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Analysis of a challenging subset of WADA-banned steroids and anti-estrogens by LC/MS/MS

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OVERVIEW An LC/MS/MS method for the analysis of 22 World Anti-Doping Agency (WADA)-banned anabolic agents and anti-estrogens in urine that are refractory to analysis by GC/MS is presented. Reaction of ketone groups with Girard's Reagent P (1-(2-Hydrazino-2-oxoethyl)pyridinium chloride, GRP) introduces a quaternary amine functional group that dramatically enhances the sensitivity of nitrogen lacking steroids when analyzed by ESI-LC/MS. This simple derivatization with GRP and analysis by Q-q-TOF mass spectrometry provide sensitivity and selectivity well beyond that required by the WADA.

EXPERIMENTAL *Sample preparation:* Sample extracts were prepared based upon a previously published method [1]:To 3 mL of urine add 1 mL of 0.8 M potassium phosphate buffer, pH 7.0. Add 3.5 IU of β-glucuronidase (from *e. coli*) and incubate at 50 °C for 1 hour. Add 750 mcL of a 20% (w/v) solution of a K_2CO_3/$KHCO_3$ (1:1) mixture. Extract with 6 mL of t-butyl methyl ether. Remove and dry organic layer at 40° C under air. *Sample Reconstitution/Derivatization:* Reconstitute samples in 20 mcL of methanol followed by 80 mcL of 1 M Girard's reagent P (GRP) in 50 mM ammonium acetate buffer, pH 4.2. Incubate at room temperature for 1 hour prior to injection. *LC/MS/MS Parameters:* Instrument: Agilent 1100 HPLC connected via a TurboIonSpray® (ESI) source to a QStar-XL (Q-TOF) mass spectrometer (Applied BioSystems); Injection Volume: 20 mcL; Flow rate: 250 mcL/minute; Column: Phenomenex Luna C18(2), 150 mm x 2.1 mm, 3 µm particle, 100 Å pore; Column Temp.: 50 °C; HPLC Gradient: (A = 0.1% Formic Acid; B = Methanol) 75% A to 5% A in 12 min. hold for 2 minutes, return to 75% A in 0.5 min., equilibrate for 6 min. (total time = 20.5 min.); Turboionspray Position: 4.5 mm left, 0.5 mm back; Mode: Positive ion; Nebulizer Gas: 45 units; Auxiliary Gas: 40 units; Curtain Gas: 45 units; ESI

Voltage: 4000 V; Ion Source Temp.: 200 °C; Targeted MS/MS: Eight periods, one experiment per analyte; Q2 Pulsing: On, with one region of MS/MS spectrum selected.

RESULTS The LOD's, precursor and product ions of all analytes detected by this method are presented in Table 1 and representative extracted ion chromatograms in Figure 1.

Table 1

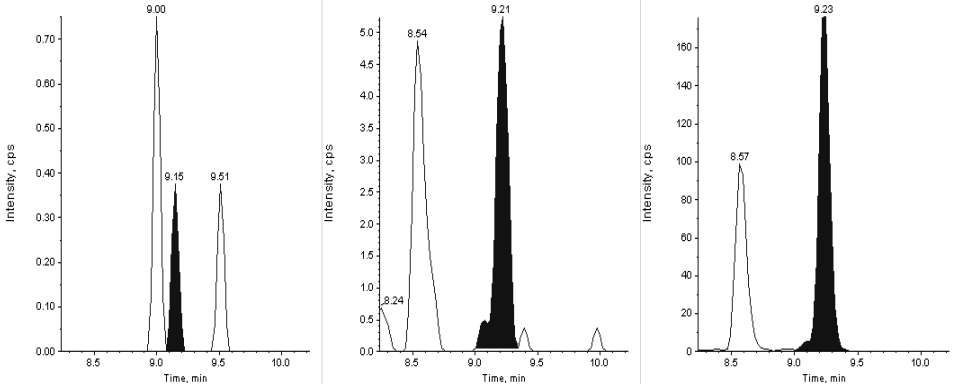
Analytes detected by this method (* indicates a GRP derivative)

