Analyst

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2025, **150**, 773

Screening analysis of doping agents in horse urine and plasma with dilute and shoot using liquid chromatography high resolution mass spectrometry†

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Received 2nd December 2024,
Accepted 19th January 2025

DOI: 10.1039/d4an01501k

rsc.li/analyst

Various technical methodologies are required to accurately detect substances of different chemical and pharmacological properties in biological samples, which are increasing in number and variety daily. Therefore, laboratories where many samples and different factors are analyzed simultaneously need methods with easy sample preparation, short analysis times and low analysis costs. In this study, the objective was to scan substances susceptible to chemical degradation, amenable to analysis without hydrolysis, and exhibiting short-term stability by employing a straightforward, expeditious, and cost-efficient method. For this purpose, a high-throughput dilute and shoot screening protocol was developed and validated utilizing high-performance liquid chromatography coupled with high-resolution mass spectrometry to analyze various pharmacological compounds in horse urine and plasma. Over 200 prohibited substances across multiple categories were scanned within a 13 minute run. Chromatographic separation was performed on a C18 column using an elution gradient of mobile phase A, 5 mM ammonium bicarbonate at pH 9, and mobile phase B, methanol, at a flow rate of 0.3 mL min^{-1}. The method was validated according to the specifications of 2002/657/EC multi-screening requirements. The detection capability ranged from ≤1 to 200 ng mL^{-1} for prohibited substances. The implementation of the screening method in doping analysis, and the analysis of real positive case samples served to underscore the practical applicability of the developed method. To the best of our knowledge, this is a rare method that can be applied to both urine and plasma samples and provides a rapid, practical, broad-spectrum, and high-throughput analysis of prohibited substances in horse plasma and urine cost-effectively.

Introduction

In equine sports, both performance-enhancing and performance-impairing substances (or methods) can be used to manipulate race.[1] Therefore, the number of substances to be analyzed in horseracing is much greater than that of in human sports. In addition, the number of test samples for drug screening by anti-doping laboratories continues to grow. Thus, the challenge faced by horse and human doping control laboratories is the increase in easily available drugs that are capable of affecting the performance of horses and human athletes.[2] Doping laboratories must also analyze the substances used for the treatment of horses, dietary, environmental, and endogenous as well as prohibited substances according to the IFHA rules.[3] The diversity of the structural and pharmacological properties of drugs has made it necessary to adopt sensitive, economical, and simple sample preparation methods that can detect a wider range of drugs during a single analysis method due to several limitations, such as limited sample volume, large number of samples, *etc.*[4–6]

For high-throughput doping analysis, liquid–liquid and solid-phase extraction techniques are commonly used, but poor extraction can be an issue for many polar substances.[7] The technique called ‘direct injection’ or “dilute and shoot (DS)” has been applied.[8–11] It has been reported that DS has been applied using LC MS–MS for the detection of many different substances in bio-

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† Electronic supplementary information (ESI) available. See DOI: **https://doi.org/10.1039/d4an01501k**

logical samples of humans and horses.[12–18] LC-HRMS offers high resolution and stable mass accuracy without the need for internal mass calibration. By coupling with external devices, such as a linear ion trap or a quadrupole, hybrid Orbitrap instruments can provide sensitive HRMS full scans as well as tandem HRMS/MS scans, thereby permitting both nontargeted screening and targeted screening in a single analytical method. Data obtained in the full scan and HRMS/MS scan can be evaluated for accurate mass, retention time, isotope pattern, selected fragment ions and HRMS/MS spectra, thereby greatly improving screening selectivity. Several studies have described the use of UHPLC for screening of doping agents[4,19–22] and for toxicological analysis.[7,23] The DS technique in combination with HRMS has shown successful results, but few studies have been conducted simultaneously on both urine and plasma samples, and these studies have applied different sample preparation steps for plasma and urine.[7]

In the literature, some studies highlighted the key advantages of DS-LC-MS (*i.e.* the lack of sample preparation, chemical or enzyme application), the universal applicability of the methodology and the ability to combine different classes of compounds in a single DS-LC-MS method.[9] The dilute and shoot technique can detect a wide range of acidic, neutral and basic drugs in a single analysis. This method can also be useful for scanning polar glucuronides, sulphate-bound substances that are difficult to extract, substances that can degrade by enzymatic or chemical interactions, and compounds with low stability that need to be analyzed quickly. For instance, diuretics have different physicochemical properties, complicating extraction procedures.[9] Therefore, some diuretics are susceptible to degradation[24] and may be hydrolyzed in solution.[4] Some substances contain easily exchangeable proton groups, such as opiates and stimulants, so that efficient ionization can be achieved and they can be suitable for analysis by the DS method.[9] Additionally, doping agents with a basic nitrogen group, such as narcotic substances are easily ionized. Therefore, these aforementioned compounds were previously detected using the DS methods.[15,25,26] In some studies, substances determined by IFHA as feed contaminants, substances with IRL values and substances with screening limits were analyzed by direct injection technique (such as salicylic acid,[27] AICAR,[14] 3-methoxytyramine,[28] and hydrocortisone[29]). However, there is a continuing need for an accelerated process for the analysis of large numbers of urine/plasma samples.[6]

In this study, more than 200 performance altering substances were simultaneously screened in urine and plasma for the first time using a sensitive mass spectrometer equipped with Orbitrap technology. This method permits the detection of substances in a short time (13 min) with a single analysis without the need for complex sample preparation. Following the multi-screening method specifications of 2002/657/EC, selectivity, specificity, ruggedness, carry-over, and matrix effects were investigated. The screening protocol was applied to retrospective analysis, real positive samples and hundreds of authentic post-race samples.

Experimental section

Chemicals and reagents

Acetonitrile (ACN) (LC-MS grade) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Methanol (MeOH) (LC-MS grade) was obtained from Carlo Erba (Emmendingen, Germany). Ammonium bicarbonate (LC-MS) was purchased from Honeywell, (Charlotte, North Carolina, USA). Ammonium hydroxide solution (25–30% NH_3 basis) was obtained from Sigma Aldrich (St Louis, Missouri, USA). Deionized water was obtained from a water purification system (Elga-Veolia Water Solutions & Technologies, UK). All the reagents were of analytical grade.

Materials

The reference standards used were obtained from various pharmaceutical companies and summarized in Table 1 according to the supplier: (1) LGC (Middlesex, UK), (2) EDQM (Strasbourg, France), (3) Santa Cruz, (4) USP (MD, USA), (5) Cayman (Michigan, USA), (6) Dr Ehrenstorfer (LGC) (Middlesex UK), (7) TRC (Toronto, Canada), (8) CHIRON AS (Trondheim, Norway), (9) Sigma (Schnelldorf, Germany), (10) TCI (Merseyside, UK), (11) Clearsynth (Hyderabad, INDIA), (12) HPC (Cunnersdorf, Germany), (13) Lipomed (Cambridge, MA), (14) Carbosynth (UK), (15) Steraloids (Newport, USA), (16) LKT (Minnesota, USA), (17) Witega (Berlin, Germany), (18) Deva Pharma (Istanbul, Türkiye), (19) HIKAL (Navi Mumbai, India), (20) MCE, (Sollentuna, Sweden), (21) Spoluka Chemical (Kyiv, Ukraine) (22) The following substances were kindly provided by the Veterinary Control Central Research Institute (Ankara, Türkiye). All reference standards were of pharmaceutical grade.

Microcentrifuge tubes were purchased from VWR (Leicestershire, England), the vortex mixer was purchased from QLAB MVM25 (Istanbul, Türkiye), and the ultracentrifuge was purchased from Beckman Coulter Allegra X-30R (Brea, California, USA).

Instrumentation

Analysis was carried out using a Dionex UltiMate3000 UHPLC coupled with a Thermo Scientific Q-Exactive Orbitrap MS (Thermo Scientific, Bremen, Germany) equipped with a heated electrospray ionization (HESI-II) source operated in the positive–negative polarity switching mode. The liquid chromatography system consisted of a degasser, an Ultimate 3000 RS pump, an autosampler, thermostated at 10 °C and a heated column compartment. The separation of analytes was carried out on a Waters (USA) XBridge® C18 column (2.1 mm, 150 mm, 3.5 µm 80 Å particle size). The temperature of the column oven was 55 °C. The mobile phases were 5 mM ammonium bicarbonate (pH 9) in deionized water (A) and MeOH (B). The gradient program was run with 97% solvent A at initial conditions (t = 0–3 min), decreased to 5% solvent A at t = 6.5 min and held there for up to 10.0 min. Then, the gradient was returned to 97% solvent A at t = 11.5 min and stabilized to t = 13 min. The flow rate was kept constant at 0.3 mL

Table 1 The compound names, therapeutic class, chemical formula, exact mass (m/z), polarity, normalized collision energy, retention time, internal standards, suppliers of the targets and screening validation results

	Compound name	IFHA class name	Therapeutic class	Chemical formula	Precursor ion	Measured mass of precursor ion (m/z)	RT (min)	Collision energy	Internal standard	Urine CCβ (ng mL^{-1})≤	Plasma CCβ (ng mL^{-1})≤	Matrix effect (urine)	Matrix effect (plasma)	Manufacturer group
1	2-(1-Hydroxyethyl) promazine sulfoxide	ISL	Tranquiliser	$C_{19}H_{24}N_2O_2S$	$[M + H]^+$	345.16313	9.32		Albuterol-D9	5	10	−6.1	37.9	1
2	3-Chlorophenylpiperazine		Psychoactive	$C_{10}H_{13}N_2Cl$	$[M + H]^+$	197.084	9.59	50	Albuterol-D9	1	5	−0.4	−76.8	8
3	3-Hydroxy detomidine	ISL	Sympatholytic	$C_{12}H_{14}N_2O$	$[M + H]^+$	203.11789	8.53		Albuterol-D9	5	5	−18.0	−71.0	7
4	3′-Hydroxy lidocaine	ISL	Local anaesthetic	$C_{14}H_{22}N_2O_2$	$[M + H]^+$	251.1754	9.49	80	3-Hydroxy lidocaine-D5	1	1	−7.4	−16.0	7
5	3-Hydroxy mepivacaine	ISL	Local anaesthetic	$C_{15}H_{22}N_2O_2$	$[M + H]^+$	263.1754	9.07		3-Hydroxy mepivacaine-D3	1	1	0.5	−2.8	7
6	3′-Hydroxy ropivacaine		Local anaesthetic	$C_{17}H_{26}N_2O_2$	$[M + H]^+$	291.2067	9.59		Albuterol-D9	1	1	2.4	−1.5	7
7	3-Methoxytyramine	TL (urine)	Stimulant	$C_9H_{13}NO_2$	$[M + H]^+$	168.10191	7.41		Albuterol-D9	10	10	12.2	−84.0	8
8	4-Fluorophenethylamine		Psychedelic	$C_8H_{10}FN$	$[M + H]^+$	140.087	8.96		Albuterol-D9	1	5	1.2	−29.4	9
9	4-Methylaminoantipyrine	ISL (urine)	NSAID	$C_{12}H_{15}N_3O$	$[M + H]^+$	218.12879	8.68	50	4 MAA-D3	10	10	−0.9	−98.1	7
10	5′-Hydroxy dantrolene	ISL	Muscle relaxant	$C_{14}H_{10}N_4O_6$	$[M + H]^+$	331.06731	8.56		Albuterol-D9	25	200	−28.9	9.8	7
11	5′-Hydroxy omeprazole		Proton-pump inhibitor	$C_{17}H_{19}N_3O_4S$	$[M + H]^+$	362.1169	8.70		Albuterol-D9	25	5	−17.3	1.3	7
12	5′-Hydroxy piroxicam		NSAID	$C_{15}H_{13}N_3O_5S$	$[M + H]^+$	348.06487	8.11		Albuterol-D9	25	25	−33.5	−38.4	9
13	7-(β-Hydroxyethyl) theophylline		Phosphodiesterase inhibitor	$C_9H_{12}N_4O_3$	$[M + H]^+$	225.09822	7.70	50	Caffeine-D3	10	1	5.0	10.7	5
14	7-Amino nitrazepam		Tranquilizer	$C_{15}H_{13}N_3O$	$[M + H]^+$	252.11314	8.40		Albuterol-D9	5	1	−32.5	−63.1	8
15	7-Aminoflunitrazepam		Tranquilizer	$C_{16}H_{14}FN_3O$	$[M + H]^+$	284.11937	8.61	50	Albuterol-D9	5	1	−23.2	−64.1	8
16	Acetopromazine		Psychotropic	$C_{19}H_{22}N_2OS$	$[M + H]^+$	327.15256	10.37		Albuterol-D9	1	1	4.9	−37.3	6
17	Adrenochrome semicarbazone		Hemostatic	$C_{10}H_{12}N_4O_3$	$[M + H]^+$	237.09822	7.49		Albuterol-D9	100	25	−26.9	15.1	9
18	AICAR		AMPK activator	$C_9H_{14}N_4O_5$	$[M + H]^+$	259.1037	1.34		Albuterol-D9	5000	625	−99.9	−88.1	9
19	Ajmaline		Psychotropic	$C_{20}H_{26}N_2O_2$	$[M + H]^+$	327.2067	9.41		Albuterol-D9	1	1	0.2	−23.8	8
20	Alfentanyl		Opioid	$C_{21}H_{32}N_6O_3$	$[M + H]^+$	417.26087	9.81		Albuterol-D9	5	5	−2.6	−60.1	8
21	Alimemazine		Psychotropic	$C_{18}H_{22}N_2S$	$[M + H]^+$	299.15765	10.90		Albuterol-D9	1	1	12.5	−57.9	8
22	Alminoprofen		NSAID	$C_{13}H_{17}NO_2$	$[M + H]^+$	220.13321	8.50		Albuterol-D9	10	1	−27.6	−34.8	11
23	Alpha methylfentanyl		Opioid analgesic	$C_{23}H_{30}N_2O$	$[M + H]^+$	351.24309	10.44		Albuterol-D9	1	1	2.7	−57.5	8
24	Altrenogest		Progestogen	$C_{21}H_{26}O_2$	$[M + H]^+$	311.20056	11.04		Albuterol-D9	25	1	38.7	−6.6	9
25	Ambroxol	ISL (urine)	Mucolytic	$C_{13}H_{18}Br_2N_2O$	$[M + H]^+$	376.98586	9.97	70	Albuterol-D9	20	20	−50.3	−52.2	8
26	Amiloride		Diuretic	$C_6H_8ClN_7O$	$[M + H]^+$	230.05516	7.33		Albuterol-D9	25	1	−18.1	−16.7	2
27	Amitriptyline		Antidepressant	$C_{20}H_{23}N$	$[M + H]^+$	278.19033	10.94		Albuterol-D9	1	1	9.3	−12.1	9
28	Amoxapine		Antidepressant	$C_{17}H_{16}ClN_3O$	$[M + H]^+$	314.10547	10.16		Albuterol-D9	1	5	−3.0	−55.6	8
29	Antipyrine		NSAID	$C_{11}H_{12}N_2O$	$[M + H]^+$	189.10224	8.37		Albuterol-D9	25	1	−16.8	−18.3	9
30	Atenolol		β-Blocker	$C_{14}H_{22}N_2O_3$	$[M + H]^+$	267.17032	8.37	65	Albuterol-D9	10	1	−14.9	−8.8	7
31	Atorvastatin		Lipid-lowering	$C_{33}H_{35}FN_2O_5$	$[M + H]^+$	559.26028	9.31		Albuterol-D9	25	5	−13.5	0.2	11
32	Atropine	IRL (urine)	*m*-R blocker	$C_{17}H_{23}NO_3$	$[M + H]^+$	290.17507	9.28		Atropine-D3	6	6	3.1	−9.5	8
33	Azaperone		Psychotropic	$C_{19}H_{22}FN_3O$	$[M + H]^+$	328.18197	9.84		Albuterol-D9	1	1	−2.1	−12.0	6
34	Baclofen		Muscle relaxant	$C_{10}H_{12}ClNO_2$	$[M + H]^+$	214.06293	7.74		Albuterol-D9	25	5	−34.1	9.3	12
35	Bamifylline		Stimulant	$C_{20}H_{27}N_5O_3$	$[M + H]^+$	386.21867	9.52		Albuterol-D9	5	1	−7.1	−5.6	7
36	Benazepril		ACE inhibitor	$C_{24}H_{28}N_2O_5$	$[M + H]^+$	425.2071	9.13		Albuterol-D9	5	1	−5.5	−55.5	8
37	Benazeprilat		ACE inhibitor	$C_{22}H_{24}N_2O_5$	$[M + H]^+$	397.1758	8.02		Albuterol-D9	50	1	−26.6	−9.9	7
38	Bendroflumethiazide		Diuretic	$C_{15}H_{14}F_3N_3O_4S_2$	$[M - H]^-$	420.03051	8.86		Albuterol-D9	50	10	−21.3	30.5	2

