters.

No	Compound	Class	RT	RRT	Ionization Mode[a]	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision Energy (eV)	Declustering Potential (V)
1	Acetazolamide	Diuretic	4,22	0,78	-	221	83	−26	−50
2	Althiazide	Diuretic	5,52	1,02	-	382	341	−30	−150
3	Amiloride	Diuretic	4,12	0,76	+	230	171	30	100
4	Azosemide	Diuretic	5,76	1,06	-	369	326	−30	−100
5	Bemethiazide	Diuretic	5,70	1,05	-	400	294	−30	−150
6	Bendroflumethiazide	Diuretic	5,74	1,06	-	420	289	−28	−150
7	Benzthiazide	Diuretic	5,62	1,03	-	430	308	−30	−100
8	Bumetanide	Diuretic	5,79	1,07	-	363	80	−30	−100
9	Buthiazide	Diuretic	5,56	1,02	-	352	269	−40	−150
10	Chlorazanil	Diuretic	5,41	1,00	-	220	151	−30	−50
11	Chlorothiazide	Diuretic	4,31	0,79	-	294	214	−30	−150
12	Chlorthalidone	Diuretic	5,12	0,94	-	337	146	−30	−100
13	Clopamide	Diuretic	5,28	0,97	-	344	78	−75	−150
14	Clorexolone	Diuretic	5,58	1,03	-	327	78	−60	−150
15	Cyclopenthiazide	Diuretic	5,76	1,06	-	378	269	−30	−150
16	Cyclothiazide	Diuretic	5,67	1,04	-	388	269	−30	−150
17	Diclofenamide	Diuretic	5,12	0,94	-	303	78	−40	−100
18	Epithiazide	Diuretic	5,58	1,03	-	424	300	−30	−150
19	Ethacrynic acid	Diuretic	5,86	1,08	-	301	243	−30	−50
20	Ethiazide	Diuretic	5,13	0,94	-	324	78	−50	−150
21	Furosemide	Diuretic	5,50	1,01	-	329	205	−34	−25
22	Hydrochlorothiazide	Diuretic	4,50	0,83	-	296	269	−25	−100
23	Hydroflumethiazide	Diuretic	4,94	0,91	-	330	239	−30	−150
24	Indapamide	Diuretic	5,59	1,03	-	364	189	−30	−100
25	Mefruside (IS)	Diuretic	5,43	1,00	-	381	78	−80	−100
26	Mefruside metabolite (5-oxo-mefruside)	Diuretic	5,24	0,97	-	395	78	−60	−100
27	Methyclothiazide	Diuretic	5,42	1,00	-	358	322	−30	−100
28	Meticrane	Diuretic	4,94	0,91	-	274	210	−32	−120
29	Metolazone	Diuretic	5,48	1,01	-	364	257	−30	−50
30	Muzolimine	Diuretic	5,50	1,01	-	270	97	−30	−50
31	Piretanide	Diuretic	5,68	1,05	-	361	80	−30	−100
32	Polythiazide	Diuretic	5,73	1,06	-	438	204	−50	−100
33	Torasemide	Diuretic	5,25	0,97	-	347	262	−30	−50
34	Trichlormethiazide	Diuretic	5,36	0,99	-	378	242	−30	−100
35	Xipamide	Diuretic	5,76	1,06	-	353	127	−30	−100
36	AICAR	AMPK agonist	1,62	0,30	+	259	110	30	100
37	AICAR-$^{13}c_5$ (Is)	AMPK agonist	1,62	0,30	+	264	110	30	100
38	5OH-MEHP-gluc	Marker of blood transfusion	5,15	0,95	-	469	293	−20	−70
39	5oxo-MEHP-gluc	Marker of blood transfusion	5,18	0,95	-	467	291	−20	−70
40	Fentanyl	Narcotic	4,92	0,91	+	337	105	47	120
41	Methadone	Narcotic	5,12	0,94	+	310	265	21	96
42	Oxycodone	Narcotic	4,28	0,79	+	316	241	47	120
43	Pethidine	Narcotic	4,72	0,87	+	248	220	29	151
44	RSR 13 (Efaproxiral)	Oxygen transfer enhancer	5,80	1,07	-	340	254	−30	−50
45	GHRP-2 Metabolite (Aa-3)	Peptide hormone	4,74	0,87	+	358	170	37	50
46	Dextran	Plasma volume expander	3,76	0,69	+	487	325	15	300
47	Hydroxyethyl starch	Plasma volume expander	4,02	0,74	+	369	207	15	300
48	Andarine	SARMs	5,56	1,02	-	440	261	−30	−100
49	Andarine metabolite	SARMs	5,32	0,98	-	307	205	−30	−100
50	Benzoylecgonine	Stimulant	4,75	0,87	-	288	121	−22	−50
			4,75	0,87	+	290	168	27	50
51	Ephedrine	Stimulant	3,84	0,71	+	166	148	15	15
52	Ethamivan-sulf	Stimulant	5,02	0,92	-	302	207	−40	−95
53	Modafinilic acid	Stimulant	5,39	0,99	-	273	167	−30	−100

(*Continues*)

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Table 1. *(Continued)*

No	Compound	Class	RT	RRT	Ionization Mode[a]	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision Energy (eV)	Declustering Potential (V)
54	Norephedrine	Stimulant	2,68	0,49	+	152	134	15	15
55	Norpseudoephedrine	Stimulant	3,34	0,62	+	152	134	15	15
56	P-OHAM-sulf	Stimulant	3,85	0,71	-	230	106	−40	−80
57	P-OHMA-sulf	Stimulant	3,87	0,71	-	244	106	−40	−40
58	Pseudoephedrine	Stimulant	3,96	0,73	+	166	148	15	15
59	Ritalinic acid	Stimulant	4,48	0,83	-	218	174	−30	−100
60	Probenecid	Uricosuric	5,81	1,07	-	284	140	−30	−50
61	Formoterol	β2-agonist	4,63	0,85	+	345	149	25	15
62	Salbutamol	β2-agonist	3,93	0,72	+	240	148	20	15
63	Salmeterol	β2-agonist	5,15	0,95	+	416	232	30	15

[a]– negative ionization mode, + positive ionization mode

Quality control samples

Urinary quality control samples were prepared with every batch at a concentration level of 125 ng/ml for diuretics and at the minimum required performance levels (MRPL) for all the other analytes. The MRPL is defined as the minimum concentration a laboratory is required to detect for a certain analyte.[49] The quality control samples of HES and dextran were prepared separately at the concentration level of 500 μg/ml. As described elsewhere, stability of urinary solution of dextran and HES was demonstrated over four weeks at 4 °C.[21] For the phthalate glucuronides, quantified post-transfusion samples were analyzed as QCs.

Urine samples

Excretion urine samples were collected for mefruside, GHRP-2, dextran, HES, andarine and ethamivan. Mefruside metabolite was measured in a urine sample collected from a male volunteer (aged 64) after administration of 25 mg of mefruside (Baycaron, Bayer). GHRP-2 metabolite was investigated in a specimen collected from a male volunteer (aged 37) 5 h after oral administration of a common dose of 10 mg GHRP-2 (HEMOGEX®, Vital Pharmaceuticals). Dextran post-administration samples were provided by the Johanna-Etienne Hospital (Neuss, Germany) accumulated by catheter for approximately 10 h after infusion of 500 ml Longasteril® (Dextran 70, Fresenius) containing 6% of dextran. HES post-administration samples were received from a male volunteer (age 46) collected 14 h after intravenous application of 500 ml HAES-steril® (Fresenius) containing 6% hydroxylethyl starch. Andarine metabolite was measured in urine specimen collected from a male volunteer (aged 58) 36 h after oral administration of andarine (dosage 75 mg) purchased from an internet provider. Post-transfusion samples provided by Surface-Zentrum für Orthopädie (Neuss, Germany) were analyzed for glucuronidated mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP-gluc) and mono (2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP-gluc). Urine samples were collected from 5 volunteers from 0 to 24 h after blood-transfusion (2 female/3 male, aged 44–79). Official doping control sample tested positive for amphetamine and methamphetamine were analyzed for p-hydroxyamphetamine sulfate (p-OHAM-sulf) and p-hydroxymethamphetamine sulfate (p-OHAM-sulf) and a controlled ethamivan reference excretion urine sample received from the World Anti-Doping Agency (WADA; Montreal, Canada) was tested for ethamivan sulfate. An informed consent was obtained from all medicated patients and an ethical approval for studies with volunteers was obtained from the ethical committee of the German Sport University Cologne.

