APPLICATION NOTE

Sensitive and robust analysis of anabolic steroids, steroid esters and other banned substances in equine plasma

Bob Gray, Eleanor Menzies, James Scarth, Sport and Specialized Analytical Services, LGC Ltd, Fordham, UK

Key Words

Anabolic steroids, doping control, equine plasma, steroid esters, TraceFinder, TSQ Quantiva, UltiMate

Application benefits

- **Qualitative screening of a broad range of banned substances in equine plasma**
- **Sensitive and robust screening method for high-throughput testing laboratories**

Goal

To develop a robust and sensitive method for the detection of a broad range of banned substances in equine plasma for doping control purposes.

Introduction

Anabolic steroids and related substances, such as anabolic steroid esters, are important compounds for equine doping control laboratories. Traditionally, the detection of these compounds has been undertaken by the analysis of urine samples using gas chromatography-mass spectrometry (GC-MS). Recently there has been a move towards alternative sample matrices, such as blood and hair, and this has coincided with a shift towards the increased use of liquid chromatography-mass spectrometry (LC-MS) as an analytical technique. LC-MS offers significant advantages in sensitivity and throughput. Methods for the detection of these banned substances necessitate excellent sensitivity to detect the very low concentrations of drug present and robustness to ensure the successful analysis of a high number of samples in short timeframes.

Experimental

Sample Preparation

Equine plasma samples were augmented with an internal standard mix (testosterone-D3, testosterone propionate-D3, testosterone phenylpropionate-D3, and testosterone decanoate-D3), pH adjusted with 0.1 M NaOH and extracted with 4 mL of methyl tert-butyl ether/ethyl acetate (1:1). The organic layer was evaporated, and the residue was reconstituted in a 100 mM solution of methoxyamine hydrochloride in methanol/water (80:20). The sample was heated to 100 °C for 60 minutes to form methyloxime derivatives. A 10 µL aliquot of derivatized sample was analyzed by LC-MS.

Liquid chromatography

Samples were chromatographed using a Thermo Scientific™ UltiMate™ 3000RS liquid chromatography pump with OAS autosampler. Mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in methanol (Fisher Chemical™ Optima™ grade) for phases A and B. Compounds were separated using a gradient elution, starting at 20% B, rising to 80% B at 2.75 minutes and then to 99% B at 8.25 minutes. At 9.45 minutes, the mobile phase was returned to initial conditions for 2 minutes. Separation was achieved on a C18 UPLC column (2.1 x 100 mm, 1.7 µm) which was held at 60 °C.

Mass spectrometry

MS analysis was performed on a Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer equipped with an Ion Max NG source and heated electrospray ionization (HESI) in positive ion mode. Two SRM transitions for each compound were monitored in order to provide a screening and qualifier ion (Table 1).

Table 1. Retention time, SRM transitions, and estimated LOD for each compound (if applicable, RT shown is for most prominent isomer).

