Analysis of a Challenging Subset of World Anti-Doping Agency-Banned Steroids and Antiestrogens by LC–MS–MS

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Abstract

A qualitative liquid chromatography–tandem mass spectrometry method for the analysis of 22 sporting federation-banned anabolic agents (or their metabolite markers) and anti-estrogens in urine that are refractory to analysis by gas chromatography–mass spectrometry is presented. In addition, a quantitative method built around World Anti-Doping Agency (WADA) guidelines for the confirmatory analysis of 19-norandrosterone, the primary metabolite of nandrolone with a WADA-specified minimum required performance limit of 1 ng/mL, is included. Hydrolysis of glucuronide conjugates, liquid–liquid extraction, no clean-up derivatization with Girard's Reagent P, and analysis by quadrupole-time-of-flight mass spectrometry provide sensitivity and selectivity well beyond that required by the WADA.

Introduction

Anabolic agents are banned for athletic use by the International Olympic Committee and many other sporting federations. With the exception of a few difficult-to-detect steroids, most urine drug testing for banned anabolic agents is carried out by gas chromatography–mass spectrometry (GC–MS) (1). Exceptional, difficult-to-detect steroids and the anabolic agents for which the World Anti-Doping Agency (WADA) has set exceptionally low detection limit requirements (2) present a detection challenge to the traditional GC–MS-based screening assay for banned anabolic agents. This challenge generally finds its root cause in poor heat stability, poor GC characteristics, and/or inadequate sensitivity by GC–MS.

To help overcome this challenge in routine urine sample screening, we have developed a highly sensitive liquid chromatography–moderate-resolution tandem mass spectrometry [LC–MS–MS via quadrupole-time-of-flight (Q-TOF)]-based method for the analysis of this problematic set of banned anabolic agents. The method is based on hydrolysis of steroid glucuronide conjugates followed by a common liquid–liquid extraction procedure and simple derivatization (one step, room temperature, no purification) with Girard's Reagent P (GRP), a ketone and aldehyde-reactive hydrazine agent containing a quaternary pyridinium amino group (Figure 1). The major advantage of this method over other LC–MS–MS-based methods for the analysis of banned anabolic agents is its sensitivity in detecting anabolic agents extracted from authentic urine, sensitivity that is brought about through the optimal employment of GRP as a derivatizing reagent and the extreme selectivity of moderate-resolution TOF MS. Sensitivity is improved from a point of barely meeting WADA minimum required performance limits (MRPL) requirements for most underivatized anabolic agents to providing completely unambiguous positive identification of banned steroids in urine well below WADA's specified MRPLs. This GRP steroid derivative (and its tri-methyl amine counterpart Girard's Reagent T) has been known for years and its gas phase fragmentation pathways recently characterized (3–6), but few attempts have been made to use it for routine analytical purposes (7,8). To our knowledge it has not been used for routine forensic purposes.

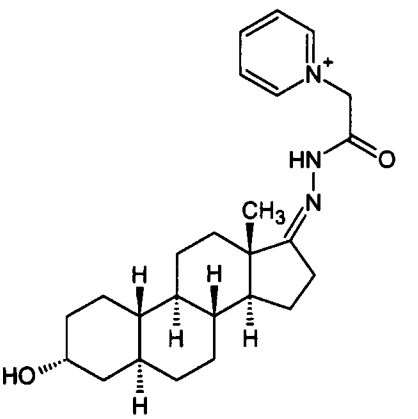

Figure 1. Structure of GRP-derivatized 19-norandrosterone.

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Perhaps this has been because of its relative obscurity and the problems imparted by the equilibrium nature of its reaction with ketones and aldehydes, problems such as quantitatively inconsistent loss of the derivative following post-derivation purification [leading to the loss of sensitivity gains, perhaps as experienced and lamented by Shackleton et al. (7)] or, conversely, feared high-performance liquid chromatography (HPLC) and MS contamination with the introduction of excessive quantities of GRP.