Sample preparation

After an aliquot of 90 μl of urine sample was fortified with 50 ng of the internal standards mefruside and $^{13}C_5$-AICAR (corresponding to 10 μl of a 5 μg/ml solution of the IS in methanol) samples were mixed, and an aliquot of 5 μl was injected into the instrument.

Validation of the method

For validation the parameters specificity, ion suppression, intra- and inter-day precision, limit of detection (LOD) and robustness were determined. Additionally, for quantification of dextran the items linearity and accuracy were investigated. The validation for identification of target analytes was performed according to the guidelines of the International Conference on Harmonization and WADA.[22,23] All calibration samples were prepared and analyzed as described above.

Specificity

Evaluation of specificity was carried out by analyzing six different spiked and six different blank urine samples collected from healthy volunteers (5 female, 1 male) to test for interfering signals in the selected MRM chromatograms at expected retention times of the analytes.

Ion suppression/ion enhancement

The extent of ion suppression or enhancement was investigated by analysing six different blank urine samples via post-column continuous infusion of a mixture of the reference compounds (1 μg/ml, 20 μl/min).[24]

Precision

Intra-day precision was determined at three concentration levels for each compound (QC_{low}, QC_{medium}, QC_{high}) using six replicates of spiked urine samples. Concentrations were chosen considering the different MRPLs of the analytes (Table 2). The corresponding inter-assay precision was calculated from samples prepared and analyzed at three different days. The precision of the method was determined by calculation of the coefficient of variation

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Table 2. Validation results.

No.	Compound	MRPL	LOD	Precision	Concentration			Intra-day precision			Inter-day precision		
				RRT ($n = 6$)		(ng/mL)			CV (%) ($n = 6/6/6$)			CV (%) ($n = 18/18/18$)	
		(ng/ml)	(ng/ml)	CV (%)	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}
1	Acetazolamide	250	25	1,55	100	250	500	5,84	7,24	5,25	4,78	2,23	2,5
2	Althiazide	250	10	0,28	100	250	500	10,82	10,53	6,67	6,26	1,26	6,74
3	Amiloride	250	25	0,37	100	250	500	9,85	5,59	16,64	5,93	3,4	7,22
4	Azosemide	250	2	0,48	100	250	500	10,11	8,04	5,57	5,15	1,75	3,22
5	Bemethiazide	250	5	0,23	100	250	500	7,56	8,63	7,19	2,96	1,07	4,51
6	Bendroflumethiazide	250	2	0,17	100	250	500	7,76	7,62	5,36	5,83	1,45	7,8
7	Benzthiazide	250	1	0,11	100	250	500	9,95	9,81	8,84	5,42	3,86	2,2
8	Bumetanide	250	1	0,22	100	250	500	7,72	6,92	6,87	6,92	1,74	3,91
9	Buthiazide	250	5	0,18	100	250	500	10,86	7,61	4,52	2,17	2,83	4,93
10	Chlorazanil	250	5	0,15	100	250	500	9,8	10,83	10,22	18,21	6,02	15,3
11	Chlorothiazide	250	25	1,65	100	250	500	9,6	7,81	7,65	4,75	1,5	5,88
12	Chlorthalidone	250	5	0,29	100	250	500	10,5	7,27	6,62	2,02	0,95	4,43
13	Clopamide	250	2	0,21	100	250	500	4,77	10,3	7,2	5,34	10,74	8,85
14	Clorexolone	250	2	0,17	100	250	500	6,66	9,21	5,6	4,32	3,39	2,25
15	Cyclopenthiazide	250	2	0,16	100	250	500	9,32	9,83	6,39	3,47	1,7	3,55
16	Cyclothiazide	250	2	0,19	100	250	500	9,69	8,87	4,12	2,59	1,86	5,33
17	Diclofenamide	250	2	0,38	100	250	500	8,64	7,11	5,24	6,37	1,83	3,77
18	Epithiazide	250	5	0,16	100	250	500	8,62	10,43	9,47	4,17	5,64	9,45
19	Ethacrynic acid	250	5	0,35	100	250	500	5,81	8,65	6,28	3,44	3,08	1,28
20	Ethiazide	250	5	0,39	100	250	500	8,7	6,83	5,92	5,75	6,45	5,39
21	Furosemide	250	20	0,33	100	250	500	7,66	8,65	4,94	6,48	1,97	1,07
22	Hydrochlorothiazide	250	3	1,25	100	250	500	7,13	6,61	4,99	5,05	1,79	3,1
23	Hydroflumethiazide	250	2	0,58	100	250	500	11,96	6,89	5,73	8,37	2,26	5,33
24	Indapamide	250	2	0,25	100	250	500	6,89	8,23	5,24	2,5	3,16	1,18
25	Mefruside (IS)	-	-	-	-	-	-	-	-	-	-	-	-
26	Mefruside metabolite	250	-	-	-	-	-	-	-	-	-	-	-
27	Methyclothiazide	250	10	0,23	100	250	500	9,97	8,15	5,44	8,17	2,15	3,42
28	Meticrane	250	20	1,14	100	250	500	13,65	5,15	6,96	6,37	5,03	2,31
29	Metolazone	250	3	0,18	100	250	500	8,29	7,4	6,58	4,16	2,03	1,64
30	Muzolimine	250	5	0,19	100	250	500	9	7,18	4,71	5,29	1,47	1,07
31	Piretanide	250	5	0,24	100	250	500	5,63	10,03	4,62	2,9	3,3	1,57
32	Polythiazide	250	1	0,13	100	250	500	9,29	7,32	7,17	8,48	3,21	1,92
33	Torasemide	250	1	0,23	100	250	500	11,88	12,88	7,21	5,46	5	6,12
34	Trichlormethiazide	250	2	0,26	100	250	500	11,8	9,34	6,6	9,83	9,7	7,77
35	Xipamide	250	2	0,16	100	250	500	11,5	7,26	7,45	5,41	7,05	6,28
36	AICAR	-	30	-	500	1500	4500	11,7	6,8	9,7	13,5	9,3	11,5
37	AICAR-$^{13}c_5$ (Is)	-	-	-	-	-	-	-	-	-	-	-	-
38	5OH-MEHP-gluc	-	30	1,36	100	550	1010	10,88	7,85	7,33	10,09	5,51	6,35
39	5oxo-MEHP-gluc	-	20	1,1	100	550	1010	10,89	8,91	7,45	10,91	6,57	4,65

(*Continues*)