Table 1 *(Contd.)*

	Compound name	IFHA class name	Therapeutic class	Chemical formula	Precursor ion	Measured mass of precursor ion (*m*/*z*)	RT (min)	Collision energy	Internal standard	Urine CCβ (ng mL^{-1})≤	Plasma CCβ (ng mL^{-1})≤	Matrix effect (urine)	Matrix effect (plasma)	Manufacturer group
39	Benzoylecgonine		Stimulant	$C_{16}H_{19}NO_4$	$[M + H]^+$	290.13868	8.23		Albuterol-D9	5	1	−24.3	−2.4	13
40	Benztropine		Antihistamine	$C_{21}H_{25}NO$	$[M + H]^+$	308.20089	11.44	80	Albuterol-D9	5	5	1.7	−60.5	22
41	Benzydamine		NSAID	$C_{19}H_{23}N_3O$	$[M + H]^+$	310.19139	10.37		Albuterol-D9	1	1	6.2	11.7	3
42	Beta-hydroxyfentanyl		Opioid analgesic	$C_{22}H_{28}N_2O_2$	$[M + H]^+$	353.22235	9.84		Albuterol-D9	1	1	−9.7	−57.5	8
43	Boldenone[a]	TL (urine)	Anabolic steroide	$C_{19}H_{26}O_2$	$[M + H]^+$	287.20056	9.48	70	Albuterol-D9	7.5	7.5	−92.2	−28.3	7
44	Bromhexine	ISL (urine)	Mucolytic	$C_{14}H_{20}Br_2N_2$	$[M + H]^+$	375.0066	12.12	50	Albuterol-D9	2000	2000	28.6	31.8	22
45	Budesonide		Glucocorticoid	$C_{25}H_{34}O_6$	$[M + H]^+$	431.24282	9.72		Albuterol-D9	5	25	8.6	−34.9	9
46	Buflomedil		Vasoactive agent	$C_{17}H_{25}NO_4$	$[M + H]^+$	308.18563	9.50		Albuterol-D9	1	1	−1.0	−5.4	9
47	Bufotenine	IRL (urine)	Psychodisleptic	$C_{12}H_{16}N_2O$	$[M + H]^+$	205.13354	8.15		Bufotenine-D4	100	100	51.2	−94.0	18
48	Bumetanide		Diuretic	$C_{17}H_{20}N_2O_5S$	$[M + H]^+$	365.11657	8.76		Albuterol-D9	25	5	−15.1	−6.2	9
49	Bupivacaine		Local anesthetic	$C_{18}H_{28}N_2O$	$[M + H]^+$	289.22744	10.22	60	Albuterol-D9	1	1	4.0	−10.1	4
50	Buprenorphine		Opioid	$C_{29}H_{41}NO_4$	$[M + H]^+$	468.31084	11.23	60	Albuterol-D9	1	1	5.1	−96.6	13
51	Buspirone		Psychotropic	$C_{21}H_{31}N_5O_2$	$[M + H]^+$	386.25505	9.81		Albuterol-D9	1	1	−1.2	−0.4	22
52	Butorphanol	ISL	Opioid	$C_{21}H_{29}NO_2$	$[M + H]^+$	328.22711	10.60		Albuterol-D9	1	1	3.7	−11.7	8
53	Caffeine	IRL	Stimulant	$C_8H_{10}N_4O_2$	$[M + H]^+$	195.08765	7.90	70	Caffeine-D3	25	25	−2.3	−11.8	5
54	Cannabidiol		Psychodisleptic	$C_{21}H_{30}O_2$	$[M + H]^+$	315.23186	10.59		Albuterol-D9	50	5	42.4	−94.1	8
55	Capsaicin		Topical analgesic	$C_{18}H_{27}NO_3$	$[M + H]^+$	306.20637	9.84		Albuterol-D9	1	1	0.4	−35.2	9
56	Caramiphen		Parasympatholytic	$C_{18}H_{27}NO_2$	$[M + H]^+$	290.21146	10.70		Albuterol-D9	1	1	4.4	−5.8	18
57	Carbetapentane		Antitussive	$C_{20}H_{31}NO_3$	$[M + H]^+$	334.23767	10.73		Albuterol-D9	1	1	1.3	5.2	9
58	Carteolol		β-Blocker	$C_{16}H_{24}N_2O_3$	$[M + H]^+$	293.18597	9.01		Albuterol-D9	5	5	−5.9	−8.2	4
59	Carvedilol		β-Blocker	$C_{24}H_{26}N_2O_4$	$[M + H]^+$	407.19653	9.67		Albuterol-D9	1	1	−7.5	−21.4	4
60	Celecoxib		NSAID	$C_{17}H_{14}F_3N_3O_2S$	$[M + H]^+$	382.08316	9.87		Albuterol-D9	100	5	−10.7	−19.5	9
61	Cetirizine		Antihistamine	$C_{21}H_{25}ClN_2O_3$	$[M + H]^+$	389.16265	9.49		Albuterol-D9	10	1	−31.6	0.9	9
62	Chincochaine		Local anesthetic	$C_{20}H_{29}N_3O_2$	$[M + H]^+$	344.23325	10.41		Albuterol-D9	1	1	1.3	3.5	22
63	Chlorcyclizine		Antihistamine	$C_{18}H_{21}ClN_2$	$[M + H]^+$	301.1466	10.70		Albuterol-D9	1	1	2.5	−2.6	9
64	Chlorpheniramine		Antihistamine	$C_{16}H_{19}ClN_2$	$[M + H]^+$	275.13095	10.02		Albuterol-D9	1	1	1.2	−23.0	18
65	Chlorpromazine		Psychotropic	$C_{17}H_{19}ClN_2S$	$[M + H]^+$	319.10302	11.03	50	Albuterol-D9	1	5	12.8	−76.9	6
66	Cilazapril		ACE inhibitor	$C_{22}H_{31}N_3O_5$	$[M + H]^+$	418.23365	9.18		Albuterol-D9	1	1	−5.0	4.8	22
67	Cilazaprilat		ACE inhibitor	$C_{20}H_{27}N_3O_5$	$[M + H]^+$	390.20235	8.06		Albuterol-D9	1	1	−29.8	−13.8	7
68	Cimaterol		β-Agonist	$C_{12}H_{17}N_3O$	$[M + H]^+$	220.14444	8.44		Albuterol-D9	10	10	−6.3	−18.8	7
69	Cimbuterol		β-Agonist	$C_{13}H_{19}N_3O$	$[M + H]^+$	234.16009	8.75		Albuterol-D9	50	25	−5.1	−2.3	7
70	Cimetidine		Antiulcer	$C_{10}H_{16}N_6S$	$[M + H]^+$	253.12299	8.12		Albuterol-D9	50	1	−26.8	−15.5	2
71	Clenbuterol	ISL (urine)	β-Agonist	$C_{12}H_{18}Cl_2N_2O$	$[M + H]^+$	277.0869	9.71		Clenbuterol-D9	5	10	2.1	−2.1	1
72	Clencyclohexerol		β-Agonist	$C_{14}H_{20}Cl_2N_2O_2$	$[M + H]^+$	319.09746	8.87		Albuterol-D9	50	5	−2.4	−13.3	17
73	Clenpenterol		β-Agonist	$C_{13}H_{20}Cl_2N_2O$	$[M + H]^+$	291.10255	9.98		Albuterol-D9	1	1	4.6	8.1	17
74	Clenproperol		β-Agonist	$C_{11}H_{16}N_2OCl_2$	$[M + H]^+$	263.07125	9.45		Clenbuterol-D9	10	10	−0.5	−16.2	17
75	Clomipramine		Antidepressant	$C_{19}H_{23}ClN_2$	$[M + H]^+$	315.16225	11.14		Albuterol-D9	5	5	2.7	−77.3	18
76	Clonidine		α-Agonist	$C_9H_9Cl_2N_3$	$[M + H]^+$	230.02463	8.84	70	Albuterol-D9	1	1	4.0	−11.7	1
77	Cyamemazine		Antipsychotic	$C_{19}H_{21}N_3S$	$[M + H]^+$	324.15289	10.57	60	Albuterol-D9	1	1	4.3	−26.8	7
78	Danazol		Androgen receptor	$C_{22}H_{27}NO_2$	$[M + H]^+$	338.21146	10.09	40	Albuterol-D9	1	5	6.4	−20.8	4
79	Dantrolene	ISL	Striated muscle relaxant	$C_{14}H_{10}N_4O_5$	$[M - H]^-$	313.05784	8.72		Albuterol-D9	ND	200	ND	62.3	9
80	Delta-9-tetrahydrocannabinol		Psychodisleptic	$C_{21}H_{30}O_2$	$[M + H]^+$	315.23186	11.24		Albuterol-D9	25	25	−13.5	−86.8	13
81	Dembrexine	ISL	Mucolytic	$C_{13}H_{17}Br_2NO_2$	$[M + H]^+$	377.96988	9.28	50	Albuterol-D9	10	10	0.8	−27.4	2
82	Desipramine		Antidepressant	$C_{18}H_{22}N_2$	$[M + H]^+$	267.18558	11.49		Albuterol-D9	5	5	8.0	−41.8	8
83	Desmethyltramadol		Opioid analgesic	$C_{15}H_{23}NO_2$	$[M + H]^+$	250.18016	9.29		Albuterol-D9	1	1	−2.9	−21.9	8
84	Dextrorphan		Opioid	$C_{17}H_{23}NO$	$[M + H]^+$	258.18524	9.67		Albuterol-D9	1	1	2.9	−18.4	8