In summary, this paper describes a highly sensitive LC–MS–MS via Q-TOF-based method for the analysis of banned anabolic agents that takes advantage of a combination of high ionization efficiency—imparted by a derivatizing reagent with yet untapped potential for LC–MS based applications—and moderate instrumental resolving power to provide unambiguous identification of banned anabolic agents at or, in many cases, well below WADA-established detection limit requirements. The method meets all WADA-established confirmation criteria and goes beyond by employing additional mass accuracy criteria.

Materials and Methods

Materials

Anabolic drugs and metabolites were purchased from Sigma-Aldrich (St. Louis, MO), Steraloids (Newport, RI), or the

Table I. Summary of Analytes and Their Chromatographic, Mass Spectral, and Detection Limit Properties

Analyte	LC–MS Time Period (min)	Mass-to-Charge Ratio of Precursor Ion	Mass-to-Charge Ratio of Product Ions Monitored in Data Analysis Method*	Nature of Precursor Ion	Derivatization Status	Collision Energy (eV)	LOD (ng/mL)	WADA's MPRL† (ng/mL)
Aminoglutethimide	1.5–4	223.1	146.1, 160.1, 188.1	MH^+	none	23	0.3	not declared
Clenbuterol	1.5–4	277.1	203.0, 259.1	MH^+	none	17	0.13	2
6β-Hydroxyfluoxymesterone	1.5–4	486.3	379.2, 407.2	M^+	mono	46	0.63	10
9α-Fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetrol‡	4–6.7	337.2	95.1	$[M+H-H_2O]^+$	none	27	2.5	10
Raloxifene	6.7–10.5	474.2	112.1, 84.1	MH^+	none	41	0.1	not declared
Exemestane	6.7–10.5	430.25	185.1, 279.2	M^+	mono	38	0.1	not declared
Epitrenbolone§	6.7–10.5	404.2	297.2, 325.2	M^+	mono	36	0.1	10
Oxymesterone	6.7–10.5	452.3	136.1, 167.1	M^+	mono	47	0.63	10
Gestrinone§	6.7–10.5	442.2	335.2, 363.2	M^+	mono	37	0.16	10
Methyltestosterone (IS)§	6.7–10.5	436.3	sum of 436.3, 357.3, 329.3, 163.1, 151.1	M^+	mono	46	–	N/A
19-Norandrosterone#	6.7–10.5	410.3	241.2, 259.2	M^+	mono	44	0.13	1
Tetrahydrogestrinone§	6.7–10.5	446.3	264.2, 306.2, 339.2	M^+	mono	40	0.63	10
1-Methylene-5α-androstan-3α-ol-17-one**	6.7–10.5	436.3	161.1, 267.2	M^+	mono	46	0.16	10
Anastrozole	6.7–10.5	294.2	225.1	MH^+	none	24	3.3	not declared
2-Hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one††	10.5–12.2	347.2	147.1, 281.2	MH^+	none	21	2.5	10
Clomiphene§	10.5–12.2	406.2	100.1, 72.1	MH^+	none	35	0.16	not declared
3'-Hydroxystanozolol	12.2–13.6	345.3	97.0	MH^+	none	60	0.063	2
4β-Hydroxystanozolol	12.2–13.6	345.3	269.2, 309.2, 327.2	MH^+	none	20	0.16	10
Epioxandrolone	13.6–14.3	289.2	135.1, 229.2	$[M+H-H_2O]^+$	none	27	2.5	10
Mestanolone	14.3–15.1	305.2	159.1, 229.2	MH^+	none	29	2.5	10
17α-Methyl-5β-androstane-3α,17β-diol‡‡	14.3–15.1	271.2	161.1, 175.2, 189.2	$[M+H-2H_2O]^+$	none	32	2	2
Fulvestrant	15.1–20.5	607.3	467.2, 493.2, 589.3	MH^+§§	none	25	0.3	not declared
Epimetendiol##	15.1–20.5	269.2	105.1	$[M+H-2H_2O]^+$	none	30	0.25	2

* By virtue of the nature of TOF mass analyzers, mass spectral data for all ions are acquired.
† If MRPL value is not declared, experiments were carried out as if it were 10 ng/mL.
‡ A fluoxymesterone metabolite.
§ Chromatographically separable *cis* and *trans* isomers exist; the most abundant isomeric form is monitored.
# A nandrolone metabolite.
** A methenolone metabolite.
†† A formebolone metabolite.
‡‡ A methyltestosterone metabolite.
§§ The GRP derivative of the sulfoxide forms but is eliminated in the ion source in favor of the protonated MH^+ molecule.
A methandienone metabolite.

