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Implementation of gas chromatography combined with simultaneously selected ion monitoring and full scan mass spectrometry in doping analysis

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ABSTRACT

A comprehensive screening method for the detection of prohibited substances in doping control is described and validated. This method is capable of detecting over 150 components mentioned on the list of the World Anti-Doping Agency including anabolic androgenic steroids, stimulants and all narcotic agents that are currently analysed using different analytical methods. The analytes are extracted from urine by a combined extraction procedure using freshly distilled diethyl ether and *tert*-butyl methyl ether as extraction solvents at pH 9.5 and 14 respectively. Prior to GC–MS analysis the residues are combined and derivatised using a mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, NH_4I and ethanethiol. The mass spectrometer is simultaneously operated in the full scan mode (mass range varies along with GC-oven temperature program) and in the selected ion monitoring mode. The obtained limits of detection are in compliance with the requirements set by the World Anti-Doping Agency. Besides narcotics, stimulants and anabolic androgenic agents, this method is also capable of detecting several agents with anti-estrogenic activity and some beta-agonists. This comprehensive screening method reduces the amount of urine needed and increases the sample throughput without a loss in sensitivity and selectivity.

1. Introduction

The goal of doping control is to preserve the "spirit of sport", characterised by several values such as ethics, fair play and health. Therefore, the World Anti-Doping Agency (WADA) has established a list of prohibited substances including anabolic agents, stimulants and narcotics [1]. Hyphenated chromatographic techniques, i.e. GC–MS and LC–MS, play a major role in the detection of doping agents in urine samples after selective extraction, and eventual derivatisation. While GC–MS played an important role in doping control for several decades, over the last years LC–MS has gained importance and several groups of doping agents (e.g. corticosteroids, diuretics, anabolic agents) are detected using this technique [2–4]. Nevertheless, GC–MS still plays an important role in doping control, especially for the detection of anabolic androgenic steroids (AASs) after selective derivatisation [5–7] as ionisation in LC–MS is not optimal for several AASs lacking a keto-moiety [8]. Other GC-screening methods are capable of detecting narcotic agents and stimulants. While in the past GC with nitrogen–phosphorus detection (NPD) was often used to determine volatile nitrogen-containing stimulants such as amphetamines [9–11], a comprehensive GC–MS screening method is now available for the simultaneous detection of narcotic agents and stimulants using *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) as a derivatisation agent [12]. The use of this derivatisation agent is mandatory as numerous underivatised stimulants show poor mass spectra. Moreover, the availability of mass spectra of suspicious peaks offers a substantial improvement compared to analytical methods relying on NPD because the unequivocal identification of suspicious substances should be achieved by the combination of retention time and mass spectrum [13]. As the minimum required performance limits (MRPLs) for narcotic agents and stimulants are relatively high [14] (200 and 500 ng/ml, respectively) the mass spectrometer can be operated in the full scan mode for these substances. On the other hand, the current screening method for the determination of anabolic androgenic steroids, including the determination of the steroid profile, has to be performed in the selected ion monitoring mode (SIM) as the concentrations in urine are in the low nanogram/ml range. As a consequence, WADA has set the MRPL for anabolic androgenic steroids at 10 ng/ml excepting epitestosterone and the metabolites of methandienone, stanozolol, methyltestosterone (all 2 ng/ml) and norandrosterone (1 ng/ml) [14]. Besides the difference in mass spectrometric settings, both analytical methods (i.e. method for anabolising agents and method for narcotics/stimulants) have some common features. Hydrolysis is mandatory to remove the glucuronide moieties attached to several narcotic agents and anabolic androgenic steroids during

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phase II metabolism and subsequent extraction is performed at alkaline pH. In addition both methods rely on a derivatisation procedure using MSTFA as primary constituent prior to injection on the chromatographic system. The introduction of high performance electronics in the newest generation of single quadrupole mass spectrometers allows a faster data transfer and a higher scan rate. In addition, this high data transfer rate allows the alternating acquisition of SIM and scan data in a single run. This alternating acquisition offers the opportunity of combining two current screening methods in one comprehensive screening method. Therefore, the goal of this study was to explore the possibilities of combined SIM/scan data acquisition in doping control in order to improve productivity and reduce total analytical run time.

2. Experimental

2.1. Chemicals and reagents

Morphine, oxymorphone, buprenorphine, 11-nor-Δ^9-tetrahydrocannabinol carboxylic acid (THC-COOH), 6-monoacetylmorphine (6-MAM) and EDDP ((±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate, metabolite of methadone) were purchased from Cerilliant (Round Rock, TX, USA). Codeine, fenoterol, clenbuterol, etilefrine and pemoline were a gift from Boehringer-Ingelheim (Brussels, Belgium). Hydromorphone, ethylmorphine, ephedrine HCl, heroin (diacetylmorphine), dextromoramide, oxycodone, fentanyl, pethidine, dimethylamphetamine HCl, mephentermine sulphate, phendimetrazine HCl, testosterone, 11β-hydroxyandrosterone (11β-OH-androsterone), 11β-hydroxyetiocholanolone (11β-OH-etiocholanolone), 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5β-androstane-3α,17β-diol, androsterone, amiloride, epitestosterone, 4-androstene-3,17-dione and methadone were obtained from Sigma (Bornem, Belgium). Normethadone was obtained from Bios-Coutelier (Brussels), pentazocine from Whintrop Laboratories (Newcastle, UK), dehydroepiandrosterone (DHEA) from Serva (Heidelberg, Germany) and dihydrotestosterone (DHT) from Piette International Labs. (Drogenbos, Belgium). 3α-Hydroxytibolone (tibolone metabolite) was obtained from Akzo Nobel (Oss, The Netherlands).