Compound	Retention Time	SRM Transitions		Estimated LOD
	(min)	Screening	Qualifier	(pg/mL)
6-OXO	4.18	388.3 → 303.2	388.3 → 326.2	25
Altrenogest	4.08	340.2 → 212.2	340.2 → 225.1	5
Boldenone	3.45	316.1 → 120.1	316.1 → 106.1	25
Boldenone undecylenate	8.25	482.3 → 120.1	482.3 → 150.1	2
Boldione	4.30	343.1 → 120.1	343.1 → 281.1	25
Clenbuterol	1.33	277.1 → 132.1	277.1 →140.0	10
Clostebol	4.01	352.2 → 141.1	352.2 → 172.1	2
Dimethylfluoxymesterone	3.93	348.2 → 207.1	348.2 → 264.2	10
Drostanolone	4.86	334.3 → 93.1	334.3 → 288.2	25
Ethisterone	3.66	342.2 → 126.1	342.2 → 138.1	2
Fluoxymesterone	3.22	366.2 → 91.1	366.2 → 210.1	50
Fluticasone propionate	3.77	530.2 → 120.1	530.2 → 290.1	10
GW-501516	4.45	454.1 → 188.0	454.1 → 257.0	5
Hydroxyprogesterone acetate	3.98	402.3 → 126.1	402.3 → 138.2	2
Hydroxyprogesterone caproate	5.68	458.3 → 138.1	458.3 → 342.2	5
Mestanolone	4.27	334.3 → 96.3	334.3 → 105.2	25
Mesterolone	4.14	334.3 → 105.1	343.3 → 109.9	25
Methandienone	3.72	330.2 → 106.2	330.2 → 120.1	25
Methenolone	4.20	332.2 → 81.1	332.2 → 107.1	10
Methyltestosterone	3.97	332.3 → 126.1	332.3 → 138.1	5
Nandrolone	3.47	304.1 → 138.1	304.1 → 106.1	10
Nandrolone decanoate	8.48	458.4 → 138.1	458.4 → 112.2	50
Nandrolone laurate	9.19	486.4 → 126.1	486.4 → 138.1	10
Nandrolone phenylpropionate	6.31	436.3 → 105.1	436.3 → 138.1	5
Nandrolone undecanoate	8.85	472.4 → 138.1	472.4 → 79.2	10
Norethandralone	4.24	332.3 → 112.0	332.3 → 138.1	5
Norethisterone	3.45	328.2 → 112.1	328.2 → 138.1	2
Stanozolol	2.73	329.3 → 81.1	329.3 → 95.1	10
Superdrol	5.20	348.3 → 302.3	348.3 → 107.1	50
Testosterone	3.70	318.1 → 126.1	318.1 → 138.1	10
Testosterone acetate	4.96	360.3 → 126.1	360.3 → 138.1	5
Testosterone benzoate	6.49	422.3 → 126.1	422.3 → 138.1	5
Testosterone cypionate	7.67	442.3 → 126.1	442.3 → 138.1	50
Testosterone decanoate	8.71	472.4 → 126.1	472.4 → 138.1	5
Testosterone isocaproate/Caproate	6.93/7.03	416.3 → 126.1	416.3 → 138.1	25
Testosterone phenylpropionate	6.59	450.3 → 126.1	450.3 → 138.1	2
Testosterone propionate	5.56	374.3 → 126.1	274.3 → 138.1	2
Testosterone undecanoate	9.07	486.4 → 126.1	486.4 → 138.1	5
Trenbolone	3.31	300.2 → 251.2	300.2 → 197.1	5
Trenbolone acetate	4.37	342.2 → 235.9	342.2 → 254.1	10
Testosterone D3 (IM)	3.7	321.3 → 138.1	n/a	n/a
Testosterone decanoate D3 (IM)	8.71	475.3 → 126.1	n/a	n/a
Testosterone phenylpropionate D3 (IM)	7.05	453.2 → 138.1	n/a	n/a
Testosterone propionate D3 (IM)	5.55	377.3 → 138.1	n/a	n/a

Method evaluation

Method performance (limit of detection – LOD) was assessed by analyzing, in duplicate, pooled equine plasma samples spiked at concentrations of 2, 5, 10, 25, 50, and 100 pg/mL with all of the compounds detailed in Table 1. Selectivity was assessed by analyzing 20 individual plasma samples for the presence of interfering matrix peaks at the expected retention time of all compounds. Method robustness has been demonstrated by analyzing over 8,000 equine plasma samples.

Data processing

Data were processed using Thermo Scientific™ TraceFinder™ software.

Results and discussion

Limits of detection for all compounds were 2 to 50 pg/mL, with the majority (28 of 41) having LODs of 10 pg/mL or lower. Figure 1 shows chromatograms for testosterone phenylpropionate screening and qualifier ions at 2 pg/mL. The method has been shown to be robust for high-throughput screening, with over 8,000 equine plasma samples being analyzed to date with only routine instrument maintenance required. The method has been used to identify a number of presumptive positives for banned substances. Figures 2 and 3 show data from a post-race equine plasma sample that produced screening findings for two compounds. Figure 2 shows screening and qualifier ions for GW-501516, a compound that can be used to enhance endurance, and Figure 3 shows data for boldenone undecylenate, an esterified version of the anabolic steroid boldenone, which can be used to promote increases in muscle mass.

