RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2007; **21**: 2439–2446
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3103

Preventive doping control analysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids

Costas G. Georgakopoulos[1]*, Ariadni Vonaparti[1,5], Marianna Stamou[1,5], Polyxeni Kiousi[1,6], Emmanouil Lyris[1], Yiannis S. Angelis[1], George Tsoupras[2], Bernhard Wuest[3], Michel W. F. Nielen[4,7], Irene Panderi[5] and Michalis Koupparis[6]

[1]Doping Control Laboratory of Athens, Olympic Athletic Center of Athens 'Spiros Louis', 37 Kifissias Ave., 151 23 Maroussi, Greece
[2]Agilent Technologies Europe B.V. Meyrin Branch, 39 rue de Veyrot, Meyrin 1, Geneva, CH 1217 Switzerland
[3]Chemetrix (Pty), Block J, Central Park, 400, 16th Road, Midrand, South Africa
[4]RIKILT Institute of Food Safety, P.O.Box 230, 6700 AE Wageningen, The Netherlands
[5]Division of Pharmaceutical Chemistry, Department of Pharmacy, University of Athens, Panepistimiopolis-Zographou, 15771 Athens, Greece
[6]Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis-Zographou, 15771 Athens, Greece
[7]Wageningen University, Laboratory of Organic Chemistry, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

Received 22 March 2007; Revised 9 May 2007; Accepted 21 May 2007

A new combined doping control screening method for the analysis of anabolic steroids in human urine using liquid chromatography/electrospray ionization orthogonal acceleration time-of-flight mass spectrometry (LCoaTOFMS) and gas chromatography/electron ionization orthogonal acceleration time-of-flight mass spectrometry (GCoaTOFMS) has been developed in order to acquire accurate full scan MS data to be used to detect designer steroids. The developed method allowed the detection of representative prohibited substances, in addition to steroids, at concentrations of 10 ng/mL for anabolic agents and metabolites, 30 ng/mL for corticosteroids, 500 ng/mL for stimulants and β-blockers, 250 ng/mL for diuretics, and 200 ng/mL for narcotics. Sample preparation was based on liquid-liquid extraction of hydrolyzed human urine, and the final extract was analyzed as trimethylsilylated derivatives in GCoaTOFMS and underivatized in LCoaTOFMS in positive ion mode. The sensitivity, mass accuracy, advantages and limitations of the developed method are presented. Copyright © 2007 John Wiley & Sons, Ltd.

The evolution of time-of-flight mass spectrometry hardware, in terms of orthogonal acceleration and ion reflectron geometry, has allowed its application to doping control analysis for accurate and sensitive full scan acquisition.

In this study, we present validation data of qualitative gas chromatography/orthogonal acceleration time-of-flight mass spectrometry (GCoaTOFMS) and liquid chromatography/orthogonal acceleration time-of-flight mass spectrometry (LCoaTOFMS) methods for the detection of anabolic agents and corticosteroids (together with indicative β-blockers, stimulants, narcotics and diuretics) in athletes' urine samples, proving the capability of this combined screening system to detect additional designer analytes with similar molecular characteristics.

An alternative approach for the comprehensive screening of designer steroids using oaTOFMS has been developed by Nielen *et al.*[1] with the use of androgen bioassay detection and subsequent fractionation and analysis by LC quadrupole oaTOFMS for structure elucidation.

**Correspondence to*: C. G. Georgakopoulos, Doping Control Laboratory of Athens, Olympic Athletic Center of Athens 'Spiros Louis', 37 Kifissias Ave., 151 23 Maroussi, Greece.
E-mail: oaka@ath.forthnet.gr
Contract/grant sponsor: WADA.

Many approaches exist for the screening of anabolic steroid abuse in doping control analysis based either on GC (e.g.[2]) or on LCMS systems (e.g.[3]). Screening methods based on LCoaTOFMS of prohibited substances, but not of anabolic steroids[4] and of corticosteroids, β2-agonists and selected designer steroids,[5] have also been reported. Thevis *et al.*[6] reported a similar approach based on precursor ion scanning on a LC triple quadrupole mass spectrometer for the detection of unknown designer steroids with structures similar to known designer steroids (e.g. tetrahydrogestrinone[7]).

EXPERIMENTAL

LCoaTOFMS

An Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) Rapid Resolution LC system was used for the chromatographic separation. The system consisted of a vacuum degasser, a two-piston binary pump, an autosampler and a column oven. Chromatographic separation was performed at 35°C using a Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm particle size; Agilent Technologies).

The mobile phase consisted of solvent A: 5 mM ammonium formate (Acros Organics, Morris Plains, NJ, USA) in 0.01% formic acid (Riedel-de Haen, Seelze, Germany) and solvent B: a mixture of acetonitrile (Riedel-de Haen)/water (Riedel-de Haen) 90:10 (v/v) containing 5 mM ammonium formate and 0.01% formic acid (v/v). The flow rate was 0.3 mL min^{-1}. The proportion of solvent B was increased from 10% to 80% in 9 min, to 100% in 10 min, held at 100% for 1 min and then reduced to 10% in 0.5 min. The analysis run time was 11.5 min and the post-run equilibrium time was 2.5 min. The injection volume was 5 μL.

