Supplementary Online Content

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Analytical Methods

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ANALYTICAL METHODS

Reagents and Equipment

Methanol and acetonitrile were high-performance liquid chromatography (HPLC) grade or better and provided by Burdick & Jackson (Honeywell, Morris Plains, NJ, USA). Purified water was provided by an Advantage A10 system (Millipore, Billerica, MA). HPLC columns used for analysis include a Raptor Biphenyl 2.1 × 100 mm column with 2.7 µm particle size (Restek Corporation, Bellefonte, PA, USA); an Acquity BEH C18 2.1 × 50 mm column with 1.7 µm particle size (Waters Corporation, Millford, MA, USA); and an Acquity CSH C18 2.1 × 50 mm column with 1.7 µm particle size (Waters Corporation). Instruments used for liquid chromatography interfaced to tandem mass spectrometry (LC-MS/MS) based screening and confirmation include a Xevo TQ-S triple quadrupole mass spectrometer interfaced to an Acquity I-class ultra-performance liquid chromatography (UPLC; Waters Corporation), a QExactive Plus orbitrap mass spectrometer with heated electrospray ionization (HESI-II) and atmospheric pressure chemical ionization (APCI) probes interfaced to an UltiMate 3000 UPLC system (Thermo Scientific, Sunnyvale, CA, USA), and a Quantiva triple quadrupole mass spectrometer interfaced to a Vanquish UPLC system (Thermo Scientific). For gas chromatography-based analysis a ZB-1 MS column (250 µm × 30 m; 0.1 µm film thickness; Phenomenex, Torrance, CA, USA) was used. Gas chromatography interfaced with tandem mass spectrometry (GC-MS/MS) analysis was carried out using a 7890B gas chromatograph with multimode inlet interfaced to a 7000C triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

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Sample extraction

For each supplement, the equivalent of one suggested serving was extracted when that information was provided by the supplier. Otherwise one capsule, one tablet, or 1000 µL of solution was extracted, depending on the dosage form. Capsules were opened and the contents of the capsules weighed prior to suspension in 5000 µL of methanol. Tablets were placed in a single-use plastic pouch and crushed using a Silent Knight pill crusher (Medline Industries, Northfield, IL, USA); the resulting powder was weighed and then suspended in 5000 µL of methanol. Liquids were diluted with 4000 µL of methanol. After addition of methanol, the samples were shaken for a minimum of 15 min, and centrifuged at 1600 RPM for five minutes to clarify the suspension of undissolved material.

Sample Screening

Each sample was subjected to both targeted and untargeted analysis using liquid chromatography/mass spectrometry (LC-MS) and gas chromatography/mass spectrometry (GC-MS). The blank matrix used for control samples consisted of a methanol extract of the contents of a capsule of FenuGreek (General Nutrition Centers, Pittsburgh, PA, USA). For liquid chromatographic analysis, 20 µL of methanol extract was spiked with 20 µL of a 1 ng/µL solution of epitestosterone and the sample was dried under an air stream at 40°C using a TurboVap system (Biotage, Charlotte, NC, USA). The sample was reconstituted using 50 µL methanol with mixing by vortex and then 100 µL of 25 mM ammonium formate in 0.1% formic acid with mixing by vortex. For gas chromatographic analysis, 20 µL of methanol extract was treated identically to the liquid chromatography samples up to reconstitution, after which a mixture of *N*-Methyl-

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N-(trimethylsilyl) trifluoroacetamide (MSTFA) containing 0.2% (w/v) ammonium iodide and 1% (v/v) ethanethiol was added. The sample was heated at 70 °C for thirty min.

Targeted LC-MS/MS screening was carried out on Xevo TQ-S system using a method used for general screening of urine samples for the presence of anabolic and related agents in our WADA-approved testing program. For the analysis, a Raptor Biphenyl column was maintained at 40 °C. The injection volume was 5 µL. A binary mobile phase consisting of 25 mM ammonium formate in 0.1% formic acid (A) and acetonitrile (B) was used. A flow rate of 0.45 mL/min and a gradient program (with linear interpolation) of 0 min- 0.5% B; 0.5 min- 0.5% B; 5 min- 5% B; 11.5 min- 70% B; 11.6 min- 95% B; 12.5 min- 95% B; 12.6 min- 0.5% B; 14.5 min- 0.5% B was used. Mass spectrometric conditions included electrospray ionization with a capillary voltage of 500 V, a desolvation temperature of 450 °C, a desolvation gas flow rate of 1000 L/h, a cone gas flow rate of 150 L/h, and a cone potential of 30 V.

