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An ultra fast and sensitive detection of 165 drugs of abuse in human urine using polarity switching ultra performance liquid chromatography tandem mass spectrometry

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ABSTRACT

The screening of wide variety of prohibited substances in a time bound manner by adhering to latest World Anti-Doping Agency (WADA) guidelines is a challenging task for doping control laboratories. The revised criterion of detection limits (WADATD2013MRPL) has further required the doping laboratories to review their testing procedures. The present work was aimed at developing a fast, sensitive and robust analytical method based on solid phase cleanup (SPE) and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to achieve the required detection levels. The method development involved optimization of deconjugation of phase II metabolites, SPE using mixed-mode ion cartridges for extraction of analytes of wider chemistries; and fast polarity switching UPLC-MS/MS detection. The developed method was validated to detect approximately 165 compounds and/or metabolites prohibited by WADA. The eight minutes runtime allowed testing of approximately 180 samples in 24 hours at the limit of detection (LOD) of 50% below required detection levels.

KEYWORDS

Bioanalytical method;
Sports drug testing;
UPLC-MS/MS;
Polarity switching;
WADA.

INTRODUCTION

The detection and identification of prohibited compounds and methods of doping has been regulated for sports drug testing laboratories by World Anti-Doping Agency (WADA). The WADA publishes a prohibited list every year consisting of wide range of pharmacological classes of drugs[1]. WADA creates respective technical documents that outline minimum required performance limits (MRPL) as well as international standard for laboratories (ISL), which accredited doping control laboratoriesmust follow[2]. The prohibited list covers nine pharmaceutical classes of substances (e.g., anabolic steroids, corticosteroids, stimulants, diuretics, anti-estrogens etc), three forbidden doping methods (e.g., substance for enhancement of oxygen transfer, chemical and physical manipulation and gene doping), and two groups of analytes alcohol and â-blockers are prohibited in specific activities[1]. Therefore, numerous technical approaches are needed to analyse the great diversity of doping agents.

Anti-doping analysis is conducted in two steps. Initially, screening of samples is performed, in the

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case of a suspiciousresult; an additional selective confirmation is carried out[3]. As every sample has to be screened, the screening method has to be highly sensitive and specific to ensure identification of suspected sample and in the same time should minimize the probability of false suspects. Doping analysis requires the use of several different chromatographic, mass spectrometric and immunological methods[4-7] which makes it mandatory for all the doping control laboratories to have a number of separate analytical procedures, thereby making screening of each sample more complex, time-consuming and laborious. Therefore, it has become necessary to develop high-throughput techniques to screen in a single method a large set of compounds with different physicochemical properties avoiding false negatives and false positive results.

In the last decade, the suitability of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been demonstrated as a technique of choice over traditional gas chromatography mass spectrometry (GC-MS) methods for multi-target screening due to the development of electrospray ionization (ESI) sources which operates at atmospheric pressure[8-11]. This capability allows the detection of both low- and high-molecular weight compounds[12].

The ultra performance liquid chromatography (UPLC) based methods for screening analyses have emerged in the anti-doping field. The advantages of UPLC (in which columns are packed with sub-2µ particles operating at pressures up to 1000 bar) have been demonstrated to rapidly and efficiently separate drugs and related substances[13,14]. Due to the reduced analysis time, peaks become very narrow and an adapted detection device is thus mandatory. Mass spectrometers with fast scanning like triple-quadrupole (QQQ) are often coupled to UPLC and used in tandem mode by monitoring ion transitions[15,16]. Modern QQQ instruments offer very fast acquisition cycle times and polarity switching usually expanding the number of analytes which can be detected in a single run. Numerous methods have been employed in the past decade conjoining UPLC with QQQ mass spectrometer (UPLC-MS/MS)for identification of variety of doping agents[15-17].

The choice of development of a suitable sample pre-treatment for a screening purpose is a challenge as the method should provide good extraction yields for a combination of analytes with very different physico-chemical properties (neutral, basic and acidic, lipophilic and hydrophilic). Moreover, human urine is usually a very dirty matrix so it requires an extraction technique in which the impurities of the sample should be eliminated to avoid any possible unwanted interference of the matrix. The diversified categories of drugs of abuse mostly contain basic compounds with exceptions of neutrals (glucocorticosteroids) and acidics (diuretics). Besides, the huge number of different endogenous components normally found in urine makes the selective detection of analytes at low concentration very challenging. Recently, various methods have been developed to detect the banned compounds in human urine using dilute and shoot approach[18-19]. The dilute and inject approach is certainly fast, but it does not allow for the detection of analytes at very low concentrations and does not include deconjugation of glucuronides[20-21]. Hydrolysis is mandatory to remove the glucuronide moieties attached to several doping agents during phase II metabolism. Liquid – liquid extraction (LLE) may be used for the sample purification but it faces some pitfalls. To cover a wide range of different drugs, two consecutive extractions, one at basic and the other at acidic pH, is required. In addition, LLE requires careful separation of the phases and can be time consuming and tend to use large volumes of solvent. Moreover, the sample extracts are not very clean.

The most preferred technique for said requirement is solid-phase extraction (SPE) as it requires less washing and cleaning steps and blocks the impurities of the sample within the cartridge. A single or multiple-stage SPE has been applied for the sample preparation methods in various fields of doping[20-22]. In particular, a single mixed-mode cartridge is reliable for the fractionation of acid, neutral and basic drugs from biological samples because the drugs are adsorbed separately by hydrophobic or ion-exchange interaction on to the cartridge.

To ensure consistency of the measurements amongst doping control laboratories, WADA defines the MRPL, which is the concentration of a prohib-

ited substance at which the laboratories are expected to detect the prohibited drug/s. From January 2013, WADA has revised the MRPL criterion for the detection of drugs by the anti-doping laboratories wherein, the MRPL of various drugs has been reduced from 20-80% (TABLE 1)[23]. In addition, the criteria for limit of detection (LOD) is also revised (TDMRPL2013) which states that the laboratory's method validation of the initial testing procedure shall include the estimation of the LOD for each compound and the estimated LOD shall not be higher than 50% of the MRPL. The revised MRPL and LOD criterion applicable from January 2013 has further necessitated the need to review and revise testing procedures in the doping laboratories to achieve the targeted MRPLs.

The lower detection limits enforced in the new technical guidelines of WADA has required improving the existing screening method by LC-MS/MS in our lab in order to 1) develop a comprehensive and fast analytical method based on multi target approach reducing the burden of number of detection methods and analysis time 2) method sensitive enough to achieve the detection levels. Hence, the aim of this work was to develop a high throughput and sensitive screening method for the detection of various drugs at or below the WADA TD2013 MRPL. It was required to derive the extraction procedure to extract analytes with a very wide range of chemistries as well as the detection method so as to improve the LOD of drugs.

Hence, a three step strategy was made for the overall improvement of the method: i) Enzymatic hydrolysis for the deconjugation of phase II metabolites (glucuronides), ii) SPE using mixed mode cartridges for the execration of acidic, basic and neutral molecules in single step and, iii) identification of drugs on highly sensitive and upgraded instrument i.e. UPLC-MS/MS using fast polarity switching. The developed method would allow fast and

TABLE 1 : Comparison of WADA technical documents (WADA TD2010MRPL vs WADA TD2013MRPL)

WADA TD2010MRPL			WADA TD2013MRPL		
Prohibited Class	**Specific Examples/Exception**	**Concentration**	**Prohibited Class**	**Exception**	**Concentration**
		10 ng/ml			5 ng/ml
	Clenbuterol	2 ng/ml	S1 a. Exogenous anabolic androgenic steroids	Dichlormethyltestosterone	2 ng/ml
S1 a. Exogenous anabolic androgenic steroids	Methandienone	2 ng/ml		Methandienone	2 ng/ml
	Methyltestosterone	2 ng/ml		Methyltestosterone	2 ng/ml
	Stanozolol	2 ng/ml		Stanozolol	2 ng/ml
			S1.2 Other anabolic agents	Clenbuterol	0.2 ng/ml
S2. Peptide hormones, Growth factors and related substances	hCG	5 mIU/ml			
S3. β2 agonists		1oo ng/ml	S3. β2 agonists		20 ng/ml
S4. Hormone antagonists and modulators	Aromatase inhibitors, SERMs, Other anti-oestrogenic substances	50 ng/ml	S4. Hormone antagonists and modulators	Aromatase inhibitors, SERMs, Other anti-oestrogenic substances	20 ng/ml
S5. Diuretics and other masking agents	Diuretics	250 ng/ml	S5. Diuretics and other masking agents	Diuretics	200 ng/ml
S6. Stimulants		500 ng/ml	S6. Stimulants		100 ng/ml
	Strychnine	200 ng/ml		Octopamine	1000 ng/ml
		200 ng/ml			50 ng/ml
S7. Narcotics	Buprenorphine	10 ng/ml	S7. Narcotics	Buprenorphine	5 ng/ml
	Fentanyl and derivatives	10 ng/ml		Fentanyl and derivatives	2 ng/ml
			S8. Cannabimimetics		1 ng/ml
S9. Glucocorticosteroids		30 ng/ml	S9. Glucocorticosteroids		30 ng/ml
P2. Betablockers		500 ng/ml	P2. Betablockers		100 ng/ml

sensitive detection of various categories of drugs well within the requirement of WADA TD2013 MRPL guidelines with scope for inclusion of newer entities due to use of dedicated though comprehensive sample clean up and detection technique.