Bambuterol, methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA) and methylenedioxymethylamphetamine (MDMA) were a kind gift from the Portuguese doping control laboratory. 17α-Methyltestosterone was provided by the Deutsche Sporthochschule (Cologne, Germany). Fencamfamine HCl, norephedrine HCl, norpseudoephedrine HCl (cathine), pseudoephedrine HCl and methamphetamine HCl were purchased from Merck (Darmstadt, Germany), pipradrol HCl from Merrell-Dow (Cincinnati, OH, USA) and amphetamine sulphate, salmeterol xinafoate and triamterene from GlaxoSmithKline (Philadelphia, PA, USA). Phenmetrazine and prolintane HCl were a gift from Boehringer & Sohn (Ingelheim am Rhein, Germany). Heptaminol HCl was purchased from Ets. A De Bournonville (Braine L'Alleud, Belgium), norfenfluramine HCl from Eutherapie Benelux (Brussels), 5-hydroxypentoxyfylline (5-OH-pentoxyfylline) from Hoechst (Frankfurt, Germany) and fenfluramine HCl, amineptine, amineptine C5-metabolite (amineptine M-C5) and fenspiride HCl from Laboratories Servier (Orleans, France). Methylephedrine HCl was obtained from Laboratoire G.A. (Cochard, France), phentermine HCl from Certa Noville (Mehaigne, Belgium), nikethamide and methylphenidate from Ciba-Geigy (Groot-Bijgaarden, Belgium) and mefenorex from Produits Roche (Brussels). Chlorphentermine HCl was donated by Tropon (Cologne, Germany). Isopropylhexedrine was obtained from Veride (Diegem, Belgium) and ethylamphetamine HCl from Will-Pharma Benelux (Brussels). Crotethamide, cropropamide, 6-hydroxybromantan (6-OH-bromantan), carphedon, 7β,17α-dimethyl-5β-androstane-3α,17β-diol (calusterone metabolite), zilpaterol, letrozole metabolite and benzylpiperazine were purchased from NMI (Pymble, Australia). Pholedrine was from Knoll (Ludwigshaven, Germany), fenethylline from Chemiwerk Hamburg (Germany), etamivan from Sinclair Pharmaceuticals (Godalmings, UK) and benzoylecgonine from Lipomed (Arlesheim, Switzerland). Furfenorex and clobenzorex were obtained from Roussel Uclaf (Romainville, France), methoxyphenamine and benzphetamine from Upjohn (Kalamazoo, USA), amfepramone from Lab. Pharm. R.H. Trenker (Brussels, Belgium), dimefline from Recordate Industria Chemica & Farmaceutica (Milan, Italy), lidocaine from Astra Chemicals (Brussels, Belgium), propoxyphen from Park Davis (Bornem, Belgium) and formoterol from Novartis (Arnhem, The Netherlands). Aminogluthetimide was purchased from European Pharmacopeia (Strasbourg, France) and cyclopentamine was from Eli Lilly (Brussels, Belgium). Phenprometamine and excretion urines of the aromatase inhibitors clomiphene, cyclofenyl, tamoxifen, anastrazole, and letrozole as well as from the stimulants prolintane, sibutramine, isometheptene and amfepramone were obtained after the controlled administration of a therapeutic dose and provided by other doping control laboratories, the World Association of Anti-Doping Scientists (WAADS), the International Olympic Committee (IOC) and WADA.

3′-Hydroxystanozolol (stanozolol metabolite), 16β-hydroxyfurazabol (furazabol metabolite), 4-chloro-17α-methyl-androsta-1,4-diene-6β,17β-diol-3-one (oral turinabol metabolite), 6β-hydroxymetandienone (6β-OH-metandienone), 2-hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one (formebolone metabolite), 1-testosterone, 5β-androst-1-ene-17β-ol-3-one (boldenone metabolite), 5α-androst-1-ene-3,17-dione (1-androstenedione), 4-hydroxy-19-nortestosterone (4-OH-nandrolone), 4-hydroxyandrost-4-ene-3,17-dione (4-OH-androstenedione), 2α-methyl-5α-androstane-3α-ol-17-one (drostanolone metabolite), 1α-methyl-5α-androstane-3α-ol-17-one (mesterolone metabolite), 19-noretiocholanolone, 19-norandrosterone, 7β-hydroxydehydroepiandrosterone (7β-OH-DHEA), 17β-methyl-5β-androst-1-ene-3α,17α-diol (epimetenediol), 9-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol (fluoxymesterone tetrol), 17α-methyl-5α-androstane-3α,17β-diol (α-methyltestosterone metabolite), 17α-methyl-5β-androstane-3α,17β-diol (β-methyltestosterone metabolite), 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane (norboletone metabolite 1), 17α-ethyl-5β-estrane-3α,17β-diol (norethandrolone metabolite), 7α,17α-dimethyl-5β-androstane-3α,17β-diol (bolasterone metabolite), 13β,17α-diethyl-3α,17β-dihydroxy-5β-gonane (norboletone metabolite 2) and 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (fluoxymesterone dimethyl metabolite) were purchased from NMI. 6α-Hydroxyandrost-4-ene-3,17-dione (6α-OH-androstenedione), mibolerone, etiocholanolone, norclostebol and 1(5α)-androstene-3α,17β-diol (1-androstenediol) were purchased from Steraloids (Newport, RI, USA). Boldenone, 1-methylen-5α-androstan-3α-ol-17-one (metenolone metabolite), oxymesterone and 4-chloro-3α-hydroxyandrost-4-ene-17-one (clostebol metabolite) were obtained from Institut für biochemie (DSHS, Cologne, Germany). 17α-Trenbolone, salbutamol and zeranol were purchased from RIVM (Zeist, The Netherlands). Ethisterone, oxandrolone and 3α,5α-tetrahydronorethisterone (norethisterone metabolite) were kind gifts from Winthrop, Laboratório de Análises e Dopagem (Instituto do Desporto, Lisbon, Portugal), Searle & Co. (Chicago, IL, USA), Institute of Organic Chemistry and Biochemistry (Academic of Sciences of the Czech Republic, Prague, Czech Republic), respec-

tively. Anastrazole was from Astra Zeneca (Macclesfield, UK) and terbutaline from Draco (Lund, Sweden).

MSTFA was purchased from Chem. Fabrik Karl Bucher (Waldstedt, Germany) and the enzyme preparation β-glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostics (Mannheim, Germany). *tert*-Butyl methyl ether (TBME) was purchased from Biosolve (Valkenswaard, The Netherlands), Methanol (MeOH) from Fisher Scientific (Loughborough, UK) and potassium hydroxide (KOH), disodium hydrogenphosphate (Na_2HPO_4), sodium dihydrogenphosphate (NaH_2PO_4), ammonia (NH_3) (25%, H_2O), ammonium chloride (NH_4Cl), sodium chloride (NaCl) and sodium sulphate (Na_2SO_4) were all from Merck.

Ammonium buffer was prepared by the addition of 25% (v/v) NH_3 to a saturated NH_4Cl solution until pH 9.5. The phosphate buffer (pH 7) was prepared by dissolving 7.1 g $Na_2HPO_4{\cdot}2H_2O$ and 1.4 g $NaH_2PO_4{\cdot}H_2O$ in 100 ml water.

2.2. GC–MS conditions

The GC/MS analysis is carried out on an Agilent 5975 mass spectrometer directly coupled to an Agilent 6870 gas chromatograph equipped with a J&W-Ultra 1 column with a length of 17 m, internal diameter of 0.2 mm and a film thickness of 0.11 μm. The GC system is operated in constant flow mode at a flow rate of 0.6 ml/min. For GC separation the oven temperature starts at 70 °C, set at 90 °C/min to 100 °C (held for 5 min) after which the oven is set at 30 °C/min to 180 °C and consecutively set at 3 °C/min to 232 °C (held for 0 min). Finally the oven is set at 40 °C/min to 310 °C and the oven is held isothermally at 310 °C for 3 min.

