Yulia Kucherova, Borislav Starcevic, Brian D. Ahrens and Anthony W. Butch

Development of a high-throughput sample preparation procedure for gas chromatography-mass spectrometric analysis of various classes of doping agents using a 96-well plate format

UCLA Olympic Analytical Laboratory, Department of Pathology & Laboratory Medicine, Geffen School of Medicine at UCLA, Los Angeles, USA

Introduction

The volume of urine specimens tested by World Anti-Doping Agency (WADA)-accredited laboratories for sport organizations is constantly increasing. There is also a growing need for more rapid reporting of test results. Although extremely sensitive mass spectrometers and more efficient gas and liquid chromatography systems have improved the ability of laboratories to detect and identify prohibited substances faster, sample aliquoting and clean up procedures still remain the limiting factor in overall laboratory efficiency and throughput. Commonly used methods for doping control analysis typically involve manual urine aliquoting and sample cleanup procedures that are extremely labor-intensive.[1] For this reason, there is a need for developing and validating automated aliquoting and sample cleanup methods to produce high-throughput assays that meet increased testing volume demands and improved overall turnaround times. Furthermore, the introduction of automation will minimize variability among extracted samples, minimize human errors, and will result in lower testing costs by reducing the number of manual steps requiring human intervention.

In this study we evaluated the feasibility of automating the extraction of conjugated compounds from urine using a 96-well microtiter plate format for analysis by gas chromatography-mass spectrometry (GC-MS). [2,3]

Materials and Methods

Chemicals and Reagents

High performance liquid chromatography (HPLC) grade methanol, hexane and ethyl acetate, and dibasic sodium phosphate, sodium carbonate, sodium bicarbonate and sodium sulfate

were obtained from Fisher Scientific (Pittsburgh, PA, USA). Monobasic potassium phosphate was obtained from Mallinckrodt Baker (Paris, KY, USA). [$^2H_3$]-testosterone, (d^3T) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). β-glucuronidase from *E. coli*, (specific activity of ~200 U/mL at 37 °C) was obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey & Nagel (Düren, Germany), and ammonium iodide (NH4I) and dithioerythritol (DTE) from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Urine Samples

A pooled drug-free urine was fortified with various WADA-banned substances at the minimum required performance limit (MRPL), unless otherwise specified. 1-androstenedione, 6α-hydroxyandrostenedione, methyl-1-testosterone, mibolerone, 19-norandrosterone and norclostebol were obtained from Steraloids Inc. (Newport, RI, USA); methyltrienolone was from Perkin Elmer (Waltham, MA, USA); boldenone, atenolol, clostebol, metoprolol, oxprenolol, pindolol, propranolol, sotalol and tetrahydrocannabinol acid were from Alltech (Nicholasville, KY, USA); boldione, ethisterone, acebutolol, alprenolol, bambuterol, betaxolol, bisoprolol, fenoterol, morphine, terbutaline and salmeterol were from Sigma-Aldrich. 9(10)-dehydronandrolone was from AK Scientific (Mountain View, CA, USA); 17-epimethandienone, 5β-androst-1-en-17β-ol-3-one, 4-chloro-3α-hydroxy-androst-4-en-17-one, 6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone, 2α-methyl-5α-androstan-3α-ol-17-one, epioxandrolone, epitrenbolone, 16β-hydroxyfurazabol, mesterolone, methenolone and 17α-ethyl-5β-estrane-3α,17β-diol were from the National Measurement Institute (Pymble, NSW, Australia); bolasterone and norbolethone were from Pfizer (New York, NY, USA); danazol was from Sterling-Winthrop Inc. (Pittsburg, PA, USA); oxandrolone was from United States Pharmacopeia (Rockville, MD, USA); benzoylecgonine, 6β-hydroxymethandienone, 9α-fluoro-18-nor,17,17-dimethyl-androsta-4,13-diene-11β-ol-3-one, 17α-methyl-5β-androstane-3α,17β-diol and noroxycodone were from Cerillant (Round Rock, TX, USA); oxymorphone was from DuPont (Willmington, DE, USA); salbutamol was from Schering-Plough (Kenilworth, NJ, USA); hydrocodone, hydromorphone and carteolol were from Abbot (Abbot Park, IL, USA); oxycodone was from NIDA (Bethesda, MD, USA); celiprolol was from Roche Pharmaceuticals (Nutley, NJ, USA); esmolol was from Sinova (Bethesda, MD, USA); labetalol and nadolol were from Spectrum Laboratory Products Inc (Gardena, CA, USA); levobunolol was from TRC Inc (North York, Ontario, Canada); mepindolol was from