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Table 2. *(Continued)*

No.	Compound	MRPL	LOD	Precision	Concentration			Intra-day precision			Inter-day precision		
40	Fentanyl	10	1	0,07	5	10	20	5,46	7,75	10,6	17,01	18,75	13,64
41	Methadone	200	1	0,05	100	200	500	6,53	8,01	5,6	19,91	14,67	3,87
42	Oxycodone	200	5	0,29	100	200	500	6,39	6,43	7,58	15,21	15,49	13,18
43	Pethidine	200	1	0,1	100	200	500	6,24	6,04	9,7	6,85	5,42	10,47
44	RSR 13 (Efaproxiral)	-	1	0,26	100	250	500	7,44	6,42	4,77	3,35	2,95	1,99
45	GHRP-2 metabolite (AA-3)	-	3	0,1	5	10	20	10,61	8,09	8,85	11,35	11,9	2,33
46	Dextran[b]	-	30 µg/mL	0,98	99 µg/mL	480 µg/mL	975 µg/mL	10,18	11,36	11,65	10,61	11,21	12,56
47	Hydroxyethyl starch	-	10 µg/mL	0,47	100 µg/mL	500 µg/mL	1000 µg/mL	9,47	7,64	8,08	18,06	16	16,48
48	Andarine	-	1	0,31	100	250	500	5,53	10,3	7,44	1,46	0,38	4,66
49	Andarine metabolite	-	-	-	-	-	-	-	-	-	-	-	-
50	Benzoylecgonine[a]	500	1-25	0,43	100	250	500	8,5	12,87	6,98	2,91	1,83	3,4
51	Ephedrine	500	5	0,59	7.5 µg/mL	15 µg/mL	30 µg/mL	7,13	7,09	6,79	8,53	9,29	7,84
52	Ethamivan-sulf	500	25	0,05	100	500	1000	9,56	7,77	8,82	4,95	8,51	2,69
53	Modafinilic acid	250	10	0	100	250	500	8,22	3,33	3,16	3,62	1,98	2,19
54	Norephedrine	500	70	0,52	2.5 µg/mL	5 µg/mL	10 µg/mL	5,26	6,89	7,49	8,63	14,21	10,47
55	Norpseudoephedrine	500	70	0,68	2.5 µg/mL	5 µg/mL	10 µg/mL	5,42	6,95	7,7	12,12	13,18	9,69
56	P-OHAM-sulf	500	25	0,64	100	500	1000	11,12	6,49	7,92	11,73	15,88	4,35
57	P-OHMA-sulf	500	25	0,57	100	500	1000	8,64	7,64	7	13,45	13,34	6,99
58	Pseudoephedrine	500	5	0,7	5 µg/mL	10 µg/mL	20 µg/mL	7,48	11,62	13,12	8,06	11,86	18,57
59	Ritalinic acid	500	70	0,06	100	250	500	13,98	14,33	6,99	5,22	3,02	6,01
60	Probenecid	250	1	0,3	100	250	500	7,22	6,26	7,25	2,07	1,59	2,59
61	Formoterol	100	5	0,12	50	100	250	8,41	8,29	9,35	13,54	12,64	3,71
62	Salbutamol	100	5	0,57	50	100	250	12,11	8,08	10,43	14,96	17,02	4,99
63	Salmeterol	100	5	0,1	50	100	250	9,7	12,62	7,14	14,33	3,4	12,83

[a] Benzoylecgonine: The estimated LODs were below 1 ng/mL in positive ionization mode and 25 ng/mL in negative ionization mode.

[b] Dextran linearity (100–1000 µg/mL): $y = -0.203 + 0.002 \cdot x$, $r > 0.98$. The average relative recovery at the concentration level of 500 µg/mL was 104.1%.

(CV) of the area ratio of the ion transition of the analytes and the internal standard.

Limits of detection

The limit of detection (LOD) was estimated via signal to noise ratio (S/N) of the respective ion traces using ten blank samples and ten fortified samples at concentration levels of 5 and 50 ng/ml (diuretics), 5 ng/ml (narcotics, beta2-agonists, GHRP-2), 10 and 100 ng/ml (stimulants), 25 ng/ml (stimulant sulfo-conjugates), 50 ng/ml (5oxo-MEHP and 5OH-MEHP) and 10 and 50 µg/ml (dextran and HES).

Robustness

Robustness has been tested by comparison of the relative retention times of the analytes in the QC samples over a month. The calculated CVs are presented in Table 2.

Results and discussion

Sample preparation aspects

Direct injection of native urine specimens provides a wide applicability of the assay as well as a very simple and rapid sample preparation without hydrolysis, purification, or further sample preparation steps. In addition to substances that are excreted unchanged into urine – i.e. diuretics, beta2-agonists and narcotics – compounds were implemented that are mainly excreted metabolized. As new target analytes 5oxo-MEHP-gluc and 5OH-MEHP-gluc, phase II metabolites of DEHP and selected stimulant sulfoconjugates were analyzed, avoiding time-consuming hydrolysis steps.

LC-MS

The chromatographic run was optimized taking into account the chemical versatility of the analytes, resulting in a wide range of polarities. A gradient starting at 100% aqueous buffer (5 mM ammonium acetate, 0.1% glacial acetic acid, pH 3.5) was required to ensure sufficient retention for hydrophilic compounds, for example acetazolamide and amiloride (Figure 1). For the first eluting compound AICAR, the use of a labelled analogue ($^{3}C_5$-AICAR) is obligatory to control retention and matrix interferences. To avoid column blockage a pre-column has been used. Over 2000 analyses were conducted with the same analytical column without any loss in chromatographic performance. As presented in Table 2, the relative retention times of all analytes proved to be stable with coefficients of variation (CVs) less than 2%, fulfilling the recommended identification criteria.[25]

The possibility of fast polarity switching (50 ms) ensures an optimized ionization, regarding differences in the acidic or basic character of the molecules. The detection of a wide range of doping agents was accomplished in one analytical run. While 18 analytes were detected in positive ion mode mainly as protonated quasi-molecular ions $[M+H]^+$, 46 analytes were detected in negative ion mode as deprotonated quasi-molecular ions $[M\text{-}H]^-$. All analytes were clearly detectable at required concentration levels.[22]

Diuretics, beta2-agonists, narcotics, and stimulants

As depicted in Figure 1, the developed method enables the detection of 36 diuretics with LODs ranging from 1 to 25 ng/ml, fulfilling the required concentration levels. As a proof of principle three beta2-agonists, four narcotics, and six stimulants were chosen as model compounds to investigate the possibility to screen for these classes of compounds via direct injection of native urine.

Analytes that produce ions with the same mass-to-charge ratio were separated, such as norephedrine-norpseudoephedrine (*m/z* 152/134) and ephedrine-pseudoephedrine (*m/z* 166/148) (Figure 1). It is noteworthy that samples with high concentrations should be diluted to ensure adequate chromatographic resolution. Due to the lack of reference material for the mefruside metabolite, a reliable screening was tested using mefrusid post-administration samples.

AICAR

The adenosine monophosphate activated protein kinase activator 5-amino-4-imidazolecarboxyamide ribonucleoside (AICAR) was found to significantly improve endurance of untrained mice.[26] Considering the fact that due to endogenous production of AICAR in healthy humans considerable amounts are excreted into urine, a semi-quantitative approach is required to screen for abnormally high urinary concentrations.