Half a microliter is injected in the splitless mode. The split valve remains closed during the whole analysis. The mass spectrometer is operated in the SIM/scan mode. Scan parameters are as follows: 2.3 min → 10 min: *m/z* 50–390 (4.15 scans/s); 10 min → 16.5 min: *m/z* 50–525 (3.06 scans/s); 16.5 min → 25.78 min: *m/z* 50–650 (2.46 scans/s).

The sampling rate was set at 2 and the threshold value was set at 0.

In the SIM mode 15 groups were created with a dwell time for the individual ions of 10 ms (scan rate 3.42–24.32 scans/s). For each component one or more diagnostic ions were selected (Table 1). The SIM group which they are part of is indicated between brackets in the fourth column of Table 1.

2.3. Urine extraction

Extraction was performed with 4 ml of urine divided in aliquots of 3 and 1 ml for the extraction of the conjugated and non-conjugated components, respectively. One ml of phosphate buffer (pH 7), 50 μl of β-glucuronidase (*E. coli* K12) and 50 μl of the internal standard 17α-methyltestosterone (2 μg/ml, MeOH) were added to the aliquot of 3 ml of urine after which the sample is hydrolysed overnight at 42 °C. After the hydrolysate was made alkaline with 1-ml ammonium buffer (pH 9.5) and extraction was performed with 5 ml of freshly distilled diethyl ether by rolling for 20 min. Following centrifugation (1200 × *g*, 5 min) the organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated under oxygen-free nitrogen at 40 °C. To the aliquot of 1-ml urine, 50 μl of the internal standard cyclopentamine (100 μg/ml), 1 ml of KOH (5 M, pH 14), 1 g of NaCl and 1 ml of TBME were added. After rolling for 20 min and centrifugation (1200 × *g*, 5 min) the organic layer was added to the residue of the extraction performed at pH 9.5 and evaporated under oxygen-free nitrogen at room temperature. The final residue was derivatised with 100 μl MSTFA/NH_4I/ethanethiol (640/1/2; v/w/v) for 1 h at 80 °C.

2.4. Method validation

The analytical method validation was performed according Eurachem guidelines [15] and was divided in a qualitative and a quantitative part.

For the qualitative part, the limits of detection (LODs) were determined by spiking 10 different urine samples with reference mixtures at different levels in concentrations varying from 50 to 200% of the WADA MRPL level. The LOD was defined as the lowest concentration where a substance can be detected in all analysed samples ($n = 10$). Repeatability was assessed through the analysis of multiple samples spiked at different levels during the determination of the LOD. Selectivity and specificity were tested by the analysis of a reference mixture of corticosteroids ($n = 20$) and 10 different blank urine samples. The corticosteroids used are those described by Deventer and Delbeke [4].

A quantitative method validation was performed for the substances that are part of the urinary steroid profile and the substances with a urinary threshold level. An equal weighted linear calibration curve (not forced through zero) was established in the concentration range suitable for each component (Table 3) by plotting the relative abundances of the most intense ion of each component (quantitation ion) to the ion 301 of the internal standard 17α-methyltestosterone. Therefore, steroid-free urine was prepared by pouring blank urine over a preconditioned XAD2 column. Afterwards this urine was checked for the absence of endogenous steroids and subsequently spiked at 5 different levels with methanolic solutions of the reference substances. Each concentration was analysed in triplicate and all points were used to construct the calibration curve (least sum of squares). The precision was evaluated by the determination of the repeatability and the reproducibility. To measure the repeatability 6 samples at three different concentrations (lowest, medium and highest concentrations of the calibration curve) were analysed. The reproducibility was examined by analysing three times 6 samples at the same concentrations as for the repeatability. This was done by different analysts at different times. The trueness was calculated in terms of percentage as the difference between the obtained average value and the true value. A maximal value of 20% was allowed for the trueness.

A one-point calibration procedure was evaluated by the analysis of 21 samples fortified at the levels mentioned in Table 4. The first sample was used for the one-point calibration. The results of the other 20 samples were used to determine average, trueness, standard deviation and relative standard deviation (RSD). Confirmation limits were determined by subtracting three times the standard deviation from the obtained average concentration.

3. Results and discussion

As a result of the introduction of high performance electronics in GC–MS, faster data transfer is achieved and scan rates up to 10,000 units/s can be reached. Consequently, more data points can be acquired per chromatographic peak allowing improved peak integration. In addition it allows the alternative acquisition of SIM and scan spectra in one analytical run. While SIM offers a higher sensitivity, unknown peaks can now be identified using the scan trace in combination with commercial or self-developed libraries.

The combination of SIM and scan in a single run could be useful in doping control since it could allow the combination of multiple methods into one single method. However, the MRPLs for prohibited substances in doping control are at the cutting-edge of analytical technology and hence such a combination can only be used if there is no significant loss of sensitivity,

Table 1
Retention times (t_R), relative retention times (RRT), diagnostic ions and LODs of the investigated components