The mass spectrometer was an Agilent Technologies 6210 orthogonal acceleration time-of-flight mass spectrometer, equipped with an electrospray ionization (ESI) source operated in the positive ion mode. Nitrogen was used as the drying and nebulizing gas. The drying gas flow and temperature were 10 L min^{-1} and 350°C, respectively, and the nebulizer gas pressure was 45 psi. The applied capillary voltage was 4000 V. The fragmentor voltage was optimized at 140 V in order to reduce the fragmentation of the $[M+H]^+$ ions of the analytes and thus maximize the mass accuracy calculation of the molecular masses. Mass spectral data were collected from *m/z* 100 to 1100 at 9300 transients per second, i.e. 9300 spectra are summed in one spectrum. All the other MS parameters (transfer optic voltages, voltages of the ion focus and octapole lens for optimizing the beam shape as it enters the TOF analyzer, TOF voltages, and detector voltage) were automatically optimized by the instrument autotuning procedure, performed on a weekly basis. The mass calibration was performed daily before starting the analysis over a mass range of *m/z* 118.0863–2721.8950 using a calibration solution provided by the manufacturer (G1969-85000, Agilent Technologies). The full width at half maximum (FWHM)[8] mass resolution ranged from 4500 (at *m/z* 118.0863) to 14500 (at *m/z* 2721.8948). Reference mass correction was used during the analysis, to achieve better mass accuracies, by continuously introducing two reference compounds, hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy) phosphazine and purine (Agilent Technologies), simultaneously with the samples into the ESI source from a second orthogonal nebulizer. All aspects of instrument control, tuning, method setup and parameters, sample injection and sequence operation were controlled by the Agilent Technologies Mass Hunter software (A.02.02).

GCoaTOFMS

The chromatographic separation was performed on an Agilent 6890N gas chromatograph and the oaTOFMS analysis on a Waters Micromass GCT (Manchester, UK) orthogonal acceleration-reflectron TOF mass spectrometer in electron ionization mode at 70 eV at a mass resolution of 7000 FWHM. The GCT was operated with Waters Masslynx V4.SP4 software. The chromatographic column was Agilent Ultra 1, coated with methyl silicone gum, 12 m length, 0.200 mm i.d. and 0.33 μm film thickness, and helium was used as the carrier gas. The temperature program was: 180°C initial temperature, 3°C/min to 235°C and, after 3.2 min, 15°C/min to 310°C where it was held for 5 min. The injection volume was 1 μL. The GCT averaged scans every 0.45 s with an interscan delay of 0.05 s and a mass range *m/z* 80–800. Instrument tuning and calibration was performed using perfluorotributylamine (PCR Research Chemicals, Gainesville, FL, USA). Perfluorotributylamine was subsequently pumped out and replaced with 2,4,6-tris(trifluoromethyl)-1,3,5-triazine (also named metri, Aldrich, Milwaukee, WI, USA) for sample acquisition, in order to improve the dynamic range of the GCT. During acquisition runs, metri was continuously introduced into the ion source.

Materials and sample preparation

A detailed description of the materials and the sample preparation methodology of the anabolic steroids screening procedure is presented elsewhere,[2,9] so only a brief description of the sample preparation is presented here. A 5.0 mL volume of urine was mixed with phosphate buffer (pH 7.0). After the addition of methyltestosterone (internal standard) and enzyme β-glycuronidase from *Escherichia coli*, the urine sample was hydrolyzed. The pH was then adjusted to 9.0–10.0 with a mixture of sodium hydrogen carbonate and sodium carbonate. Extraction was carried out with diethyl ether, using anhydrous sodium sulfate as the salting-out agent. After being shaken, the mixture was centrifuged. The organic phase was split into two separate conical tubes. For the LCTOF analysis the organic phase was dried under a stream of nitrogen and reconstituted with 100 μL of 9:1 solvent A/solvent B (v/v). For the GCTOF analysis the organic phase was dried under a stream of nitrogen and derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)/NH_4I/dithioerythritol (DTE). A urine excretion study of the designer steroid prostanozol,[10] kindly provided by the Doping Control Laboratory of Moscow, Russia, was used as to test the ability of the method to acquire mass spectra from unknown compounds.

Validation parameters

The validation parameters for the qualitative determination of the analytes presented herein are related to the GCoaTOFMS and LCoaTOFMS instrumentation and not to the sample preparation.

Matrix interference

Ten different drug-free urine specimens were prepared in order to prove that no interfering peaks exist in the selected ion chromatograms. A drug-free urine sample, prepared every working day, was injected on six different days.

Identification capability

The identification capability ensures the capability of the method to detect the analytes at a concentration of 10 ng/mL for anabolic steroids, 30 ng/mL for corticosteroids, 500 ng/mL for stimulants and β-blockers, 250 ng/mL for diuretics, and 200 ng/mL for narcotics. These concentrations are related to the concentration specifications of the World Antidoping Agency (WADA[11]) and not to the capability of the method to detect lower concentrations. The experiments for the identification capability were conducted on six days, analyzing two different aliquots, each fortified with a different group of analytes and prepared as above.[2,9] Chromatographic retention time reproducibility was also checked in the same experiments.