Table 1 *(Contd.)*

	Compound name	IFHA class name	Therapeutic class	Chemical formula	Precursor ion	Measured mass of precursor ion (*m*/*z*)	RT (min)	Collision energy	Internal standard	Urine CCβ (ng mL^{-1})≤	Plasma CCβ (ng mL^{-1})≤	Matrix effect (urine)	Matrix effect (plasma)	Manufacturer group
85	Diazepam		Psycotropic	$C_{16}H_{13}ClN_2O$	$[M + H]^+$	285.07892	9.78		Albuterol-D9	25	25	10.3	−10.8	13
86	Diclofenac	ISL (urine)	NSAID	$C_{14}H_{11}Cl_2NO_2$	$[M + H]^+$	296.02396	9.19		Albuterol-D9	5	5	−19.2	−11.6	8
87	Diflunisal		NSAID	$C_{13}H_8F_2O_3$	$[M - H]^-$	249.03687	8.88	70	Albuterol-D9	5	1	23.1	41.1	9
88	Dihydrocapsaicin		Topical analgesic	$C_{18}H_{29}NO_3$	$[M + H]^+$	308.22202	10.03		Albuterol-D9	5	5	−8.3	−65.6	7
89	Dimethyl sulfoxide	IRL	Pain reliever	C_2H_6OS	$[M + H]^+$	79.02121	1.39		Albuterol-D9	1.5	1.5	−32.1	−14.5	22
90	Diphenhydramine		Antihistamine	$C_{17}H_{21}NO$	$[M + H]^+$	256.16959	10.25		Albuterol-D9	1	1	8.1	1.1	6
91	Diprenorphine		Opioid antagonist	$C_{26}H_{35}NO_4$	$[M + H]^+$	426.26389	10.64		Albuterol-D9	1	1	2.0	−93.8	22
92	Domperidone		Antiemetic	$C_{22}H_{24}ClN_5O_2$	$[M + H]^+$	426.16913	9.71	60	Albuterol-D9	5	5	−5.8	−15.6	8
93	Droperidol		Antiemetic	$C_{22}H_{22}FN_3O_2$	$[M + H]^+$	380.17688	9.80		Albuterol-D9	10	1	−5.3	−8.9	8
94	Dyphylline		Bronchodilator	$C_{10}H_{14}N_4O_4$	$[M + H]^+$	255.10878	7.57		Albuterol-D9	100	5	41.8	3.3	9
95	Ecgonine methyl ester		Stimulant	$C_{10}H_{17}NO_3$	$[M + H]^+$	200.12812	8.02	60	Albuterol-D9	ND	10	ND	−30.1	8
96	Edrophonium		ACE inhibitor	$C_{10}H_{16}NO$	$[M + H]^+$	166.12264	2.22		Albuterol-D9	ND	100	ND	−41.3	3
97	Eltenac	ISL (urine)	NSAID	$C_{12}H_9Cl_2NO_2S$	$[M + H]^+$	301.98038	9.10		Albuterol-D9	12.5	2.5	−33.2	−83.1	7
98	EMDP		Opioid	$C_{19}H_{21}N$	$[M + H]^+$	264.17468	10.61	50	Albuterol-D9	1	5	18.2	−58.8	8
99	Enalapril		ACE inhibitor	$C_{20}H_{28}N_2O_5$	$[M + H]^+$	377.2071	8.88		Albuterol-D9	1	1	−10.5	−2.1	9
100	Enalaprilat		ACE inhibitor	$C_{18}H_{24}N_2O_5$	$[M + H]^+$	349.1758	4.00		Albuterol-D9	ND	50	ND	−52.6	9
101	Etamiphylline		Bronchodilator	$C_{13}H_{21}N_5O_2$	$[M + H]^+$	280.1768	9.22		Albuterol-D9	5	1	−1.9	−8.5	21
102	Ethacrynic acid		Diuretic	$C_{13}H_{12}Cl_2O_4$	$[M + H]^+$	303.01854	8.96		Albuterol-D9	ND	50	ND	12.5	3
103	Ethyl glucuronide		Metabolite of ethanol	$C_8H_{14}O_7$	$[M - H]^-$	221.06668	1.25		Albuterol-D9	100	25	−97.0	126.0	7
104	Etilefrine		Stimulant	$C_{10}H_{15}NO_2$	$[M + H]^+$	182.11756	7.94		Albuterol-D9	25	5	−18.4	−31.8	7
105	Etoricoxib		NSAID	$C_{18}H_{15}ClN_2O_2S$	$[M + H]^+$	359.06155	9.34	60	Albuterol-D9	5	5	−2.6	4.5	9
106	Fadrazole		Aromatase inhibitor	$C_{14}H_{13}N_3$	$[M + H]^+$	224.11822	9.02		Albuterol-D9	1	1	−7.2	−7.1	7
107	Famprofazone		Stimulant	$C_{24}H_{31}N_3O$	$[M + H]^+$	378.25399	10.57		Albuterol-D9	1	1	8.9	5.4	1
108	Fenspiride		Antitussive	$C_{15}H_{20}N_2O_2$	$[M + H]^+$	261.15975	9.26		Albuterol-D9	5	1	−10.3	−1.9	9
109	Fexofenadine		Antihistamine	$C_{32}H_{39}NO_4$	$[M + H]^+$	502.29519	9.37		Albuterol-D9	1	10	6.6	−1.1	18
110	Flunitrazepam		Psychotropic	$C_{16}H_{12}FN_3O_3$	$[M + H]^+$	314.09355	9.31		Albuterol-D9	10	25	−9.6	−14.7	7
111	Flunixin	ISL	NSAID	$C_{14}H_{11}F_3N_2O_2$	$[M + H]^+$	297.08454	9.06		Albuterol-D9	10	50	−6.6	−32.3	9
112	Flupentixol		Psychotropic	$C_{23}H_{25}F_3N_2OS$	$[M + H]^+$	435.17125	10.64		Albuterol-D9	1	1	−0.7	−12.2	2
113	Fluphenazine		Psychotropic	$C_{22}H_{26}F_3N_3OS$	$[M + H]^+$	438.18214	10.53		Albuterol-D9	1	1	5.4	−29.5	16
114	Fluspirilene		Psychotropic	$C_{29}H_{31}F_2N_3O$	$[M + H]^+$	476.2508	10.67		Albuterol-D9	10	10	9.1	−97.5	9
115	Fluvoxamine		Antidepressant	$C_{15}H_{21}F_3N_2O_2$	$[M + H]^+$	319.16279	10.22		Albuterol-D9	1	1	9.2	−49.3	8
116	Formoterol		β-Agonist	$C_{19}H_{24}N_2O_4$	$[M + H]^+$	345.18088	9.08		Albuterol-D9	5	5	−9.1	−16.3	2
117	Furosemide	ISL	Diuretic	$C_{12}H_{11}ClN_2O_5S$	$[M - H]^-$	329.00044	8.21		Furosemide-D5	25	25	−28.6	67.5	2
118	Gabapentin		Antiepileptic	$C_9H_{17}NO_2$	$[M + H]^+$	172.13321	6.95		Albuterol-D9	200	100	−7.6	5.6	19
119	Guaifenesin		Expectorant	$C_{10}H_{14}O_4$	$[M + H]^+$	199.09649	8.56		Albuterol-D9	5	5	−20.2	−29.7	9
120	Halcinonide		Glucocorticoid	$C_{24}H_{32}ClFO_5$	$[M + H]^+$	455.19951	10.0		Albuterol-D9	5	10	−4.8	−35.2	7
121	Haloperidol		Psychotropic	$C_{21}H_{23}ClFNO_2$	$[M + H]^+$	376.14741	10.02		Albuterol-D9	1	1	−7.6	−16.9	8
122	Hordenine	IRL (urine)	Sympathomimetic	$C_{10}H_{15}NO$	$[M + H]^+$	166.12264	8.60	60	Albuterol-D9	10	10	−3.6	−41.4	8
123	Hydrocortisone[a]	IT (urine)	Glucocorticoid	$C_{21}H_{30}O_5$	$[M + H]^+$	363.2166	9.22		Albuterol-D9	5	5	−16.4	−24.2	6
124	Hydroxy homosildenafil		Phosphodiesterase inhibitor	$C_{23}H_{32}N_6O_5S$	$[M + H]^+$	505.22277	9.45		Albuterol-D9	5	1	−6.8	18.4	7
125	Hydroxyflutamide		Nonsteroidal antiandrogen	$C_{11}H_{11}F_3N_2O_4$	$[M - H]^-$	291.05981	9.46		Albuterol-D9	1	5	6.3	6.0	7
126	Hydroxyzine		Antihistamine	$C_{21}H_{27}ClN_2O_2$	$[M + H]^+$	375.18338	10.34		Albuterol-D9	1	1	2.1	−56.2	1
127	Imidapril		ACE inhibitor	$C_{20}H_{27}N_3O_6$	$[M + H]^+$	406.19726	8.87		Albuterol-D9	5	1	−12.3	−15.0	7
128	Imipramine		Antidepressant	$C_{19}H_{24}N_2$	$[M + H]^+$	281.20123	10.83		Albuterol-D9	5	5	7.0	−15.4	13