	t_R (min)	RRT	SIM/scan	Diagnostic ions (m/z)	LOD (ng/ml)
Stimulants (MRPL 500 ng/ml)					
Dimethylamphetamine	3.58	0.176	Scan	72; 91; 148	250
Mephentermine	3.78	0.186	Scan	72; 91; 148	250
Cyclopentamine (IS)	4.58	0.225	Scan	130; 198; 116	nd
Amphetamine	4.98	0.245	Scan	116; 192; 91; 100	250
Norfenfluramine	5.15	0.254	Scan	116; 260; 159	250
Isopropylhexedrine	6.20	0.305	Scan	130; 212; 116	250
Phentermine	6.32	0.311	Scan	91; 114; 130; 154	250
Phenprometamine	6.35	0.313	Scan	116; 206	250
Methamphetamine	6.38	0.314	Scan	130; 206; 91	250
Heptaminol	6.86	0.338	Scan	116; 274; 131	250
Methylephedrine	6.90	0.340	Scan	72; 236; 102	250
Phendimetrazine	7.03	0.346	Scan	85; 57; 191; 70	250
Fenfluramine	7.19	0.354	Scan	144; 159; 288	250
Ethylamphetamine	7.24	0.356	Scan	144; 220; 91	250
Cathine	7.42	0.365	Scan	116; 147; 280	250
Norephedrine	7.48	0.368	Scan	116; 147; 280	250
Nikethamide	7.49	0.369	Scan	106; 177; 78; 51	500
Methoxyphenamine	7.80	0.384	Scan	236; 130; 91	250
Amfepramone	7.83	0.385	Scan	277; 233	–
Mefenorex	7.88	0.388	Scan	120; 91; 56	250
Ephedrine	7.92	0.390	Scan	130; 147; 249	250
Chlorophentermine	7.92	0.390	Scan	130; 114; 240	250
Pseudoephedrine	7.98	0.393	Scan	130; 147; 249	250
Prolintane	8.18	0.403	Scan	91; 126; 127; 174	250
MDA	8.20	0.404	Scan	116; 236; 135	250
Phenmetrazine	8.30	0.409	Scan	100; 249; 234	250
Crothetamide	8.39	0.413	Scan	69; 86; 154; 181	250
Benzylpiperazine	8.40	0.413	Scan	91; 102; 157; 248	100
Furfenorex	8.41	0.414	Scan	81; 138; 53	250
Fencamfamine	8.44	0.415	Scan	215; 186; 98	250
Propoxyphen artefact	8.52	0.419	Scan	91; 193; 208; 115	100
MDMA	8.58	0.422	Scan	130; 250; 73	250
Cropropamide	8.64	0.425	Scan	100; 168; 69; 195	250
Pholedrine	8.74	0.430	Scan	130; 294; 179	250
Lidocaine	8.84	0.435	Scan	86; 220; 235	100
MDEA	9.05	0.445	Scan	144; 264; 135	250
Benzphetamine	9.22	0.454	Scan	65; 91; 148	250
Methylphenidate	9.45	0.465	Scan	156; 118; 280	250
Etilefrine	9.90	0.487	Scan	130; 147; 382	1000
Ethamivan	10.02	0.493	Scan	193; 264; 223; 295	250
Pemoline	10.28	0.506	Scan	178; 392; 163	250
Carphedon	10.45	0.514	Scan	272; 257; 104	250
Isometheptene metabolite 1	11.15	0.549	Scan	115; 130; 286	EU
Isometheptene metabolite 2	11.30	0.556	Scan	130; 286; 301	EU
Clobenzorex	11.36	0.559	Scan	240; 316; 125; 91	250
Pipradrol	11.38	0.560	Scan	84; 165; 239	250
N-Ethylaminopropiophenone	11.65	0.574	Scan	144; 234; 243	EU
N-Ethylnorpseudoephedrine	12.33	0.607	Scan	144; 207; 308	EU
Benzoylecgonine	12.6	0.620	Scan	86; 240; 361	–
Prolintane metabolite 5a	15.20	0.748	Scan	117; 184; 304	EU
5-OH-Pentoxyphylline	15.24	0.750	Scan	337; 352; 237; 181	250
Nor-sibutramine	15.35	0.756	Scan	102; 158; 238	EU
Prolintane metabolite 5b	15.40	0.758	Scan	117; 184; 304	EU
Fenspiride	15.48	0.762	Scan	241; 317; 105; 154	250
D-Hydroxy-nor-sibutramine	15.60	0.768	Scan	156; 246; 376	EU
L-Hydroxy-nor-sibutramine	15.70	0.773	Scan	156; 246; 376	EU
Prolintane metabolite 9	15.80	0.778	Scan	138; 228; 304	EU
Prolintane metabolite 14	16.80	0.827	Scan	140; 179; 322	EU
6-OH-Bromantan	17.48	0.860	Scan	395; 393; 91	100
Dimefline	18.22	0.897	Scan	279; 323; 163; 308	250
Fentanyl	19.03	0.937	Scan	245; 189; 146	100
Amineptine M-C5	21.65	1.066	Scan	192; 115; 178; 218	250
Amineptine	22.12	1.089	Scan	192; 115; 178; 218	250
Fenethylline	22.32	1.099	Scan	322; 220; 250; 91	250
Agents with anti-estrogenic activity (no MRPL defined)					
Letrozole	12.70	0.625	Scan	291; 217; 190	25
Aminogluthetimide	14.37	0.707	Scan	491; 520; 505	500
Anastrazole	16.20	0.797	Scan	209; 224; 293	EU
Hydroxy-bis-desacetyl-cyclofenyl	19.30	0.950	Scan	343; 422; 512	EU
Hydroxy-methoxy-tamoxifen	19.85	0.977	Scan	58; 72; 489	EU
Hydroxy-clomiphene	20.48	1.008	Scan	58; 86; 100	EU

Table 1 (*Continued*)