In earlier studies, urinary AICAR concentrations in an athletes' population ($n = 499$) were determined, with a mean AICAR level of 2186 ng/ml. Concentrations were found to differ depending on gender, type of sport and type of sample collection (in- and out of-competition). In order to fix reference values for further investigations quantile 95 and 99 values of AICAR levels have been determined, which could be used to identify 'abnormally high' concentrations of AICAR in doping control samples.[27]

The dissociation pathways of AICAR after positive electrospray ionization have been described in earlier reports[27,28] and match the pathways found in the present study. The quantifier ion transition used in this study was *m/z* 259/110. To ensure reliable semi-quantification $^{13}C_5$-labelled AICAR was used as internal standard (*m/z* 265/110). According to its polar character, AICAR was detected at the beginning of the chromatographic run at approx. 1.7 min. Therefore, the use of the labelled internal standard $^{13}C_5$-AICAR is strongly recommended to control for any matrix or ion suppression effects. The validation results of AICAR are received from an earlier study utilizing direct injection and the same instrumental set-up.[27]

GHRP-2

According to earlier studies, unchanged GHRP-2 and its main metabolite (AA-3) were excreted in urine after intravenous administration of GHRP-2 as 0.2–5.9% and 2.2–5.7% of a dose, respectively. Although the maximum concentration of AA-3 being one-tenth that of GHRP-2, the detection of AA-3 is likely to be indicative of GHRP-2 because of its longer half-life.[29] By quantification of GHRP-2 metabolite after a single oral administration of 10 mg GHRP-2, it was detectable for more than 20 h with maximum urinary concentrations of 15 ng/ml (after 5 h) while the intact peptide or additional metabolites were not detected in the samples.[19]

In this study, AA-3 was observed as protonated molecular ion $[M+H]^+$ at *m/z* 358 and the main product ions were 287 (y2), 269 (b2), 241 (a2) and 170 (immonium ion of β-naphthylalanine) as published earlier.[29] The ion transition used for screening was *m/z* 358/170. The applicability of the method was demonstrated with the investigation of GHRP-2 post administration specimens. As illustrated in Figure 2, the GHRP-2 metabolite was detectable in samples 5 h after oral administration of a common dose of 10 mg GHRP-2. Nevertheless, a longer detection window would be advantageous, but is difficult to achieve without pre-concentration steps. Therefore, special procedures are available allowing a detection of GHRP-2 up to 20 h after application.[19]

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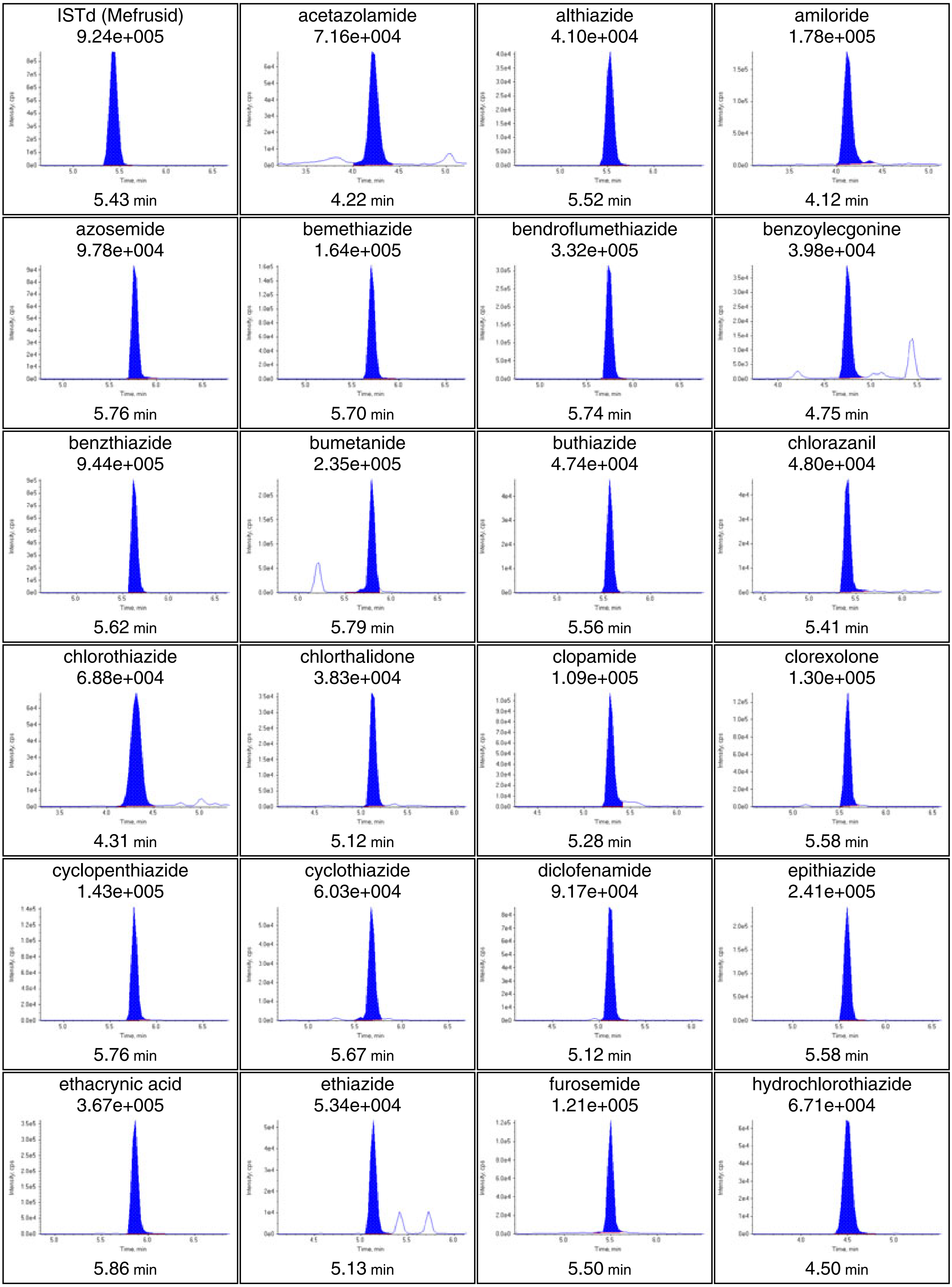
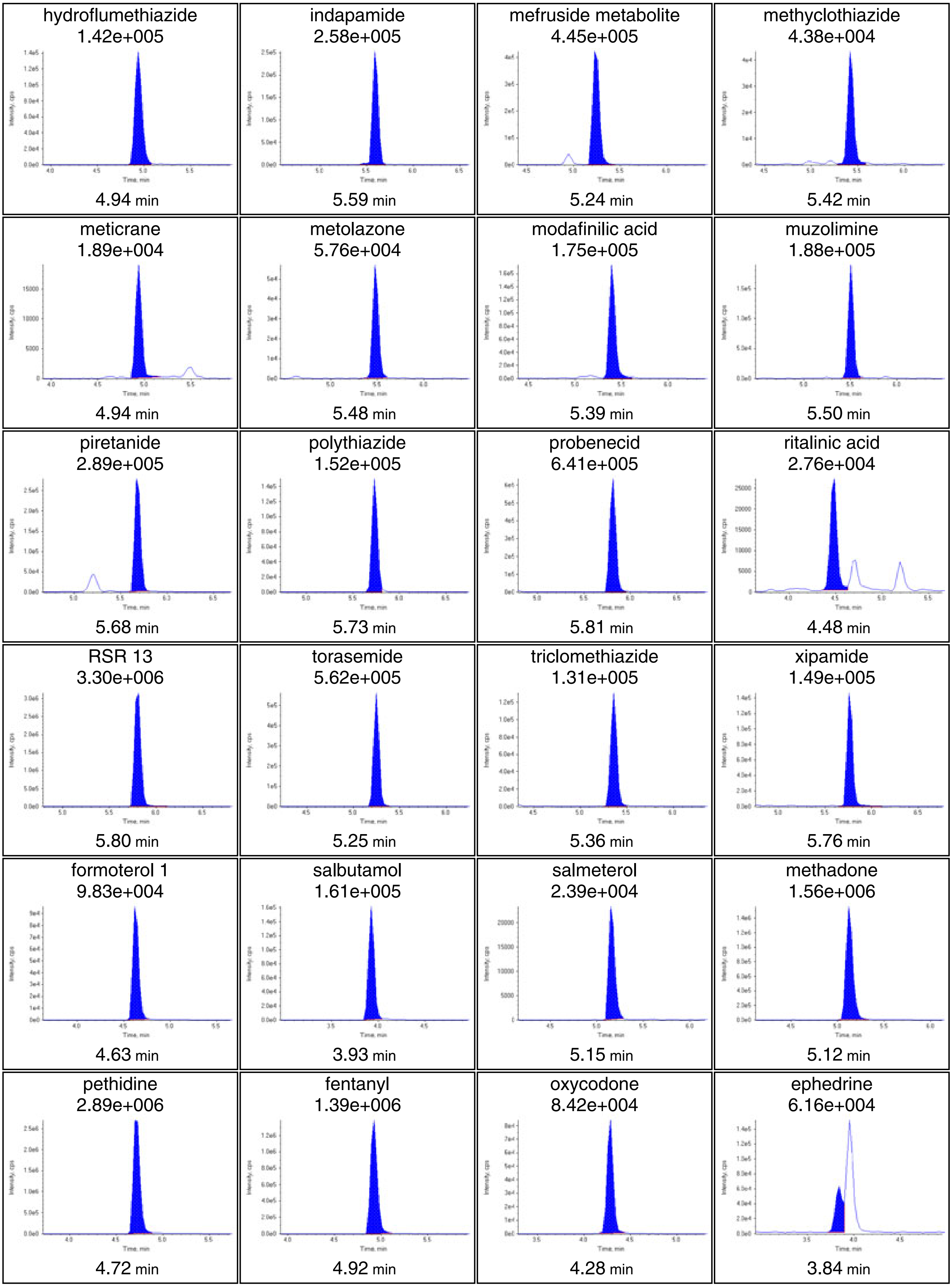