MATERIALS & METHODS

Chemicals and reagents

All reagents were of analytical grade or HPLC grade: acetonitrile,and ethyl acetate were purchased from Qualigens Mumbai, India. Tertiarybutyl methyl ether, and formic acid 98% were supplied by Merck, Mumbai, India. Out of the 165 compounds screened in the method, the certified reference materials of 152 drugs were available in the laboratory. The certified reference compounds were purchased mainly from National Measurement Institute (NMI, Sydney, Australia), Cerilliant (Round Rock, Texas) and Sigma-Aldrich (St. Louis, MO), or from the pharmaceutical manufacturer; several were kindly donated by other anti-doping laboratories. In some cases, where reference material was not available, urine samples obtained from drug administration studies were used. The mobile phases for UPLC were filtered through a 0.2 µm PTFE filter. Ultra high purity nitrogen was obtained from nitrogen generator plant installed at the laboratory. Water was purified using a Milli-Q water purification system installed in the laboratory (Millipore, Bedford, USA).

Solutions

Stock standard solutions of the 152 substances were prepared separately at a concentration of 1 mg/ml in suitable solvent depending on the solubility and stored at -20 ºC in glass vials fitted with PTFE caps. The standard mixtures were prepared and were spiked afresh in quality controls (QCs) every time. Allsolutions are evaluated periodically for degradation by comparing peak areas, peak area ratios and peak shapes to historical values.

System suitability standards

The internal standards (ISTDs) were used as the system suitability standards (SSS) containing six selected analytes of different molecular weights, polarities and chemical classess viz. 17-α methyltestosterone (AAS), mefruside (diuretic), formoterol d-6 (β2 agonist), *dl*-amphetamine d6 (stimulant), bupranolol (beta-blocker) and diphenylamine (stimulant). These ISTDs were added to each sample and were monitored in terms of peak area and retention time. All ISTDs were monitored in positive ionization except mefruside which was monitored in negative ionization.

Quality control samples

Drug-free urine samples were collected from 20 different volunteers divided into 2 ml aliquots and kept frozen at <10 °C in polypropylene tubes prior to use. The Quality Control (QC) samples which were run in each assay were prepared by spiking the blank urine samples (2 ml) with theworking standard mixture solutions to achieve the necessary concentration at MRPL of each category of prohibited class. Negative quality control (NQC) samples were spiked with only with the ISTDs. The ISTDs were added at the following concentration; 17-α methyltestosterone (50 ng/ml), mefruside (50 ng/ml), formoterol d-6 (30 ng/ml), dl amphetamine d6 (50 ng/ml), bupranolol (50 ng/ml) and diphenylamine (50 ng/ml).

Sample preparation

To two ml of urine sample aliquots internal standards at defined concentration were added. The urine samples were hydrolysed by β –glucuronidase *(E.coli)* enzyme at 60°C for an hour after optimizing pH 7.0 using 0.2 M phosphate buffer. Hydrolyzed samples were loaded on to the Oasis HLB cartridges pre-equilibrated with 2 ml methanol and 2 ml water. After application of the samples, the cartridges were washed with water. Elution of analytes was performed with 3 ml methanol. Samples were evaporated under a gentle nitrogen flow at 60 °C and then were reconstituted into 100 ìl of a solution of mobile phase (acetonitrile : 1 % formic acid ; 50:50 ; v/v) and transferred into conical autosampler vials for analysis.

Instrumentation

The liquid chromatographic system was Waters® Aquity UPLC equipped with degasser, binary pump,

TABLE 2: UPLCMS/MS operating conditions

UPLC	
Column	AcquityBEH C18, 2.1 mm X 100 mm X 1.7 µ
Mobile Phase	1% Formic acid (Solvent A), Acetonitrile (Solvent B)
Flow rate	300 µl/min
Gradient	95% A to 0% A in 5.00 min and then back to 95% A by 7 min followed by equilibration at 95% A for 1 min
Injection volume	5 µl
MASS SPECTROMETER	
Ionization mode	Electrospray ionization
Polarity	+/- Polarity switching (50 ms)
Ion spray voltage	+ve 5500 V -ve 4500 V
Ion source temperature	550ºC

autosampler thermostated at 5ºC and column compartment. The column employed was Acquity BEH C18, 2.1mm X 100mm X 1.7µ particle size from Waters (Millford, USA). Samples were stored at 4 °C in the autosampler prior to analysis.

The LC effluent was pumped to an Atmospheric Pressure Ionization (API) 5500 QQQ mass spectrometer (AB Sciex, Darmstadt, Germany). The ion source was operated under fast polarity switching (50 ms) electro spray ionization mode. The analytes and the ISTDs were detected utilizing multiple reaction monitoring (MRM) of diagnostic precursor-product ion transitions at dwell times of 5 ms. The instrumental conditions are depicted in TABLE 2. For optimization of the declustering potential and the collision energy solutions of pure reference compounds of each analyte were directly injected using a 1 ml syringe at flow rate of 10 ml/min. Nitrogen was used as collision gas delivered from a nitrogen generator (Anest Iwata Motherson, Japan). Target MRMs and compound dependant parameters for each analyte are listed in TABLE 3. Data acquisition, data handling, instrument control and data processing were performed using Analyst 1.5.2® Software (AB Sciex).

Method development and validation

The analytical method was developed and validated as per the WADA guidelines for the anti-doping laboratories[26]. For validation the parameters recovery percentage, specificity, ion suppression, intra and inter-day precision, LODand robustness were determined.

Recovery

The recoveries of all target compounds tested in urine were determined at the MRPL as regulated by WADA. Ten drug-free urine samples were fortified at the MRPL concentration with all of the compounds tested, another ten drug-free urine specimens were extracted according to the described SPE protocol, and all compounds tested were added to the elution solvent before evaporation. The direct standards (without extraction) corresponded to 100% recovery. Recovery was evaluated by comparing the mean peak-area ratio of the analyte and the ISTD in spiked and direct samples.

Specificity

Evaluation of specificity was carried out by analyzing six spiked (at MRPL) and six different blank urine samples collected from six different healthy volunteers to test for interfering signals in the selected MRM chromatograms at expected retention times of the analytes.The specificity was also demonstrated by studying 100 urine samples from anti-doping controls that had previously tested negative with reference methods to demonstrate that no interferences were detected at the retention time of the analytes under investigation. Amongst these few samples containing common over-the-counter medicaments, such as paracetamol, ibuprofen or salicylates were also analysed. The specific gravity of these samples ranged between 1.001-1.030.

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TABLE 3: The target MRMs & compound dependent parameters for UPLC-MS/MS analysis