	t_R (min)	RRT	SIM/scan	Diagnostic ions (m/z)	LOD (ng/ml)
Narcotic agents (MRPL 200 ng/ml)					
Pethidine	8.88	0.437	Scan	71; 178; 247; 218	100
EDDP	10.45	0.514	Scan	277; 262; 220	–
Normethadone	10.95	0.539	Scan	58; 72; 165; 224	100
Methadone	12.34	0.607	Scan	72; 296; 85	100
Pentazocine	12.72	0.626	Scan	245; 357; 289; 342	100
Codeine	14.84	0.730	Scan	371; 234; 178; 196	100
Ethylmorphine	15.42	0.759	Scan	385; 357; 234; 192	100
Hydromorphone	15.75	0.775	Scan	429; 414; 234	100
Oxycodone	15.88	0.782	Scan	459; 444; 368; 297	100
Morphine	16.02	0.789	Scan	429; 414; 236	100
6-MAM	16.58	0.816	Scan	339; 340; 287; 204	100
Oxymorphone-tris-TMS	16.90	0.832	Scan	517; 502; 355; 412	100
Heroin	17.28	0.851	Scan	369; 327; 310; 268	–
Oxymorphone-bis-TMS	17.40	0.857	Scan	445; 430; 517; 357	100
Dextromoramide	21.88	1.077	Scan	265; 128; 100; 165	100
Buprenorphine	24.12	1.187	Scan	554; 506; 173; 438	100
β_2-Agonists (no MRPL defined)					
Terbutaline	9.98	0.491	SIM (1)	86; 356	50
Salbutamol	10.45	0.514	SIM (1)	86; 369; 440	250
Bambuterol	17.42	0.857	Scan	86; 354; 72; 439	250
Fenoterol	20.48	1.008	Scan	322; 412; 356; 236	–
Formoterol	22.10	1.088	Scan	383; 349; 265	500
Salmeterol	24.12	1.187	SIM (15)	334; 369	100
Anabolic agents (MRPL 10 ng/ml)					
Clenbuterol	11.05	0.544	SIM (1)	86; 300; 335; 337	1
Zilpaterol	13.32	0.656	SIM (2)	98; 308; 291; 405	20
19-Norandrosterone	15.10	0.743	SIM (3)	420; 405; 315; 225	1
Epimetenediol	15.28	0.752	SIM (3)	143; 216; 358	1
Boldenone metabolite	15.30	0.753	SIM (3)	194; 417; 432	5
19-Noretiocholanolone	15.90	0.783	SIM (4)	420; 405; 315; 225	1
Androsterone	16.35	0.805	SIM (5)	434; 419	nd
Etiocholanolone	16.50	0.812	SIM (5)	434; 419	nd
5α-Androstane-3α,17β-diol	16.65	0.820	SIM (5)	241; 421; 129	nd
5β-Androstane-3α,17β-diol	16.75	0.825	SIM (5)	241; 421; 129	nd
Norethisteron metabolite	16.90	0.832	SIM (5)	431; 446; 343	10
Drostanolone metabolite	16.92	0.833	SIM (5)	169; 343; 433; 448	5
1-Androstenedione	17.38	0.856	SIM (6)	415; 430	5
Metenolone metabolite	17.40	0.857	SIM (6)	341; 431; 446	5
DHEA	17.52	0.862	SIM (6)	432; 417; 327	nd
1-Testosterone	17.70	0.871	SIM (7)	194, 432; 206	5
Fluoxymesterone dimethyl metabolite	17.70	0.871	SIM (7)	462; 208	5
1-Androstenediol	17.80	0.876	SIM (7)	434; 143; 405	1
α-Methyltestosterone metabolite	17.82	0.877	SIM (7)	143; 435; 270; 255	1
β-Methyltestosterone metabolite	17.94	0.883	SIM (7)	143; 435; 270; 255	1
Epitestosterone	17.95	0.884	SIM (7)	432; 417	1
5α-Androstane-3β,17β-diol	17.95	0.884	SIM (7)	241; 421; 129	nd
17α-Trenbolone	18.05	0.889	SIM (7)	307; 412	10
7β-OH-DHEA	18.15	0.893	SIM (7)	430; 415; 169	10
DHT	18.18	0.895	SIM (7)	434; 419; 405	nd
Boldenone	18.30	0.901	SIM (8)	206; 415; 430	5
4-Androstene-3,17-dione	18.40	0.906	SIM (8)	430; 415	nd
Tibolone metabolite	18.60	0.916	SIM (8)	443; 353; 143	5
Testosterone	18.70	0.921	SIM (8)	432; 417	nd
Mesterolone metabolite	18.71	0.921	SIM (8)	169; 343; 433; 448	5
Calusterone metabolite	18.80	0.925	SIM (8)	143; 374; 284	–
11β-OH-Androsterone	19.00	0.935	SIM (9)	522; 417	nd
Norethandrolone metabolite	19.18	0.944	SIM (9)	157; 331; 421	5
11β-OH-Etiocholanolone	19.18	0.944	SIM (9)	522; 417	nd
Bolasterone metabolite	19.20	0.945	SIM (9)	143; 374; 284	5
Clostebol metabolite	19.35	0.952	SIM (9)	466; 451; 169; 431	5
Mibolerone	19.78	0.974	SIM (9)	301; 446; 356	5
Norboletone metabolite 1	20.10	0.989	SIM (10)	157; 435; 144	5
17α-Methyltestosterone (IS)	20.32	1.000	SIM (10)	446; 301	nd
Ethisterone	20.65	1.016	SIM (11)	456; 441; 157	5
Oxandrolone	20.70	1.019	SIM (11)	308; 321; 363	–
Norboletone metabolite 2	20.92	1.030	SIM (11)	157; 435; 144	5
4-OH-Nandrolone	21.10	1.039	SIM (11)	506; 147	5
6α-OH-Androstenedione	21.25	1.046	SIM (12)	518; 503; 413	5
Zeranol	21.52	1.059	SIM (12)	433; 523; 538	5
4-OH-Androstenedione	21.54	1.060	SIM (12)	518; 147; 503	10
Norclostebol	21.64	1.065	SIM (12)	452; 454	5
Fluoxymesterone tetrol	21.70	1.068	SIM (12)	143; 462; 552; 642	–
6β-OH-Metandienone	21.92	1.079	SIM (12)	517; 532; 143	5

Table 1 (*Continued*)

	t_R (min)	RRT	SIM/scan	Diagnostic ions (*m/z*)	LOD (ng/ml)
Oxymesterone	22.25	1.095	SIM (13)	534; 389	5
Oral-turinabol metabolite	22.50	1.108	SIM (13)	315; 317; 143	10
Oxandrolone-S-artefact	22.60	1.112	SIM (13)	510; 481; 143	10
Formebolone metabolite	22.78	1.121	SIM (13)	143; 361; 439	–
Furazabol metabolite	23.06	1.135	SIM (14)	218; 231; 143	10
Stanozolol metabolite	23.22	1.143	SIM (14)	254; 545; 560	20
Diuretics (MRPL 250 ng/ml)					
Amiloride	12.32	0.606	Scan	388; 390; 298; 117	250
Triamterene	21.38	1.052	Scan	459; 469; 382; 171	125
Cannabinoids (MRPL 15 ng/ml)					
THC-COOH	20.40	1.004	SIM (10)	371; 473; 488	7,5

nd: not determined; EU: excretion urine; (–): no LOD obtained.

Table 2
Overview of all critical parameters in the presented method compared to previous methodology

	Old methodology		**New methodology**
Parameter	SIM method	Scan	**SIM/scan method**
Target components	Anabolic steroids	Narcotics/stimulants	**Anabolic steroids/narcotics/stimulants**
Urine volume	2 ml	3 ml / 1 ml	**3 ml / 1 ml**
Enzymatic hydrolysis	2,5 h , 56°C	Overnight , 42°C / /	**Overnight , 42°C / /**
Extraction buffer	Carbonate buffer pH 9.2	Ammonium buffer pH 9.5 / KOH, 1N, pH14	**Ammonium buffer pH 9.5 / KOH, 1N, pH14**
Extraction solvent	Diethylether	CH_2Cl_2/MeOH (9/1) / TBME	**Diethylether / TBME**
Extraction time	20 min	20 min / 60 min	**20 min / 20 min**
		Combination of extracts	**Combination of extracts**
Derivatisation reagent	MSTFA/NH_4I/ethanethiol (320/1/2, v/w/v)	MSTFA	**MSTFA/NH_4I/ethanethiol (640/1/2, v/w/v)**
GC oven Temp Progr.	120°C (0 min), 60°C/min → 183°C (0 min), 3°C/min → 232°C (0 min), 40°C/min → 310°C (3 min)	60°C (0 min), 90°C/min → 100°C (5 min), 20°C/min → 300°C (3 min)	**70°C (0 min), 90°C/min → 100°C (5min), 30°C/min → 180°C (0min), 3°C/min → 232°C (0 min), 40°C/min → 310°C (3 min)**
Total runtime	22.33 min	18.44 min	**25.8 min**
MS mode	SIM	Scan	**SIM/scan**

as claimed by the manufacturers. In order to check this claim 17α-methyltestosterone-bis-TMS, an internal standard frequently used in screening methods for the detection of anabolic androgenic steroids [5,16], was analysed with both settings of the mass spectrometer. As can be seen in Fig. 1, SIM/scan analysis results only in a relatively small loss of sensitivity (<16%) compared to results obtained in a SIM method proving the possibilities of SIM/scan.