DOI: 10.1002/rcm

Table 1. LCESIoaTOFMS analytes

Substance	Category	Ion	Theoretical *m/z*	S/N ratio	Mass accuracy/ mass error (ppm)
16a-Hydroxyprednisolone	Corticosteroid	$[M+H]^+$	377.1959	144	1.7
16β-Hydroxystanozolol	Anabolic steroid	$[M+H]^+$	345.2537	18	4.0
17-Epioxandrolone	Anabolic steroid	$[M+H]^+$	307.2268	28	2.7
17-Epitrenbolone	Anabolic steroid	$[M+H]^+$	271.1693	429	1.7
19-Norandrosterone	Anabolic steroid	$[M+NH_4]^+$	294.2422	39	0.4
19-Noretiocholanolone	Anabolic steroid	$[M+NH_4]^+$	294.2422	34	1.0
2-Hydroxymethyl-17α-methylandrosta-1,4-diene-diol-3-one Formebolone met	Anabolic steroid	$[M+H]^+$	347.2217	116	0.9
4-Hydroxytestosterone	Anabolic steroid	$[M+H]^+$	305.2111	44	0.2
6α-Hydroxyandrost-4-ene-3,17-dione	Anabolic steroid	$[M+H]^+$	303.1955	91	6.5
6β-Hydroxybromantane	Stimulant	$[M+H]^+$	322.0801	298	1.4
6β-Hydroxymethandienone	Anabolic steroid	$[M+H]^+$	317.2111	81	1.1
9α-F-17α-methyl androst-4-en-3α,6β,11β,17β-tetrol Fluoxymesterone metabolite (met.)	Anabolic steroid	$[M+H-H_2O]^+$	337.2173	77	1.0
9α-F-18-nor-17,17-dimethyl-4,13-dien-11β-ol-3-one Fluoxymesterone met.	Anabolic steroid	$[M+H]^+$	319.2068	560	0.8
Acebutolol	β-Blocker	$[M+H]^+$	337.2122	>1000	1.1
Alprenolol	β-Blocker	$[M+H]^+$	250.1802	>1000	0.7
Aminoglutethimide	Aromatase Inhibitor	$[M+H]^+$	233.1285	249	1.6
Atenolol	β-Blocker	$[M+H]^+$	267.1703	>1000	1.3
Beclomethasone	Corticosteroid	$[M+H]^+$	409.1776	94	0.6
Betamethasone	Corticosteroid	$[M+H]^+$	393.2072	164	0.2
Betaxolol	β-Blocker	$[M+H]^+$	308.2220	>1000	0.3
Bisoprolol	β-Blocker	$[M+H]^+$	326.2326	>1000	1.2
Bolasterone	Anabolic steroid	$[M+H]^+$	317.2475	257	1.0
Boldenone	Anabolic steroid	$[M+H]^+$	287.2006	19	2.5
Boldenone metabolite	Anabolic steroid	$[M+H]^+$	289.2162	24	0.1
Calusterone	Anabolic steroid	$[M+H]^+$	317.2475	124	1.7
Carteolol	β-Blocker	$[M+H]^+$	293.1860	>1000	1.4
Carvedilol	β-Blocker	$[M+H]^+$	407.1965	977	0.7
Celiprolol	β-Blocker	$[M+H]^+$	380.2544	>1000	3.1
Clenbuterol	β2-Agonist	$[M+H]^+$	277.0869	115	0.3
Cortisol	Endogenous corticosteroid	$[M+H]^+$	363.2166	97	2.2
Cortisone	Endogenous corticosteroid	$[M+H]^+$	361.2009	438	1.0
Desonide	Corticosteroid	$[M+H]^+$	417.2272	157	1.6
Dexamethasone	Corticosteroid	$[M+H]^+$	393.2072	192	2.6
Esmolol	β-Blocker	$[M+H]^+$	296.1856	>1000	2.8
Ethisterone Danazol met.	Anabolic steroid	$[M+H]^+$	313.2162	142	0.4
Fludrocortisone	Corticosteroid	$[M+H]^+$	381.2072	475	0.9
Flumethasone	Corticosteroid	$[M+H]^+$	411.1978	179	1.3
Flunisolide	Corticosteroid	$[M+H]^+$	435.2177	536	0.6
Fluocortolone	Corticosteroid	$[M+H]^+$	377.2123	140	2.8
Formoterol	β2-Agonist	$[M+H]^+$	345.1809	852	0.7
Gestrinone	Anabolic steroid	$[M+H]^+$	309.1849	602	1.7
Labetalol	β-Blocker	$[M+H]^+$	329.1860	>1000	1.3
Levobunolol	β-Blocker	$[M+H]^+$	292.1907	>1000	2.3
Methylprednisolone	Corticosteroid	$[M+H]^+$	375.2166	327	5.4
Methyltestosterone	Internal standard	$[M+H]^+$	303.2319	>1000	0.5
Methyltrienolone	Anabolic steroid	$[M+H]^+$	285.1849	107	2.4
Metipranolol	β-Blocker	$[M+H]^+$	310.2013	>1000	0.7
Metoprolol	β-Blocker	$[M+H]^+$	268.1907	>1000	2.4
Nadolol	β-Blocker	$[M+H]^+$	310.2013	>1000	1.1
Oxandrolone	Anabolic steroid	$[M+H]^+$	307.2268	204	1.7
Oxprenolol	β-Blocker	$[M+H]^+$	266.1751	>1000	0.6
Pindolol	β-Blocker	$[M+H]^+$	249.1598	>1000	0.9
Prednisolone	Corticosteroid	$[M+H]^+$	361.2009	104	0.4
Prednisone	Corticosteroid	$[M+H]^+$	359.1853	245	2.1
Propranolol	β-Blocker	$[M+H]^+$	260.1645	>1000	2.3
Salbutamol	β2-Agonist	$[M+H]^+$	240.1594	483	0.1
Salmeterol	β2-Agonist	$[M+H]^+$	416.2795	673	0.9
Sotalol	β-Blocker	$[M+H]^+$	273.1267	>1000	2.3
Terbutaline	β2-Agonist	$[M+H]^+$	226.1438	288	0.0
Tetrahydrogestrinone	Anabolic steroid	$[M+H]^+$	313.2162	102	0.7
Timolol	β-Blocker	$[M+H]^+$	317.1642	509	1.5
Trenbolone	Anabolic steroid	$[M+H]^+$	271.1693	433	7.7
Triamcinolone	Corticosteroid	$[M+H]^+$	395.1864	129	2.8
Triamcinolone acetonide	Corticosteroid	$[M+H]^+$	435.2177	>1000	2.3

DOI: 10.1002/rcm

Table 2. GCEIoaTOFMS analytes. Mass accuracy/mass error and theoretical *m/z* have been calculated for the ions with the highest S/N ratio of the per-trimethylsilyl (TMS) derivatives