Figure 1. (*Continued*).



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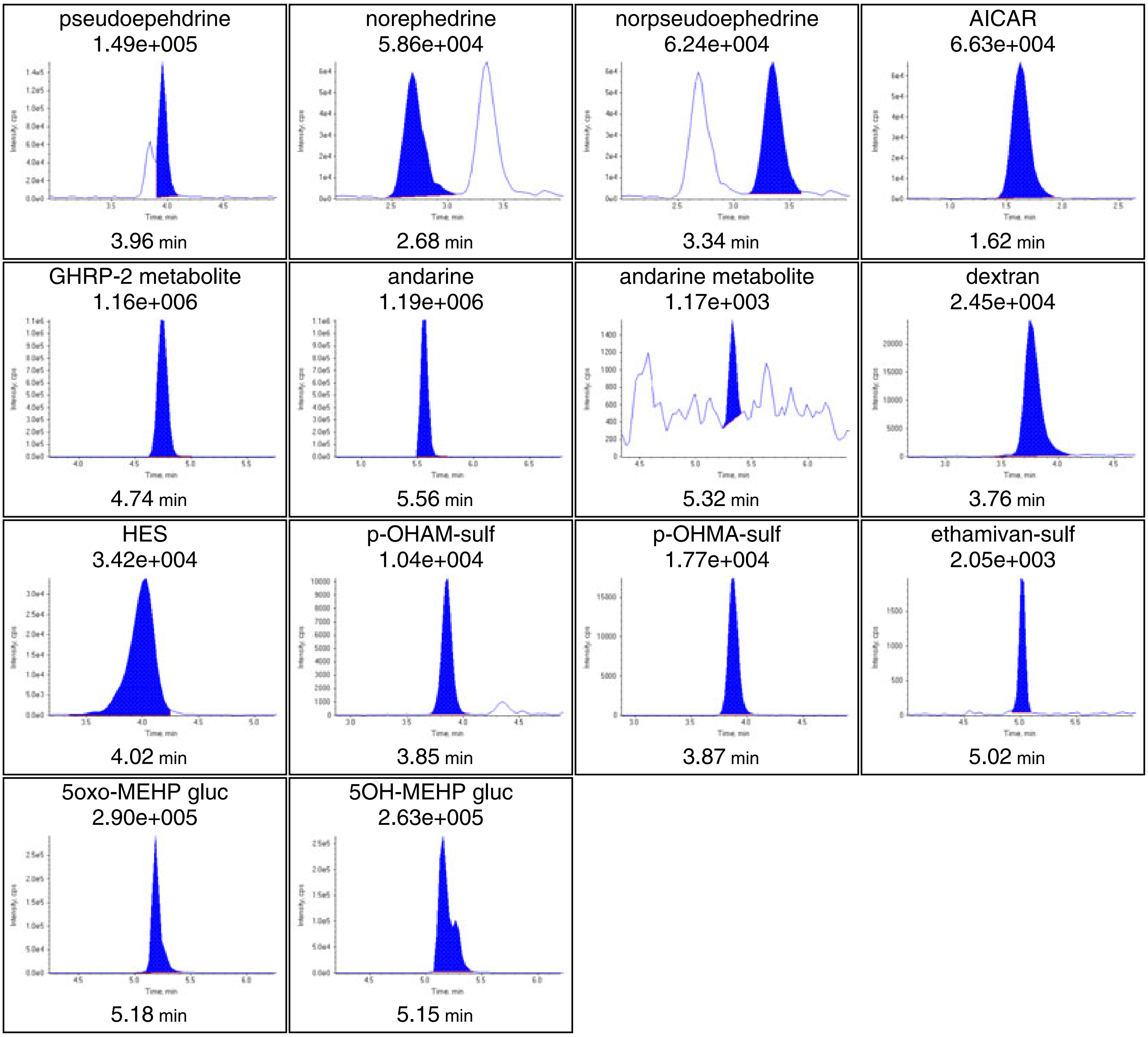

Figure 1. Extracted ion chromatograms of the analytes in a quality control sample; concentration levels are as follows: 125 ng/mL for diuretics, andarine and RSR-13; 100 ng/mL for beta2-agonists; 200 ng/mL for narcotics; 500 ng/mL for stimulants and stimulant sulfo-conjugates; 500 µg/mL for dextran and HES.

Phthalate glucuronides

As published earlier, increased urinary concentrations of DEHP metabolites can be useful markers to indicate homologous or autologous blood transfusion.[30,31] The phthalate metabolites are excreted into urine mainly as conjugates following phase-II glucuronidation.[32] Commonly, these conjugates were enzymatically hydrolyzed and determined with LC-MS/MS. [30,31] To avoid time-consuming hydrolysis of conjugates and to enable a comprehensive monitoring of concentration levels in routine doping control samples the implementation of glucuronidated DEHP metabolites as target analytes into a multi-target approach was required.

Due to the lack of reference material the glucuronidated DEHP metabolites were characterized and identified using liquid chromatography coupled to high resolution / high accuracy mass spectrometry (Exactive OrbiTrap®, Thermo Fisher). Therefore, a post-transfusion sample was analyzed containing known amounts of 5OH-MEHP (665 ng/ml) and 5oxo-MEHP (421 ng/ml). Glucuronidated conjugates of 5OH-MEHP and 5oxo-MEHP were identified as deprotonated molecular ions $[M-H]^-$ at *m/z* 469.1708 Da and 467.1559 Da with the calculated error of 1.48 ppm and 0.03 ppm, respectively. Additionally, the glucuronides of 5OH-MEHP and 5oxo-MEHP (5OH-MEHP-gluc and 5oxo-MEHP-gluc) were investigated with LC-MS/MS in enhanced (linear ion trap) product ion mode. The most abundant product ions at *m/z* 291 and 293 resulted from the unconjugated molecules by the loss of glucuronic acid (-176 Da) and the corresponding glucuronic acid ion was observed at *m/z* 175. The fragment of 5oxo-MEHP-gluc at *m/z* 143 and the corresponding fragment of 5OH-MEHP-gluc at *m/z* 145 were identified as the deprotonated analogues of 5-(hydroxymethyl)heptan-2-one and 2-ethylhexane-1,5-diol resulting from the loss of 2-formylbenzoic acid from the unconjugated molecule. Fragment at *m/z* 113 can be formed by elimination of CH_2O or a CH_3OH from the