Novartis Pharmaceuticals Basel, Switzerland); timolol was from Tocris Bioscience (Ellisville, MO, USA); RSR-13 was from Allos Therapeutics (Westminster, CO, USA). Methasterone was extracted from a Superdrol pill from Anabolic Xtreme (USA); and metipranolol was extracted from an ophthalmic solution from Alcon Pharmaceuticals (Fort Worth, TX, USA).

Aliquoting and Extraction of Urine Samples

A Quad-Z 215 Liquid Handler (Gilson, Middleton, WI, USA) was used to transfer 1 mL of urine from collection containers into wells of a 96-well sample collection plate (Phenomenex, Torrance, CA, USA). Custom sample trays were manufactured to hold 'A' bottle urine collection containers provided by most clients. One sample tray can be loaded on the liquid handling system at a time and each sample tray holds 32 urine containers. The system has four independently operated sample probes that use disposable pipette tips. After aspiration of 1 mL of urine, the sample probe is raised out of the urine container and a small amount of air is aspirated to prevent pipette tip leakage and cross-contamination of urine samples. The liquid handling system takes approximately one hour to aliquot 96 urines into a microtiter plate and the only operator-assisted task is to switch out urine sample trays twice during the aliquoting process.

After aliquoting is complete, the 96-well plate is transferred to a Zephyr automated solid-phase extraction (SPE) Workstation (Caliper, Hopkinton, MA, USA). The Zephyr SPE Workstation adds 8 µL of internal standard (40ng/mL of d^3T), 20 µL of β-glucoronidase (~200 U/mL) and 400 µL of 0.2M phosphate buffer to each well of the 96-well plate containing urine samples. The plate is then removed and incubated at 50ºC for 1 hour. Solid phase extraction of the urine samples is performed using Strata-X polymeric reversed-phase 96-well SPE plates (Phenomenex). Prior to the addition of urine samples, the workstation conditions the extraction plate by adding 500 µL of methanol to each of the wells, followed by 500 µL of water. After the wells drain, urine samples are added to the wells followed by washing with water, then 30% methanol in water followed by hexane. Bound material is eluted twice with 500 µL of ethyl acetate into a 96-well recovery plate. The Zephyr SPE workstation has a clog detection system that examines each well in the plate to verify that they were emptied during the extraction procedure before moving the extraction plate to the next area of the workstation. Elution solvent is evaporated using a 96-well TurboVap (Caliper). Derivatizing agent (25 µL of MSTFA/NH4I/Dithioerythritol, 1000:2:5, v/w/w)[4] is manually added to each well of the 96-well plate, the plate is incubated for 15 min at 60 ºC, and the

samples are loaded onto a GC/MS for analysis. The entire extraction procedure using the Zephyr takes about 45 minutes and does not require any operator time.

For comparison, urine samples were processed by our laboratory's current method using Caliper Rapid Trace Liquid handler modules and SPE sorbent material-C18 based Empore disc cartridges (3M, St. Paul, MN , USA).[5,6] The method uses 2.5 mL of urine and each module sequentially processes 10 urine samples. Briefly, 20 µL of internal standard (40 ng/mL of d^3T), 50 µL of β-glucoronidase (~200 U/mL) and 1000 µL of 0.2M phosphate buffer were added to each 2.5 mL urine aliquot and incubated for 1 hour at 50ºC. The hydrolyzed urine was then applied to a SPE column that was preconditioned with 500 µL of methanol, followed by 500 µL of water. After rinsing the column with water, 10% methanol in water was added, followed by hexane, then the retentate was eluted twice with 1 mL of ethyl acetate. The entire extraction process requires approximately 20 minutes per sample. Solvent was evaporated to dryness and the extracts were derivatized for 15 min at 60ºC with 50 µL of MSTFA/NH4I/DTE (1000:2:5, v/w/w).