Table 1 *(Contd.)*

	Compound name	IFHA class name	Therapeutic class	Chemical formula	Precursor ion	Measured mass of precursor ion (*m*/*z*)	RT (min)	Collision energy	Internal standard	Urine CCβ (ng mL^{-1})≤	Plasma CCβ (ng mL^{-1})≤	Matrix effect (urine)	Matrix effect (plasma)	Manufacturer group
129	Indoprofen		NSAID	$C_{17}H_{15}NO_3$	$[M + H]^+$	282.11247	8.52	60	Albuterol-D9	25	1	−12.9	−9.9	9
130	Isothipendyl		Antihistamine	$C_{16}H_{19}N_3S$	$[M + H]^+$	286.13724	10.44		Albuterol-D9	1	5	5.6	−30.6	8
131	Isoxicam		NSAID	$C_{14}H_{13}N_3O_5S$	$[M + H]^+$	336.06487	8.34		Albuterol-D9	25	5	−27.6	−25.7	9
132	Isoxsuprine		β-Agonist	$C_{18}H_{23}NO_3$	$[M + H]^+$	302.17507	9.54		Albuterol-D9	25	5	−2.4	−40.1	9
133	Ketamine		Anesthetic	$C_{13}H_{16}ClNO$	$[M + H]^+$	238.09932	9.58		Albuterol-D9	25	25	3.8	−21.6	7
134	Ketoprofen	ISL	NSAID	$C_{16}H_{14}O_3$	$[M + H]^+$	255.10157	8.71		Ketoprofen-D3	10	10	−7.6	−23.9	8
135	Lamotrigine		Antiepileptic	$C_9H_7Cl_2N_5$	$[M + H]^+$	256.01513	8.65		Albuterol-D9	10	25	−17.1	−9.5	6
136	Levomepromazine		Psychotropic	$C_{19}H_{24}N_2OS$	$[M + H]^+$	329.16821	10.85		Albuterol-D9	1	1	4.2	−65.0	1
137	Lidocaine		Local anesthetic	$C_{14}H_{22}N_2O$	$[M + H]^+$	235.18049	9.89	70	3-Hydroxy lidocaine-D5	1	1	1.9	−21.9	6
138	Loratadine		Antihistamine	$C_{22}H_{23}ClN_2O_2$	$[M + H]^+$	383.15208	10.40		Albuterol-D9	5	1	11.3	9.8	7
139	Mabuterol		β-Agonist	$C_{13}H_{18}ClF_3N_2O$	$[M + H]^+$	311.11325	9.84		Albuterol-D9	1	1	4.4	−1.6	17
140	Mapenterol		β-Agonist	$C_{14}H_{20}ClF_3N_2O$	$[M + H]^+$	325.1289	10.06		Albuterol-D9	1	5	6.4	8.1	17
141	Meclofenamic acid	ISL	NSAID	$C_{14}H_{11}Cl_2NO_2$	$[M - H]^-$	294.00941	9.20		Albuterol-D9	125	625	−10.4	−6.1	4
142	Medetomidine		Sedative	$C_{13}H_{16}N_2$	$[M + H]^+$	201.13863	9.70	100	Albuterol-D9	1	1	3.9	−19.9	8
143	Meldonium		Anticonvulsant	$C_6H_{14}N_2O_2$	$[M + H]^+$	147.1128	1.20	50	Albuterol-D9	25	25	−66.3	−4.8	20
144	Meloxicam	ISL	NSAID	$C_{14}H_{13}N_3O_4S_2$	$[M + H]^+$	352.04202	8.56		Meloxicam-D3	25	1	−16.6	1.3	8
145	Memantine		NMDA receptor antagonist	$C_{12}H_{21}N$	$[M + H]^+$	180.17468	11.01	60	Albuterol-D9	25	50	62.4	10.8	18
146	Mephentermine		Stimulant	$C_{11}H_{17}N$	$[M + H]^+$	164.14338	9.71		Albuterol-D9	5	25	2.3	−54.8	22
147	Mepivacaine	ISL	Local anaesthetic	$C_{15}H_{22}N_2O$	$[M + H]^+$	247.18049	9.54	60	3-Hydroxy mepivacaine-D3	10	1	−2.3	−20.7	2
148	Methadone		Opioid	$C_{21}H_{27}NO$	$[M + H]^+$	310.21654	10.91		Albuterol-D9	1	1	−0.9	−60.9	8
149	Methapyrilene		Antihistamine	$C_{14}H_{19}N_3S$	$[M + H]^+$	262.13724	9.89		Albuterol-D9	1	1	2.0	−24.2	6
150	Methocarbamol	ASL (urine)	Striated muscle relaxant	$C_{11}H_{15}NO_5$	$[M + H]^+$	242.1023	8.55		Albuterol-D9	100	10	−23.4	−30.8	10
151	Metolazone		Diuretic	$C_{16}H_{16}ClN_3O_3S$	$[M + H]^+$	366.06737	8.81		Albuterol-D9	25	5	−14.9	−35.8	2
152	Moexipril		Antihypertensive	$C_{27}H_{34}N_2O_7$	$[M + H]^+$	499.24388	9.08		Albuterol-D9	5	5	−8.6	−0.7	7
153	Morphine	IRL (urine)	Opioid	$C_{17}H_{19}NO_3$	$[M + H]^+$	286.14377	8.76		Morphine-D3	15	3	7.6	−10.3	13
154	Morphine-3-glucuronide		Opioid	$C_{23}H_{27}NO_9$	$[M + H]^+$	462.17586	7.60	70	Morphine-D3	150	3	−37.6	−15.2	8
155	Morphine-6-glucuronide		Opioid	$C_{23}H_{27}NO_9$	$[M + H]^+$	462.17586	8.05	70	Morphine-D3	150	3	−51.5	−60.8	8
156	Nalorphine		Opioid	$C_{19}H_{21}NO_3$	$[M + H]^+$	312.15942	9.41		Albuterol-D9	10	10	4.2	−57.9	8
157	Naproxen	ISL (urine)	NSAID	$C_{14}H_{14}O_3$	$[M + H]^+$	231.10157	8.65		Albuterol-D9	250	10	−24.5	−26.6	2
158	*N*-Desmethylvenlafaxine		Antidepressant	$C_{16}H_{25}NO_2$	$[M + H]^+$	264.19581	9.49		Albuterol-D9	1	1	7.4	−37.2	22
159	Nebivolol		β-Blocker	$C_{22}H_{25}F_2NO_4$	$[M + H]^+$	406.18244	10.13	60	Albuterol-D9	1	1	−0.5	−33.7	18
160	Niflumic acid		NSAID	$C_{13}H_9F_3N_2O_2$	$[M - H]^-$	281.05434	9.06		Albuterol-D9	5	1	−7.6	−31.7	2
161	Nimesulid		NSAID	$C_{13}H_{12}N_2O_5S$	$[M + H]^-$	307.03942	8.77		Albuterol-D9	10	25	−18.4	26.0	2
162	Nitrazepam		Psychotropic	$C_{15}H_{11}N_3O_3$	$[M + H]^+$	282.08732	9.29		Albuterol-D9	25	5	−11.8	−63.4	8
163	*N*-*N*-Dimethyltryptamine	IRL (urine)	Psychodisleptic	$C_{12}H_{16}N_2$	$[M + H]^+$	189.13863	9.32		Albuterol-D9	2.5	2.5	−1.7	−71.1	8
164	Norfenfluramine		Stimulant	$C_{10}H_{12}F_3N$	$[M + H]^+$	204.09946	9.86		Albuterol-D9	5	5	5.5	−7.9	1
165	Norfentanyl		Opioid Analgesic	$C_{14}H_{20}N_2O$	$[M + H]^+$	233.16484	9.30		Albuterol-D9	1	1	−7.5	−62.9	8
166	Norketamine		NMDA receptor antagonist	$C_{12}H_{14}ClNO$	$[M + H]^+$	224.08367	9.34		Albuterol-D9	10	1	−0.3	−15.4	8
167	Normorphine		Opioid	$C_{16}H_{17}NO_3$	$[M + H]^+$	272.12812	7.65	60	Albuterol-D9	50	5	3.1	−19.1	8
168	Norpethidine		Opioid	$C_{14}H_{19}NO_2$	$[M + H]^+$	234.14886	9.81	40	Albuterol-D9	5	25	−1.8	−54.9	8

Table 1 *(Contd.)*

	Compound name	IFHA class name	Therapeutic class	Chemical formula	Precursor ion	Measured mass of precursor ion (*m*/*z*)	RT (min)	Collision energy	Internal standard	Urine CCβ (ng mL^{-1})≤	Plasma CCβ (ng mL^{-1})≤	Matrix effect (urine)	Matrix effect (plasma)	Manufacturer group
169	Nortriptyline		Antidepressant	$C_{19}H_{21}N$	$[M + H]^+$	264.17468	11.38		Albuterol-D9	1	5	10.4	−44.1	5
170	Oxazepam		Tranquilizer	$C_{15}H_{11}ClN_2O_2$	$[M + H]^+$	287.05818	9.48		Albuterol-D9	5	5	−10.3	−14.7	13
171	Oxprenolol		β-Blocker	$C_{15}H_{23}NO_3$	$[M + H]^+$	266.17507	9.81		Albuterol-D9	1	5	5.1	0.5	18
172	Pemoline		Stimulant	$C_9H_8N_2O_2$	$[M + H]^+$	177.06585	7.92		Albuterol-D9	25	5	−35.8	−14.5	13
173	Pentoxifylline		Vasodilator	$C_{13}H_{18}N_4O_3$	$[M + H]^+$	279.14517	8.56		Albuterol-D9	5	5	−16.0	−20.5	6
174	Perindopril		ACE inhibitor	$C_{19}H_{32}N_2O_5$	$[M + H]^+$	369.2384	8.99		Albuterol-D9	1	1	−1.7	5.9	8
175	Perindoprilat		ACE inhibitor	$C_{17}H_{28}N_2O_5$	$[M + H]^+$	341.2071	7.36		Albuterol-D9	100	5	−57.4	−33.1	7
176	Phenacetin		NSAID	$C_{10}H_{13}NO_2$	$[M + H]^+$	180.10194	8.83		Albuterol-D9	50	25	5.0	−9.7	6
177	Piroxicam		NSAID	$C_{15}H_{13}N_3O_4S$	$[M + H]^+$	332.06995	8.32		Albuterol-D9	200	10	−28.7	−19.2	2
178	Prazepam		Tranquilizer	$C_{19}H_{17}ClN_2O$	$[M + H]^+$	325.11022	10.13		Albuterol-D9	1	5	−3.3	−65.8	8
179	Prednisolone	IT (urine)	Glucocorticoid	$C_{21}H_{28}O_5$	$[M + H]^+$	361.20095	9.13		Albuterol-D9	50	50	−12.3	−30.5	6
180	Prednisone		Glucocorticoid	$C_{21}H_{26}O_5$	$[M + H]^+$	359.1853	8.99		Albuterol-D9	50	25	−45.5	−15.3	6
181	Pregabalin		Antiepileptic	$C_8H_{17}NO_2$	$[M + H]^+$	160.13321	5.15		Albuterol-D9	ND	50	ND	4.3	9
182	Probenecid		Diuretic	$C_{13}H_{19}NO_4S$	$[M + H]^+$	286.11076	8.70		Albuterol-D9	25	25	−12.6	−18.5	9
183	Procaine	ISL (plasma)	Local anesthetic	$C_{13}H_{20}N_2O_2$	$[M + H]^+$	237.15975	9.10	40	Albuterol-D9	10	10	−5.6	−51.3	6
184	Promazine		Psychotropic	$C_{17}H_{20}N_2S$	$[M + H]^+$	285.142	10.64		Albuterol-D9	1	1	8.2	−72.6	6
185	Propionylpromazine		Psychotropic	$C_{20}H_{24}N_2OS$	$[M + H]^+$	341.16821	10.64		Albuterol-D9	10	5	4.3	−50.5	6
186	Propranolol		β-Blocker	$C_{16}H_{21}NO_2$	$[M + H]^+$	260.16451	10.16		Albuterol-D9	1	1	3.1	8.3	6
187	Pyrilamine		Antihistamine	$C_{17}H_{23}N_3O$	$[M + H]^+$	286.19139	9.96		Albuterol-D9	1	5	6.4	−13.1	9
188	Quinapril		ACE inhibitor	$C_{25}H_{30}N_2O_5$	$[M + H]^+$	439.22275	9.19	40	Albuterol-D9	1	1	−9.8	−60.8	22
189	Quinaprilat		ACE inhibitor	$C_{23}H_{26}N_2O_5$	$[M + H]^+$	411.19145	8.20		Albuterol-D9	25	1	−23.8	−14.7	14
190	Quinidine		Antiarrhythmic	$C_{20}H_{24}N_2O_2$	$[M + H]^+$	325.19105	9.92		Albuterol-D9	1	1	−9.5	−62.0	22
191	Ramipril		ACE inhibitor	$C_{23}H_{32}N_2O_5$	$[M + H]^+$	417.2384	9.19		Albuterol-D9	1	1	−4.4	9.8	7
192	Ramiprilat		ACE inhibitor	$C_{21}H_{28}N_2O_5$	$[M + H]^+$	389.2071	8.05		Albuterol-D9	10	1	−24.9	−8.2	12
193	Ranitidine		H2-R blocker	$C_{13}H_{22}N_4O_3S$	$[M + H]^+$	315.14854	8.65		Albuterol-D9	100	25	0.4	−8.8	3
194	Ritalinic acid		Stimulant	$C_{13}H_{17}NO_2$	$[M + H]^+$	220.13321	8.29	60	Albuterol-D9	25	5	−27.3	−8.6	9
195	Romifidine	ISL (urine)	Sympatholytic	$C_9H_9BrFN_3$	$[M + H]^+$	258.00366	8.65		Albuterol-D9	5	25	−7.7	−20.9	8
196	Salbutamol	ISL (urine)	β-Agonist	$C_{13}H_{21}NO_3$	$[M + H]^+$	240.15942	8.36		Albuterol-D9	1	1	27.2	−19.2	9
197	Salicylic acid	IT	Analgesic	$C_7H_6O_3$	$[M - H]^-$	137.0244	2.99		Salicylic acid-D6	500	500	64.8	42.8	9
198	Scopolamine		*m*-R blocker	$C_{17}H_{21}NO_4$	$[M + H]^+$	304.15433	8.95		Albuterol-D9	6	6	−1.8	−64.2	9
199	Sertraline		Antidepressant	$C_{17}H_{17}Cl_2N$	$[M + H]^+$	306.08108	11.10		Albuterol-D9	5	5	6.9	−58.9	18
200	Sildenafil		Vasodilator	$C_{22}H_{30}N_6O_4S$	$[M + H]^+$	475.2122	9.65		Albuterol-D9	1	1	−3.3	32.9	18
201	Stanozolol		Anabolic steroide	$C_{21}H_{32}N_2O$	$[M + H]^+$	329.25874	10.06	80	Albuterol-D9	1	1	13.1	−13.7	15
202	Sufentanyl		Opioid	$C_{22}H_{30}N_2O_2S$	$[M + H]^+$	387.21008	10.47		Albuterol-D9	1	1	3.3	−56.7	22
203	Sulindac		NSAID	$C_{20}H_{17}FO_3S$	$[M + H]^+$	357.09552	8.61		Albuterol-D9	25	1	−22.6	−13.5	3
204	Temazepam		Psychotropic	$C_{16}H_{13}ClN_2O_2$	$[M + H]^+$	301.07383	9.58		Albuterol-D9	25	5	12.2	−1.5	13
205	Temocaprilat		ACE inhibitor	$C_{21}H_{24}N_2O_5S_2$	$[M + H]^+$	449.11994	8.17		Albuterol-D9	25	10	−1.3	1.4	7
206	Tenoxicam		NSAID	$C_{13}H_{11}N_3O_4S_2$	$[M + H]^+$	338.02637	8.23		Albuterol-D9	10	1	6.8	−13.0	2
207	Testosterone[a]	IT	Anabolic steroide	$C_{19}H_{28}O_2$	$[M + H]^+$	289.21621	9.65		Albuterol-D9	10	10	−0.7	−40.5	6
208	Testosterone enanthate		Anabolic steroide	$C_{26}H_{40}O_3$	$[M + H]^+$	401.30502	11.49		Albuterol-D9	ND	25	ND	−51.8	1
209	Testosterone propionate		Anabolic steroide	$C_{22}H_{32}O_3$	$[M + H]^+$	345.24242	10.61		Albuterol-D9	25	10	2.4	4.6	15
210	Tiletamine		Dissociative anesthetic	$C_{12}H_{17}NOS$	$[M + H]^+$	224.11036	9.69		Albuterol-D9	10	10	5.2	−28.2	7
211	Timolol		β-Blocker	$C_{13}H_{24}N_4O_3S$	$[M + H]^+$	317.16419	9.71		Albuterol-D9	5	5	20.9	−10.4	4
212	Tixocortol pivalate		Corticosteroid	$C_{26}H_{38}O_5S$	$[M + H]^+$	463.25127	10.03		Albuterol-D9	5	5	2.7	−13.2	7
213	Tolmetin		NSAID	$C_{15}H_{15}NO_3$	$[M + H]^+$	258.11247	8.72		Albuterol-D9	100	100	−11.7	−5.7	3
214	Torsemide		Diuretic	$C_{16}H_{20}N_4O_3S$	$[M + H]^+$	349.13289	8.91		Albuterol-D9	5	5	−12.4	−12.1	2
215	Tramadol		Opioid	$C_{16}H_{25}NO_2$	$[M + H]^+$	264.19581	10.22		Albuterol-D9	1	5	3.4	−25.2	13

Table 1 (Contd.)