National Analytical Reference Laboratory of Australia (NARL, Sydney, Australia). Girard's Reagent P was from Sigma-Aldrich.

β-Glucuronidase from *E. coli* was from Roche (Indianapolis, IN). All other chemicals and solvents were of either analytical or HPLC grade.

Sample preparation

Samples were extracted using a liquid–liquid extraction procedure common to anti-doping applications (9). Three milliliters of urine was added to a silanized glass test tube containing 300 ng of methyltestosterone (for use as an internal standard). To the urine was added 1 mL of 0.8M potassium phosphate buffer, pH 7.0, followed by 25 µL of β-glucuronidase solution (minimum 140 IU/mL, according to Roche package insert). The samples were then incubated at 50°C for 1 h. Following glucuronide hydrolysis 750 µL of a 20% (w/v) solution of a $K_2CO_3/KHCO_3$ (1:1) mixture was added to each sample followed by 6 mL of methyl t-butyl ether (MTBE). The samples were shaken for 10 min then centrifuged for 5 min at 2000–2500 rpm in a swing-bucket centrifuge. The organic layer was transferred to a silanized 13 × 100-mm glass tube and dried under a gentle stream of air at 40°C. Samples were reconstituted in 20 µL of methanol followed by 80 µL of 1M Girard's Reagent P in 50mM ammonium acetate buffer (pH 4.2). Samples were then transferred to autosampler vials, and 20 µL was injected after a 1-h incubation time at room temperature.

LC–MS–MS

LC–MS–MS equipment consisted of an Agilent 1100 series HPLC with inline degasser, column heater, and autosampler equipped with a 100-µL injection loop connected to an Applied Biosystems QStar-XL tandem MS equipped with a Turboionspray® (heated electrospray) ion source. The HPLC column employed was a Phenomenex Luna C18(2) column with 150- × 2.0-mm dimensions and 3-micron particles with 100-angstrom pore size. A guard frit was employed to keep particulates out of the column. The column compartment was kept at 50°C during analysis. HPLC mobile phase A consisted of 0.1% formic acid in deionized water and mobile phase B consisted of methanol. A mobile phase flow rate of 250 µL/min was employed with the following gradient: the column was equilibrated at 75% A/25% B. Upon injection the mobile phase composition was ramped to 5% A/95% B over 12 min and held at 5% A/95% B for 2 min, followed by a return to 75% A/25% B over 0.5 min and reequilibration for 6 min. Total run time was 20.5 min. HPLC pump pre-column dead volume was approximately 1.5 mL, thus there was an effective 6 min of initial isocratic chromatographic conditions at the beginning of each run. Baseline separation of the nandrolone metabolites 19-norandrosterone and 19-noretiocholanolone was achieved by starting with a mobile phase composition of 70% A/30% B and ramping to 59% A/41% B over 11 min.

The mass spectrometer was operated in positive ion mode under the following conditions: ESI probe position was 4.5 mm off axis to the left and 0.5 mm back from the closest possible setting. Nebulizer gas (Gas 1) was set at 45 arb units, auxiliary gas (Gas 2 or heated gas) at 40 arb units, curtain gas at 45 arb units, auxiliary gas temperature at 200°C, and ESI voltage at 4000 V. For the first 1.5 min of each run, flow was diverted to waste via an automated built-in post-column valve to avoid contamination of the ion source with excessive amounts of GRP derivatizing reagent. The MS was operated in targeted MS–MS mode with Q1 set to unit resolution, a collision gas (nitrogen) pressure of 8 arb units, and 'enhance' mode activated for the mass-to-charge ratio of the most abundant product ion. Time periods containing separate MS–MS experiments were set up as shown in Table I. (Note that for all compounds only the mono-GRP derivative is formed and that the derivative is not protonated because of the permanent positive charge imparted by the quaternary pyridinium functional group of the GRP molecule.)