Analyte (MS/MS transition)	Nature of Precursor Ion	LOD (ng/mL)
Aminoglutethimide (233→188,160,146)	MH+	0.3
Clenbuterol (288→259,203)	MH+	0.13
6β-Hydroxyfluoxymesterone* (486→407,379)	M+	0.63
9α-Fluoro-17α-methyl-androst-4-en-3α,6β,11β,17β-tetrol (337→95)	$[M+H-H_2O]+$	2.5
Raloxifene (474→112,84)	MH+	0.1
Exemestane* (430→279, 185)	M+	0.1
Epitrenbolone* (404→325,297)	M+	0.1
Oxymesterone* (452→187,179,167)	M+	0.63
Gestrinone* (442→363,335)	M+	0.16
Methyltestosterone* (IS)	M+	-
19-Norandrosterone* (410→259,241,159)	M+	0.13
Tetrahydrogestrinone (THG)* (446→339,306,264)	M+	0.63
1-Methylene-5α-androstan-3α-ol-17-one* (436→267,161)	M+	0.16
Anastrozole (294→225)	MH+	3.3
2-Hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (347→281,147)	MH+	2.5
Clomifene (406→100,72)	MH+	0.16
3'-Hydroxystanozolol (345→97)	MH+	0.063
4β-Hydroxystanozolol (345→327,309,269)	MH+	0.16
Epioxandrolone (289→229,135)	$[M+H-H_2O]+$	2.5
Mestanolone (305→229,159)	MH+	2.5
17α-Methyl-5β-androstane-3α,17β-diol (271→189,175,161)	$[M+H-2H_2O]+$	2
Fulvestrant (607→467,493,589)	MH+	0.3
Epimetendiol (269→105)	$[M+H-2H_2O]+$	0.25

Figure 1

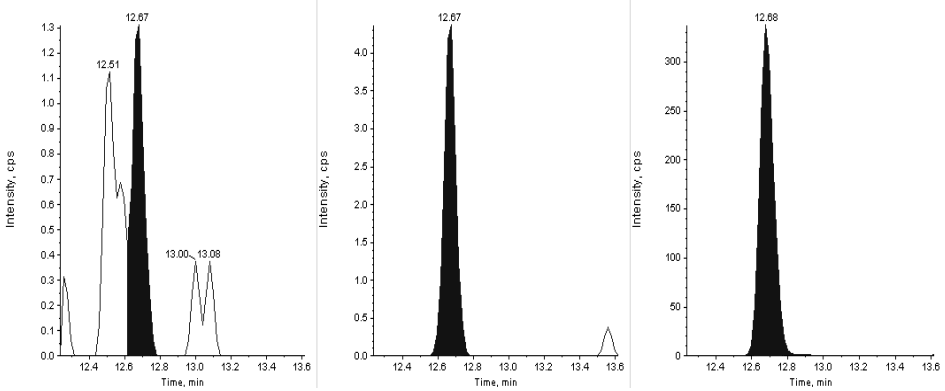
Tetrahydrogestrinone (THG) – Designer steroid; 9.2 minutes

Negative Urine LOD = 0.63 ng/mL MRPL = 10 ng/mL



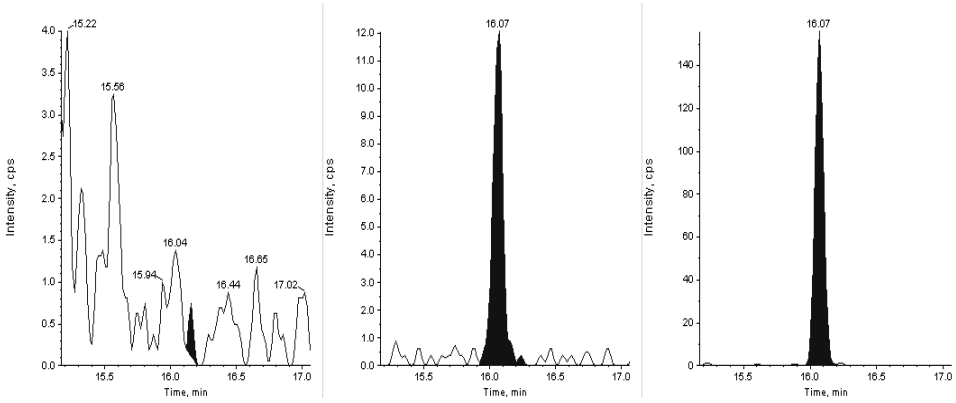
3'-Hydroxystanozolol – A primary metabolite of stanozolol; 12.7 minutes