Gas Chromatography-Mass Spectrometry (GC-MS) Conditions

Analysis was conducted using an Agilent 6890N gas chromatograph (GC) fitted with a J & W Scientific Ultra 1 column (17m, 0.2mm i.d., 0.11µ film thickness) coupled to an Agilent 5975 mass spectrometer (EI-MS). Samples were injected onto the column using a GC PAL autosampler (Leap Technologies, Carrboro, NC, USA). The GC oven temperature program starts at 180°C followed by a 3°C per minute ramp to 230°C, a 40°C per minute ramp to 310°C, and a final time of 2 minutes. Data was collected in selected ion monitoring mode using ions specific for the per-trimethylsilylether derivative of each compound. Signal to noise (S/N) ratio estimates were defined as the root mean square, with noise being the square root of the average of the squares of deviation from the average baseline. S/N estimate calculations were based on a single measurement.

Results

We examined the fully-automated 96-well extraction procedure for the ability to detect a wide range of compounds encompassing several drug classes on the WADA prohibited list. The sensitivity of the automated method for each compound was compared to our current extraction procedure using C-18 based Empore disc cartridges. Negative urine samples were fortified with representative parent compounds or metabolites at the MRPL or at cut-off concentrations for compounds defined as threshold compounds by the WADA.[7,8]

WADA requires an MRPL of 10 ng/mL for most anabolic agents.[8] As shown in Table 1, the S/N ratio was either enhanced or similar for numerous compounds and associated metabolites when comparing automated extraction with the extraction method using C-18 Empore disc cartridges. Improved sensitivity was obtained for parent compounds such as boldenone and boldione, and metabolites of boldenone, furazabol, methandienone and androstenedione.

Table 1. Sensitivity of the automated 96-well microtiter plate urine extraction procedure for detecting selected anabolic steroids based on signal to noise (S/N) ratios.

Parent Compound (metabolite)[a]	**S/N ratio using 96-well extraction**	**S/N ratio using Empore disc cartridge extraction**
Improved sensitivity using the 96-well extraction procedure		
Boldenone	16	6
Boldenone (5ß-androst-1-en-17ß-ol-3-one)	53	29
Boldione	9	4
Furazabol (16β-hydroxyfurazabol)	14	6
Methandienone (6 ß-hydroxymethandienone)	107	25
Methandienone (17-epimethandienone)	24	Not detected
Androstenedione (6α-hydroxyandrostenedione)	95	8
Similar sensitivity compared with the Empore disc extraction procedure		
1-androstenedione	112	125
9(10)-dehydronandrolone	82	78
Bolasterone	36	47
Clostebol (4-chloro-3α-hydroxy-androst-4-en-17-one)	44	33
Danazol	12	15
Dehydrochlormethyltestosterone (6β-hydroxy-4-chloro-1,2-dehydro-17α-methyltestosterone)	9	10
Ethisterone	96	83
Mesterolone (1α-methyl-5α-androstan-3α,17β-diol)	22	25
Methenolone (3α-hydroxy-1-methylen-5α-androstan-17-one)	68	64
Methasterone[b]	56	47
Norbolethone	45	39
Norethandolone (17α-ethyl-5β-estrane-3α,17β-diol)	92	68
Trenbolone (epitrenbolone)[b]	16	20
Oxandrolone (17-epioxandrolone)	26	28
Decreased sensitivity using the 96-well plate extraction procedure		
17α-methyl-5β-androstane-3α,17β-diol	24	165
Clostebol	257	1340
Drostanolone (2α-methyl-5α-androstan-3α-ol-17-one)	19	27
Fluoxymesterone (9α-fluoro-18-nor,17,17-dimethylandrosta-4,13-diene-11β-ol-3-one	86	143

Methyl-1-testosterone	6	9
Methyltrienolone	8	20
Mibolerone	9	75
Nandrolone (19-norandrosterone)[c]	5	31
Norclostebol[b]	46	215
Oxandrolone	4	6

[a]Metabolites used to identify parent compounds are provided in parentheses. Parent compounds and metabolites were tested at the WADA MRPL of 10 ng/mL, unless otherwise indicated.
[b]Tested at 25 ng/mL.
[c]Tested at the WADA threshold concentration of 2 ng/mL.