Table 3
Results of the quantitative method validation

Component	Calibration range (ng/ml)	R^2	Trueness (%)	Repeatability (%)	Reproducibility (%)
Androsterone	300–4800	0.997	81.95–106.08	1.78–10.85	9.90–11.92
Etiocholanolone	300–4800	0.998	82.10–103,19	2.05–8.67	7.68–18.33
Testosterone	25–400	0.998	85.70–107.58	1.23–8.00	5.23–8.11
Epitestosterone	25–400	0.999	93,32–111.47	1.77–5.57	5.57–12.57
5α-Androstane-3α,17β-diol	25–400	0.998	91.59–106.99	2.84–14.18	11.12–14.06
5β-Androstane-3α,17β-diol	25–400	0.999	89.49–102.40	2.29–9.39	8.94–16.62
5α-Androstane-3β,17α-diol	25–400	0.998	95.01–105.95	2.05–9.82	6.23–8.01
DHEA	25–400	0.999	86.57–104.55	1.30–7.20	7.46–13.33
DHT	25–400	0.999	90.75–111.77	2.44–9.37	5.07–15.03
4-Androstene-3,17-dione	25–400	0.999	87.48–109.08	0.80–7.53	10.17–14.83
11β-OH-Androsterone	250–4000	0.998	81.88–112.00	1.59–12.71	9.60–17.60
11β-OH-Etiocholanolone	250–4000	0.999	89.79–108.44	2.21–9.34	5.21–17.46
Morphine	250–2000	0.985	92.69–101.60	2.39–10.57	22.77–37.86
THC-COOH	5–100	–	–	–	–
Salbutamol	250–2000	–	–	–	–

Table 4
Results of one-point calibration

	Andro	Etio	T	E	11β-OH-Andro	11β-OH-Etio	DHEA	DHT	Adion	T/E
Theoretic concentration (ng/ml)	1200	1200	50	50	1000	1000	100	100	100	1
Average concentration (ng/ml)	1020.71	1015.41	51.37	51.92	951.37	937.21	97.91	110.73	114.55	0.99
SD (ng/ml)	104.78	105.30	2.14	2.60	53.18	57.41	4.00	6.88	13.25	0.02
RSD (%)	10.27	10.37	4.17	5.01	5.59	6.13	4.08	6.21	5.79	1.79
RSD_{max} (%)	10.38	10.38	16.74	16.74	10.67	10.67	15.08	15.08	15.08	–
Trueness (%)	−14.94	−15.38	2.74	3.85	−4.86	−6.28	−2.09	10.73	14.55	−1.02
Lower limit (ng/ml) (=average − 3 × SD)	706.37	699.50	44.94	44.12	791.83	764.98	85.92	90.10	74.79	0.94
Upper limit (ng/ml) (=average + 3 × SD)	1335.05	1331.32	57.79	59.73	1110.91	1109.44	109.90	131.37	154.31	1.04

	5α-Asten-3α,17β-diol/ 5β-Asten-3α,17β-diol	DHT/Epi	Andro/Etio	Adion/Epi	Salbutamol	Morfine	THC	19-Norandrosterone
Theoretic concentration (ng/ml)	1	2	1	2	500	1000	15	2
Average concentration (ng/ml)	0.99	2.13	1.01	2.20	599.08	1130.16	16.75	1.88
SD (ng/ml)	0.02	0.05	0.01	0.18	47.67	72.23	1.94	0.12
RSD (%)	1.92	2.47	0.77	8.38	7.96	6.39	11.56	6.50
RSD_{max} (%)	–	–	–	–	11.84	10.67	20.07	27.18
Trueness (%)	−1.35	6.59	0.55	10.10	19.82	13.02	11.65	−5.92
Lower limit (ng/ml) (=average − 3 × SD)	0.93	1.98	0.98	1.66	456.06	913.45	12.57	1.51
Upper limit (ng/ml) (=average + 3 × SD)	1.04	2.29	1.03	2.74	742.09	1346.86	20.37	2.25

T = testosterone, E = epitestosterone, Andro = androsterone, Etio = etiocholanolone, Adion = 4-androstene-3,17-dione.

At present, GC–MS plays an important role in doping control for the detection of anabolic androgenic steroids, narcotic agents and stimulants. Since the development of an analytical method combining the detection of narcotic agents and (volatile) stimulants [12] two screening methods were used, i.e. a SIM (anabolic steroids) and a scan (narcotic agents/stimulants) method. As both methods require hydrolysis, alkaline extraction and derivatisation theoretically they should be suitable for the combination in one analytical SIM/scan method.

An analytical problem however is derivatisation. While in the previous methodology narcotics and stimulants are derivatised using MSTFA, anabolic steroids are derivatised with MSTFA/NH_4I/ethanethiol (320/1/2; v/w/v) to create TMS-enol ethers [17]. The latter derivatisation mixture however results in bad chromatography and decreased sensitivity for narcotic agents such as morphine in comparison to a 50% diluted derivatisation agent (i.e. MSTFA/NH_4I/ethanethiol 640/1/2; v/w/v) (Fig. 2a). In addition, this diluted derivatisation agent does not influence peak shape and sensitivity for anabolic steroids as tested for 17α-methyltestosterone (Fig. 2b) and therefore can be used in the combined analytical procedure.

In general, methods for the detection of anabolic androgenic steroids use a fast initial increase in temperature from approximately 120–180 °C after which anabolic steroids are chromatographically separated at a slow rate of 3–4 °C/min [6,7,18]. For narcotics and stimulants on the other hand temperature needs to be isothermal during the first part of the chromatographic run in order to avoid the loss of the very volatile substances, such as methylamphetamine, in the solvent front. Afterwards the temperature can be increased at a slightly higher temperature rate of ca. 20 °C/min allowing the separation of narcotic agents [12]. In general, run times for anabolic agents are 20–30 min and for narcotic and stimulating agents 15–20 min.