Compound	Mol. Weight	Polarity	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (eV)
S1- ANABOLIC AGENTS						
16-β-OH-STANOZOLOL	344.5	+	345	81;121	40	45;45
3-OH-STANOZOLOL	344.5	+	345	97;121	40	50;45
4-β-OH-STANOZOLOL	344.5	+	345	145;269	40	45;35
STANOZOLOL	328.4	+	329	81	60	55
3-OH-PROSTANOZOLOL	328.4	+	329.3	97.1;111.2	40	40;40
16-β-OH-PROSTANOZOLOL	328.4	+	329.1	81.1;107.2	40	40;40
5β-ANDROST-1-EN-17 β-OL-3-ONE (BOLDENONE METABOLITE)	288.4	+	289	121;187	40	40;40
BOLDENONE	286.1	+	287	121;135	40	40;40
CLENBUTEROL	276.1	+	276.7	203;168	40	30;40
EPIOXANDROLONE	306.4	+	307	121;289	40	40;30
OXANDROLONE	306.2	+	307	121;289	35	30;20
EPITRENBOLONE	270.4	+	271	199;227	40	40;35
TRENBOLONE	270.4	+	271	199;227	40	40;35
FORMEBOLONE	344.4	+	347	173;147	45	45;45
GESTRINONE	308.4	+	309	241;199	45	35;35
METHYLTRIENOLONE	284.1	+	285	198;227	40	40;30
METHYLDIENOLONE	286.4	+	287.2	159.4;135.1	40	40;40
TETRAHYDROGESTRINONE	312.4	+	313	241;159	45	35;40
9α-FLURO-17,17-DIMETHYL-18 NOR-ANDROSTAN-4,13-DIENE-11β,OL-3-ONE (FLUOXYMESTERONE-MET-3)	318.4	+	319	225.2;299.3	40	35;35
M17β-HYDROXYMETHYL-17α-METHYL ANDROST-18 NOR 1, 4,13-TRIENE-3-ONE (METHANDIENONE MET-4)	298	+	299	269;135	40	20
17-B-METHYL OXANDROLONE	304	+	305.2	275.2;133.1	40	30;40
D4 ANDROSTERONE GLUCURONIDE	470.6	+	471.3	413.5;301.2	50	15;31
ISTD (17α METHYLTESTOSTERONE)	302.5	+	303	109	35	35
S3- β-2 AGONISTS						
TERBUTALINE	225.2	+	226.3	152;125.1	35	35;35
FENOTEROL	303.1	+	304	152;135	40	40;30
FORMOTEROL	344.1	+	345	327;149	40	20;40
D6-FORMOTEROL	350.4	+	351.2	155.3	40	40
S4- HORMONE AND METABOLIC MODULATORS						
AMINOGLUTETHIMIDE	232.1	+	233.1	205.3;188.1	35	20;40
ANASTRAZOL	293.1	+	294	225.1;210	40	30;35
CLOMIPHENE	405.1	+	406	100;72	40	45
HYDROXY CLOMIPHENE	421	+	422	100;72	40	45;45
3- METHOXY 4-OH CLOMIPHENE	451	+	452	100;72	45	45;45
HYDROXY EXEMESTANE	298.4	+	299	135;121	40	45;45
EXEMESTANE	296.1	+	297	149;121	40	45;45
17-KETO FULVESTRANT	604.7	+	605.4	377;587.7	50	45;45
RALOXIFENE	473.1	+	474	269;112	45	40;45
HYDROXY TAMOXIFENE	417.5	+	418	72;346.1	40	45;35
TOREMIFENE	405.1	+	406	205;72	40	40;45
α -OH-TOREMIFENE	421	+	422	386;404	45	25;15
CARBOXYTOREMIFENE	401	+	402	72;45	45	45;45
D1 HYDRACARBOXYTORMIFENE	403	+	404	72;45	45	45;45

Compound	Mol. Weight	Polarity	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (eV)
S5-DIURETICS AND OTHER MASKING AGENTS						
AMILORIDE	229	+	230	171;116	35	40;40
CANRENONE	340.4	+	341	187;107	40	35;35
SPIRONOLACTONE	416.5		341	187;107	40	35;35
PROBENECID	285.1	+	286	244;185	40	25;40
TRIAMTERENE	253.1	+	254	237;195	40	20;40
ACETAZOLAMIDE	222.2	-	221	83;142	40	50;40
BENDROFLUMETHIAZIDE	421.1	-	420	289;328	45	40;35
4-AMINO-6-CHLORO-1,3-BENZENEDISULFONAMIDE (BENDROFLUMETHIAZIDE-DEGRADATION PRODUCT-1)	287.7	-	286	207;169	40	35;40
4-AMINO 6 TRIFLURO METHYLBENZENE1,3DISULPHONAMIDE (BENDROFLUMETHIAZIDE- DEGRADATION PRODUCT -2)	319.27	-	318	214;239	45	40;40
BENZTHIAZIDE	431.9	-	430	228;308	40	45;35
BUMETANIDE	364.4	-	363	319;80	45	30;50
CHLORTHALIDONE	338.8	-	337	190;146	45	40;45
CHLORTHIAZIDE	295.7	-	294	214;179	45	30;40
CYCLOTHIAZIDE	389.8	-	388	205;269	40	40;40
ETACRYNIC ACID	303.1	-	301	243;206.9	35	40;35
EPITHIAZIDE	425.8	-	424	269;404	45	40;35
EPLERENONE	414.5	+	415	163.5;337.2	45	45;35
HYDROXY EPLERENONE	430	-	431	337;355	40	30;30
HYDROCHLOROTHIAZIDE	297.7	-	296	269	45	30
HYDROFLUMETHIAZIDE	331.2	-	330	239;302	45	35;30
INDAPAMIDE	365.8	-	364	189;132	45	40;45
METHYLCHLOTHIAZIDE	360	-	358	322	40	20
POLYTHIAZIDE	439	-	438	324.5;418	45	30;25
METOLAZONE	365.06	+	366	259;377	40	40;35
ALTHIAZIDE	383.1	-	382.2	340.8;269.2	45	26;38
CLOPAMIDE	345.1	-	344.1	167.3;77.9	47	48;52
CYCLOPENTHIAZIDE	379.2	-	378.1	205;269	40	36;38
DICLOFENAMIDE	305.1	-	305.2	240.8;77.7	39	36;48
METICRANE	275	-	274.2	210;78.1	38	38;47
TRICHLORMETHIAZIDE	380.6	-	379	242.7;307.3	42	41;26
ISTD (MEFRUSIDE)	382.9	-	381	189	45	40
S6. STIMULANTS						
2- AMINO N ETHYL PHENYL BUTANE	177	+	178	91;113.1	30	30;25
AMIPHENAZOLE	191.2	+	192	117;106	30	25;25
ADRAFINIL	289.3	-	288	121;74	45	35;45
AMPHETAMINE	135.2	+	136.1	91.1;119.1	30	30;25
BENFLUOREX	351.3	+	351.9	230;149	40	25;35
BENZOYLECGONINE	289.3	+	290	168.2;105.1	40	35;35
1-BENZYLPIPERAZINE	176.3	+	177.2	91;65	30	40;50
β -METHYL PHENYL AMINE	135	+	136.1	111;91.2	30	15;30
DIMETHYLAMPHETAMINE	163.3	+	164	91;119	30	30;25
CROPROPAMIDE	240.3	+	240.9	196.2;100.1	40	15;25
CROTETAMIDE	226.3	+	227	182;85.9	40	15;30
COCAINE	303.1	+	304	150;105	35	35;40

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Compound	Mol. Weight	Polarity	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (eV)
S6. STIMULANTS						
CYCLAZODONE	216	+	217	79.4;106	40	40;35
ETILEFRINE	181.2	+	182	135;107	35	30;35
FAMPROFAZONE	377.5	+	378	175;229	40	45;35
FENBUTRAZATE	367.5	+	368	119;234	40	45;35
FENCAMFAMINE	215.3	+	216	129;171	40	40;40
FENETYLLINE	341.4	+	342	119;181	45	40;40
FENFLURAMINE	231.2	+	232	159;109	35	18;45
HEPTAMINOL	145.2	+	146.1	128;69	30	20;25
ISOMETHEPTENE	141.1	+	142	69;55.3	30	25;20
MECLOFENOXATE	257.7	+	258	213;141	40	25;40
MEPREDINE	247.3	+	248.2	220.1;174.1	35	30;30
MEPHEDRONE	177.2	+	178	145.2;160.1	30	25;15
p-OH-MESOCARB	338	+	339	193;119	35	36;38
METHYL PHENIDTAE	233.2	+	234	84;56	45	45;45
METHYLECGONINE ESTER	181	+	182	117.9,91.2	35	30;40
N-ETHYL AMPHETAMINE	163.3	+	164	91;119	30	35;15
MODAFINIL	273	+	274	167;152	40	35;40
NORFENEFRINE	153.2	+	154	136;91.2	30	20;30
NORFENFLURAMINE	203.2	+	204	187;159	35	17;40
OCTOPAMINE	153.1	+	154	91;136	35	30;20
ORTETAMINE	149.2	+	150	105;133	30	30;20
OXILOFRINE	181.2	+	182	164;105	30	20;30
p-OH-AMPHETAMINE	151.1	+	152	135;107	35	20;30
p-METHYL AMPHETAMINE	149.2	+	150.6	133;105	30	10;15
PENTERAZOL	138.1	+	139	96;68.9	30	25;27
PHENPROMETHAMINE	149.2	+	150.2	119;90.7	30	15;15
PHOLEDRINE	165.2	+	166	107;77	30	30;35
PRENYLAMINE	329.2	+	330.1	90.9;118.9	35	30;30
PROPYLHEXEDRINE	155.3	+	157	69;84	30	25;30
RITALINIC ACID	219.2	+	220	84;56.1	40	40;40
SIBUTRAMINE	279.8	+	280.2	124.8;138.8	35	30;30
STRYCHNINE	334.41	+	335	184;264	45	40;35
TAUMINOHEPTANE	115.18	+	116	57;41	30	30;30
METHYLHEXANEAMINE	115.18	+	116	57;41	30	30;30
DIMETHYAMINOETHANOL (DMAE)	89.1	+	90	72;57	30	20;25
MECLOFENOXATE-DP-1 *p*-CHLORPHENOXYACETIC ACID (4-CPA)MECLOFENOXATE-DP-2	186.6	-	185	127;111	30	30;30
METHAMPHETAMINE	149.2	+	150	91;115		
AMFEPRAMONE	205.1	+	206.2	105.1		
MODAFINILIC ACID	274	-	273	167;105.8	35	35;35
NICOTINE	162.2	+	163	132;117	30	15;15
COTININE	176.2	+	177	143;98	30	25;30
TETRA-OH-COTININE	192	+	193	134;86	30	30;35
NICOTINE-N-OX	178	+	179	132;117	30	30;35
D6-AMPHETAMINE	141.2	+	142	125.1	30	11
DIPHENYLAMINE (ISTD)	169.2	+	170.1	93	30	30