	Compound name	IFHA class name	Therapeutic class	Chemical formula	Precursor ion	Measured mass of precursor ion (*m*/*z*)	RT (min)	Collision energy	Internal standard	Urine CCβ (ng mL^{-1})≤	Plasma CCβ (ng mL^{-1})≤	Matrix effect (urine)	Matrix effect (plasma)	Manufacturer group
216	Trandolapril		ACE inhibitor	$C_{24}H_{34}N_2O_5$	$[M + H]^+$	431.25405	9.21		Albuterol-D9	25	1	−9.4	5.6	8
217	Trandolaprilat		ACE inhibitor	$C_{22}H_{30}N_2O_5$	$[M + H]^+$	403.22275	8.41		Albuterol-D9	10	1	−21.8	−16.2	14
218	Triamcinolone acetonide	ISL (urine)	Corticosteroid	$C_{24}H_{31}FO_6$	$[M + H]^+$	435.21774	9.45		Albuterol-D9	5	5	−2.6	−14.1	9
219	Triamcinolone hexacetonide		Corticosteroid	$C_{30}H_{41}FO_7$	$[M + H]^+$	533.29091	10.52		Albuterol-D9	5	5	19.7	13.8	2
220	Triazolam		Psychotropic	$C_{17}H_{12}Cl_2N_4$	$[M + H]^+$	343.05118	9.45		Albuterol-D9	5	1	−5.5	6.2	13
221	Tripelennamine		Antihistamine	$C_{16}H_{21}N_3$	$[M + H]^+$	256.18082	10.02		Albuterol-D9	1	1	3.3	−20.4	9
222	Tuaminoheptane		Stimulant	$C_7H_{17}N$	$[M + H]^+$	116.14338	10.19	100	Albuterol-D9	ND	100	ND	−5.9	3
223	Tulobuterol		β-Agonist	$C_{12}H_{18}ClNO$	$[M + H]^+$	228.11497	9.96		Albuterol-D9	1	1	7.0	7.7	17
224	Valdecoxib		NSAID	$C_{16}H_{14}N_2O_3S$	$[M + H]^+$	315.07979	9.12		Albuterol-D9	50	25	−17.8	−41.2	8
225	Valsartan		Antihypertensive	$C_{24}H_{29}N_5O_3$	$[M + H]^+$	436.23432	8.55		Albuterol-D9	10	5	−11.6	−15.3	18
226	Vardenafil		Vasodilator	$C_{23}H_{32}N_6O_4S$	$[M + H]^+$	489.22785	9.70		Albuterol-D9	1	25	−8.5	50.9	3
227	Xipamide		Diuretic	$C_{15}H_{15}ClN_2O_4S$	$[M + H]^+$	355.05138	8.32		Albuterol-D9	100	25	−61.6	36.2	9
228	Xylazine	ISL	Sedative	$C_{12}H_{16}N_2S$	$[M + H]^+$	221.1107	9.67	60	Albuterol-D9	1	5	2.7	−79.0	6
229	Zilpaterol		β-Agonist	$C_{14}H_{19}N_3O_2$	$[M + H]^+$	262.155	8.57		Albuterol-D9	5	1	−17.2	−19.8	7
230	Zolazepam		Psychotropic	$C_{15}H_{15}FN_4O$	$[M + H]^+$	287.13027	8.89		Albuterol-D9	5	10	−7.8	−25.9	7
231	Zolpidem		Psychotropic	$C_{19}H_{21}N_3O$	$[M + H]^+$	308.17574	9.43		Albuterol-D9	1	1	2.2	0.9	13
232	Zomepirac		NSAID	$C_{15}H_{14}ClNO_3$	$[M + H]^+$	292.0735	8.93		Albuterol-D9	50	5	−19.0	−3.5	9
233	3-Hydroxy lidocaine-D5		Internal standard	$C_{14}H_{17}D_5N_2O_2$	$[M + H]^+$	256.20679	9.45							7
234	3-Hydroxy mepivacaine-D3		Internal standard	$C_{15}H_{19}D_3N_2O_2$	$[M + H]^+$	266.19423	9.07							7
235	4 Methylamino-D3-antipyrine		Internal standard	$C_{12}H_{12}D_3N_3O$	$[M + H]^+$	221.14762	8.65							7
236	Albuterol-D9		Internal standard	$C_{13}H_{12}D_9NO_3$	$[M + H]^+$	249.21591	8.22							7
237	Atropine-D3		Internal standard	$C_{17}H_{20}D_3NO_3$	$[M + H]^+$	293.1939	9.24							8
238	Bufotenine-D4		Internal standard	$C_{12}H_{12}D_4N_2O$	$[M + H]^+$	209.15865	8.08							7
239	Caffeine-D3		Internal standard	$C_8H_7D_3N_4O_2$	$[M + H]^+$	198.10648	7.91							8
240	Clenbuterol-D9		Internal standard	$C_{12}D_9H_9Cl2N2O$	$[M + H]^+$	286.14339	9.71							8
241	Furosemide-D5		Internal standard	$C_{12}H_6ClD_5N_2O_5S$	$[M - H]^-$	334.03183	8.21							8
242	Ketoprofen-D3		Internal standard	$C_{16}D_3H_{11}O_3$	$[M + H]^+$	258.1204	8.69							7
243	Meloxicam-D3		Internal standard	$C_{14}H_{10}D_3N_3O_4S_2$	$[M + H]^+$	355.06085	8.53							7
244	Morphine-D3		Internal standard	$C_{17}H_{16}D_3NO_3$	$[M + H]^+$	289.1626	8.74							8
245	Salicylic acid-D6		Internal standard	$C_7D_4O_3H$	$[M - H]^-$	141.04952	3.21							9

[a] Endogenous compounds. ISL: international screening limit. IRL: international residue limit. IT: international threshold. ASL: asian section limit. AICAR: 5-aminoimidazole-4-carboxamide-1-β-d-Ribofuranoside. EMDP: 5-ethyl-2-methyl-4-4-diphenyl 3-4-dihydro-2*H*-pyrrole. 4MAA: 4-methylaminoantipyrine. NSAID: non-steroidal anti-inflammatory drug. NMDA: *N*-methyl ᴅ-asparate. ACE: angiotensin converting enzyme. AMPK: activated protein kinase.

min^{-1}, and the injection volume was 5 μL. The nitrogen sheath flow rate and the auxiliary gas flow rate were set to 50 and 10 arbitrary units (arb), respectively. The sweep gas flow rate was 2 arb. The capillary temperature was 350 °C, the spray voltage was +3.0 kV, the maximum spray current was 11.8 μA during analysis and the S-lens RF level was 40%. The capillary voltage was set in positive or negative ion mode. The instrument operated in full scan mode in both positive and negative ion modes from *m*/*z* 53.6 to 804 Da at 140 000 resolution. The automatic gain control (AGC) was set to 1×10^6 and the maximum injection time (IT) was set to 100 ms. The data acquisition rate was 2 Hz. The following parameters were also recorded for product ion mass spectra (dd-MS2): resolution 17 500 in positive and negative ionization modes, AGC target 2×10^5 for positive ionization mode and 1×10^5 for negative ionization mode, maximum IT 60 ms, $(_N)$CE 17.5, 35, 55, isolation window and offset 3.0 and 1.0 *m*/*z* respectively. The dd setting minimum was set as an AGC target of 2×10^3, an intensity threshold of 3.3×10^4, and dynamic exclusion of 10.0 s in both positive and negative ionization modes. Data processing was performed using Thermo Finnigan Xcalibur software (version 4.1.31.9) with a mass tolerance window of ±5 ppm. Orbitrap performance in both positive and negative ionization modes was evaluated every 3 days and external calibration of the mass spectrometer was performed using Exactive Calibration Kit solutions (Sigma-Aldrich, St Louis, USA and ABCR GmbH & Co. KG, Karlsruhe, Germany). The chemical formula, exact mass (*m*/*z*), polarity, normalized collision energy and retention time of the targets are listed in Table 1.

Preparation of working solutions

Stock standard solutions of substances were prepared at a concentration of 1 mg mL^{-1} or 100 μg mL^{-1} in an appropriate solvent (for example, if the solvent was specified on the certificate of the standard, it was prepared using that solvent; if not, MeOH, ethanol, deionized water or other solvents were used.) and stored at −20 °C. The standards were divided into 5 pools and finally a single mixture was prepared by diluting the pools in water. The concentrations were in accordance with the recommended limit values, residues and screening limited substances listed in Table 1.[3] The working concentration range of the other prohibited substances was established as 1, 5, 10, 25, 50, 100 and 200 ng mL^{-1} in the urine and plasma samples.

Stock internal standard (IS) solutions were prepared in appropriate solvents at a concentration of 1 mg mL^{-1}. The internal standard mixture was prepared in appropriate volumes corresponding to a concentration of 50 ng mL^{-1} in the sample by using stock solutions.

Preparation of the calibrator working solution and reagents

The quality control (QC) samples were prepared by spiking blank urine and plasma samples with a pool corresponding to 2 times the detection capability (CCβ) value for each substance after the CCβ values were determined. It was used for robustness, selectivity and matrix effect studies.

The aqueous mobile phase containing 5 mM ammonium bicarbonate was prepared in a 2.5 L amber glass bottle by dissolving 988.0 mg ammonium bicarbonate in water and adjusting the pH to 9.0 (±0.2) by adding ammonium hydroxide solution.

Urine and plasma specimens

All biological samples used in the study were anonymous samples sent to the Pendik Veterinary Control Institute Doping Laboratory for analysis. Blank urine samples were obtained from post-race samples that were free of target analytes. The samples were stored in a deep freezer at −20 °C prior to use. Blood samples from post-race samples were also provided. Real positive samples were obtained in the same way. After transfer to the laboratory, the samples were centrifuged and the drug-free plasma samples were stored in a deep freezer (−20 °C).

Sample preparation

A 100 μL aliquot of urine/plasma was transferred to a microcentrifuge tube and 10 μL of internal standard mixture was added. 100 μL ACN was added for protein participation and the volume was adjusted to 500 μL with mobile phase A (5 mM ammonium bicarbonate, pH 9), mixed for 3 min by vortexing and then centrifuged at 30 000*g* (18 000 rpm) for 10 min. Finally, 5 μL of the supernatant was injected into the UHPLC-HRMS.

Validation

The method was validated according to European Commission guidelines. Detection capability (CCβ), selectivity/specificity, applicability, and ruggedness were investigated for qualitative screening validation.[30] The method includes more than 200 compounds that are prohibited or have limited screening values (including the International Screening Limit (ISL), International Residue Limits (IRL), and international thresholds (IT) established by the International Federation of Horseracing Authorities, in addition to that the Asian Screening Limits (ASLs) established by the Asian Racing Federation[31]). Threshold or limited substances at or below the specified level were selected for this method. The limit level was considered for method validation, but the detection limit was defined as low as possible for prohibited substances. Blank samples were selected notably drug-free samples. However, for endogenous compounds (such as hydrocortisone, hordenine, testosterone, salicylic acid, methoxytyramine, *etc.*), the CCβ value relies on negative urine samples with a typical endogenous concentration.

In screening validation, CCβ is the smallest analyte content that may be detected in a sample with an error probability of β.[30] The β error is the probability that the tested sample is truly non-compliant, even though a compliant measurement has been obtained and should be <5% for the screening test. Therefore, CCβ is the most important criterion for validation.

The first screening target concentration (STC) was calculated for each analyte by the use of a series of calibration levels

(1, 2, 5, 10, 25, 50, 100, and 200 ng mL^{-1}). Four criteria were used to select the STC. At least 3 fragment ions, retention times (RTs), and library results must be matched for reference standards and spiked samples. For prohibited substances, the STC must be at or below the reference point of action.[30] The STC must be below the regulatory/action limit for limited substances. After calculating the STC, 20 blank and 20 spiked samples (at the screening target limit concentration) were analyzed to determine the CCβ. The highest response of the blank sample and the smallest response of the spiked samples were noted. If the maximum response of the blank is less than the minimum response of the spiked sample, it means that the CCβ of the method is less than or equal to the spiked concentration (which is the screening target limit concentration and $\frac{1}{2}$ of the regulatory/action limit).

The cut-off level defines the response or signal from a screening test indicating that a sample contains an analyte at or above the STC. If the cut-off level is exceeded, suspect sample processing should be performed. The cut-off level of the method corresponds to the smallest response of the spiked sample among the 20 samples.