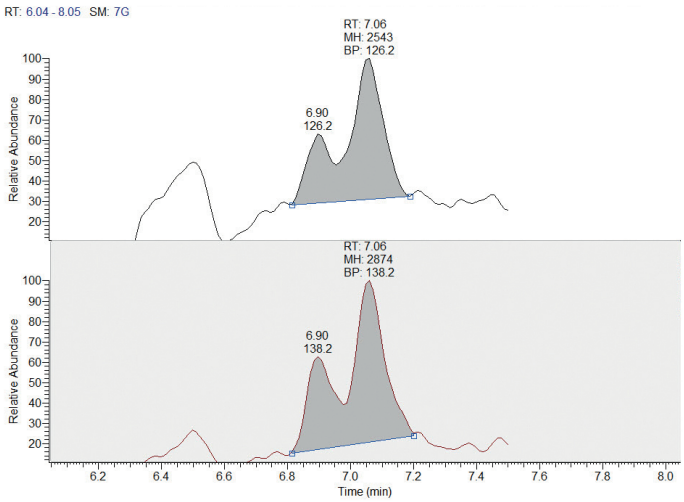

Figure 1. Chromatograms for testosterone phenylpropionate screening and qualifier ions at 2 pg/mL (two isomers observed due to use of methoxyamine HCL derivatization).

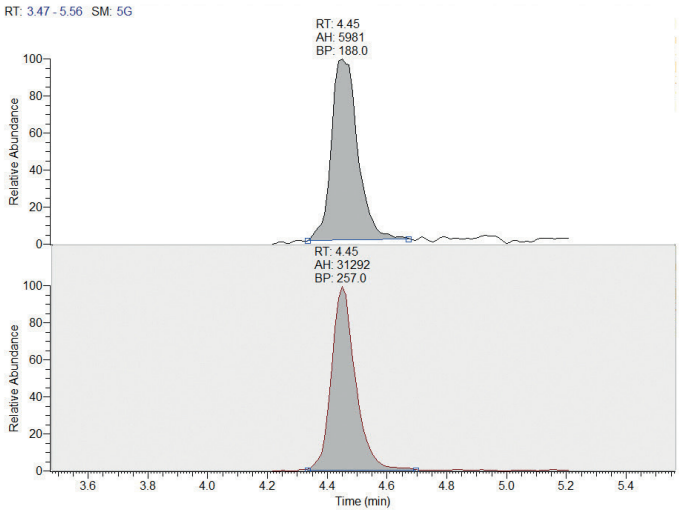

Figure 2. Chromatograms for GW-501516 screening and qualifier ions.

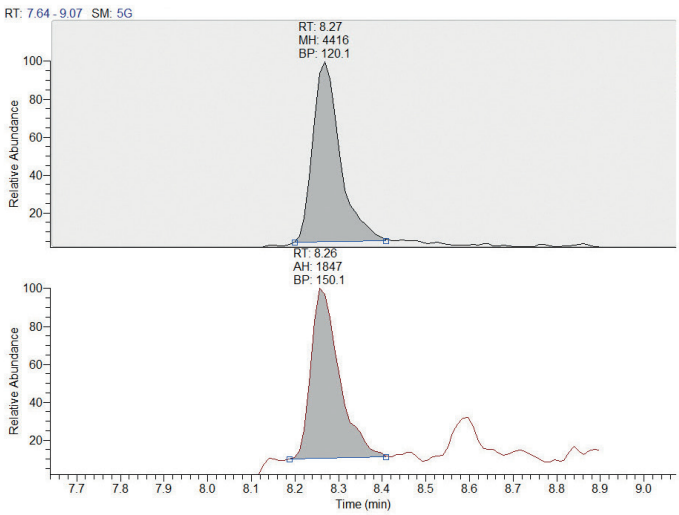

Figure 3. Chromatograms for boldenone undecylenate screening and qualifier ions.

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Conclusion

Using the Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer, a sensitive and robust method was developed for the qualitative screening of a broad range of banned substances in equine plasma. The method was used to successfully analyze over 8,000 equine plasma samples, resulting in a number of presumptive positive findings for banned substances.

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