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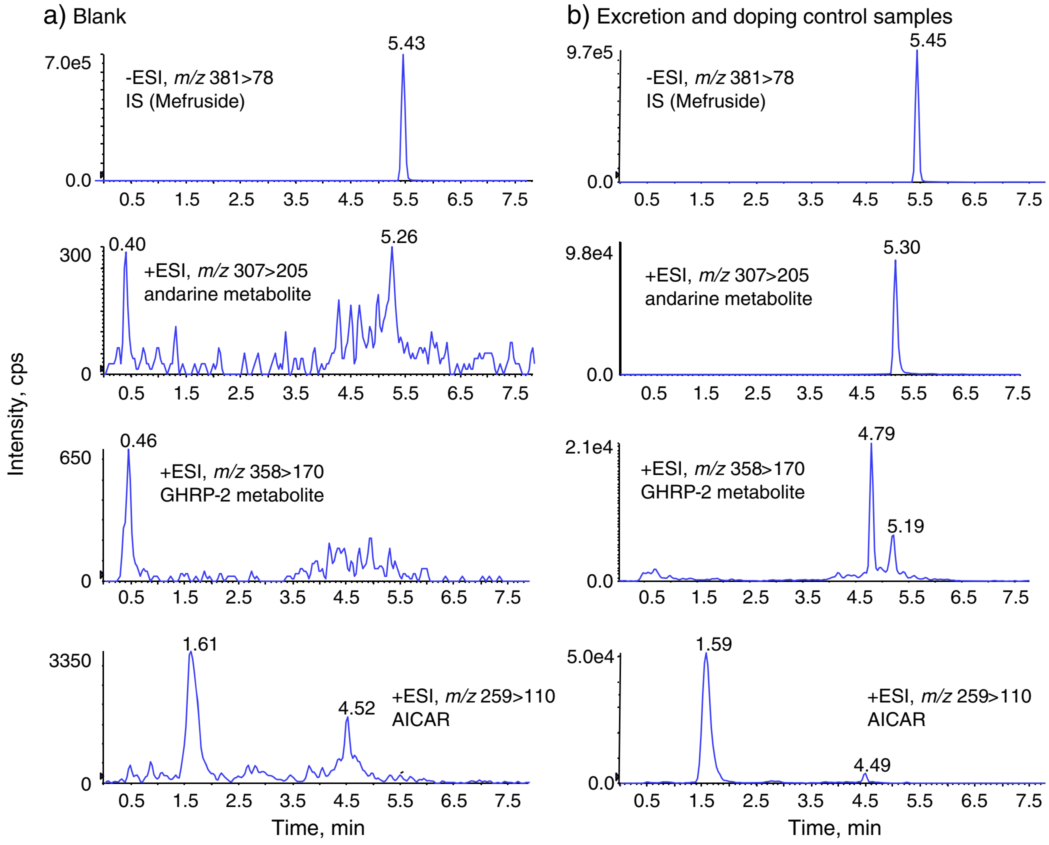

Figure 2. Extracted ion chromatograms of andarine metabolite, GHRP 2 metabolite and low endogenous level of AICAR in a blank sample (a), andarine and GHRP 2 excretion urine samples and the endogenous level of AICAR in a doping control sample (b).

fragment at *m/z* 143 or 145 or by elimination of H_2O and CO_2 from the glucuronic acid fragment[33]. Product ion at *m/z* 85 was characterized as a specific fragment of the glucuronic acid[33] (Figure 3). For screening purposes the ion transitions at *m/z* 469/293 for 5OH-MEHP-gluc and at *m/z* 467/291 for 5oxo-MEHP-gluc were monitored. As depicted in Figure 4, the peak shapes suggested more glucuronide forms of the analytes. This was proven with optimized chromatographic conditions (data not shown) indicating different locations of the glucuronic moiety.

The method has been validated for 5OH-MEHP-gluc and 5oxo-MEHP-gluc using post-transfusion samples. To ensure the ability of the method for semi-quantitative purposes the correlation of 5OH-MEHP and 5oxo-MEHP concentrations and the relative intensities of the corresponding 5OH-MEHP-gluc and 5oxo-MEHP-gluc was determined. The phthalate concentrations of the samples were measured according to a method published earlier. [31] The relative intensities of phthalate glucuronides were highly correlated to the corresponding phthalate concentrations ($r = 0.993$ for 5oxo-MEHP, $r = 0.990$ for 5OH-MEHP) suggesting that the method provides reliable data to screen for abnormal high concentrations of DEHP metabolites indicating for homologous or autologous blood transfusion.

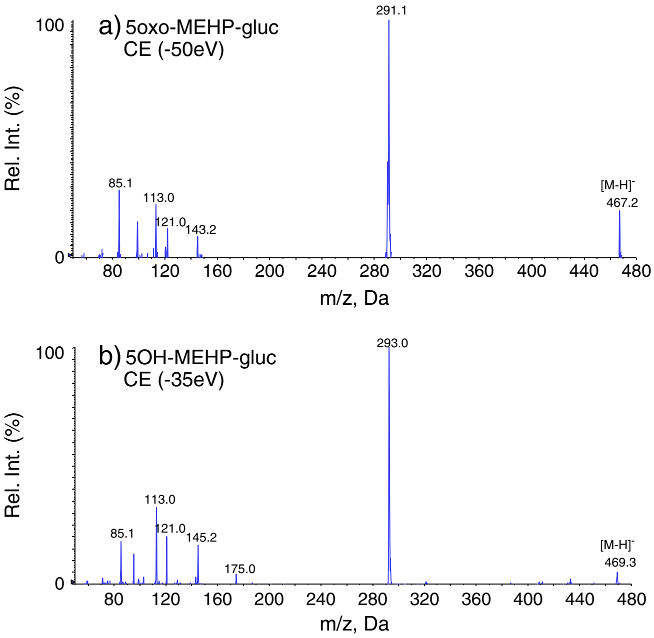

Figure 3. Negative ESI enhanced (linear ion trap mode) product ion spectra of the deprotonated molecules [M-H]- of 5oxo-MEHP-gluc (a) and 5OH-MEHP-gluc (b) obtained by analyzing a post-transfusion urine sample (5oxo-MEHP: 421 ng/mL, 5OH-MEHP: 665 ng/mL).

Plasma volume expanders

Detection methods for PVEs, such as HES and dextran, have been established utilizing LC-MS, MALDI-TOF-MS or GC-MS after essential hydrolysis of the polymers and further sample preparation steps.[15,16,34] A comprehensive assay for the determination of the analytes in human urine without hydrolysis or further sample preparation steps was published as well. The in-source fragmentation strategy of this method allows the use of tandem mass spectrometry for the determination of polysaccharide-based PVEs.