Compound	Mol. Weight	Polarity	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (eV)
S.7- NARCOTICS						
BUPRENORPHINE	467.6	+	468	414;396.1	45	30;35
FENTANYL	336.2	+	337	105;188	40	40;40
NOR-BUPRENORPHINE	413.5	+	414.3	101;396	45	40;20
NORFENTANYL	232.3	+	233	84.2;56.1	40	40;45
D3 CODEINE	302.3	+	302.9	215.1;165.1	40	35;45
D3 CODEINE GLUCURONIDE	478	+	479.2	303.1;165.2	45	40;55
D3 MORPHINE	288	+	289	201; 152	35	35; 55
D3 MORPHINE 6 BETA GLUCURONIDE	464	+	465.2	289.1,74	45	40;55
S8-CANANBINOIDS						
JWH-122	213	+	214	169;141	35	35;40
S9-GLUCOCORTICOSTEROIDS						
20β-OH-PREDNISOLONE	362	+	363	267;345171	45	20;45
BECLOMETHASONE	408.9	+	409	391; 279	60	25; 35
BETAMETHASONE	392.1	+	393.1	373.3;337.4	45	20;25
DEXAMETHASONE	392.4	+	393.1	373.3;337.4	45	20;25
16-α-OH PREDNISOLONE	376.4	+	377	359;323	40	25;30
BUDESONIDE	430.2	+	431	173;323	50	40;35
CORTISONE	360.4	+	361	163;105	35	35;35
DESACETYLDEFLAZACORT	399.4	+	400	124;147	45	45;45
DESONIDE	416.2	+	417	399;147	40	20;40
FLUDROCORTISONE	380.1	+	381	181;105	45	40;45
FLUDROCORTISONE ACETATE	422.4	+	423	239;343	50	45;35
FLUMETHASONE	410.4	+	411	253;121	45	25;35
FLUNISOLIDE	434.5	+	435	321;121	45	35;40
FLUOCORTOLONE	376.4	+	377	303;171	45	30;40
CARBOXYFLUTICASONE	452.4	+	453	293;275	45	40;35
FLUTICASONE	500.5	+	501	293;313	45	45;40
HYDROCORTISONE	362.4	+	363	121	45	40
METHYL PREDNISOLONE	374.2	+	375	161;357	45	40;20
PREDNISOLONE	360.1	+	361	343;147	35	20;40
PREDNISONE	358.1	+	359	171;341	45	40;20
TRIAMCINOLONE ACETONIDE	434	+	435	415	45	20
TRIAMCINOLONE	394.4	+	395	375;357	45	20;30
TETRA HYDROXY CORTISOL	366	+	367	331;313	40	20,30
TETRA HYDROXY CORTISONE	364	+	365	347;329	40	20;30
P.2-BETA BLOCKERS						
ACEBUTOLOL	336.2	+	337	116;72	45	40;45
ALPRENOLOL	249.1	+	250	116;147	40	40;40
ATENOLOL	266.1	+	267	190;145	40	40;40
BETAXOLOL	307.2	+	308	121;133	40	40;40
BISOPROLOL	325.2	+	326	116;72	40	40;45
BUNOLOL	291.3	+	292	236;201	40	35;35
CARVEDILOL	406.1	+	407	100;222	45	40;35
CELIPROLOL	379.4	+	380	251;74	40	35;40
CARTEOLOL	292.1	+	293	237;202	40	30;30

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Compound	Mol. Weight	Polarity	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (eV)
ESMOLOL	295.1	+	296	219;145	40	30;40
LABETALOL	328.1	+	329	162;311	40	40;20
METIPRANOLOL	309.1	+	310	116;191	40	45;45
METOPROLOL	267.1	+	268	116;191	40	40;40
NADOLOL	309.1	+	310	254;201	45	30;40
OXPRENOLOL	265.1	+	266	72;116	40	40;35
PINDOLOL	248.1	+	249	116;172	40	35;35
PROPRANOLOL	259.1	+	260	116;183	40	40;35
SOTALOL	272.1	+	273	255;213	35	20;30
TIMOLOL	316.1	+	317	261;244	40	30;35
ISTD (BUPRANOLOL)	271.7	+	273	217	40	30
M.1- ENHANCEMENT OF OXYGEN TRANSFER						
EFAPROXIRAL METABOLITE	341.4	-	340	120;254	45	45;40

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 (10 µg/mL at a flow rate of 7 µL/min)[24].

Precision

Intra-day precision was determined at MRPL for each compound using five replicates of spiked urine samples. The corresponding inter-assay precision was calculated from samples prepared and analyzed on three different days (n=5/day). The precision of the method was determined by calculation of the coefficient of variation (CV%) of the area ratio of the ion transition of the analytes and the internal standard.

Limit of detection

The LOD was defined as the lowest concentration of analyte (S/N>3) that can be identified, measured and reported. The LOD was estimated via signal to noise ratio (S/N) of the lowest abundant MRM transition using ten blank samples and ten fortified samples at concentration levels from 2-50 % of MRPL for different compounds.

Applicability to routine doping control samples

The method was applied to fifty urine samples previously declared positive for one of the substances included in this screening using earlier method. In addition, hundred urine samples which were already reported negative by previous method were also reanalyzed.

RESULTS AND DISCUSSION

A sensitive and high-throughput screening method for the determination of 165 prohibited substances from 10 different classes viz: S1. anabolic agents including clebuterol (21), S2. β-2 agonists (03), S4. hormones and metabolic modulators (14), S5. diuretics and masking agents (30), S6. stimulants (51), S7. narcotics (04), S8. cannabinoids (01), S9.glucocorticosteroids (21), P2. beta blockers (19) and M1. Method for oxygen enhancement (01) was developed and validated for qualitative analysis.

To achieve the MRPL levels as per WADA TD2013MRPL it became necessary to have a more sensitive method. The use of UPLC-MS/MS system to achieve the high sensitivity level proved to be beneficial but at the same time it required a specific enough sample preparation method with cleaner extracts.

Sample preparation

A generic sample preparation method was developed which was able to isolate and preconcentrate analytes of different classes i.e. corti-

costeroids, anabolic steroids, β 2 agonists, diuretics, stimulants, narcotics, betablockers and hormone and metabolic modulators in one procedure from urine samples. The classical sample preparation method previously employed in the laboratory consisted of hydrolysis by β-glucuronidase (*E.coli*) followed by two step liquid-liquid extraction: extraction in TBME at pH 9-10 and extraction in ethyl acetate at pH 4[25,26]. While this method gave optimum results and was in-use in laboratory for 5 years, but few impediments were observed after the applicability of WADA TD2013MRPL which gave rise to the need of shifting sample preparation method to solid phase extraction procedure.

The approach towards sample extraction in this study was aiming to recover wide range of chemistries of the acidic, basic and neutral in one single sample. The protocol used for the SPE in this study was based on mixed mode cartridges optimized to provide best extraction recoveries. Several mixed mode cartridges were tested. Higher extraction yields were obtained with Oasis HLB cartridges as compared with other cartridges. The pH dependence of the recoveries of acidic, basic and neutral drugs was examined to set the condition of charging the sample into the column. It was found that an effective recovery of all the compounds could be achieved while keeping the sample pH neutral prior to loading the sample on cartridge. The greatest impediment of SPE of urine samples is the high rate of column blockage experienced during sample extraction. Sample blockage could result in the loss of significant throughput. Centrifugation of urine sample prior to sample preparation has beenused to overcome this problem.

In addition, the method uses only 2 ml of urine volume for the analysis of 165 drugs from 10 different categories. In general the volumeis 2–4 times lower than the volume normally used for screening of such high number of drugs in routine doping control procedures. This is useful since in doping control a limited amount of urine is available for screening and confirmation of a wide range of substances. The effectiveness of the de-conjugation step was evaluated in every sample by monitoring the signal of D3-codeine glucuronide and D3-6β morphine glucuronide, together with their deconjugation products D3-codeine and D3-6β morphine. ISTDs were selected to correct random or systematic errors in the positive and negative ESI modes. Stable isotopically labeled standards are generally preferred for biological matrices (especially inquantitative assays), as they are structurally similar to the analyteand eluted at similar retention time. However, since this study was based on qualitative analysis the use of isotopically labeled internal standards was not a mandate. Hence, the choice of internal standards from various categories viz. 17-α methyltestosterone (AAS), mefruside (diuretic), formoterol d-6 (β2 agonist), dl amphetamine d6 (stimulant), bupranolol (beta-blocker) and diphenylamine (stimulant) aided in keeping a quality check on system suitability.