Additionally, the threshold value (T) and cut-off factor (F_m) were calculated as follow:

Threshold value T:

$$T = B + 1.64 \times \mathrm{SD_b}$$

B is the mean response, and "$\mathrm{SD_b}$" is the standard deviation of the blank samples.

The cut-off factor F_m is:

$$F_m = M - 1.64 \times \mathrm{SD_s}$$

M is the mean response, and "$\mathrm{SD_s}$" is the standard deviation of spiked samples.

The values obtained were plotted on the graph of the number of responses to analysis, and the "possible positive suspect sample range" was determined for each analyte. The graphs for quinapril in urine and benazeprilat in plasma are shown as examples in Fig. 1.

Selectivity and specificity

The selectivity/specificity was verified by analyzing 20 different blanks and spiked urine/plasma samples in the presence of various groups of different substances (nonsteroidalanti-inflammatory drugs (NSAIDs) anabolic steroids, narcotics, corticosteroids, stimulants, beta-agonists, *etc.*).

Matrix effect

The matrix effect for all the target analytes was evaluated by comparing the drug response in matrix sample and the water sample (n = 3).[32] Twenty different blank urine/plasma samples were pooled and spiked for the calculation of the ME. Distilled water was also spiked in the same way. The formula used was %matrix effect = (urine or plasma area/water area) × 100 − 100. The results are shown in Table 1.

Ruggedness

To determine the ruggedness and retention time stability of the method, 3 different drug-free urine and plasma samples were spiked at the QC level. The samples were prepared and analyzed on day 0, and re-injected after 72 hours (after re-calibration of HRMS) (n = 3). The change in retention time was calculated according to the initial retention time for each injection.[15,33]

Ruggedness was tested by checking retention times and responses of analytes spiked at QC levels on plasma and urine samples for minor changes in different parts of the method. Minor changes were defined as different grades of MeOH (HPLC or LCMS grade), centrifugation times (15 min or 30 min), column temperatures (55 °C or 50 °C) and dilution solvents (with mobile phase A or deionized water). CV% values were calculated for the RTs and responses.

Carry over

The carry-over effect was assessed by injecting blank urine samples after injecting the urine/plasma samples with the highest concentration. The results of the successive blanks were examined for the presence of substances.

Application to real samples

The method was used for routine analysis for one year. Before each batch was started, the blank and QC samples were analyzed and checked. The suitability of the method was verified by previously declared positive samples.

Retrospective analysis

To demonstrate the performance of the full scan method, a plasma sample previously detected orphanadrine (unavailable and unvalidated compound in our method) by different methods was analysed and reprocessed to demonstrate the applicability of retrospective analysis with the developed method. Prior to processing, the masses of the samples were added to the full scan HRMS database for library detection, retention time and isotope pattern matching.

Results and discussion

Method development

Sample preparation. With the increasing use of the DS technique in toxicological analysis, its advantages, such as simplicity, time and cost efficiency, are being evaluated along with its disadvantages. These disadvantages include the fact that the diluted sample contains many matrix components, the detection limit is high due to dilution, and the matrix effect occurs.[10] Therefore, the focus of this study was to overcome these disadvantages. The sample preparation step included dilution to reduce non-active contaminants such as salts, acids, bases, xenobiotics, proteins and nucleic acids naturally present in urine/plasma, and protein precipitation. The effects of ACN, TCA and MeOH, which were used in previous studies for protein precipitation in biological samples, were evalu-

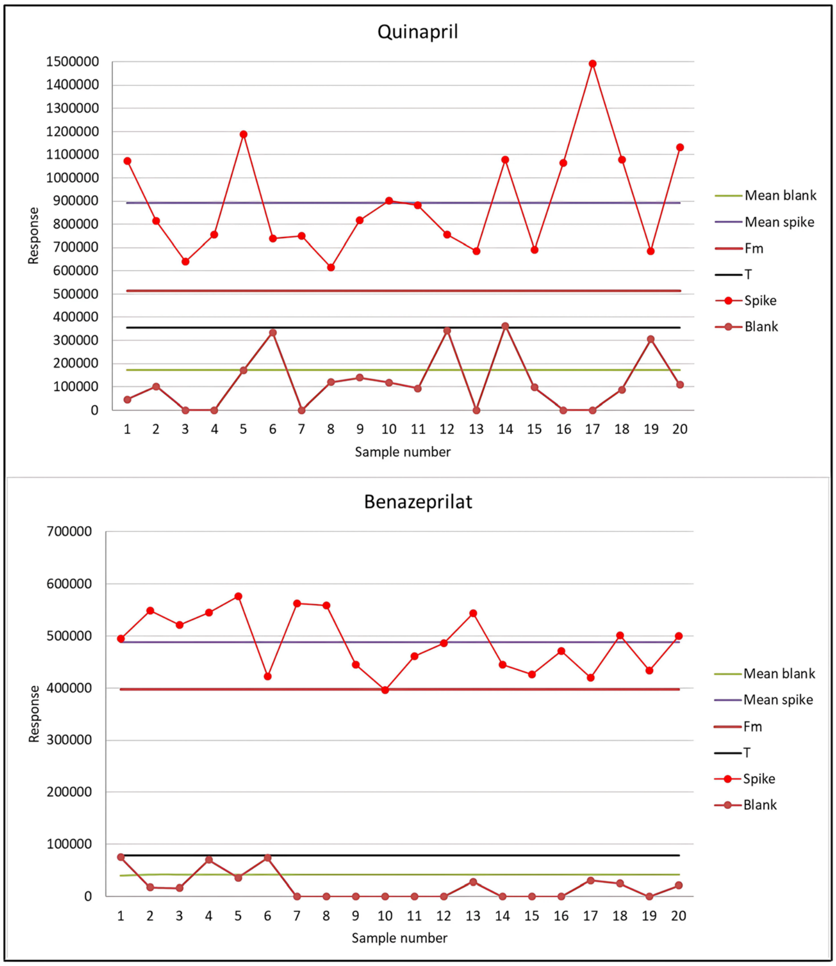

Fig. 1 Example graph for the calculation of CCβ according to the guidelines for quinapril in the urine (top) and benazeprilat (bottom) in the plasma.

ated.[7] The precipitation of proteins or macromolecules with ACN in urine and TCA in plasma was more effective than that with MeOH, but the use of ACN was convenient in terms of the number of drugs detected. For this reason, ACN was used in both urine and plasma for ease of application. Ultracentrifugation was used to reduce physical and/or chemical background noise from precipitation. Dilution was also the best choice to reduce the matrix effect and mitigate the effects of non-active contaminants such as salts, acids, bases, xenobiotics, proteins and nucleic acids on the detection of analytes (the results of the matrix effect study are presented and discussed in detail in the matrix effect section). Thereby, the sample preparation step was minimized by ACN addition, dilution and ultracentrifugation.

Chromatographic conditions. To ensure effective separation, in the study conducted by Görgens *et al.* 2016,[21] a trial was performed in both urine and plasma using 5 mM ammonium acetate with 0.1% acetic acid (A) and ACN (B). However, a total of 195 active substances in urine and 207 active substances in plasma were detected. An increase in peak width and significant peak tailing were observed in this study. It was observed that methadone had a greater response in both urine and plasma, and mephentermine gave higher response in only plasma when these mobile phases were used. This can be considered an example of the analyzed substances being more effectively separated and detected at basic pH (pH 9) than at acidic pH (pH 3.5). To improve chromatographic separation, for mobile phase B, trial studies were carried out in various combinations with MeOH, MeOH-ACN (50–50), MeOH-ACN (70–30), MeOH-ACN (90–10) and ACN only. The selection of MeOH as a mobile phase was found to be superior to ACN in terms of retention behavior peak shapes and improved ionization for several analytes. After assessment, MeOH and ammonium bicarbonate buffer (pH 9) were selected because of their high MS signals and good chromatographic separation without deterioration of peak shapes. The ammonium bicarbonate (5 mM, pH 9) mobile phase was freshly prepared once or twice a week, pH controls were performed, and no deterioration in stability was detected during the analyses. As a result of the experiments, the compounds with the highest response values were detected when only MeOH was used in the B mobile phase.

Since the method did not involve extraction, it was highly probable that dilution and application of the sample would cause blockage and high pressure in the column. In particular, the fact that horse urine is a much more complex matrix than human urine is one of the most important factors that could cause blockage in the column. The pH working range of the existing column was wide (1–12). In the method in which we used another mobile phase above pH 9, the reproducibility and stability of the current column was high, other studies support this,[14] and this study was continued with current the column.

For effective separation in the column, an attempt was made to ensure separation by taking into account the analyte properties and diversity. The gradient for the adhesion of hydrophilic compounds was initiated with a highly aqueous mobile phase. In this way, it aims to remove pollutant components, which are mostly polar. Although this situation causes some substances with highly polar properties (AICAR, edrophonium, ethyl glucuronide, *etc.*) to hold less in the column and to be detected at higher concentrations compared to previous studies,[15] a wide spectrum can be achieved considering the number of substances detected. After a certain polarity equilibrium, effective separation was achieved by increasing the amount of organic solvent.

The main aim of the study was to screen a large number of compounds in a short period of time. Reducing the gradient and re-equilibration time can make chromatography more cost-effective, thus increasing the number of runs in a given period and still generating reproducible chromatography even when the stationary phase is not precisely recalibrated.[34] One of the most important factors affecting the re-equilibration time is the choice of mobile phase. In order to reduce the equilibration time, studies showed that methanol binds less strongly to the stationary phase than dose acetonitrile and it is easier to removed from the column, and studies aldo showed that the use of a mobile phase containing an organic solvent reduces the equilibration time compared with a purely aqueous phase.[34] The column conditions were the same for the initial 0–3 min and the final 1.5 min, at the start of the column to remove unwanted polar components that may have been introduced with the sample, particularly during the initial time. The equilibration time in the last part was increased by 1 min additions until optimized to a total of 18 min, and the deviations in the RTs, peak shapes and response values were evaluated. For meldonium, ethyl glucuronide, and DMSO, no change in peak shape, RT and response value was detected depending on the equilibration time. For AICAR, the RT value changed by 0.3 seconds on average due to the prolongation of the equilibration time, but the peak shape deteriorated with prolonged equilibration time. For edrophonium and salicylic acid, RT changed similarly, but the response values decreased. Only enalaprilat and pregabalin improved in peak shape with increasing equilibration time. Other than these, there was no change in RT in other peaks. At this time, the shortest maximum time at which reproducible results were obtained was 13 min. The RT deviations in ESI Table S1† also indicate that the equilibration time was sufficient.

Previous chromatographic seperation studies reported that ACE inhibitors and their active metabolites produced a split peak and this negativity was prevented by increasing the column temperature (55 °C).[7] Therefore, this study also demonstrated that the asymmetry persisted and broadened for perindoprilat. However, depending on the characteristics of the column used and increasing the temperature, it was observed that these polar substances were more symmetrical, especially in the plasma samples. This was demonstrated by comparing the effects of even a 5 °C minor difference in column temperature in the durability study (Fig. 2).

The detection time of the analytes was accomplished within 13 min. Screening ion chromatograms of 225 target analytes in urine and analytes that can be detected only in plasma at their

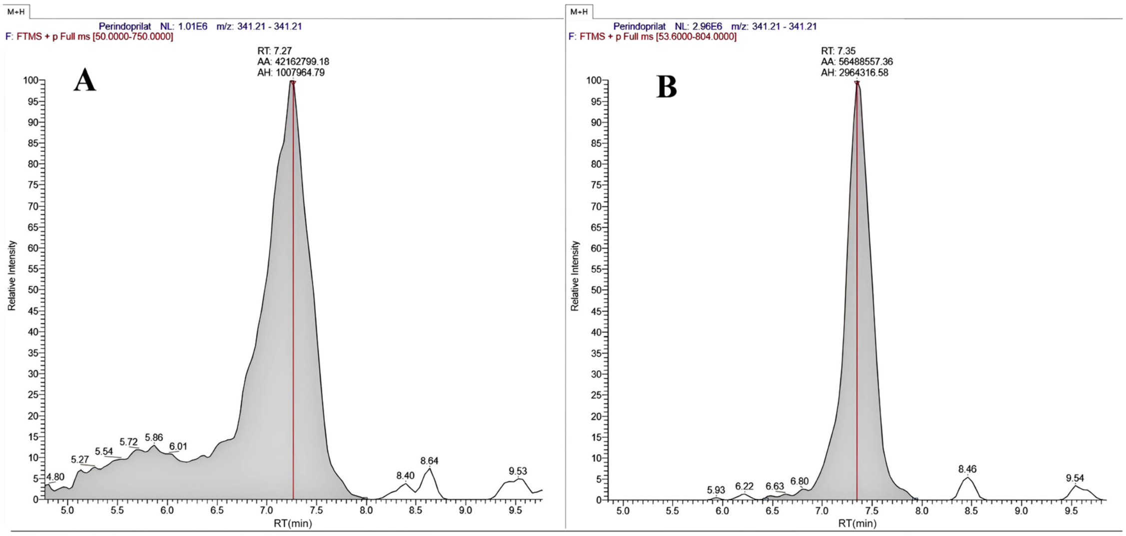

Fig. 2 Effects of column temperature on perindoprilat detection at (A) 55 °C (B) 50 °C.

detection capability value (CCβ) are shown in ESI Fig. S1 and S2.† A continuation series of studies for the separate analyses of endogenous hydrocortisone,[29] 3-methoxytyramine[28] and salicylic acid, which have been analyzed by hydrophilic interaction liquid chromatography (HILIC) and have problems in RPLC separation owing to their polar properties, could have been combined in this study.