Substance	Derivative	Category	Theoretical *m/z*	S/N ratio	Mass accuracy/ mass error (ppm)
ISTD (methyltestosterone)	di-OTMS	Anabolic steroid	446.3036	162	5.6
Acebutolol	diOTMS, diNTMS	β-Blocker	437.2238	192	10.1
Alprenolol	OTMS, NTMS	β-Blocker	378.2285	55	6.6
Amiloride	diNTMS	Diuretic	316.0817	15	55.0
Amiphenazole	triNTMS	Stimulant	407.1703	90	5.4
6α-Hydroxyandrost-4-ene-3,17-dione	triOTMS	Anabolic steroid	518.3068	84	6.0
Androst-4-en-3,17-dione met.					
Benzoyl ecgonine Cocaine met.	OTMS	Stimulant	240.1420	4	12.9
Betaxolol	OTMS, NTMS	β-Blocker	144.1208	255	1.4
7α,17α-Dimethyl-5β-androstane-3α,17β-diol	diOTMS	Anabolic steroid	143.0892	9	14.0
Bolasterone met.					
Bolasterone	diOTMS	Anabolic steroid	445.2958	39	10.3
5β-Androst-1-en-17β-ol-3-one Boldenone met.	diOTMS	Anabolic steroid	194.1127	11	12.9
Buprenorphine	diOTMS	Narcotic	554.3123	1336	5.4
7β,17α-Dimethyl-5β-androstane-3α,17β-diol	diOTMS	Anabolic steroid	143.0892	9	16.8
Calusterone met.					
Calusterone	diOTMS	Anabolic steroid	445.2958	12	4.5
Carteolol analyte	OTMS, NTMS	β-Blocker	86.0970	130	7.0
Celiprolol	OTMS	β-Blocker	86.0970	287	7.0
Chlortalidone	diNTMS,OTMS	Diuretic	554.1314	221	3.4
Clenbuterol	diOTMS	β2-Agonist	335.0695	165	4.2
Clopamide	diOTMS	Diuretic	474.1470	1638	0.8
4-Chloroandrost-4-en-3α-ol-17-one Clostebol met.	diOTMS	Anabolic steroid	451.2255	10	11.1
Ethisterone Danazol met.	diOTMS	Anabolic steroid	456.2880	36	1.3
2α-Methyl-5α-androstan-3α-ol-17-one Drostanolone met.	diOTMS	Anabolic steroid	433.2958	51	10.6
Esmolol	OTMS,NTMS	β-Blocker	424.2340	39	14.4
9α-F-18-nor-17,17-dimethylandrosta-4,13dien-11β-ol-3-one	diOTMS	Anabolic steroid	208.1283	26	16.8
Fluoxymesterone met.					
Formestane	triOTMS	Aromatase inhibitor	518.3068	133	8.1
Furazabol	OTMS	Anabolic steroid	143.0892	3	13.3
Hydromorphone	diOTMS	Narcotic	429.2156	141	0.0
Indapamide	diOTMS	Diuretic	509.1392	36	4.3
Labetalol diastereomer 1	triTMS	β-Blocker	234.1678	59	2.6
Labetalol diastereomer 2	triTMS	β-Blocker	234.1678	67	0.9
1α-Methyl-5α-androstan-3α-ol-17-one Mesterolone met.	diOTMS	Anabolic steroid	433.2958	10	12.5
Epimetenediol Methandienone met.	diOTMS	Anabolic steroid	143.0892	4	12.6
6β-Hydroxymethandienone Methandienone met.	triTMS	Anabolic Steroid	517.2990	45	6.0
1-Methylen-5α-androstan-3α-ol-17-one Methenolone met.	diOTMS	Anabolic steroid	431.2802	35	15.3
Methenolone	diOTMS	Anabolic steroid	208.1283	9	2.9
Metipranolol	OTMS, NTMS	β-Blocker	266.1339	68	3.4
Metoprolol	OTMS, NTMS	β-Blocker	224.1233	45	8.0
Mibolerone	diOTMS	Anabolic steroid	431.2802	16	15.1
Nadolol	triOTMS	β-Blocker	510.2892	521	9.0
19-Noretiocholanolone Nandrolone met.	diOTMS	Anabolic steroid	405.2645	134	7.4
13β,17α-Diethyl-3α,17β-dihydroxy-5α-gonane Norbolethone met.	diOTMS	Anabolic steroid	157.1049	17	5.1
13β,17α-Diethyl-3α,17β-dihydroxy-5β-gonane Norbolethone met.	diOTMS	Anabolic steroid	157.1049	13	21.0
17α-Ethyl-5α-estrane-3α,17β-diol Norethandrolone met.	diOTMS	Anabolic steroid	157.1049	21	14.6
17α-Ethyl-5β-estrane-3α,17β-diol Norethandrolone met.	diOTMS	Anabolic steroid	157.1049	10	4.5
Tetrahydronorethisterone Norethisterone met.	diOTMS	Anabolic steroid	431.2802	12	9.0
Oxprenolol	OTMS,NTMS	β-Blocker	222.1076	34	0.5
Oxycodone	diOTMS	Narcotic	459.2261	83	4.4
Oxymesterone	triOTMS	Anabolic steroid	534.3381	53	7.5
Pemoline	triOTMS	Stimulant	392.1772	226	2.0
Pindolol	OTMS,NTMS	β-Blocker	205.0923	37	11.7
Probenecid	OTMS	Diuretic	328.1039	1009	0.0
Propranolol	OTMS,NTMS	β-Blocker	216.1024	43	13.9
Salbutamol	triOTMS	β2-Agonist	86.0970	4	11.6
3′- and 4β,17β-dihydroxy-17α-methyl-5α-androst-	triOTMS	Anabolic steroid	143.0892	6	7.0
2-eno-[3,2-c]pyrazole Stanozolol mets.					
Terbutaline	triOTMS	β2-Agonist	86.0970	7	11.6
Testosterone-4-OH-isomer 1	triOTMS	Anabolic steroid	505.2990	7	2.2
Testosterone-4-OH-isomer 2	triOTMS	Anabolic steroid	505.2990	5	0.8
Timolol	OTMS	β-Blocker	373.1730	161	7.0
Triamterene	triOTMS	Diuretic	469.2261	136	3.0
Zeranol	triOTMS	Anabolic agent	433.2231	17	5.5

DOI: 10.1002/rcm

Specificity

Specificity was checked by injecting prepared urine samples fortified with substances chemically close to the substances under determination.