Chromatographic conditions

UPLC improves chromatographic resolution, speed and sensitivity, and when coupled to fast scanning mass spectrometry, facilitates rapid, high-throughput analysis. Therefore, a UPLC system with Acquity BEH C18, 2.1mm X 100mm X 1.7µ column was used. The chromatographic optimizations allowed to separate and detect a mixture of 165 doping substances within 8 min. Majority of compounds were basic (68%) (e.g., stimulants,β-blockers) followed by acidic substances (22%) (e.g., diuretics, stimulants) with the remainder being neutral analytes (e.g., few diuretics,anti-estrogens).

The chromatographic conditions were chosen in an appropriate way and were found to be compatible with the API source. The choice for the mobile phase was adopted from the previous screening method employed in the laboratory[25-26]. A gradient starting at 95% aqueous buffer (1% Formic acid, pH 3.5) was required to ensure sufficient retention for hydrophilic compounds. To avoid column blockage a pre-column has been used. Over 1500 analyses were conducted with the same analytical column without any loss in chromatographic performance. In the beginning of the gradient, mostly amphoteric compounds with low logD values (log P at defined pH) eluted, reflecting their hydrophilicity. Peak shapes were generally good although some splitting of peaks was observed in the beginning of the gradient. Compounds with the same retention time were readily identified by their mass spectrum.

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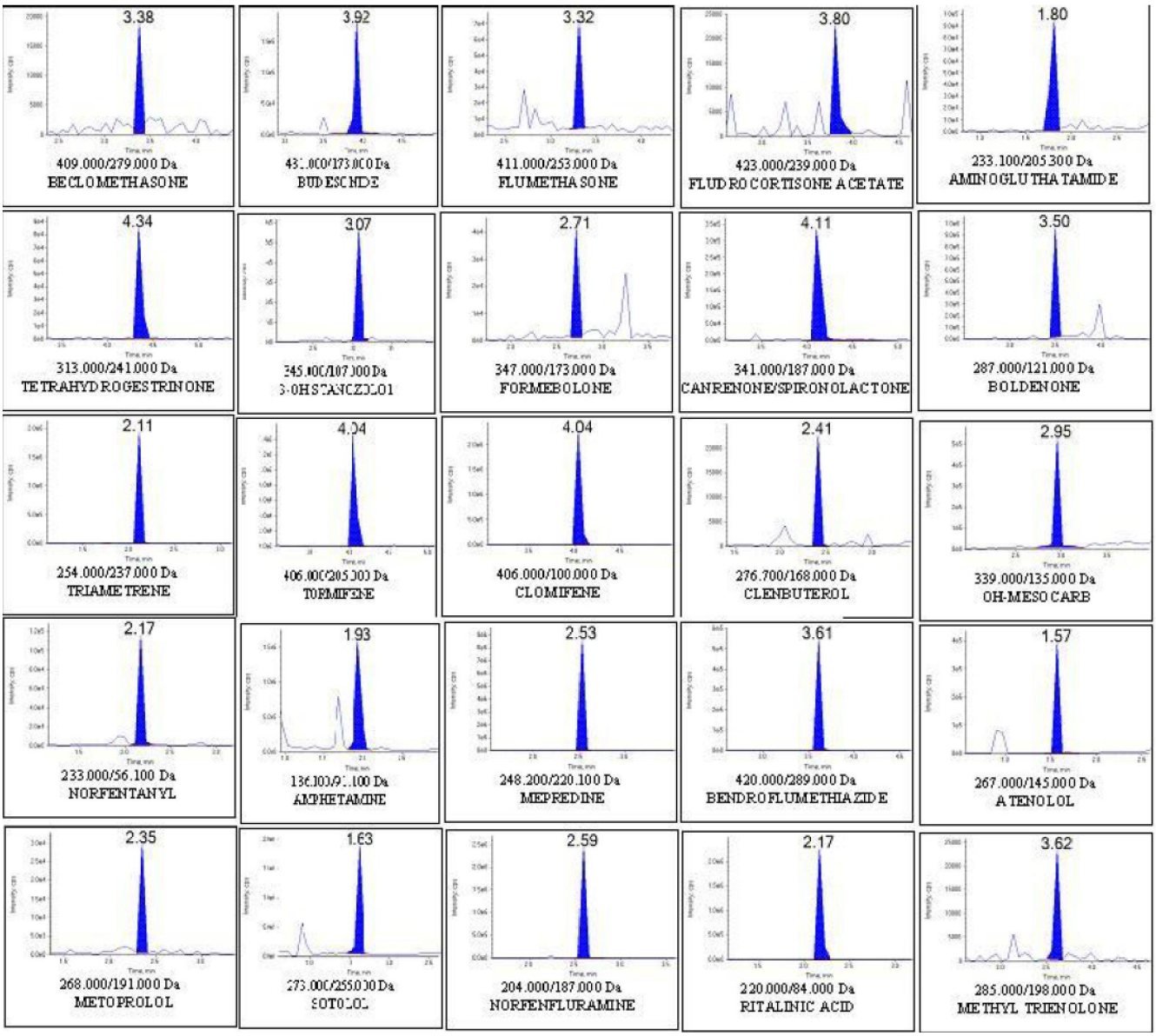

Figure 1 : Multiple reaction monitoring (MRM) ion chromatogram peaks of various analytes showing identification capability of the method

Mass spectrometry

For mass spectrometric method development individual standard solutions of all drugs were optimized in both positive and negative ESI modes. As expected, higher signal intensity was obtained in positive ESI mode for basic and neutral compounds and, negative ionization mode was preferred for acidic molecules. The method was optimized to detect the drugs, using the MRM pair comprising of precursor and product ions. The product ions (Q3) were obtained during the collision of the precursor ions (Q1) in collision cell (Q0). The corresponding retention times, MS and MS/MS spectra were then used to obtain structural information. All the compounds showed good sensitivity and were separated in 8 minutes of runtime. Analyte identification in the screening step was based both on retention time (*t*R) and m/z of a diagnostic ion (MRM).

The capability of inclusion of fast polarity switching (50 ms) in the method ensured an optimized ionization, of acidic or basic analytes in the same analytical run. While 142 analytes were detected in positive ionization mode mainly as protonated molecule $[M+H]^+$, 23 analytes were detected in negative ionization mode as deprotonated molecule $[M-H]^-$.

Method validation

The screening method developed in the study aimed at qualitative analysis. The identification was based on the compound's chromatographic and mass spectrometric properties. For every batch of urine samples, cleaning of curtain plate with methanol was performed before sample analysis. A QC sample was injected at the beginning and end of the analytical sequence to verify that the analytical process was in control. No significant change in sensitivity was observed between the two QC sample injections throughout this study, indicating that the method is robust for routine use. No remarkable variation in results of inter day, intraday & inter personal studies was observed, confirming that the method is sufficiently reliable and reproducible.

Specificity

The evaluation of specificity in six different spiked and six different blank urine samples showed no interfering signals in the selected MRM chromatograms at expected retention times of the analytes. Moreover, the analysis of hundred independent negative urine samples allowed extensive evaluation of the specificity of the method. Co-elutions with endogenous substances were observed for prednisolone, boldenone, methyldeinolone, formebolone, amphetamine, oxiloferine and methylphenidate. Therefore, more specific fragments were obtained for these analytes and were incorporated in the method.

Identification capacity

All the 165 compounds showed good identification capacity yielding good peak shapes with maximum possible number of dwell times. The effect of dwell time on sensitivity has been reported by Herrin et al.[27]. The longer dwell times led tobetter sensitivity, although the gain in sensitivity was moderate beyond 20ms. The pitfall of longer dwell times resulted in a longer duty cycle time that had a negative impact onchromatographic data points, particularly for the narrow peaks. All 165target compounds could be easily detected with sufficient sensitivity at the dwell time of 5 ms which was used in subsequent MRM experiments (Figure 1).

Ion suppression/ion enhancement

Ion-suppression/ion enhancement, sometimes referred to as matrix effect, is acommon problem in API mass spectrometry[28,29]. No significant ion suppression or enhancement was observed on six different blank urines which were analyzed with continuous co-infusion of the target analytes (10 µg/mL at a flow rate of 7 µL/min) via T connector.