Negative Urine LOD = 0.063 ng/mL MRPL = 2 ng/mL



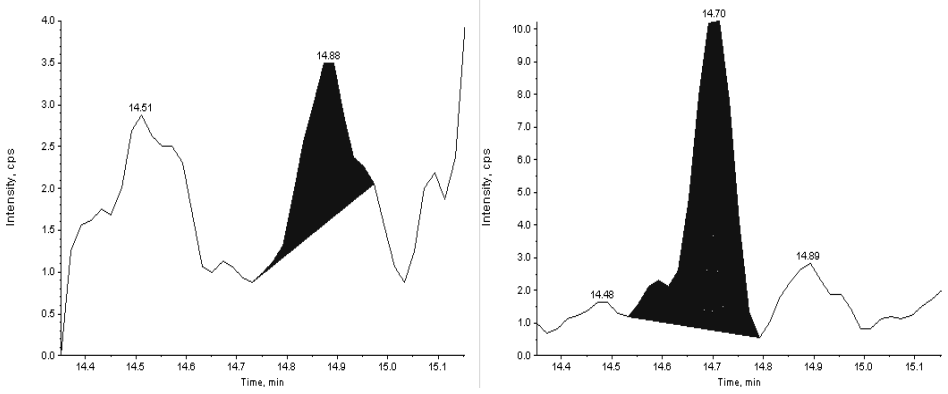
Epimetendiol – A major metabolite of metandienone; 16 minutes

Negative Urine LOD = 0.25 ng/mL MRPL = 2 ng/mL

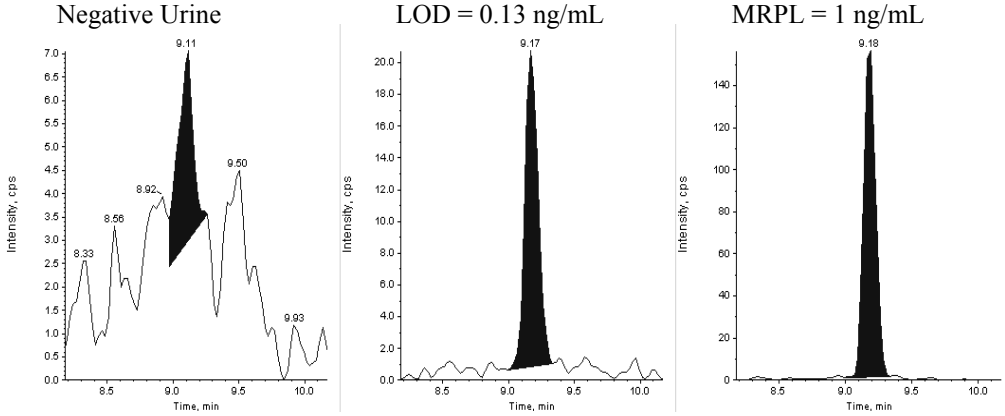


Methyltestosterone Metabolite(17α-Methyl-5β-androstane-3α,17β-diol); 14.7 min.

Negative Urine LOD (& MRPL) = 2 ng/mL



19-Norandrosterone – Primary metabolite of nandrolone; 9.2 minutes



DISCUSSION Sample derivatization with GRP results in the incorporation of a pre-ionized, readily desolvated quaternary amine, providing enhanced sensitivity for steroids by ESI-LC/MS (this concept is not new). Derivatization with GRP also results in the formation of cis/trans isomers which chromatographically resolve in many cases and serve as an aid in confirming analyte identity (see non-integrated peaks in THG). Successful utilization of GRP as part of this routine sample screening procedure hinged upon recognition of the fact that the imine formation between GRP and ketone-containing molecules was reversible. Thus, we found it best not to purify analyte molecules from excess GRP prior to injection. Unreacted GRP eluted within the first 1.5 minutes and, therefore, was automatically diverted to waste. The derivatization reaction is no more complicated than the ordinary reconstitution of the samples in HPLC mobile phase.

CONCLUSION A method is presented for the no-effort-added incorporation of the Girard's reagent P derivative (a quaternary amine) to a routine LC/MS/MS application for the detection of anabolic agents in urine, providing excellent sensitivity for difficult-to-detect steroids.

REFERENCE Geyer H., Schänzer W., Mareck-Engelke U., Nolteernsting E., Opfermann G. Screening procedure for anabolic steroids – The control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. *In*: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.) Recent Advances in Doping Analysis (5) Sport und Buch Strauβ, Köln (1998): 99-102.