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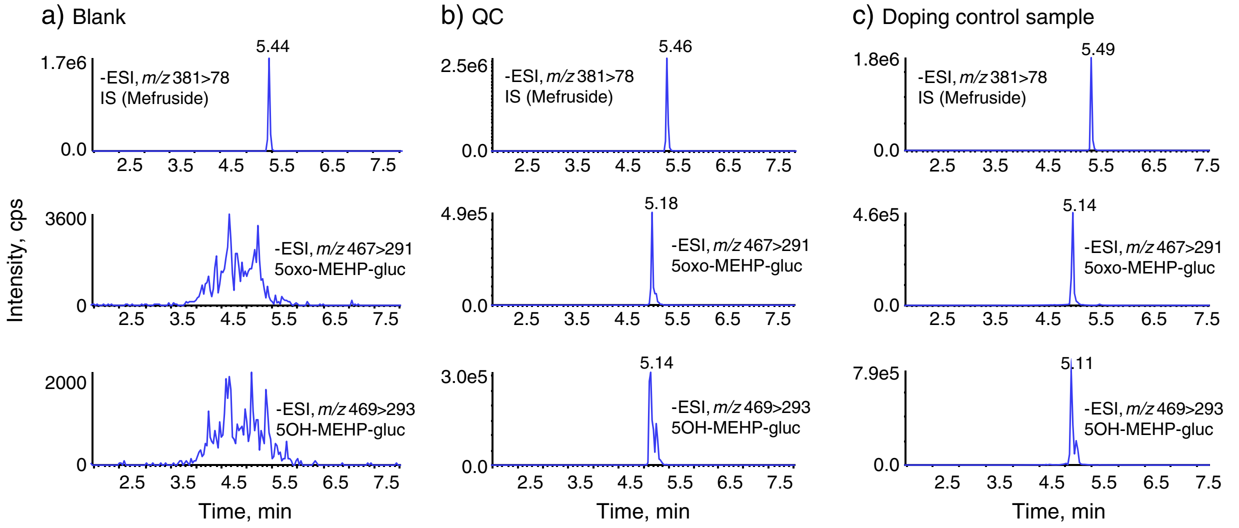

Figure 4. Extracted ion chromatograms of 5oxo-MEHP-gluc and 5OH-MEHP-gluc in a blank sample (a), a quality control sample (QC, post-transfusion sample: 5oxo-MEHP: 421 ng/mL, 5OH-MEHP: 667 ng/mL) (b) and in a suspicious doping control sample (5oxo-MEHP: 410 ng/mL 5OH-MEHP: 905 ng/mL) (c).

Protonated fragment ions of dextran and HES were produced due to in-source fragmentation by applying a high orifice potential.[14]

The diagnostic ion transitions at *m/z* 369/207 for HES corresponding to $[HE(gluc)_2 + H]^+$ and at *m/z* 487/325 for dextran corresponding to $[gluc_3 + H]^+$ were analyzed. To ensure the ability of the method to reveal an intravenous administration of the plasma volume expanders, HES and dextran post-administration samples were analyzed. As illustrated in Figure 5, the analysis of post-administration samples demonstrated abundant signals for the diagnostic ion-transitions *m/z* 369/207 (HES) and *m/z* 487/325 (dextran). The presented quality control sample represents the suggested threshold of 500 μg/ml for dextran. Less abundant interferences were observed in the negative sample, as low physiological levels of oligo- or polymeric glucose were frequently detected. In this study, the LODs calculated for dextran (30 μg/ml) and HES (10 μg/ml) were comparable to those described earlier.[14]

SARMs

Selective androgen receptor modulators (SARMs) are potent anabolic agents with tissue-selective properties. Due to their potential misuse in elite sport, the entire class of androgen receptor modulators has been prohibited in sports according to WADA's anti-doping regulations since January 2008.[35] In doping analysis, screening of SARMs was achieved utilizing GC-MS[36] as well as LC-MS/MS.[20,37–42] Andarine (S-4) is one of several structurally related arylpropionamide-derived SARMs which is available on the black market. Human urinary metabolites of the SARM S-4 (andarine) were characterized using mass spectrometric approaches. The dephenylated aglycon (M1) and its glucuronide conjugate were two of the most prominent metabolites found in the spot urine sample collected after administration of andarine.[43]

In this study S-4 and the dephenylated aglycon of S-4 (M1) were integrated into the screening procedure. The analytes were observed

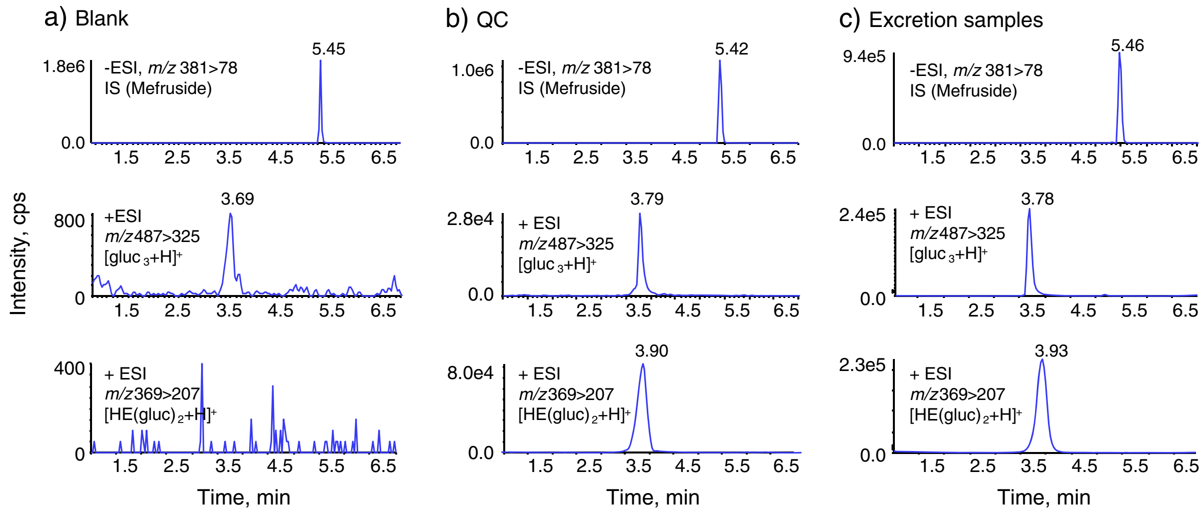

Figure 5. Extracted ion chromatograms of dextran and HES in a blank sample (a), a quality control (QC) sample (dextran: 500 μg/mL; HES: 500 μg/mL) (b) and in post administration urine samples of patients receiving 500 mL Longasteril® (Dextran 70) containing 6% of dextran and 500 mL HAES-steril® containing 6% hydroxylethyl starch (c), respectively.

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as deprotonated molecular ions $[M-H]^-$ at *m/z* 440 (S-4), 307 (M1) and under collision induced dissociation the analytes produced the product ions observed in earlier studies.[42,44] The transitions used for screening were 440/261 and 307/205 for S-4 and M1, respectively.

Due to lack of reference material for M1, validation was performed on andarine (S-4). The applicability of the method for the detection of the metabolite M1 was proven with the analysis of urine specimens collected after oral administration of andarine (dosage of 75 mg) purchased from an internet provider. The metabolite M1 was clearly detectable up to 36 h after administration (Figure 2).

Stimulant sulfo-conjugates

In sports drug testing, stimulants are commonly determined after acidic hydrolysis and subsequent analysis by LC-MS/MS.[45] Existing methods are quite laborious, thus an alternative approach was followed using model compounds of stimulant-conjugates synthesized in-house to test for a possible screening without complex sample preparation. One major metabolic pathway consists of sulfo-conjugation of the compounds, such as methamphetamine (MA), ethamivan, and to a minor extent, amphetamine. [46] In earlier studies, it was reported that the transformation of methamphetamine into p-hydroxy-methamphetamine (p-OHMA) was the main metabolic pathway in humans.[47] In samples from MA users, p-OHMA, p-OHMA-gluc and p-OHMA-sulf were detected. The level of p-OHMA-sulf reached approximately 5.5–9.7 times those of p-OHMA-gluc in the samples. These data suggested that sulfo-conjugation is a major pathway in the MA phase II metabolism.[46] Ethamivan sulfate has been also detected in excretion urine sample obtained from a healthy male volunteer collected 6–12 h after oral administration of 40 mg of ethamivan.[6]

Within this study, the model compounds p-OHAM-sulf, p-OHMA-sulf and ethamivan-sulf were investigated. It is noted, that p-OHAM itself is marketed as stimulating agent. After optimization the ion transitions at *m/z* 230/106 for p-OHAM-sulf at *m/z* 244/106 for p-OHMA-sulf and at *m/z* 302/207 for ethamivan-sulf were monitored (Figure 1).