Extracted ion chromatograms of mass-to-charge (m/z) 206 and 430 ions for boldenone are shown in Figure 1, illustrating the increased ion intensity achieved using the automated extraction method.

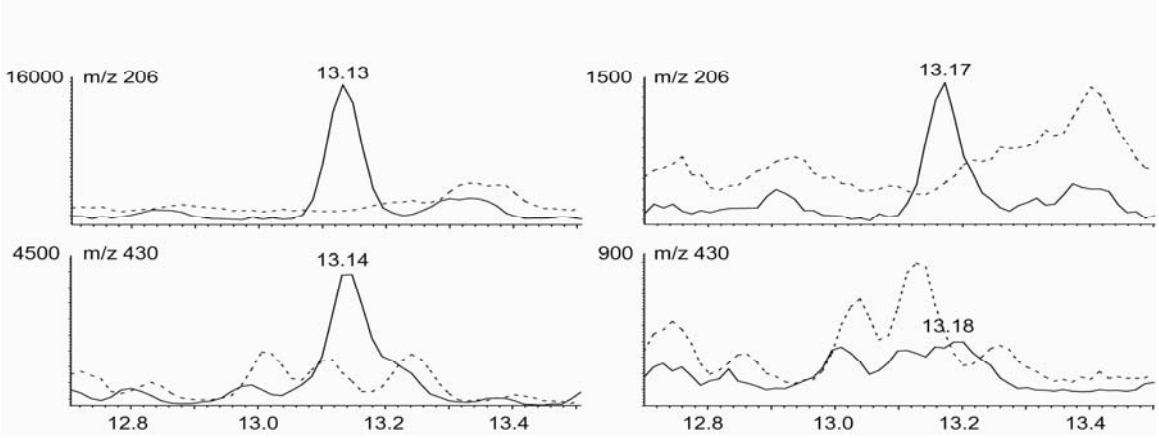

Figure 1. Extracted ion chromatograms showing boldenone eluting at 13.1 minutes for m/z 206 and 430 ions using the automated (left panels) or Empore disc cartridge (right panels) extraction procedures. Solid and dashed lines represent urine fortified with boldenone and drug-free negative urine, respectively. The y-axis represents ion abundance.

The 17-epi metabolite of methandienone was readily detected by the automated extraction method despite not being detected by the C-18 disc cartridge method (ion chromatograms not shown). Further refinement of the automated extraction procedure is needed as several anabolic agents displayed reduced recovery (Table 1). For instance, 19-norandrosterone, a major metabolite of nandrolone with a threshold concentration of 2 ng/mL, had a 6-fold reduction in S/N ratio using the automated extraction procedure when monitoring m/z 405 and 420 ions (Figure 2). Oxandrolone also exhibited a low S/N ratio of 4 using the automated extraction procedure; however, this compound is also poorly recovered using C-18 Empore disc cartridges (S/N ratio of 6).