Analytes producing ions with the same mass-to-charge ratio were separated chromatographically by their retention time (edrophonium-hordenine, tramadol-*N*-desmethyl venlafaxine, cannabidiol-delta-9-THC, morphine-3-glucuronide, and morphine-6-glucuronide) or by the difference between the fragment ions (EMDP-nortriptyline, alminoprofen-ritalinic acid, and metolazone-indapamide). Indapamide was not included in the validation study because it was detected at the same RT as metolazone and cannot be accurately separated, so it was shown only in a chromatogram (ESI Fig. S1†). Although the peak shape of enalaprilat in plasma is not symmetrical, it fulfills the retention time, fragment ions, isotopic pattern and library matching, like gabapentin and amiloride in urine.

Bendroflumethiazide, dantrolene, diflunisal, ethyl glucuronide, furosemide, hydroxyflutamide, meclofenamic acid, niflumic acid, nimesulide and salicylic acid were detected by negative ionization, whereas other substances were detected by positive ionization. Some substances detected by negative ionization in previous studies were better detected by positive ionization in this study (metolazone, bumetanide, probenecid, xipamide and others).[19] Protonated ions were used for positive ion mode, and only deprotonated ions were used for negative ion mode.

Internal standards were used to detect the effects of sample preparation, changes in chromatographic conditions, monitoring retention time shifts and instrumental variations such as ESI signals, sensitivity, *etc*. Therefore, the CV% values of the interday retention time and response values of the available internal standards ($n = 9$) were evaluated (albuterol d9: 0.14% and 6.14%, 3-hydroxy lidocaine-d5: 0.16% and 6.67%, 3-hydroxy mepivacaine-d3: 0.19% and 9.13%, 4 methylamino-d3-antipyrine: 0.18% and 13.22%, atropine d3 0.15% and 7.69%, bufotenine-d4 0.28% and 14.20%, caffeine d3: 0.46% and 7.80%, clenbuterol d9: 0.16% and 6.56%, furosemide d5: 0.18% and 10.95%, ketoprofen d3 0.17% and 12.13%, meloxicam d3: 0.14% and 13.64%, morphine d3: 0.21% and 14.34%, and salicylic acid-d6: 0.84% and 9.94%). Albuterol d9, which has the lowest deviation from the internal standards used, was chosen because it provides sensitive, stable and reproducible results. Albuterol d9 was used as an internal standard for all substances, except for substances with specific internal standards. It also facilitated the application. Other internal standards were selected for substances with similar analytical behavior. For example, the internal standards furosemide-d5 and salicylic acid-d6 were studied with negative ionization and furosemide and salicylic acid were also negatively ionized; therefore, these substances were used since deuterated internal standards were available. Analyte concentrations in the samples were estimated by the area ratio of the target analyte to the internal standard using a one-point calibration curve if a limit reference compound was detected.

Narrower mass isolation windows allow longer collection times and therefore higher sensitivities, but it is also necessary to limit the maximum ion capacity. This is because a low isolation window may cause the signal to drop; therefore, substances with low detection limits may not be detected. For this purpose, the isolation window was optimized using isolation windows from 1 to 4. For most compounds and for compounds with the same product ion, an isolation window of 3 m/z with a collision energy ramp (17.5, 35, 55) gave adequate results for fragmentation ions, ion raitos and signals.

The identification of the analytes was controlled with the spiked sample peak by checking the retention time (±2% of the reference), at least 3 fragments, isotopic pattern matching and library matching. A mass tolerance of 5 ppm and a full MS resolution of 140 000 were applied in the method, which has previously been shown to have reproducible results in studies.[19,35] In addition, according to the AORC acceptance criteria defined in the minimum criteria for identification by chromatography and mass spectrometry, at least three product ions were monitored and 20% relative abundance was carried out for each drug for confirmation.[36]

The concentrations of various metabolites in plasma are lower than that of in urine. Therefore, it is important to detect trace concentrations as much as possible in plasma. When the data of the study were compared with other studies, it was found that some substances could be detected at lower concentrations.[19,37,38] As the method saves considerable time in preparation and application, it is suitable for use in situations where rapid results are required, such as pre-race analysis. It can be considered an important alternative, especially when compared with the results obtained with limited and threshold substances in pre-race analysis using hydrolysis and SLE.[6] It was concluded that the method was sufficient for both pre-screening and cross-checking.

The absence of a preconcentration step in the DS technique can be seen as a disadvantage in the detection of substances with low limit values (acepromazine, butorphanol, procaine, xylazine and others). However, the detection of most of the limited substances by this method is important, as it avoids reanalysis. In general, the method can detect more substances in plasma, and for some substances, lower concentrations can be detected in plasma compared to the urine matrix. In addition, the method can be used for the analysis of a wide range of substances with different polarities, chemical structures and pharmacological activities. As a result, in the present study, 225 substances in horse urine and 232 substances in horse plasma were fully detected and validated. The developed method can screen 26 international screening limits, 7 residue limits, 5 thresholds, 1 Asian section limit and 193 prohibited substances. In addition to that an unlimited number of new substances can be added to the method if the target can be recovered from the sample.

Nearly 5000 anti-doping samples (approximately 4000 urine and 1000 plasma samples) sent to the laboratory were successfully analyzed using the developed method in a year. These analyses were performed on the same column regardless of the physical characteristics of the samples. During these analyses, mobile phases were freshly prepared once or twice a week. Positive and negative mass calibrations of mass spectrometry were also repeated every three days. The cone is cleaned every week before positive and negative optimization, and the needle was replaced if a problem with optimization or an excessively low signal is detected during analysis. In routine analysis using this method, the MS source was cleaned only 2–3 times during the analysis of 4000–5000 samples, and detailed maintenance was carried out annually by an authorized company representative.

Method validation

Detection capability. The detection capability was in the range of ≤1 ng mL^{-1} to 200 ng mL^{-1} for prohibited substances. Seventy-nine of the analytes in urine and 96 of the analytes in plasma had CCβ values at or below 1 ng mL^{-1}, 48 analytes in urine and 66 analytes in plasma had CCβ values at or below 5 ng mL^{-1} and 31 analytes in urine, and 27 analytes in plasma had CCβ values at or below 10 ng mL^{-1}. The highest CCβ value was detected for AICAR, but the chromatographic reasons for this have already been explained and previous studies have indicated that it can be detected at high concentrations due to its endogenous characteristics.[14]

The threshold, residue and screening target limited subtances are shown in Table 1. Among the detected threshold substances, only prednisolone in the urine and plasma and testosterone in the plasma could not meet the expected concentrations. Apart from these two compounds, other substances can be screened according to their threshold levels. For international residue-limited substances except methylsulfonylmethane, theobromine and theophylline, CCβ levels are sufficient in both urine and plasma. In international screening, a limited category of 23 substances in urine and 14 substances in plasma can be screened by the current method. Additionally, 13 substances in urine and 1 substance in plasma could be validated at the ISL level.

The CCβ value was calculated for all the substances and graphs were created by determining the values in Fig. 1. If $F_m > T$, CCβ was approved as valid, as well as it means that the false negative rate is ≤5% and that CCβ is equal to STC. Therefore, calculating T and F_m is important for deciding whether the method is valid. The graphs in Fig. 1 clearly show these values and the working range of the method.

Selectivity and specificity. The specificity was satisfactory as no interfering substances were detected at the retention times in 20 blank urine/plasma samples. For selectivity, other common doping agents (NSAIDs, anabolic steroids, narcotics, corticosteroids, stimulants, beta-agonists, *etc.*) were spiked and analyzed simultaneously. With respect to the results, no interferences were detected and concluded that the method was selective. Additionally, more than 5000 samples were analyzed by our method within one year, and either blank or positive samples, no interferences or false positives were detected.

Ruggedness. Stable retention times are necessary for a correct interpretation of the results. For evaluation of retention time deviation QC samples were reinjected after 72 hours (ESI Table S1†). The retention time of gabapentin in both plasma

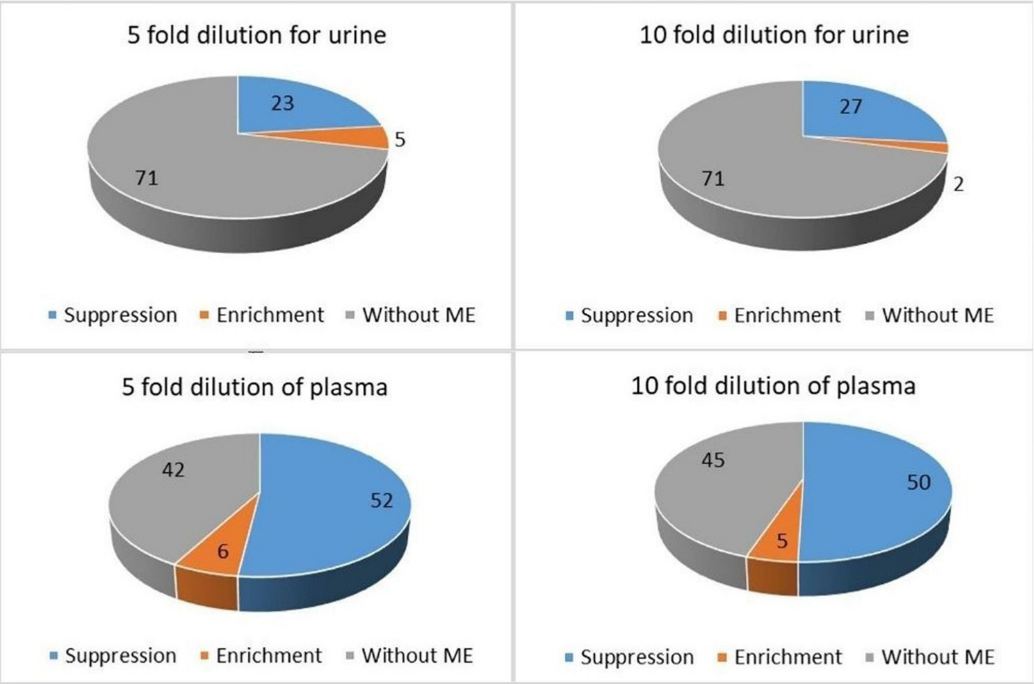

Fig. 3 Effects of dilution on the urine and plasma matrix.

and urine differed, while the CV% value of adrenochrome semicarbazone in the urine sample was greater than 2%. Except for these substances, no variation above 2% was detected in the reanalyzed samples.

To assess robustness, the effects of small changes in the different steps of the routine method on the retention time and response were evaluated according to the CV% (ESI Table S2†). A deviation of 2% in retention time and 15% in response was considered as a significant effect. When analyzing the results, it was shown that minor changes did not have a major effect on the detection of the majority of target analytes, but these changes did make a difference for a few target analytes. The differences in retention time and area were mainly due to the change in column temperature. At 55 °C, the peaks were more symmetrical, and the response values were greater. However, for 3-methoxytyramine and cetirizine, the area was much greater at 50 °C, and there was a change in retention time. For 5 hydroxy omeprazole, ambroxol, eltenac, hydroxy homosildenafil, and mephentermine the response values at 50 °C were almost 50% lower in both the urine and plasma in comparison to values observed at 55 °C. For AICAR, the response deviated in urine at 50 °C, for bromhexine, the response decreased in urine at 30 min of centrifugation and 50 °C column temperature, and the response increased after