As proof of principle, official doping control samples tested positive for amphetamine, methamphetamine, and ethamivan were investigated. The corresponding sulfo-conjugates of the stimulants were clearly identified in all of the samples, indicating that screening of stimulant sulfo-conjugates provides an alternative strategy in doping control, without complex sample pretreatment (Figure 6). Furthermore, ethamivan-sulf was detected in controlled reference urine sample of ethamivan (Figure 6).

Assay validation

In order to test for assay suitability, the parameters specificity, ion suppression/enhancement, intra- and inter-day precision, LOD, and robustness, and additionally for dextran accuracy and linearity were determined.

Regarding specificity, no interfering signals of the matrix were detected at the expected retention times of the analytes except of the physiological levels of AICAR and low basal levels of phthalate metabolites as it was found in earlier studies. [26,30] Concerning dextran, low physiological urinary concentrations of oligo- or polymeric glucose were observed which were clearly differentiable from the suggested threshold level of 500 µg/ml in order to identify a dextran administration.[21] Stable retention times are of utmost importance for reliable evaluation, especially if native urine is injected. Analysis of QC samples over four weeks yielded stable retention times (CV $<2\%$) for all of the compounds (Table 2).

Concerning ion suppression or enhancement there was no significant decrease or increase of the electrospray response at

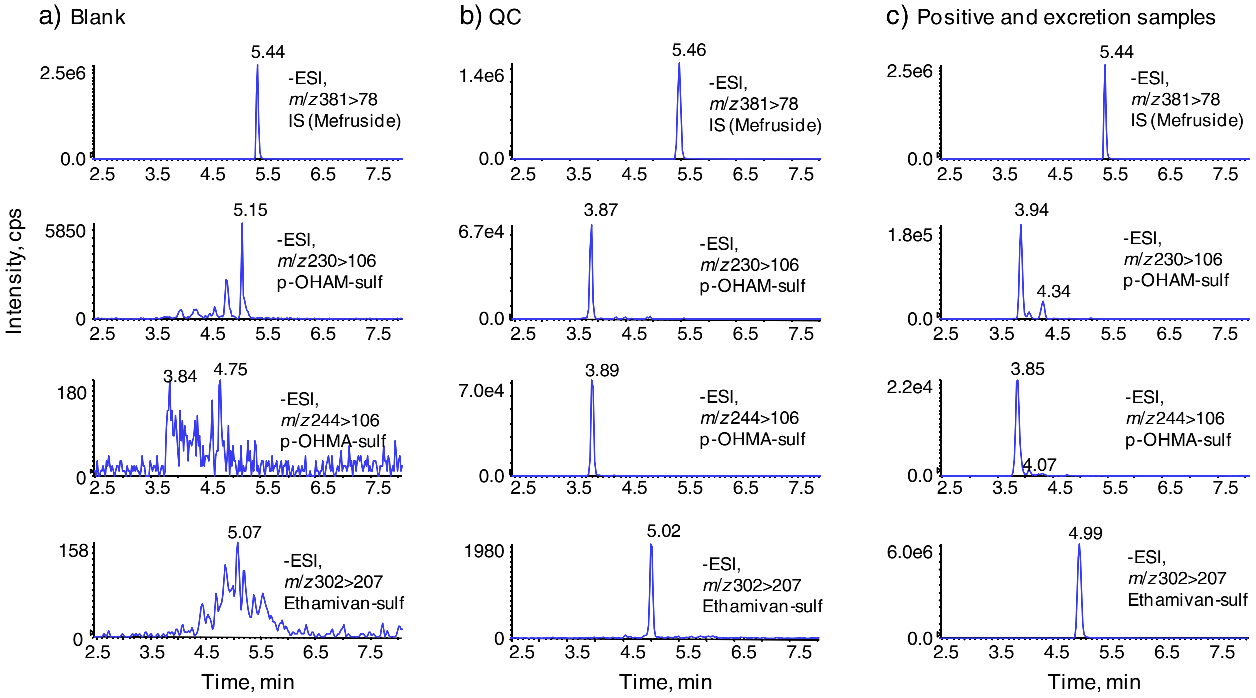

Figure 6. Extracted ion chromatograms of p OHAM sulf, p OHMA sulf and ethamivan-sulf in a blank sample (a), a quality control (QC) sample (b) and in official doping control samples tested positive for amphetamine and methamphetamine and in a controlled ethamivan post administration urine sample (c).

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the expected retention times of the analytes when urinary matrix was injected.

Intra-and inter-day precisions were determined at three concentration levels with coefficients of variation less than 20% for all analytes. It is noteworthy that the thiazide-based diuretics are unstable in aqueous media.[48] To increase their stability, a thermostated autosampler is required.

The assay demonstrated a linear correlation between dextran concentration and response within the given concentration range (100–1000 μg/ml) with the correlation coefficients (R) above 0.98. The slope and the intercept of the calibration curve were 0.002 and -0.203, respectively. The relative recovery was determined in six fortified samples at the concentration of 500 μg/ml by means of comparison of theoretical and calculated concentration ranging from 96.7 to 109.6% with an average recovery of 104.1%.

At the required signal-to-noise ratio of 3, the estimated LODs ranged from 1 to 70 ng/ml (Dextran: 30 μg/ml, HES: 10 μg/ml) making the method suitable to detect all of the compounds below the MRPLs. [22,49]

Conclusion

The ongoing increase of prohibited compounds and samples in sports drug testing forces doping control laboratories to develop more comprehensive multi-target assays that combine high-throughput, simplified sample preparation, and a reliable detection for different classes of compounds. In this work, a very comprehensive, simple and robust method based on LC-(ESI)-MS/MS is presented enabling the detection of diuretics, beta2-agonists, narcotics, stimulants and stimulant sulfo-conjugates, plasma volume expanders, andarine (S4), GHRP-2, AICAR and glucuronidated DEHP metabolites after direct injection of urine specimens far below the required limits. The combination of different procedures reduces costs and speed up sample turn-around time.

It is the first time that a reliable screening of the polysaccharide-based PVEs dextran and HES, commonly analyzed in time-consuming stand-alone procedures, has been accomplished within a multi-target assay. Additionally – as new target analytes for the class of stimulating agents – selected stimulant sulfo-conjugates were investigated demonstrating a great potential to avoid complex sample preparation and allowing the implementation of numerous additional compounds. As a completely novel approach, the monitoring of glucuronidated di(2-ethylhexyl) phthalate (DEHP) metabolites has demonstrated its applicability to detect abnormally high levels of plasticizers indicating illicit blood transfusion. Using the presented method, screening for such markers in each doping control sample is quite straightforward and offers a valuable tool in the fight against blood doping.

The combination of a highly sensitive new generation mass spectrometer using between-scan polarity switching and direct injection of urine specimens offers a wide applicability and flexibility of the current method, as demonstrated by the successful analysis of a wide range of compounds with different physico-chemical properties and accordingly, different chromatographic and mass spectrometric behaviour. Accordingly, as a future perspective, the implementation of new emerging classes of prohibited compounds seems promising. However, every method has its limitations and not all prohibited compounds can be analyzed easily via direct injection of native urine, for example, peptides/proteins or substances which are detectable mainly in blood.

Acknowledgement

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