Carryover

Carryover was examined twice in drug-free samples injected in sequence after fortified samples.

RESULTS AND DISCUSSION

Tables 1 and 2 give lists of the analytes that fulfilled the specifications of the validation parameters, as described above, and met the criteria of the quality system. Analytes that failed to be detected, either by LCoaTOFMS or GCoaTOFMS, were 16-OH-furazabol, 17-epimethandienone, 6β-OH-turinabol, 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol. In relation to a detection sensitivity parameter, the criterion for the introduction of the analytes to the quality system was that the signal-to-noise (S/N) ratio was greater than 3. This threshold did not allow inclusion in the quality system of analytes that had been detected at a S/N ratio less than 3. However, their detection was facilitated in most cases by the lack of background matrix noise in both instruments in the respective ion chromatograms, when a printout of ion chromatograms with high m/z accuracy was used.

LCoaTOFMS method

During the method development, methanol and acetonitrile were tested in the LC mobile phase. For most analytes there was no difference observed between the two solvents but acetonitrile was preferred because it gave better chromatographic peak shape. In addition, methanol significantly increased the pressure of the chromatographic system. Formic acid was used as an acidic modifier, at a concentration of 0.01%, to optimize the chromatography and increase the formation of the protonated molecules of the analytes. The use of ammonium formate buffer was important for the formation of ammonium adducts for some steroids (e.g. 19-noretiocholanolone and 19-norandrosterone), which gave very low signals for the protonated molecules. Various gradient elution programs were tested in order to achieve chromatographic separation of analytes with similar molecular formulae (e.g. cortisone-prednisolone).

The LCoaTOFMS method specificity was proven from the different adducts and/or different relative retention times of the analytes. The drug-free urine samples showed no matrix interferences in the chromatographic windows of the analytes. The identification capability was proven by the detection of the analytes during the six working days of the validation, as presented in Table 1. Carryover signal was not detected in drug-free samples that were injected in sequence after the analysis of the fortified urine samples. The retention times proved to be very stable: the relative standard deviations (RSDs) of the relative retention times of the analytes were better than 0.2%.

The results of the mass accuracy estimation are also shown in Table 1 and a mass accuracy profile along the chromatographic peak range is presented in Fig. 1, as an indication of the influence of the signal intensity on mass accuracy. Since high mass accuracy is a main target of the present approach, a low fragmentor voltage was used for two reasons: (a) to enhance the $[M+H]^+$ signal intensity in order to improve accuracy and (b) to apply the internal algorithm of the Molecular Feature Extractor (MFE) of Mass Hunter software to propose possible molecular formulae, after estimating the molecular mass from various adducts – $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$ and their isotopes. In addition, the software compares sample mass spectra and retention time data against a database containing accurate MS data, retention times and molecular formulae of known substances.

The mass accuracy for the LCoaTOFMS method is estimated as better than 2 ppm using an internal reference. In a day-to-day experiment this can be achieved for ions with a S/N ratio better than about 10:1. For less abundant ions the background noise can influence the mass accuracy in some rare cases. A mass calibration can be done on a daily basis. Routine measurements show that the instrument is, however, stable for a week without tuning. For automatic operation, tuning or calibration functions can be

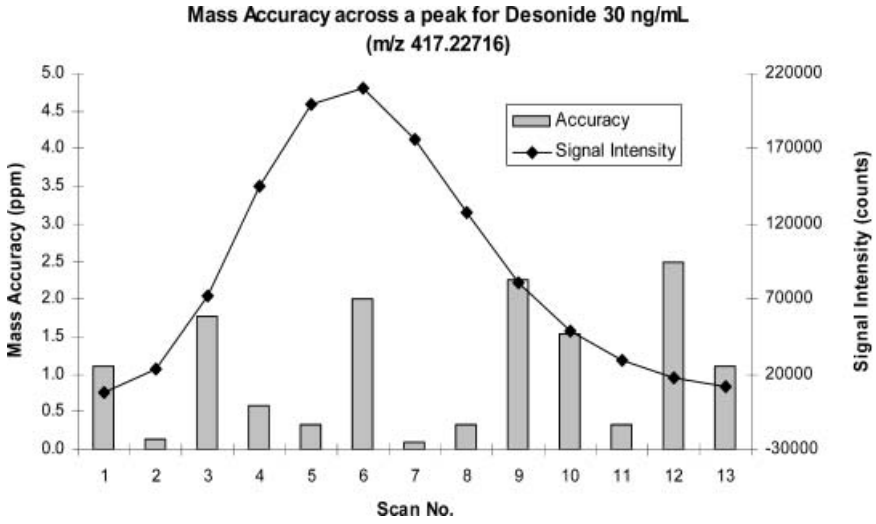

Figure 1. Mass accuracy profile along the chromatographic peak range for desonide fortified at 30 ng mL^{-1} in urine in LCoaTOFMS.