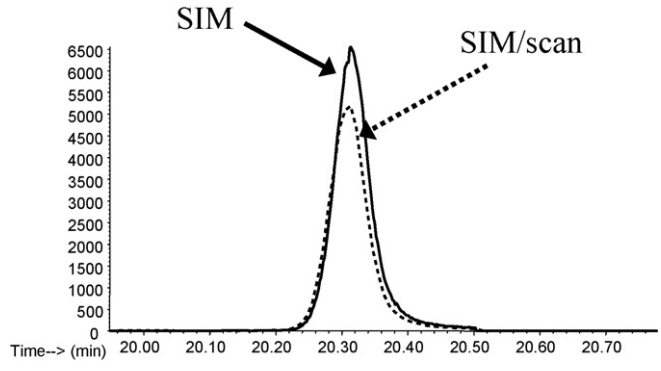

Fig. 1. Acquisition of 17α-methyltestosterone-bis-TMS in SIM mode compared to SIM/scan mode.

In order to detect both groups, the isothermal part was retained to allow the detection of stimulants after which temperature was rapidly increased to 180 °C followed by the slow gradient of 3 °C/min to allow the separation of the anabolic agents. The total run time is 25.8 min is comparable to the run time of most methods for anabolic androgenic steroids [6,7,18].

In order to obtain as many scans as possible scan parameters were divided into three groups. During the first 10 min only low molecular weight components such as stimulants elute and the mass range is restricted to *m/z* 50–390, while later in the chromatographic run the highest mass is increased to 525 and 650, respectively.

In the SIM trace, 15 groups are created with a maximum of 25 ions in one group (Table 1). Dwell times were set at 10 ms resulting in SIM scan rates between 3.42 and 24.32 scans/s. The dwell time could be decreased resulting in more scans cycles, but this might decrease the sensitivity.

The mass spectrometer is alternatively operated in the SIM and scan mode and the combined SIM/scan rate can be calculated as follows:

$$\frac{1}{((1/SR_{SIM}) + (1/SR_{scan}) \times 1.05)}$$

In this equation both individual scan rates (SR_{SIM} = scan rate in SIM mode and SR_{scan} = scan rate in scan mode) are incorporated just as an arbitrary constant (1.05) reflecting the time needed to switch from one setting to another.

In this method, the lowest total scan rate is 1.36 scans/s resulting in about 8 data points during a normal GC peak of 6 s. Compared to the current anabolic screening method this is 0.3 scans/s higher (unpublished data) which corresponds to two additional data points per chromatographic peak.

To allow the extraction of the stimulants the procedure was divided into two independent extractions at pH 9.5 and 14 respectively, similar to the combined screening method for narcotics and

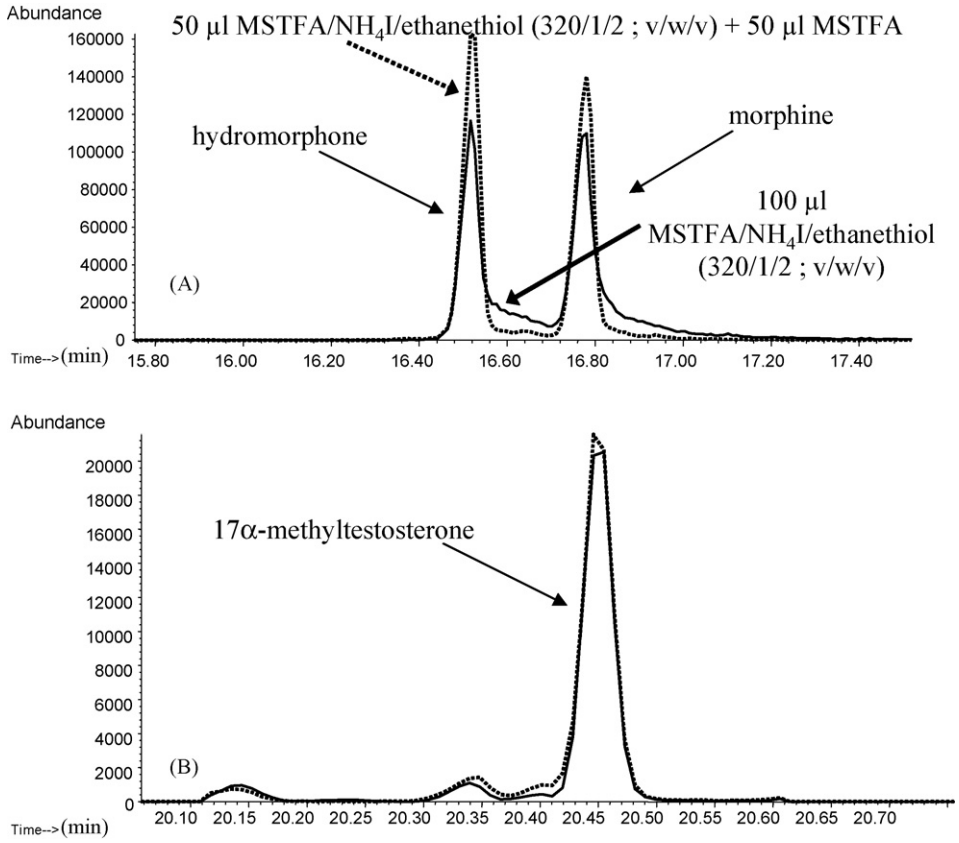

Fig. 2. Comparison of MSTFA/NH_4I/ethanethiol (320/1/2) (—) and MSTFA/NH_4I/ethanethiol (640/1/2) (- - -) as derivatisation agent for morphine and hydromorphone (A) and 17α-methyltestosterone (B).

stimulants [12]. For the conjugated components 3 ml of urine was hydrolysed overnight permitting the complete hydrolysis of morphine [12] and norandrosterone. After the hydrolysis, ammonium buffer (pH 9.5) was used instead of a solid carbonate buffer frequently used for the extraction of AAS [16,19] and several organic solvents were tested. For anabolic steroids and narcotic agents ethyl acetate provided the best results but very high interferences of urea and glycerol in the first part of the chromatographic run hampered the detection of the volatile stimulants. The combination of dichloromethane and methanol as currently used for conjugated narcotics and stimulants [12] gave bad extraction recoveries for the anabolic steroids. The best option was the use of diethyl ether although the use of this solvent resulted in the loss of benzoylecgonine, the urinary marker of cocaine. Nevertheless, diethyl ether was chosen as the extraction solvent at pH 9.5 because benzoylecgonine can be readily detected via LC–MS(MS).

Extraction at pH 14 is as usual with *tert*-butyl methyl ether. Table 2 summarises all critical parameters of this method compared to current screening methods.