Results

MS

MS–MS spectra for representative underivatized and GRP derivatized steroids are shown in Figures 2A and 2B, respectively. In addition to less abundant fragmentations, GRP-derivatized steroids frequently show a loss of 79 Da (from loss

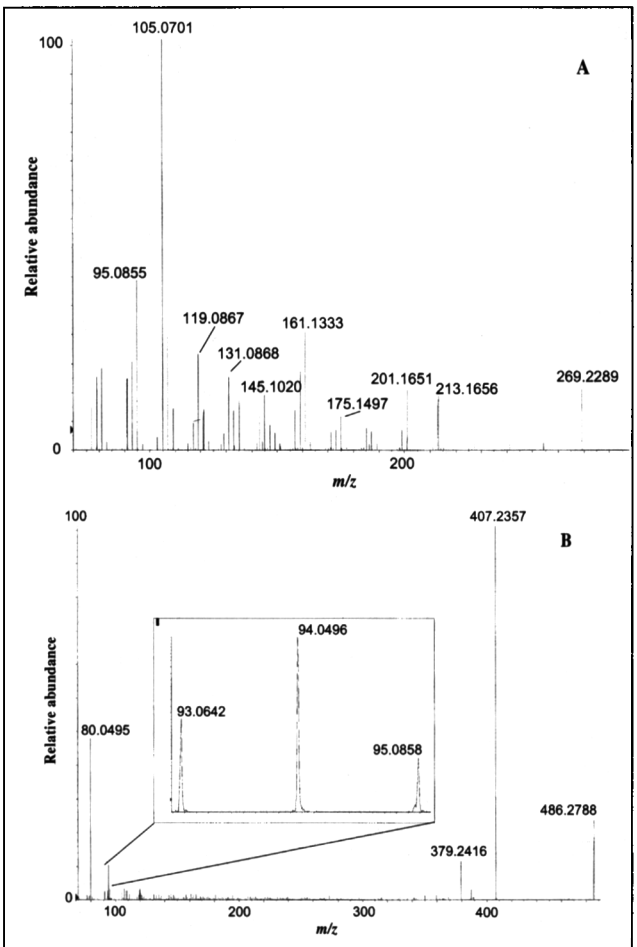

Figure 2. Low collision energy MS–MS spectra representative of an underivatized steroid (epimetendiol) (A) and a GRP-derivatized steroid (6β-hydroxyfluoxymesterone) (B). The inset of panel B is shown to demonstrate resolution.

of the pyridine portion of the GRP derivative; *m/z* 407 in Figure 2B) and a pyridine fragment that retains the charge (*m/z* 80 in Figure 2B). In order to minimize the chance of obtaining ambiguous results during the analysis of authentic samples, the elemental composition of most monitored product ions was determined via accurate mass measurement and knowledge of the elemental composition of the precursor ions. This permits the proper centering of narrow (± 20 ppm) product ion extracted ion chromatograms (XICs) in the data analysis method and establishes the accurate mass by which to compare data obtained during confirmational analyses as described. TOF technology provides the advantage of monitoring as many product ion XICs as desired without having to change instrument acquisition parameters; in essence, all product ions are mass analyzed and detected all the time. XICs must simply be specified in the MS data analysis method.

Method validation

Table I provides a summary of the parameters for the LC–MS–MS method, including the limits of detection (LODs) for each compound. The LOD here is defined as the concentration in authentic urine at which a signal-to-noise ratio of at least 5 is obtained for the most abundant product ion's ± 20 ppm XIC. For each LOD experiment, a set of urine samples fortified at decreasing concentrations (in half-concentration steps from twice the MRPL down to 1/256 of the MRPL) were analyzed. LODs reported are the highest single LOD value determined for a given analyte from two different batches analyzed on two different days by two different analysts with blank urine from two different authentic urine pools. One of the two urine pools was quite cloudy and dirty in appearance and produced LODs (which are reported here) for several analytes that were 2–4 times those observed in the first experiment. Thus, we consider the LODs reported here to be rather robust. (Because of the extreme selectivity of the analytical method, the XICs for many analytes show no baseline signal whatsoever for extracts of blank urine samples. Thus, LODs could not be determined by multiplying the standard deviation of the mean observed signal for blank samples by a Z-value of 5.) WADA-established minimum required performance limits (MRPLs) (i.e., minimum required detection limits) are far surpassed in most cases (Table I), providing unambiguous analyte identification in samples containing concentrations of banned substances that are well below WADA-specified performance criteria. To illustrate, XICs (± 20 ppm) of select product ions for several analytes (as extracted out of 3 mL of authentic urine) from unfortified samples, samples fortified at their LOD, and samples fortified at their WADA-specified MRPL concentrations are shown in