DOI: 10.1002/rcm

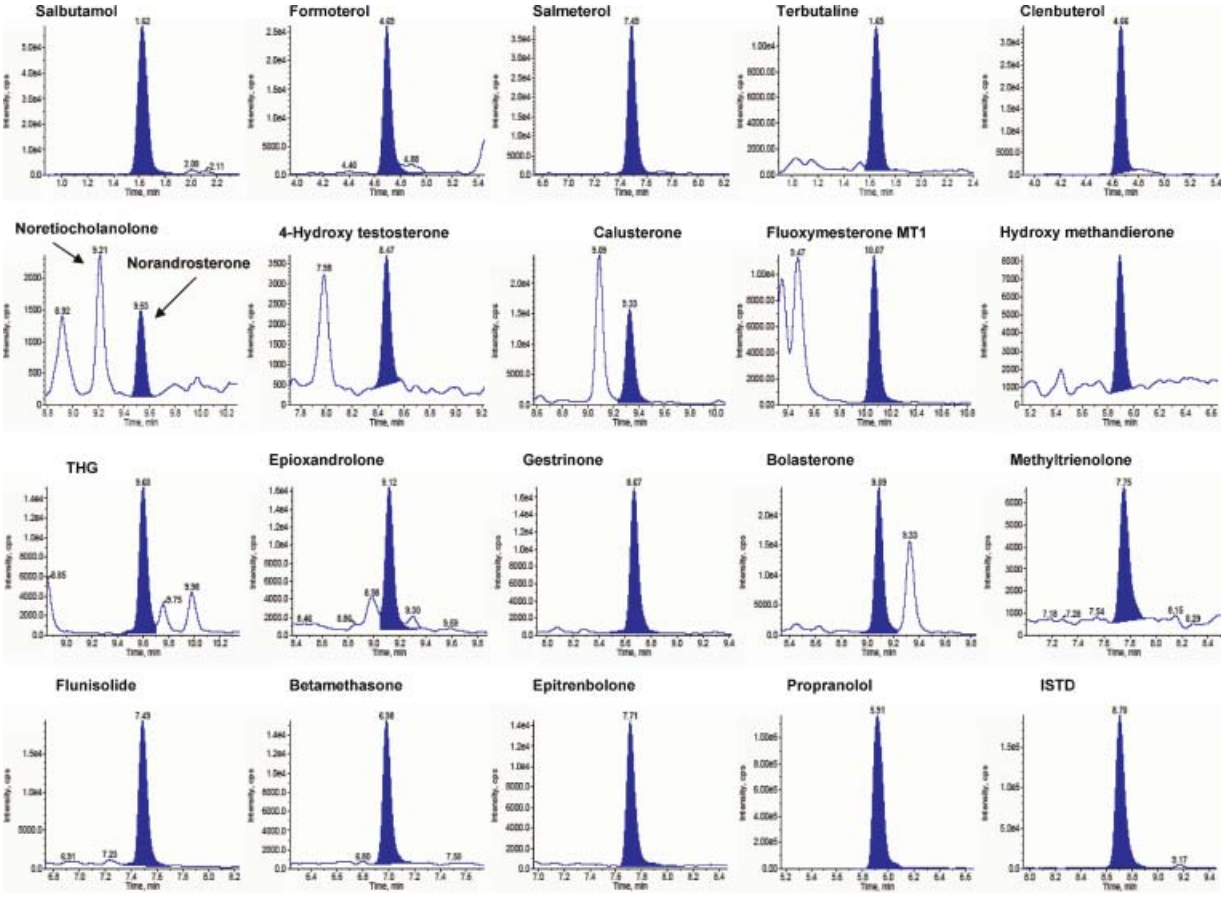

Figure 2. Extracted ion chromatogram at a 10 ppm mass window, of urine sample fortified with the substances, in LCoaTOFMS.

integrated in worklists to be done automatically during the sequences.

In Fig. 2, ion chromatograms of a number of analytes are presented as an example of the data collected during the validation process at a 10 ppm mass window.

GCoaTOFMS method

A thorough description of the GCT instrument, especially concerning the limitations of the time-to-digital converter, is presented elsewhere.[12] As in the LCoaTOFMS validation, specifications for the specificity, matrix interferences and

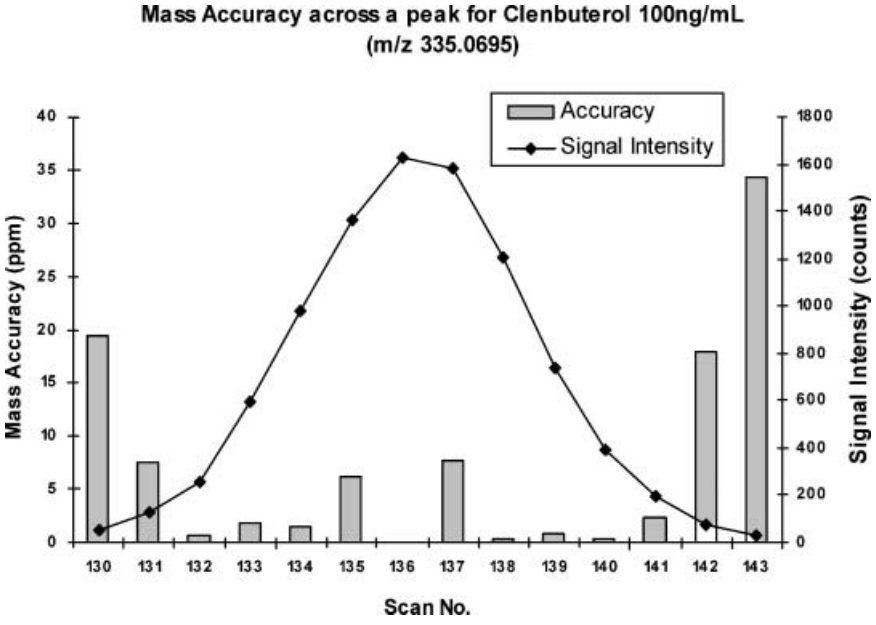

Figure 3. Mass accuracy profile along the chromatographic peak range for clenbuterol fortified at 100 ng mL^{-1} in urine in GCoaTOFMS.