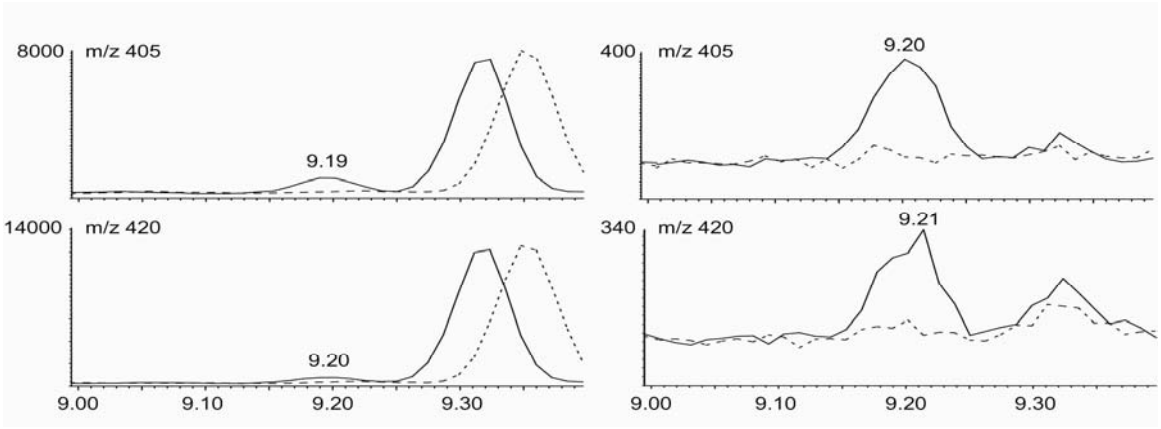

Figure 2. Extracted ion chromatograms showing 19-norandrosterone eluting at 9.2 minutes for m/z 405 and 420 ions using the automated (left panels) or Empore disc cartridge (right panels) extraction procedures. Solid and dashed lines represent urine fortified with 19-norandrosterone and drug-free negative urine, respectively. The y-axis represents ion abundance.

As shown in Table 2, beta-blockers were easily detected at 250 ng/mL, which is 50% below the MRPL. Many of the compounds had increased S/N ratios using the automated extraction method when compared to the method using Empore disc cartridges. The S/N ratio was greater than 30 for all of the beta-blockers examined except for mepindolol which had a S/N of 5.

Table 2. Sensitivity of the automated 96-well microtiter plate urine extraction procedure for detecting selected beta-blockers based on signal to noise (S/N) ratios.

Compound[a]	**S/N ratio using 96-well extraction**	**S/N ratio using Empore disc cartridge extraction**
Improved sensitivity using the 96-well extraction procedure		
Alprenolol	1503	511
Atenolol	130	21
Betaxolol	1365	192
Carteolol	1850	922
Metoprolol	1806	673
Propranolol	193	80
Sotalol	2028	1208
Timolol	209	67
Similar sensitivity compared with the disc extraction procedure		
Bambuterol	523	586
Esmolol	1958	1447
Fenoterol	465	540
Levobunolol	370	333
Decreased sensitivity using the 96-well plate extraction procedure		

Acebutolol	66	167
Bisoprolol	63	283
Celiprolol	209	407
Labetalol	34	122
Mepindolol	5	25
Metipranolol	560	984
Nadolol	41	131
Oxprenolol	281	760
Pindolol	42	120

[a]All compounds were tested at 50% of the MRPL (250 ng/mL).

All of the narcotics examined were readily detected at the MRPL of 200 ng/mL using the automated extraction procedure (Table 3). There was significant improvement in the detection of hydrocodone, morphine and oxycodone using the automated extraction method. Noroxycodone and oxymorphone had very high S/N ratios using the automated extraction procedure, but were undetectable by the method using Empore disc cartridges.

Detection of cocaine (benzoylecgonine) at 150 ng/mL (30% of the MRPL) was significantly improved using the automated extraction method. Although the S/N ratio of the THC metabolite was reduced, there was still enough sensitivity to detect it at the threshold level of 15 ng/mL. The beta-2 agonist salbutamol was easily detected using the automated extraction method at the WADA threshold of 1000 ng/mL. Terbutaline was detected at 200 ng/mL, a concentration twice the MRPL, whereas Salmeterol was not detected using the automated extraction method. Lastly, the synthetic allosteric modifier of hemoglobin, RSR13, could be readily detected at a concentration of 40 ng/mL

Table 3. Sensitivity of the automated 96-well microtiter plate urine extraction procedure for detecting selected narcotics, street drugs, and other compounds based on signal to noise (S/N) ratios.

Compound	**S/N ratio using 96-well plate extraction**	**S/N ratio using Empore disc cartridge extraction**
Hydrocodone[a]	1318	672
Hydromorphone[a]	110	374
Morphine[a]	2814	233
Noroxycodone[a]	874	Not detected
Oxycodone[a]	2725	48
Oxymorphone[a]	426	Not detected
Cocaine (benzoylecgonine)[b]	1362	41
THC metabolite (tetrahydrocannabinol acid)[b]	59	112

Salbutamol	698	1485
Salmeterol	Not detected	138
Terbutaline	56	1518
RSR13	79	254

[a]l Compounds were tested at the WADA MRPL of 200ng/mL.
[b]Metabolites used to identify parent compounds are provided in parentheses.