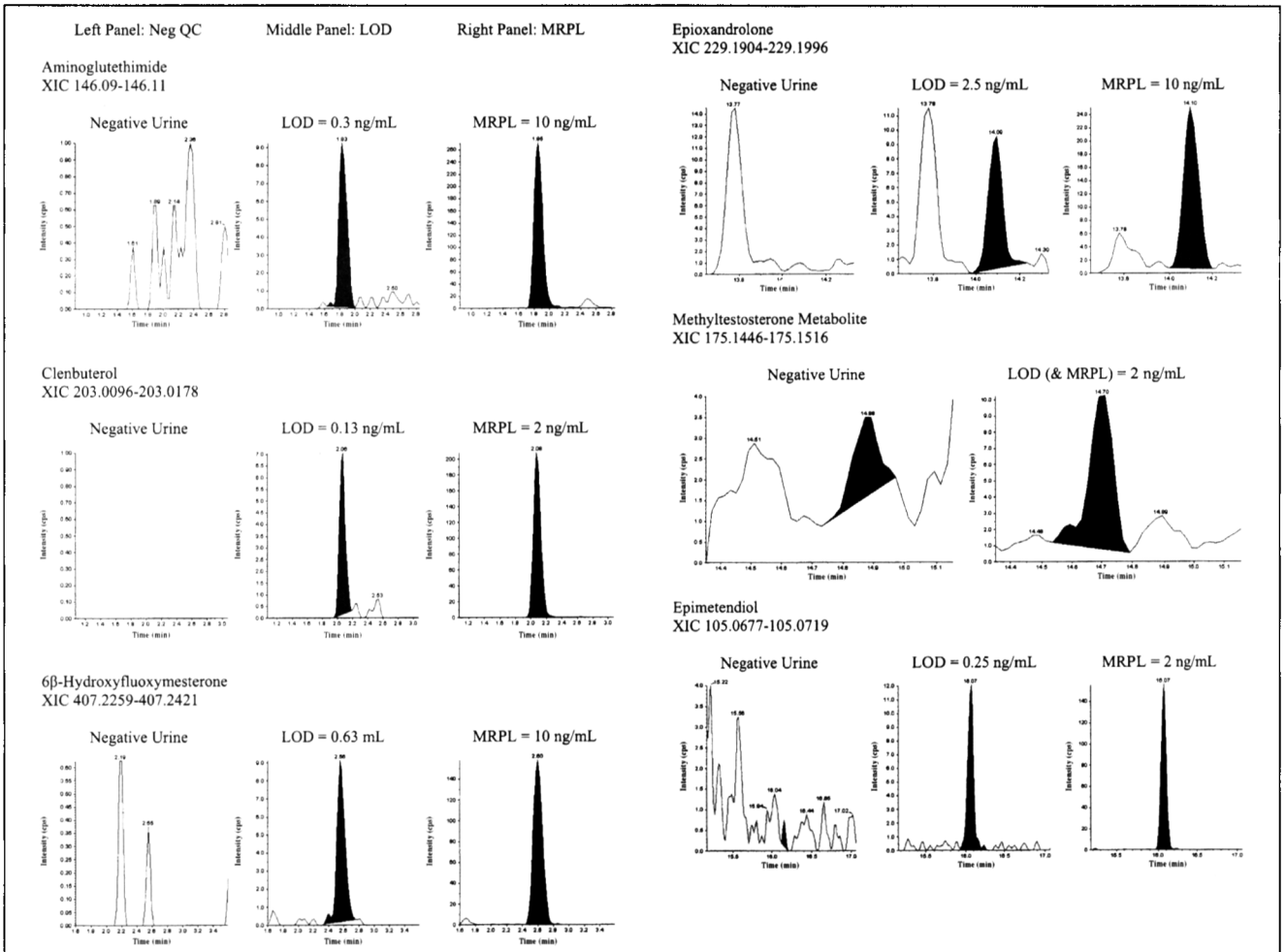

Figure 3. Extracted ion chromatograms for blank urine, urine fortified at the LOD, and urine fortified at the WADA MRPL for selected compounds analyzed. XIC indicates the mass range of the extracted ion chromatogram.