DOI: 10.1002/rcm

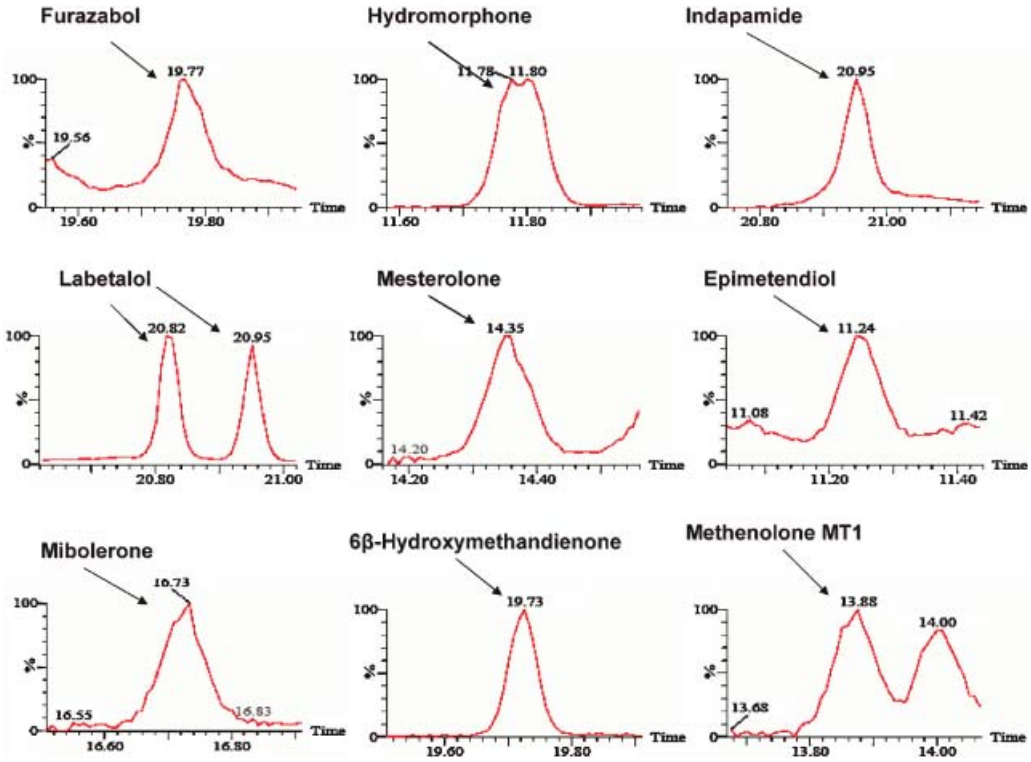

Figure 4. Extracted ion chromatogram at a window of 0.05 *m/z* units, of urine sample fortified with the substances, in GCoaTOFMS.

carryover were met, after the analysis of the same samples prepared for the LCoaTOFMS validation but derivatized accordingly.[2] Retention times proved to be very stable: the RSDs of the relative retention times of the analytes were better than 0.1%. In Table 2, data concerning the identification capability of the analytes of interest in the GCoaTOFMS are presented, together with the mass accuracy estimation. The mass accuracy profile along the chromatographic peak range in the GCT is presented in Fig. 3. In Fig. 4, ion chromatograms of a number of analytes are presented as an example of the data collected during the validation process at a 0.05 mass unit window.

The major concern during method development was to improve the dynamic range of the GCT. Various experiments were performed dealing with injection volume (1 or 0.2 μL), dilution of the final injectable extract with cyclohexane, and chromatographic run time. Best results were obtained from the above described experimental conditions. Dynamic range proved to be a limiting feature of the GCT, since it influences the chromatographic peak shape and height, in

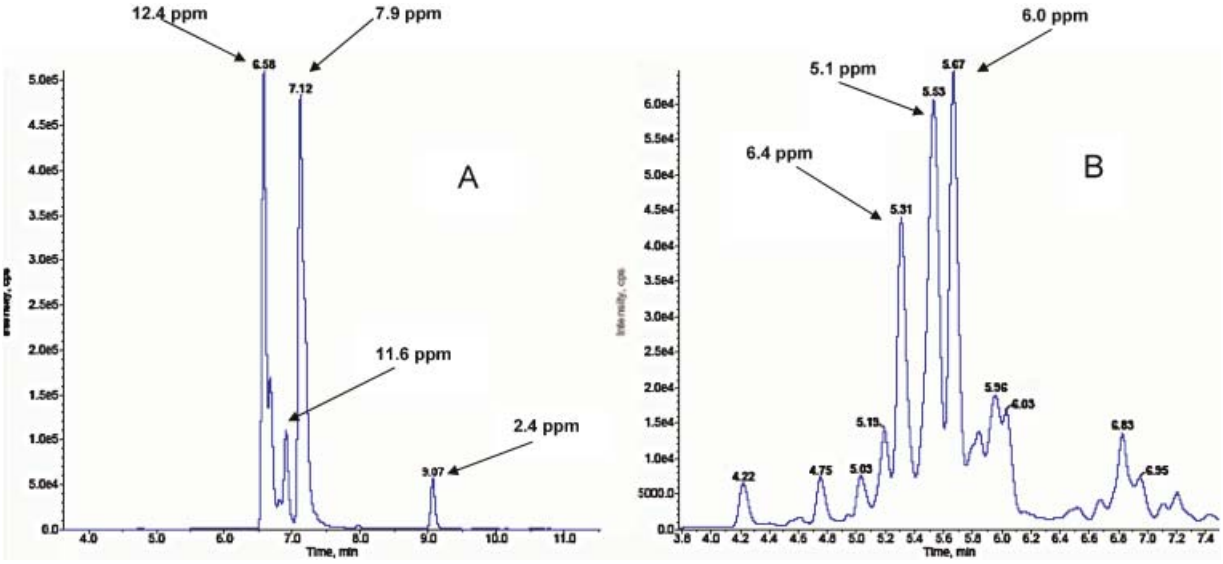

Figure 5. The LCoaTOFMS extracted ion chromatograms for (A) *m/z* 329.2224 (protonated molecule of prostanozol hydroxy-17-keto metabolites[10]) and (B) *m/z* 345.2173 (protonated molecule of the prostanozol dihydroxy-17-keto metabolites[10]). The mass accuracy of the base peaks ranges from 2.4 to 12.4 ppm.