Carryover

Carry-over, which is the appearance of an analyte signal in ablank (drug-free extracted matrix) injection subsequent to analysis of high concentration samples, is a common problem in LC–MS/MS methods[30,31]. This problem occurs due toretention of analytes by adsorption on active surfaces of the auto injector system, solvent lines, SPE, or the analytical column. The carryover is also dependenton the type of analyte and the dynamic range of an assay. Hence, the issue becomes exacerbated after the injection of an analyte at high concentrations. The carry-over effect was evaluated by injecting the analytes spiked in urine at 1 µg/ml, followed by injection of three blank samples. The carry over effect of less than 0.5% in the first blank sample was demonstrated by the following analytes viz.benfluorex, fencamfamine, fenethylline and timolol, but no analyte was found in the subsequent blankurine samples. Therefore, it was concluded that if any of the targeted analyteis found to be positive in two subsequent samples (if ≤ 1% of peak area than in the preceding sample), they shouldbe re-injected with two urine blanks in between.

Precision

For intra-day precision the relative peak area responses for samples spiked at the MRPL level and ana-

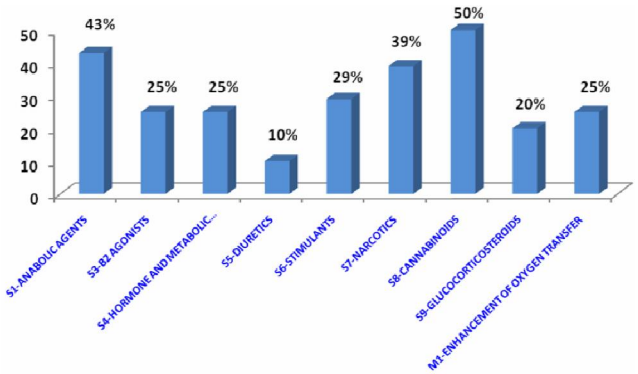

Figure 2 : Limit of detection (LOD) % as compared to WADA MRPL of various categories of banned drugs.

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TABLE 4: Method validation results showing recovery%, precision and LOD

Compound	Target conc. (ng/ml)	LOD (s/n>3)	Mean recovery at target concentration	Recovery %	Intraday Precision* (CV %)	Interday Precision* (CV %)	RRT (CV %)
16 β-OH-STANOZOLOL	2	0.5	1.9	95	7.6	9.3	0.08
3-OH-STANOZOLOL	2	0.8	1.4	70	5.8	6.5	0.22
4 β-OH-STANOZOLOL	2	1	1.1	55	5.1	8.5	0.52
STANOZOLOL	2	1	1.6	80	6.1	9.5	0.11
5 β-ANDROST-1-EN-17 β-OL-3-ONE (BOLDENONE METABOLITE)	5	2	5.1	102	5.4	7.4	0.41
BOLDENONE	5	2	6.2	124	7.1	9.5	0.56
CLENBUTEROL	0.2	0.1	0.2	100	2.2	4.1	0.11
EPIOXANDROLONE	5	2.5	5	100	3.6	5.9	0.21
OXANDROLONE	5	2.5	4	80	4.5	5.2	0.25
EPITRENBOLONE	5	2.5	3	60	8.5	9.1	0.52
TRENBOLONE	5	2.5	4	80	7.4	5.6	0.41
FORMEBOLONE	5	2	3	60	4.8	6.5	0.14
GESTRINONE	5	2	3	60	5.8	6.2	0.41
METHYL TRIENOLONE	5	2	4	80	7.1	8.5	0.46
METHYLDIENOLONE	5	2	4	80	5.6	6.5	0.65
TETRAHYDROGESTRINONE	5	2	4	80	5.9	9.1	0.25
9α-FLURO-17,17-DIMETHYL-18 NOR-ANDROSTAN-4,13-DIENE-11β,OL-3-ONE (FLUOXYMESTERONE METABOLITE-3)	5	1.5	4	80	6.5	8.2	0.45
AMINOGLUTETHIMIDE	20	5	15	75	5.4	8.5	0.26
ANASTRAZOL	20	5	16	80	7.4	8.6	0.36
CLOMIPHENE	20	5	12	60	8.5	6.3	0.54
4-OH CLOMIPHENE	20	5	20	100	5.3	6.2	0.41
EXEMESTANE	20	5	24	120	5.5	6.8	0.65
HYDROXY EXEMESTANE	20	5	10	50	8.5	4.2	0.23
FENOTEROL	20	5	6	120	5.4	6.8	0.25
FORMOTEROL	20	5	17	85	5.6	6.1	0.25
TERBUTALINE	20	5	18	90	7.8	8.9	0.54
17-KETO FULVESTRANT	20	5	22	110	5.2	8.1	0.26
RALOXIFENE	20	5	14	70	5.3	6.5	0.35
OH-TAMOXIFENE	20	5	11	55	5.6	9.5	0.41
TORMIFENE	20	5	10	50	5.5	8.5	0.65
EFAPROXIRAL	20	5	16	80	8.5	9.6	0.65
AMILORIDE	200	20	156	78	7.4	8.5	0.23
CANRENONE	200	20	186	93	5.6	9.6	0.58
SPIRONOLACTONE	200	20	186	93	4.8	8.5	0.21
METOLAZONE	200	20	164	82	5.4	7.8	0.35
PROBENECID	200	20	138	69	4.5	6.3	0.69
TRIAMTERENE	200	20	122	61	8.5	9.5	0.32
ACETAZOLAMIDE	200	20	130	65	9.4	9.5	0.22
BENDROFLUMETHIAZIDE	200	20	84	42	6.8	9.6	0.45
BENZTHIAZIDE	200	20	144	72	7.8	8.5	0.85
BUMETANIDE	200	20	193	97	5.6	8.5	0.82

Compound	Target conc. (ng/ml)	LOD (s/n>3)	Mean recovery at target concentration	Recovery %	Intraday Precision* (CV %)	Interday Precision* (CV %)	RRT (CV %)
CHLORTHALIDONE	200	20	122	61	5.5	7.4	0.41
CHLOROTHIAZIDE	200	20	188	94	8.6	9.6	0.12
CYCLOTHIAZIDE	200	20	174	87	4.5	5.8	0.47
ETACRYNIC ACID	200	25	190	95	5.9	6.7	0.25
EPITHIAZIDE	200	20	168	84	5.4	6.8	0.48
EPLERENONE	200	20	184	92	8.9	9.7	0.78
FUROSEMIDE	200	20	91	46	5.5	8.6	0.54
HYDROCHLOROTHIAZIDE	200	25	76	38	6.5	6.3	0.26
HYDROFLUMETHIAZIDE	200	20	80	40	5.8	7.8	0.57
INDAPAMIDE	200	25	86	43	8.5	8.4	0.41
METHYLCHLORTHIAZIDE	200	20	158	79	6.5	6.6	0.21
METOLAZONE	200	20	108	54	7.8	8.5	0.21
POLYTHIAZIDE	200	20	194	97	4.8	7.4	0.54
ALTHIAZIDE	200	20	186	93	7.5	9.8	0.54
CLOPAMIDE	200	20	154	77	5.8	7.6	0.86
CYCLOPENTHIAZIDE	200	20	168	84	4.8	9.6	0.54
DICLOFENAMIDE	200	20	196	98	8.6	9.6	0.57
METICRANE	200	20	148	74	8.7	8.9	0.45
TRICHLORMETHIAZIDE	200	20	152	76	9.6	8.4	0.87
AMIPHENAZOLE	100	50	86	86	6.3	6.6	0.21
2 AMINO N ETHYL PHENYL BUTANE	100	30	84	84	7.5	7.9	0.47
ADRAFINIL	100	20	50	50	5.4	8.6	0.21
AMPHETAMINE	100	30	68	68	5.4	6.8	0.45
BENFLUOREX	100	30	54	54	8.6	11.3	0.58
BENZOYLECGONINE	100	20	60	60	5.8	6.9	0.54
1-BENZYLPIPERAZINE	100	20	72	72	8.4	9.4	0.25
β METHYL PHENYL AMINE	100	20	80	80	8.4	9.4	0.85
DIMETHYLAMPHETAMINE	100	20	55	55	8.5	9.6	0.24
CROPROPAMIDE	100	20	65	65	5.4	6.5	0.52
CROTETAMIDE	100	20	66	66	7.4	7.5	0.25
COCAINE	100	20	78	78	6.4	8.3	0.24
CYCLAZODONE	100	20	74	74	6.9	9.6	0.35
ETILEFRINE	100	50	32	32	5.7	7.9	0.45
ETILAMPHETAMINE	100	20	78	78	8.9	9.6	0.47
FAMPROFAZONE	100	10	60	60	5.8	6.9	0.86
FENBUTRAZATE	100	10	76	76	7.5	8.2	0.56
FENCAMFAMINE	100	10	55	55	8.6	9.5	0.65
FENETYLLINE	100	20	48	48	6.5	8.9	0.57
FENFLURAMINE	100	20	65	65	6.6	7.5	0.52
HEPTAMINOL	100	50	35	35	7.8	9.9	0.47
ISOMETHEPTENE	100	20	45	45	8.5	9.6	0.54
MECLOFENOXATE	100	50	34	34	5.2	8.6	0.25
MEPRIDINE	100	20	65	65	8.4	9.4	0.58