Figure 3. These data were derived from extractions in which the 6 mL of MTBE extract was evaporated and reconstituted in the GRP derivatization solution. In routine practice, we find it efficient to split the 6 mL of MTBE into three equal-volume aliquots for three different screening procedures. The LODs reported here are thereby increased by a maximum of threefold if this practice is employed but, with the exception of the methyltestosterone metabolite, remain well below the WADA-specified MPRLs.

For use as a nominal identification test (i.e., sample screening method), this method was validated with regard to LODs and specificity. Because this method is employed for qualitative trace analysis, validation of accuracy, precision, limits of quantitation, linearity, and range are not applicable (10). As stated, LODs were established at a signal-to-noise ratio of 5:1 for selected product ion XICs (Figure 3). In a separate set of experiments from those used to determine the LODs, specificity was validated at the WADA MRPL concentration for each analyte by fortifying the analyte into six different authentic urine sources (n = 1 each of three different male and three different female samples) and comparing the nominal analytical results (positive or negative) to another aliquot of the same urine specimens fortified with internal standard only and with a third aliquot of the same urine specimens left completely unfortified. Data were analyzed, and nominal positive results were assessed based on a retention time within 0.1 min of the expected retention time and a signal-to-noise ratio of 5:1 or greater for ± 20 ppm wide-product ion XICs. Whenever possible, at least three different XICs of product ions were used to assess the absence or presence of a given analyte. For sample screening, product ion abundance ratios were also required to approximate WADA's criteria specified in Table II, relative to those of a pure authentic standard. All six fortified samples were clearly positive for the fortified analytes and all unfortified samples were clearly negative for the analytes in question, according to the given criteria (data not shown bcause of space limitations; see Figure 3 for typical XIC data at MPRL concentrations). As additional confirmation of method specificity, this experiment was repeated with 15 MPRL-concentration-fortified urine samples (along with 8 unfortified samples) from an authentic urine pool from multiple drug-free donors. The 15 fortified samples were clearly positive for the fortified analytes, and the 8 unfortified samples were clearly negative for the analytes in question, according to the given criteria.

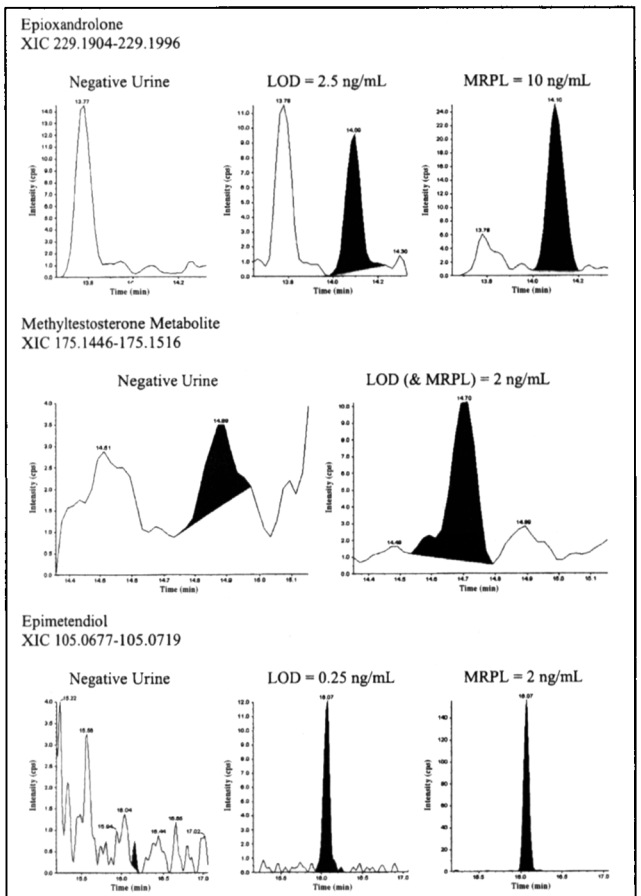

Figure 3. (continued) Extracted ion chromatograms for blank urine, urine fortified at the LOD, and urine fortified at the WADA MRPL for selected compounds analyzed. XIC indicates the mass range of the extracted ion chromatogram.