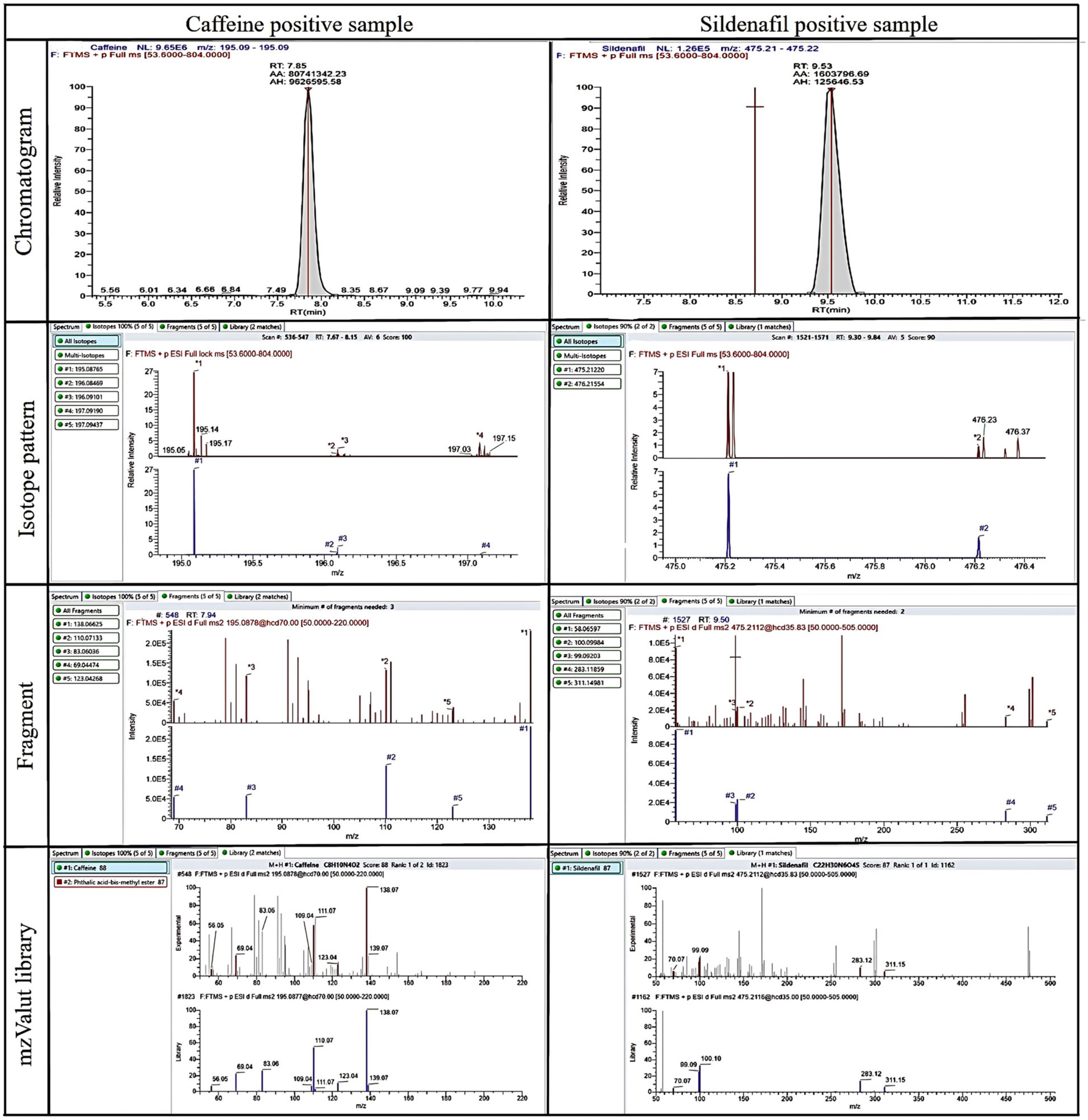

Fig. 4 The screening data of two positive declared samples in horse urine; caffeine (left) and sildenafil (right).

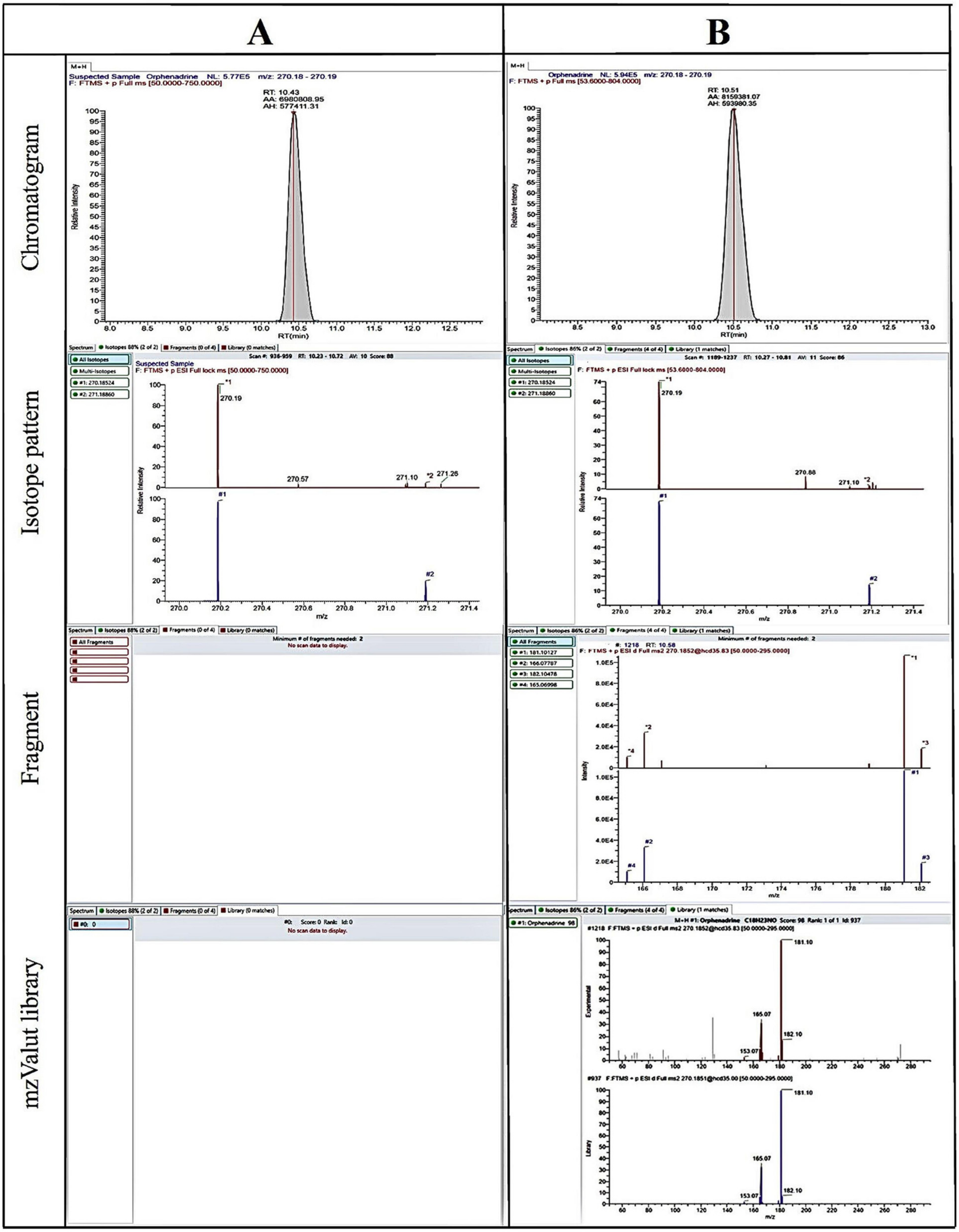

Fig. 5 Retrospective analysis of orphanadrine in plasma, (A) results of reprocessing after it had been added to the database (left) (B) results of re-analyzed after being added to the inclusion list (right).

dilution with water, whereas the response decreased after 30 min of centrifugation, 50 °C column temperature and dilution with water for plasma. For ketamine, the response decreased in plasma under all conditions, while for fluspirilene, the response decreased at 50 °C column temperature and after 30 min of centrifugation. For amoxapine, levopromazine, promazine, propionyl promazine and tuaminoheptane, minor changes in the plasma samples caused significant decreases in response. It was thought that dilution with mobile phase instead of water might be more effective in determining the shape of the peaks. However, no significant effect was observed in the urine. In plasma, no deviation in the field value was observed except for 4 MAA, amitriptyline, ketamine, enalaprilat, bromhexine and tuaminoheptane. By evaluating these differences, it was revealed that dilution with the mobile phase was more effective.

Carry over. After injection of highly concentrated spike samples, 4-methylaminoantipyrine (0.1%), benzydamine (0.4%), carvedilol (1.42%), chlorpromazine (0.85%), cymemazine (0.28%), levopromazine (0.42%), nebivolol (1.35%) and promazine (0.58%) had a carry-over effect, with a lower response in the first urine blank. However, no interfering analyte was detected in the second blank. For plasma, 4-methylaminoantipyrine (1.84%), benzydamine (0.42%), carvedilol (1.76%) and imipramine (0.4%) showed carry-over to the first blank urine but not in the second blank. Therefore, a solvent solution should be injected after the QC and/or positive sample to avoid carry-over.

Matrix effect. One of the disadvantages of the dilute and shoot technique is the matrix effect. The matrix effect of horse urine varies from one sample to another; therefore, signal enhancement and suppression vary.[29] The matrix effect can also cause a shift in retention time. To compensate for the matrix effect, suitable internal standards with properties similar to those of analytes were used. The matrix effect was evaluated by changing and optimizing the sample volume and dilution factor. For this purpose, the water blank and a spiked reference sample were included in the same calculation to assess the matrix effect (Fig. 3). Ion suppression and enrichment were observed for 23% and 5% of the target analytes for urine and 52% and 6% of the target analytes for plasma, respectively, using the current method. For urine and plasma, respectively, 71% and 42% of the analytes were within the matrix effect criteria (±15%). These results indicate that the matrix effect is more dominant in plasma than in urine.

The % matrix effect was evaluated by performing 10-fold and 5-fold dilution study. The response values obtained due to 2-fold dilution decreased by half in both matrices for substances other than cannabidiol and xipamide in urine and 4 MAA, cetirizine, chlorpromazine, dihydrocapsaicin, diprenorphine, fluvoxamine, isoxsuprine, nalorphine, and promazine in plasma.

At the 10-fold dilution, 15 substances in the urine and 20 substances in the plasma could not be detected compared with the 5-fold dilution. In addition, dilution did not significantly reduce the matrix effect in either sample. When the matrix and dilution effect results were compared with those of the study by Badoud *et al.*,[26] the percentage of matrix-unaffected analytes was greater. This important result shows that the method is suitable for the detection of analytes.

Significant ion suppression and enrichment did not interfere with the identification of prohibited substances for screening analysis. For limited substances, the matrix effect may be significant for quantitative analysis, but these screening analyses were used to provide information for confirmation analysis. The results revealed that ion suppression resulted in more matrix effects than ion enrichment in both the urine and plasma, but the resulting matrix effect was almost 50% less in the urine than in the plasma.

Application to positive case samples. The presence or absence of a substance with this method was based on chromatographic and mass spectrometric determination. The suitability of the method was demonstrated with previously positively declared samples. The present method was applied for routine analysis in our laboratories, and positive cases were detected using the DS method. The screening data of two positive declared samples (caffeine and sildenafil) are shown in Fig. 4. For these suspected samples, the specified procedure was followed and if (1) the mass tolerance was ±5 ppm, (2) the retention time was within ±0.4 min, (3) the isotopic pattern was greater than 70%, (4) at least three selected HRMS/MS fragment ions matched, and (5) the library score was greater than 80%, a green flag was signed. The results were in accordance with the screening criteria and matched with spiked samples.

Retrospective analysis. The importance of retrospective analysis in equine doping control has been emphasized previously.[22] To demonstrate the use of this method in retrospective analysis, a non-targeted substance previously declared positive in a plasma sample was reprocessed in the full screening data. Untargeted orphenadrine in plasma was detected by reprocessing after it had been added to the database (Fig. 5 (A)). It was then re-analyzed after being added to the inclusion list in the current method (Fig. 5(B)). Fragment, isotopic pattern and library results were compared to demonstrate the trueness of the retrospective analysis results.

Conclusion

Triggered by the increasing number of prohibited substances and the growing number of samples, a new dilute and shoot method has been developed and validated to analyze more than 200 substances rapidly, simply and cost-effectively. Substances with poor stability and degradability by chemical or hydrolysis were the main focus of the developed method. The method also allows the simultaneous detection of many doping agents by performing a wide range of scans. Therefore, this reduces the number of confirmatory analyses required.

LLE, and especially SPE are widely used as a preparation methods to improve detection limits. However, the DS method provides a significant economic contribution, particularly through the faster and easier detection of limited substances.

The ability to scan different substances in a single run exploited the advantages of LC-HRMS to scan both positive and negative polarities. This method can analyze most substances with screening limits as well as substances with international residue limits and threshold levels, avoiding the need for another quantitative method. In addition, the retrospective analysis and detection of positive cases demonstrated with successful applications.

This comprehensive method was successfully applied to both horse urine and plasma samples over a broad spectrum for the first time, providing simple and rapid sample preparation. Thanks to this simple, cost-effective, and high-throughput method, an increase in the efficiency of daily work in the laboratory can be achieved. The developed method is suitable for use by laboratories such as forensic and doping laboratories performing unknown analyses and detection of more than 200 analytes at the ng mL^{-1} level with μL level sample, low cost, labour and time consumption. In addition, this simple method allows the analysis of more prohibited substances and allows retrospective analysis.

Author contributions

E. F. Göktaş: conceptualization, methodology – analysis method, project administration, method validation, measurements and investigation, resources, interpretation of the results, writing – original draft preparation, writing – review. E. Kabil: measurements and investigation, resources, visualization, methodology – method validation, interpretation of the results, writing – review. E. Söylemez Yeşilçimen: interpretation of the results, writing – review. L. Dirikolu: supervision, interpretation of the results, writing – review and editing. All the authors approved the final version of the manuscript.

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

This study did not receive any specific grant from funding agencies in the public or private sectors. The authors thank the Pendik Veterinary Control Institute for the analytical instrument.

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