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Compound	Target conc. (ng/ml)	LOD (s/n>3)	Mean recovery at target concentration	Recovery %	Intraday Precision* (CV %)	Interday Precision* (CV %)	RRT (CV %)
MEPHEDRONE	100	25	84	84	9.5	8.6	0.58
P-OH-MESOCARB	100	25	77	77	6.4	8.6	0.25
METHLPHENIDATE	100	50	81	81	9.6	9.4	0.58
METHYLECGONINE	100	25	31	31	8.5	9.4	0.63
N-ETHYL AMPHETAMINE	100	50	78	78	4.5	8.6	0.14
MODAFINIL	100	25	55	55	6.7	9.4	0.58
NORFENEFRINE	100	50	28	28	8.5	8.6	0.25
NORFENFLURAMINE	100	30	51	51	6.8	9.4	0.47
OCTOPAMINE	100	50	25	25	7.9	8.8	0.89
ORTETAMINE	100	25	92	92	8.5	8.9	0.58
OXILOFRINE	100	25	28	28	6.5	9.6	0.65
P-OH-AMPHETAMINE	100	25	24	24	7.8	9.9	0.47
PARA METHYL AMPHETAMINE	100	50	118	118	8.5	9.7	0.54
PENTERAZOL	100	50	74	74	8.5	8.3	0.78
PHENPROMETHAMINE	100	25	55	55	6.5	9.2	0.54
PHOLEDRINE	100	25	39	39	10	10	0.12
PRENYLAMINE	100	25	69	69	5.6	6.4	0.68
PROPYLHEXEDRINE	100	20	49	49	9.6	9.5	0.87
RITALINIC ACID	100	50	51	51	6.9	10.4	0.21
SIBUTRAMINE	100	50	48	48	7.8	8.9	0.47
STRYCHNINE	100	10	97	97	5.8	9.5	0.98
TAUMINOHEPTANE	100	20	87	87	7.4	8.5	0.51
METHYLHEXANEAMINE	100	20	87	87	8.5	9.7	0.54
METHAMPHETAMINE	100	20	65	65	5.4	8.8	0.58
AMFEPRAMONE	100	25	55	55	9.5	8.8	0.24
BUPRENORPHINE	5	2	4	80	4.8	5.6	0.58
FENTANYL	2	1	2	100	8.6	9.6	0.58
NOR-BUPRENORPHINE	5	2	3	60	7.8	8.9	0.57
NORFENTANYL	2	0.5	2	100	8.5	9.5	0.21
JWH-122	1	0.5	0.5	50	8.5	9.6	0.24
20β-OH-PREDNISOLONE	30	5	27	90	4.5	5.6	0.28
BECLOMETHASONE	30	5	24	80	4.1	6.5	0.51
BETAMETHASONE	30	5	18	60	5.2	6.1	0.65
DEXAAMETHASONE	30	5	18	60	5.6	6.2	0.21
16-α-OH-PREDNSIOLONE	30	10	27	90	8.5	9.6	0.54
BUDESONIDE	30	10	21	70	7.4	8.5	0.54
DES ACETYL DEFLAZACORT	30	5	18	60	6.5	9.4	0.21
DESONIDE	30	5	24	80	9.6	9.5	0.54
FLUDROCORTISONE	30	5	27	90	6.5	8.5	0.54
FLUDROCORTISONE ACETATE	30	5	24	80	6.8	9.6	0.47
FLUMETHASONE	30	5	30	100	2.3	4.6	0.36
FLUNISOLIDE	30	5	21	70	8.5	9.3	0.24

Compound	Target conc. (ng/ml)	LOD (s/n>3)	Mean recovery at target concentration	Recovery %	Intraday Precision* (CV %)	Interday Precision* (CV %)	RRT (CV %)
FLUOCORTOLONE	30	5	27	90	6.5	9.5	0.12
CARBOXYFLUTICASONE	30	5	27	90	5.4	7.8	0.59
METHYL PREDNISOLONE	30	5	15	50	7.5	8.6	0.54
PREDNISOLONE	30	10	18	60	9.6	9.5	0.25
PREDNISONE	30	10	18	60	6.5	9.4	0.54
TRIAMCINOLONE ACETONIDE	30	5	24	80	4.6	6.5	0.21
TRIAMCINOLONE	30	5	15	50	3.6	4.5	0.25
ACEBUTOLOL	100	20	94	94	4.5	5.2	0.28
ALPRENOLOL	100	20	79	79	5.8	9.6	0.54
ATENOLOL	100	25	45	45	6.5	8.4	0.21
BETAXOLOL	100	20	94	94	6.9	8.5	0.58
BISOPROLOL	100	20	62	62	2.8	4.5	0.58
BUNOLOL	100	20	65	65	6.9	8.7	0.47
CARVEDILOL	100	20	64	64	8.7	8.9	0.74
CELIPROLOL	100	20	89	89	6.5	4.5	0.54
CARTEOLOL	100	20	89	89	6.9	8.7	0.54
ESMOLOL	100	20	85	85	5.8	9.7	0.57
LABETALOL	100	20	84	84	3.9	5.4	0.85
METIPRANOLOL	100	20	77	77	6.8	9.5	7.8
METOPROLOL	100	20	60	60	6.1	5.2	0.21
NADOLOL	100	20	38	38	7.1	8.4	0.47
OXPRENOLOL	100	20	73	73	6.5	5.4	0.74
PINDOLOL	100	20	85	85	6.8	8.5	0.47
PROPRANOLOL	100	20	85	85	7.6	8.5	0.22
SOTALOL	100	25	35	35	4.5	2.4	0.54
TIMOLOL	100	20	96	96	1.2	3.5	0.14

***Interday & intraday precision estimated at the target concentration of each analyte.**

lyzed via the screening method showed CV% values ranging from 1.2% (timolol) to 10% (pholedrine). While for inter-day precision the CV%ranged from 2.4% (sotalol) to 11.3% (benfluorex) (TABLE 4). The results indicate that the method has acceptable limits of repeatability and reproducibility for day-to-day screening analysis. This is an important aspect while proceeding for confirmation of the suspicious sample.

Recovery

The recovery for all compounds studied ranged from 25% (octopamine) to124% (boldenone). A wide variation in extraction recoveries is to be expected considering that the analytes under study are a combination of various chemistries like aliphatic, aryl, phenolic. alkaloids, phenanthreine, piprazine, thiazide, with varying functional groups like OH, NH_2, CO, COOH etc. It is notably important that the compounds showing lower recovery (25-60%) could also be detected at equal to or less than 50 % of MRPL level (TABLE 4).

Limit of detection (LOD)

The LOD estimated in ten fortified samples at concentration levels from 2-100 % of MRPL for different compounds was found satisfactory. It was possible to achieve the LOD ranging between 10-50% of MRPL values of different analyes (Figure 2). For compounds in which recovery was found to be below 50%, the LOD was found much below the MRPL yielding a good detection capability (TABLE 3). The developed method

satisfied WADA's criteria in terms of sensitivity for all the 165 compounds studied.

Relative retention time

In order to use *t*R as a parameter for LC behavior, it was importantto demonstrate its reliability. The relative retention times (RRTs) were observed in three consecutive batches and the precision was determined by calculation of the coefficient of variation (CV %). During this period, the preparation of fresh mobile phases and maintenance of the ESI source was performed daily. It was found that the CV % did not exceed 1% for any of the compound (TABLE 4). Therefore, *t*R was accepted as a relevant and reliable identification criterion for the analytes. The I.S.S were monitored in each sample to detect variance in LC performance, sensitivity or *t*R variations.

Applicability to routine doping control samples

The suitability of the developed method for target analysis was proven by analyzing 50 urine samples previously declared positive for substances included in the screening method. No false negative samples were found. However, these samples showed good sensitivity and identification capability towards the analyte of interest (Figure 1). Furthermore, the testing of 100 urine samples screened as negative earlier, using previous method were screened as negative using the present method. The inclusion of two MRM transitions in the screening method further ensured to improve the specificity.

The MS source cleaning was performed every 100 urine samples. The pre-column was changed after 1500 injections, and the column was replaced after 3000 injections based on daily SSS monitoring. The current method takes only 8 min. of runtime to analyze 1 sample against the 16 min runtime of the traditional method. This has significantly improved the throughput where 90 samples could be detected in 12 hours against 45samples per 12 hours using the old method. This method was thus considered beneficial in terms of analysis time, cost effectiveness, resources and requisite guidelines.