As described here, the method reported is employed as a screening method. However, because the Q-TOF MS generates full "scan", moderate-high mass accuracy MS–MS data, the method is readily adapted to a specific confirmation method to meet WADA-specified criteria. Besides meeting the ion ratio criteria specified in Table II, the only methodological adjustment required is to ensure that the precursor ion is represented in product ion mass spectra. In some cases this means turning off the "enhance" feature of the QStar-XL MS, which may mean a decrease in sensitivity of 3–4-fold. Confirmations, however, are carried out on a separate aliquot of urine from which the entire 6 mL of MTBE extract is used for analysis. As can be seen from the LODs in Table I, however, the sensitivity remains adequate to meet WADA MRPL requirements. In addition to the WADA requirements for reproducible product ion ratios and retention of the precursor ion in the MS–MS spectrum for confirmational analyses, the high mass accuracy of the QStar-XL instrument provides for the addition of another criterion to ensure a low false-positive reporting rate, namely, product ion mass accuracy. For confirmational analyses performed in our laboratory, we have determined that monitored product ions must be within 5 mDa or 20 ppm (whichever is greater) of their calculated mass.

WADA requires that reports for the confirmation of 19-norandrosterone, a primary nandrolone metabolite, must

Table II. Maximum Tolerance Windows Dictated by the WADA for Relative Product Ion Peak Intensities to Ensure Appropriate Specificity of Identification

Relative Abundance (% of base peak)	Maximum Deviation for LC–MS–MS*
> 50%	± 15% (relative abundance)
25–50%	± 25% (relative to relative abundance)
< 25%	± 10% (relative abundance)

* The deviations are relative to the peak intensities observed in the product ion spectrum of a pure authentic standard.

present quantitative results if the estimated concentration of 19-norandrosterone is in the range of 2–10 ng/mL urine (based on screening data). 19-Norandrosterone is frequently present simultaneously with 19-noretiocholanolone in the urine of persons who have used nandrolone. 19-Noretiocholanolone is a second nandrolone metabolite and diastereomer of 19-norandrosterone. Under the LC conditions described, the two metabolites are only partially chromatographically resolved. Because both metabolites have the same MS–MS spectrum, these nandrolone metabolites must be chromatographically resolved in order for quantitative information on 19-norandrosterone to be gathered. Chromatographic resolution of these isomers may be achieved as described in the Methods section.

In addition to specificity (described previously, but using chromatographic conditions to separate 19-norandrosterone and 19-noretiocholanolone), quantitative accuracy and precision at 2 ng/mL (the WADA-specified reporting threshold) and linearity over the relevant concentration range of 2–10 ng/mL were validated for 19-norandrosterone extracted out of authentic urine using 19-norandrosterone-d_4 as an internal standard at a concentration of 10 ng/mL; interassay accuracy, assessed on three separate days during a multiple week period with multiple analysts using 10 fortified urine samples per day, was found to be 103%, 96%, and 106% with coefficients of variation of 5.9%, 5.0%, and 9.5%, respectively. Taken together, interassay accuracy and precision (n = 30) was found to be 102% with a CV of 8.1%. Linearity was demonstrated by construction of a standard curve from 11 fortified authentic urine samples with concentrations ranging from 1 to 20 ng/mL and observation of an r^2 value of 0.9964. In addition, all product ion ratio (Table II) and mass accuracy criteria were met for each fortified sample (data not shown).