This combined method allows the detection of 154 components. These include all anabolic steroids, both exogenous and endogenous, narcotic agents, stimulants, agents with anti-estrogenic activity and beta-agonists present in current GC–MS screening methods.

The validation of this analytical method was divided into qualitative and quantitative parts. In all samples the peak height of the internal standard cyclopentamine has to be higher than 20,000 counts while for 17α-methyltestosterone the criterion is 2000 counts. The qualitative validation was performed on 10 different negative urines spiked at three different levels (MRPL/2, MRPL and 2 × MRPL). The LOD was defined as the lowest concentration for which a score of 10/10 was observed. For 4 components no satisfying results could be obtained at the MRPL level. In addition to benzoylecgonine, no satisfactory results could be obtained for fenoterol, calusterone and formebolone. Heroin itself was not detected, but its major metabolite, 6-mono-acetylmorphine, was validated at 100 ng/ml or half the MRPL level. Similar, diethylpropion (amfepramone) can be detected by three metabolites and oxandrolone as a sulphur-containing artefact instead of the component itself. For zilpaterol, a score of 10/10 could be obtained at 20 ng/ml, or double of the MRPL level of 10 ng/ml. All other component could be detected at or below the MRPL level set by WADA [14]. In addition to spiked samples, several stimulants and agents with anti-estrogenic activity were validated through the analysis of excretion urines. No LOD could be determined for those substances because of the lack of reference substances. The applicability of this analytical method for these components was examined by the detection of the parent compound and/or one or more metabolites. For diethylpropion (amfepramone) these metabolites are *N*-ethylaminopropiophenone and *N*-ethylnorpseudoephedrine [20]. Isometheptene, which is sold as a racemic mixture, is metabolised to a *cis*- and *trans*-2-methyl-6methylamino-2-hepten-1-ol (isometheptene metab 1 and metab 2) [21,22]. The metabolism of sibutramine has been reported numerous times in the literature. Here, nor-sibutramine and the 2 isomers of the hydroxyl-nor-sibutramine were monitored [23,24]. The metabolites of prolintane monitored in this screening method are 1-1(benzyl-3-hydroxybutyl)pyrrolidin-2-on (diastereomer, metabolite 5a and 5b), 1-(1-benzyl-4-hydroxybutyl)pyrrolidin-2-on (metabolite 9) and 1-[1-(3,4-dihydroxybenzyl)butyl]pyrrolidin-2-on (metabolite 14) [25]. For the aromatase inhibitors clomiphene, tamoxifen and cyclofenyl the monitored metabolites are respectively

hydroxyl-clomiphene, hydroxymethoxytamoxifen and hydroxyl-bis-desacetylcyclofenyl [26]. Selectivity was tested by the analysis of a reference mixture of exogenous corticosteroids which did not result in the detection of interferences at the retention times of the screened components. Analysis of the 10 different negative urines to evaluate specificity resulted in the detection of an interference at the retention time of 17α-trenbolone and 7β-OH-DHEA eluting within an interval of 0.1 min. The scan trace however revealed that this interference originated from nalorphine, the internal standard used for the narcotic agents [12]. After the removal of this internal standard both components could be validated at the MRPL level of 10 ng/ml.

Besides the qualitative method validation, a quantitative validation was carried out for the endogenous anabolic steroids, morphine, THC-COOH and salbutamol. As shown previously [12], quantification of ephedrines using this method is not possible due to the formation of multiple derivatives. The use of confirmation thresholds based upon relative abundances of diagnostic ions can assist in reducing the number of unnecessary confirmation procedures.

Linear calibration curves could be obtained with correlation coefficients (R^2) higher than 0.997 (Table 3) for all endogenous steroids monitored in the steroid profile. The trueness on three different concentrations of the calibration curves was always within the allowed margin of 20% just as the repeatability and reproducibility of which the margins are concentration dependent according to the Horwitz equation [15].

For morphine a linear calibration curve was obtained using both internal standards (nalorphine and 17α-methyltestosterone). This means that nalorphine can be removed as internal standard which also solves the selectivity problem associated with 17α-trenbolone and 7β-OH-DHEA. Only the reproducibility for morphine exceeds the allowed margins.

For THC-COOH and salbutamol no linear calibration curves could be obtained ($R^2 < 0.98$) due to a big dispersion in obtained results. For THC-COOH this is probably caused by a bad extraction recovery. As previously published, quantitative methods for the determination of THC-COOH rely on a double extraction at an acidic pH after an alkaline hydrolysis and clean-up step in addition to the use of a deuterated internal standard to compensate for variations in extraction recovery [27]. Therefore, the addition of a deuterated analogue could solve this problem.

As for every sample analysed for doping control a steroid profile has to be reported, and in order to avoid the frequent recalibration of the analytical method, routine methods often rely on one-point calibration. This one-point calibration was implemented in the method by reanalysing a quality control sample after 20 routine samples. In order to check this one-point calibration, 21 quality control samples were analysed following the above mentioned analytical procedure. The results obtained for this part of the method validation are summarised in Table 4. The obtained RSDs are in compliance with the values obtained using the Horwitz equation (RSD_{max}). In addition, the trueness never exceeded 20%. The individual measured values were scattered around the average and were situated in the interval average $\pm 3 \times$ SD. Besides the individual concentration of endogenous anabolic androgenic steroids several concentrations ratios can be used as an indicator for the abuse of certain compounds. For instance, the ratio testosterone/epitestosterone is indicative of the abuse of testosterone and some other endogenous steroids if higher than 4, while the ratios of androsterone/etiocholanolone, DHT/epitestosterone and 5α-androstane-3α,17β-diol/5β-androstane-3α,17β-diol can be used to detect the abuse of DHT [28]. Similar as for the concentration of the individual components these ratios fulfilled the criterion of ±20% for the trueness and were scattered around the average.

This approach allows to distinguish between samples with a concentration that can be higher than the threshold value from samples with concentration which will be lower than the threshold level during a subsequent quantitative confirmation procedure by using the obtained lower limit (i.e. average $- 3 \times$ SD) as confirmation threshold.

4. Conclusion

The development of high performance electronics in the newest generation of GC–MS instruments offers the opportunity to use alternating SIM/scan data acquisition in a single run allowing the possibility to combine current screening methods. The results obtained in this work have proven that the combination of routinely used screening methods in doping control is possible if a number of small modifications are made. These include the choice of extraction buffer and solvent and the derivatising agent used. The developed method is capable of detecting more than 150 different substances from the WADA list of prohibited substances in compliance with the minimum required performance limits as set by WADA. The quantitative nature of this analytical method has also been proven. The productivity has also been improved as the analytical run time is almost 15 min shorter than the total run time of the individual methods and because this method requires 2-ml less urine compared to the traditional screening methods.

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