Discussion

The objective of this work was to improve the efficiency of sample processing and reduce human error by automating the urine extraction procedure, while meeting WADA criteria for MRPL. Even when the urine volume was reduced from 2.5 mL down to 1.0 mL, MRPL could be met for most of the substances that were examined. The choice of the sorbent material (Strata X 96-well plates) was determined by its selectivity towards aromatic and polar compounds. Due to the high binding capacity and 2.0 mL well volume, it was possible to load 1.0 mL of urine in each well, which is a significant advantage compared with standard 3M Empore plates. The fully automated extraction procedure produced an increase in sensitivity (based on S/N ratios) for approximately one third of substances tested, when compared to our current extraction procedure using Empore disc cartridges. Anabolic agents such as stanozolol and clenbuterol were not examined because these compounds are routinely monitored by LC-MS/MS because of the increased detection sensitivity. Formebolone was also not examined because it is also routinely monitored by LC-MS/MS because of endogenous interferences when using GC-MS. Unfortunately, approximately one third of the compounds exhibited lower sensitivity using the automated method, while the remainder of the compounds had equivalent extraction efficiencies. In some cases, we noticed elevated background noise using 96-well extraction plates, possibly due to the addition of derivatizing agent to polypropylene plates. Additional studies are underway to address this issue.

In summary, we have developed a fully-automated, walk-away urine extraction procedure that is more efficient; requiring only about 45 minutes to complete SPE extraction of 96 samples. This is in contrast to our current method using Rapid Trace Liquid handler modules that takes more than three hours to extract a similar number of samples and requires manual addition of internal standard, buffer and enzyme. Furthermore, the frontend aliquoting step into 96-well plates can also be automated further reducing human error, manual pipetting steps and labor costs. Additional studies are currently underway to further improve the method in order to increase its sensitivity for detecting various compounds and for automating the derivatization step using a PAL GC autosampler (Leap Technologies) equipped with a heating block.

References

[1] Geyer H., Schaenzer W, Mareck-Engelke U, Nolteernsting E, Opfermann G. (1998) Screening procedure for anabolic steroids-The control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. *Recent Advances in Doping Analysis (5)*, Köln, pp99-102.
[2] Catlin DH, Kammerer RH, Hatton CK, Sekera MH, Merdink JL. (1987**)** Analytical chemistry at the games of the XXIIIrd Olympiad in Los Angeles, 1984. *Clin Chem*, **33**, 319-327.
[3] Andreas Breidbach, Don H. Catlin. (2001) RSR13, a potential athletic performance enhancement agent: detection in urine by gas chromatography/mass spectrometry *Rapid Commun Mass Spec* **15**, 2379-2382.
[4] Donike M, Zimmermann J (1980) Preparation of trimethylsilyl-, triethylsilyl- and tert-butyldimethylsilyl enol ethers of oxo steroids for gas chromatographic and mass spectrometric studies. *J Cromatogr* **202**, 483-486.
[5] Xu RN, Fan L, Rieser MJ, El-Shourbagy TA, (2007) Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS *J Pharm. Biomed. Anal.* 44, 342-355
[6] Walker V, Mills GA. (2002) Solid-phase extraction in clinical biochemistry. *Ann Clin Biochem***, 39**, 464-477.
[7] World Anti-Doping Agency The 2009 Prohibited List. Available at http://www.wada-ama.org/rtecontent/document/2009_Prohibited_List_ENG_Final_20_Sept_08.pdf (access date 16.09.2009)
[8] World Anti-Doping Agency, Technical Document-TD2009MRPL. Available at http://www.wada-ama.org/rtecontent/document/MINIMUM_REQUIRED_PERFORMANCE_LEVELS_TD_v1_0_January_2009.pdf (access date 16.09.2009)