Discussion

Successful utilization of GRP as part of this routine sample screening procedure hinged upon recognition of the fact that the imine formed between GRP and ketone-containing molecules is reversible and product formation is maximal when the reaction is carried out in the pH range of 4–5 (11). Thus, for the sake of sensitivity and quantitative precision, it is best to not purify analyte molecules from excess GRP reagent prior to injection. Under the HPLC conditions employed, the 8 µg of injected GRP elutes almost completely within the first 1.5 min and therefore can be automatically diverted to waste. The hydrophilic nature of GRP allows it to be easily flushed from the LC–MS instrumentation and it does not cause any buildup on, or extra required maintenance for, the inlet of the MS when used as described here. Because no post-derivatization clean-up is required, the derivatization reaction is no more complicated than the ordinary reconstitution of samples in HPLC mobile phase.

Only about half of the analytes in this method are converted to quaternary amine-containing GRP derivatives. The remaining analytes are unaffected by the GRP reagent and are merely protonated, or protonated and dehydrated during the electrospray process (Table I). Even though they remain underivatized, we have observed (during the course of method development) equivalent or better sensitivity for most underivatized analytes when GRP is added to urine extracts compared to when it is not added. The mechanism behind this observation remains unclear, but it is likely due to the derivatization of unmonitored endogenous urinary metabolites causing their retention time to shift away from the underivatized, monitored steroids leading to decreased ionization suppression and background noise. Clenbuterol is an exception to this observation: its LOD improves in the absence of GRP. This is due to its co-elution with the chromatographic tail of excess GRP reagent which, we suspect, causes ionization suppression of clenbuterol. As can be seen in Table I, however, the LOD for clenbuterol in the presence of GRP remains well below the WADA's MRPL.

Derivatization of ketone groups with GRP results in the formation of *cis/trans* isomers. For epitrenbolone, gestrinone, THG, and the methyltestosterone internal standard, these isomers chromatographically resolve under the HPLC gradient employed. Chromatographic separation of these isomers causes a dilution of analyte signal but provides a satellite chromatographic peak which can be used as an additional piece of confirmatory evidence for positive samples.

The method described here is not useful for the analysis of androstenedione and its dione-containing metabolite 6α-hydroxyandrostenedione. The bis-GRP derivative is too hydrophilic to elute after excess GRP, and insignificant quantities of the mono-GRP derivative are formed to meet sensitivity requirements. This scenario likely holds true for all other dione-containing steroids. One observation, however, suggests that it may not: 2-hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (a formebolone metabolite) and mestanolone (an oxymetholone metabolite) contain ketone functional groups, yet are best analyzed as their underivatized forms, most likely because either the reaction equilibrium association constant (K_a) is small and/or because the reaction dissociation rate constant (k_d) is excessively large (causing rapid derivative loss upon removal of excess GRP when the sample is injected onto the HPLC column). Thus, there may be dione-containing steroids that effectively acquire only one GRP derivative, allowing for their analysis by the method described here.

Under the conditions described here, fulvestrant (which does not contain a ketone or aldehyde group) is derivatized by GRP, mostly likely at the sulfoxide group. Interestingly, however, the derivative is completely removed during the ionization process resulting in an underivatized, protonated fulvestrant ion. This reaction is fortuitous because underivatized fulvestrant does not elute from our HPLC column (or from a DB-1ms GC column, which is used for conventional steroid screening) during the course of a routine chromatographic run.

Conclusions

In summary, we have presented a qualitative LC–MS–MS method that exceeds WADA sensitivity and selectivity specifications for the analysis of 22 sporting federation-banned

anabolic agents (or their metabolite markers) and anti-estrogens in urine that are refractory to analysis by GC–MS. In addition, we have included a quantitative method built around WADA guidelines for the confirmatory analysis of 19-norandrosterone, the primary metabolite of nandrolone. The key to development of this method was sample derivatization with GRP, which resulted in the incorporation of a pre-ionized, readily desolvated quaternary amine (and in the formation of *cis/trans* isomers that chromatographically resolved in most cases and served as an aid in confirming analyte identity). Successful utilization of GRP as part of this routine sample screening procedure, however, hinged upon recognition of the fact that the imine formation between GRP and ketone-containing molecules was reversible. Thus, it was best not to purify analyte molecules from excess GRP prior to injection. Unreacted GRP eluted within the first 1.5 min and was therefore automatically diverted to waste. Because no clean-up was required, the derivatization reaction was no more complicated than the ordinary reconstitution of the samples in HPLC mobile phase.

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