DOI: 10.1002/rcm

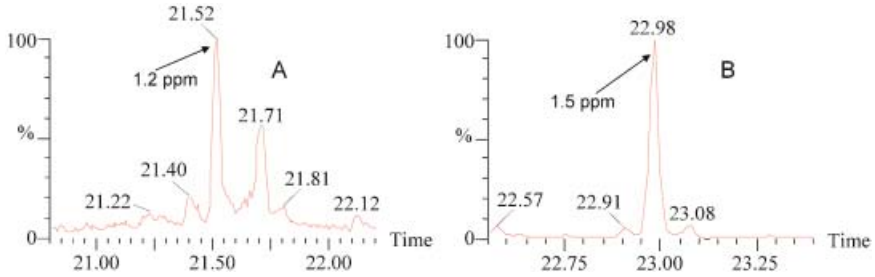

Figure 6. The GCoaTOFMS extracted ion chromatograms for (A) *m/z* 254.1271 (diagnostic ion of the prostanozol 3′-hydroxy-17-keto metabolites tri-TMS[10]) and (B) *m/z* 617.3447 (diagnostic ion of the prostanozol 6,16-dihydroxy-17-keto metabolites tri-TMS[10]). The mass accuracy range of the extracted peaks is 1–2 ppm.

the case of coelution of the peak of interest with another abundant peak.

Prostanozol excretion study analysis

The LCoaTOFMS method was applied for the analysis of the excretion urine sample of prostanozol. The extracted ion chromatograms for *m/z* 329.2224 (protonated molecules of the hydroxy-17-keto metabolites[10]) and 345.2173 (protonated molecules of the dihydroxyketo metabolites[10]) are presented in Fig. 5. The mass accuracy of the base peaks ranges from 2.4–12.4 ppm. Five different drug-free urine samples were reprocessed to show the absence of peaks at the retention times of the metabolites.

The GCoaTOFMS method was also applied for the analysis of the excretion urine sample of prostanozol. The extracted ion chromatograms for *m/z* 254.1271 (diagnostic ion of the hydroxy-17keto tri-TMS metabolites[10]) and 617.3447 (diagnostic ion of the dihydroxyketo tetra-TMS metabolites[10]) are presented in Fig. 6. The mass accuracy range of the extracted peaks is 1–2 ppm. Again, five different drug-free urine samples were reprocessed and showed no peaks at the same retention times.

CONCLUSIONS

A combined screening method based on LCoaTOFMS and GCoaTOFMS analysis was developed and validated for anabolic steroids and other representative prohibited substances in human urine. The method proved to be very sensitive for most of the substances that were tested, with high S/N ratio at the MRPL level. The proposed method is specific using accurate full scan mass spectra. LCoaTOFMS and GCoaTOFMS screening methods have both been assessed and are to be introduced into the ISO/IEC 17025:2005 Scope of Accreditation of the Athens WADA Laboratory.

The presented validation data, based on LCoaTOFMS and GCoaTOFMS preventative screening, support our proposal to use these technologies, in combination, for the acquisition of mass spectra from steroid designer drugs in a blind but accurate and generic way, which may cover a wide range of molecular features: from substances with difficulty in derivatization, but amenable to LC/ESI, to substances with difficulty in ionization, but amenable to GC conditions and/or derivatization. This dual blind data acquisition is supported by the full scan high resolution and mass accuracy data collection in low ppm mass error level at sensitivity as shown herein. Thus, doping control samples analyzed for a known repertoire of prohibited substances can also be blindly analyzed, in addition, for a wide range of unknown molecules in a sensitive and accurate way.

Acknowledgements

The authors sincerely thank WADA for financial support of the project, Agilent Technologies, Santa Clara, CA, USA for the provision of the LCoaTOFMS 6210 instrument, and the WADA Accredited Doping Control Laboratory of Moscow, Russia, for the provision of the prostanozol excretion study. CGG wishes to thank M. Stamou and A. Vonaparti for their dedication and support to this study. A. Vonaparti, PhD student, gratefully acknowledges the State Scholarships Foundation of Athens, Greece.

REFERENCES

1. Nielen MWF, Bovee TFH, van Engelen MC, Rutgers P, Hamers ARM, van Rhijn JHA, Hoogenboom LRAP. *Anal. Chem.* 2006; **78**: 424.
2. Tsivou M, Kioukia-Fougia N, Lyris E, Angelis Y, Fragkaki A, Kiousi P, Simitsek P, Dimopoulou H, Leontiou IP, Stamou M, Spyridaki MH, Georgakopoulos C. *Anal. Chim. Acta* 2006; **555**: 1.
3. Ho ENM, Leung DKK, Wan TSM, Yu NH. *J. Chromatogr. A* 2006; **1120**: 38.
4. Kolmonen M, Leinonen A, Pelander A, Ojanperä I. *Anal. Chim. Acta* 2007; **585**: 94.
5. Touber ME, Van Engelen MC, Georgakopoulos C, Van Rhijn JA, Nielen MWF. *Anal. Chim. Acta* 2007; **586**: 137.
6. Thevis M, Geyer H, Mareck U, Schanzer W. *J. Mass Spectrom.* 2005; **40**: 955.
7. Catlin DH, Sekera MH, Ahrens BD, Starcevic B, Chang Y-C, Hatton CK. *Rapid Commun. Mass Spectrom.* 2004; **18**: 1.
8. Bristow AWT. *Mass Spectrom. Rev.* 2006; **25**: 99.
9. Spyridaki MH, Kiousi P, Vonaparti A, Valavani P, Zonaras V, Zahariou M, Sianos E, Tsoupras G, Georgakopoulos C. *Anal. Chim. Acta* 2006; **573**: 242.
10. Rodchenkov G, Sobolevsky T, Sizoi V. Recent. Advances in Doping Analysis, *Proceedings of the Manfred Donike Workshop on Dope Analysis*, Cologne, Germany, June 4–9 2006, 2006; **14**: 141.
11. WADA, Technical Document TD2004MRPL. Available: http://www.wada-ama.org/rtecontent/document/perf_limits_2.pdf. Accessed 03-05-2007.
12. Dalluge J, Roose P, Brinkman UATh. *J. Chromatogr. A* 2002; **970**: 213.

DOI: 10.1002/rcm