In comparison to the earlier screening procedure a real gain in time was obtained since the sample treatment was very fast, and the screening of the 165 analytes was performed using a single generic method. It has been in routine use for more than 6months involving the analysis of over 1500 urine samples. Only one UPLC columns was needed for this period. Use of the old screening procedure has been discontinued after running both methods in parallel for 1 month.

CONCLUSION

The experiments presented in this work were based on UPLC-MS/MS. A fast, generic and sensitive method was developed for the analysis of 165 compounds achieving LOD between10 to 50% of WADA MRPL. The method was validated according to the International Standard for Laboratories (ISL) described in the World Anti-Doping Code and was selective enough to comply with the World Anti-Doping Agency recommendations. The developed method could be of significant use in bioanalytical, forensic & pharmaceutical & clinical analysis.

REFERENCES

[1] The World Anti-Doping Code:The 2013 Prohibited List. World Anti-Doping Agency Montreal, Canada.http://www.wada-ama.org. Accessed 7.01.13.

[2] WADA International Standards for Laboratories (ISL), Version 7, World Anti-Doping Agency Montreal, Canada. http://www.wada-ama.org/documents/world_anti-doping_program/wadp-islaboratories/isl/wada_int_standard_laboratories_2012_en.pdf. Accessed 7.01.13

[3] L.Rivier; Criteria for the identification of compounds by liquid chromatography–mass spectrometry and liquid chromatography–multiple mass spectrometry in forensic toxicology and doping analysis. Anal ChimActa, **492**, 69-82 **(2003)**.

[4] G.J.Trout, R.Kazlauskas; Sports Drug testing—an analyst's perspective. Chem.Soc.Rev, **33**, 1–13 **(2004)**.

[5] M.Tsivou, N.Kioukia-Fougia, E.Lyris, Y.Aggelis, A.Fragkaki, X.Kiousi, P.Simitsek, H.Dimopoulou, I.P.Leontiou, M.Stamou, M.H.Spyridaki, C.Georgakopoulos; An overview of the doping control analysis during the Olympic Games of 2004 in Athens, Greece. Anal.Chim.Acta., **555**, 1–13 **(2006)**.

[6] R.Mueller, J.Grosse, R.Lang, D.Thieme; Chromatographic techniques—the basis of doping control. J.Chromatogr A, **674**, 1–11 **(1995)**.

[7] M.Donike, H.Geyer, A.Gotzmann, M.Kraft, F.Mandel, E.Nolteernsting, G.Opfermann, G.Sigmund, W.Sch¨anzer, J.Zimmermann; In: P.Bellotti, G.Benzi, A.Ljungqvist (Eds.), Official Proceedings—International Athletic Foundation World Symposium on Doping in Sport, Florence, May 10–14, 1987, International Athletic Foundation, Italy, 53 **(1988)**.

[8] M.Kolmonen, A.Leinonen, A.Pelander, I.Ojanperä; A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/time-of-flight mass spectrometry. Anal.Chim.Acta., **585**, 94-102 **(2007)**.

[9] C.Goebel, G.J.Trout, R.Kazlauskas; Rapid screening method for diuretics in doping control using automated solid phase extraction and liquid chromatography-electrospray tandem mass spectrometry. Anal.Chim.Acta., **502**, 65-74 **(2004)**.

[10] M.W.F.Nielen, C.T.Elliott, S.A.Boyd, D.Courtheyn, M.L.Essers, H.H.Hooijerink; van Bennekom EO, Fuchs RE: Identification of an unknown β-agonist in feed by liquid chromatography/bioassay/quadrupole time-of-flight tandem mass spectrometry with accurate mass measurement. Rapid.Commun. Mass.Spectrom, **17**, 1633-1641 **(2003)**.

[11] A.Leinonen, T.Kuuranne, T.Kotiaho, R.Kostiainen; Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography–electrospray ionization tandem mass spectrometry. Steroids, **69**, 101-109 **(2004)**.

[12] M.Thevis, W.Schänzer; Current role of LC–MS (/MS) in doping control. Anal.Bioanal.Chem., **388**, 1351-1358 **(2007)**.

[13] D.Guillarme, D.T.T.Nguyen, S.Rudaz, J.L.Veuthey; Recent developments in Liquid Chromatography: Impact on qualitative and quantitative performance. J.Chromatogr.A, **1149**, 20-29 **(2007)**.

[14] D.T.T.Nguyen, D.Guillarme, S.Rudaz, J.L.Veuthey; Fast analysis in liquid chromatography using small particles size and ultra high pressure. J.Sep.Sci., **29**, 1836-1848 **(2006)**.

[15] J.O.Thörngren, F.Östervall, M.Garle; A high-throughput multi-component screening method for diuretics, masking agents, central nervous system (CNS) stimulants and opiates in human urine by UPLC–MS/MS. J.Mass.Spectrom, **43**, 980-992 **(2008)**.

[16] R.Ventura; High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. Eur.J.Mass.Spectrom., **14**, 191-200 **(2008)**.

[17] R.Ventura, M.Roig, N.Montfort, P.Sáez, R.Bergés, J.Segura; High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. Eur J Mass Spectrom (Chichester, Eng), **14(3)**, 191-200 **(2008)**.

[18] S.Guddat, E.Solymos, A.Orlovius, A.Thomas, G.Sigmund, H.Geyer, M.Thevis, Schänzer; High-throughput screening for various classes of doping agents using a new dilute-and-shoot'liquid chromatography-tandem mass spectrometry multi-target approach. Drug Test Anal, **3**, 836–850 **(2011)**.

[19] K.Deventer, O.J.Pozo, P.V.Eenoo, F.T.Delbeke; Qualitative detection of diuretics and acidic metabolites of other doping agents in human urine by high-performance liquid chromatography–tandem mass spectrometry: Comparison between liquid–liquid extraction and direct injection. J.Chromatogr.A, **1216(31)**, 5819-5827 **(2009)**.

[20] Y.Moulard, Bailly- L.Chouriberry, S.Boyer, P.Garcia, M.A.Popot, Y.Bonnaire; Use of benchtopexactive high resolution and high mass accuracy orbitrap mass spectrometer for screening in horse doping control. Anal.Chim.Acta700/1-2, **26-36**, 1873-4324 **(2011)**.

[21] C.Goebel, G.J.Trout, R.Kazlauskas; Rapid screening method for diuretics in doping control using automated solid phase extraction and liquid chromatography-electrospray mass Spectrometry. Anal.Chim.Acta., **502**, 65-74 **(2004)**.

[22] M.Kolmonen, A.Leinonen, A.Pelander, I.Ojanper; A general screening method for doping agents in human urine by solid phase extraction and liquid chromatography/time-of-flight mass spectrometry. Anal.Chim.Acta., **585**, 94–102 **(2007)**.

[23] WADA Technical Document TD2013MRPL. World Anti-Doping Agency: Montreal, Canada, 2012. http://www.wada-ama.org.Accessed 7.01.13

[24] T.M.Annesley; Ion suppression in mass spectrometry. Clin.Chem., **49**, 1041-1044 **(2003)**.

[25] I.Reddy Madhusudhana, A.Beotra, S.Jain, Ahi; A simple and rapid ESI-LC-MS/MS method for simultaneous screening of doping agents in urine samples. Indian J.Pharmacol., **41-2**, 80-86 **(2009)**.

[26] S.Ahi, A.Beotra, Jain; Detection of mono-hydroxylated metabolites of stanozolol by HPLC-ESI (+) MS/MS in Indian sports persons. Drug Test Anal, **11-12**, 538-44 **(2009)**.

[27] G.L.Herrin, H.H.McCurdy, W.H.Wall; Investigation of an LC-MS-MS (QTrap) method for the rapid

screening and identification of drugs in post-mortem toxicology whole blood samples. J.Anal. Toxico., **29**, 599-606 **(2005)**.

[28] B.K.Matuszewski; Standard line slopes as a measure of a matrix effect in quantitative HPLC–MS bioanalysis. J.ChromatogrB8, **30-2**, 293-300 **(2006)**.

[29] X.S.Tong, J.Wang, S.Zheng, J.V.Pivnichny, P.R.Griffin, X.Shen; Effect of signal interference from dosing excipients on pharmacokinetic screening of drug candidates by liquid chromatography/ mass spectrometry. Anal.Chem., **74**, 6305-6313 **(2002)**.

[30] P.T.Vallano, S.B.Shugarts, E.J.Woolf, B.K.Matusz-_ewski; Elimination of autosampler carryover in a bioanalytical HPLC–MS/MS method: A case study. J.Pharmaceut.Biomed, **36**, 1073-1078 **(2005)**.

[31] W.Zeng, D.G.Musson, A.L.Fisher, A.Q.Wang; A new approach for evaluating carryover and its influence on quantitation in highperformance liquid chromatography and tandem mass spectrometry assay. Rapid.Commun.Mass.Spectrom, **20**, 635-640 **(2006)**.