Untargeted LC-MS/MS screening was carried out on a QExactive orbitrap-based system. A Raptor Biphenyl column was maintained at 40 °C. The injection volume was 5 µL. A binary mobile phase consisting of 25 mM ammonium formate in 0.1% formic acid (A) and acetonitrile (B) was used. A flow rate of 0.45 mL/min and a gradient program (with linear interpolation) of 0 min- 5% B, 0.5 min- 5% B, 9 min- 70% B, 9.1 min- 95% B, 9.6 min- 95% B, 9.7 min- 5% B, and 11 min- 5% B was used. Samples were analyzed using both electrospray ionization (ESI) and APCI in both positive and negative ion modes. For ESI, a sheath gas flow of 45, an aux gas flow of 0, a sweep gas flow of 0, a capillary temperature of 300 °C, an aux gas heater temperature of 350 °C, a capillary voltage of 3.9 kV, an S-lens radio frequency (RF) level of 55%, a

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resolution of 35,000 Dalton (Da), an automatic gain control (AGC) target of 3×10^6 counts, a maximum ion injection time (IT) of 100 ms, and a mass range of 120-1200 Da were used. For APCI a sheath gas flow of 45, an aux gas flow of 10, a sweep gas flow of 0, a capillary temperature of 300 °C, an aux gas heater temperature of 350 °C, a discharge current of 5 µA, an S-lens RF of 55%, a resolution of 35,000 Da, an AGC target of 3×10^6 counts, a maximum IT of 100 ms, and a mass range of 120-1200 Da were used. The major peaks in the total ion current chromatograms for each analysis were identified and the accurate masses were used to predict molecular formulas compatible with the measured mass with a mass error below 5 parts per million (ppm).

Targeted GC-MS/MS screening was carried out on 7890A/7000C systems with a method used for general screening of urine samples for anabolic and related agents. Chromatography was carried out on a ZB-1MS capillary column with a flow rate of 0.9 mL/min and a temperature program consisting of an initial hold at 90 °C for 0.2 min, a 70 °C/min ramp to 125 °C with a 0.3 min hold, a 40 °C/min ramp to 188.5 °C, a 3.3 °C/min ramp to 231 °C, a 30 °C/min ramp to 310 °C with a 2 min hold, and a 30 °C/min ramp to 325 °C with 0.4 min hold. Samples were injected in pulsed splitless mode using a 0.5 µL injection and the inlet maintained at 280 °C. An initial pulse at 30 pounds per square inch (psi) was held for 0.3 min and a total purge flow of 50 mL/min was applied at 0.5 min. The interface to the mass spectrometer was maintained at 280 °C with chemical ionization (CI) using ammonia as a reagent gas. The source and quadrupoles were maintained at 300 °C, and 150 °C, respectively.

Untargeted GC-MS screening was carried out on 7890A/7000C systems using the same inlet parameters as shown above and a full scan from 100-850 Da over 0.45

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s. Ionization was achieved using electron impact (EI) and the resulting spectra for the major chromatographic peaks were compared against a library of common compounds (National Institutes of Science and Technology, Gaithersburg, MD, USA).

Confirmatory Analysis

Sample responses from the screen were used to determine dilution factors for the samples that would yield 1-50 ng of analyte for the confirmation. When necessary, the samples were diluted with methanol and 20 µL were taken for analysis. Depending on the analysis, the samples were spiked with the corresponding internal standard (methyltestosterone, clopamide, or *des*-alaninyl[3]- growth hormone-releasing peptide (GHRP)-2), evaporated to dryness under an air stream at 40 °C using a TurboVap system, then reconstituted using 50 µL methanol with mixing by vortex then 100 µL of 25 mM ammonium formate in 0.1% formic acid with mixing by vortex. The confirmation methods were more specialized for each analyte, generally using a different chromatographic separation from the screen and yielding a quantifier transition and two qualifier transitions. For a sample to meet acceptance criteria, retention times needed to be within 2% of those observed for authentic quality control samples extracted at the same time as the samples and analyzed within the same batch. Moreover, the qualifier ion ratios needed to be within 10% absolute for ion ratios greater than 50%; within 20% relative for ion ratios from 25-50%, or within 5% absolute for ion ratios between 1-25%. The amount of analyte present was roughly estimated based on the relative response compared to the quality controls and the dilution factor applied. Details for the various confirmation methods are given in eTables 1 and 2.

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eTable 1. Chromatographic methods for confirmatory analyses

Analyte	Column	Column Temp.	Mobile Phase A	Mobile Phase B	Solvent gradient
Andarine	Acquity BEH C18	35 °C	0.1% formic acid	Acetonitrile	0.3 mL/min 0.0 min- 20% B 4.0 min- 90% B 5.0 min- 90% B 5.1 min- 20% B 6.5 min- 20% B
Androstatrienedione	Acquity BEH C18	35 °C	0.1% formic acid	Acetonitrile	0.5 mL/min 0.0 min- 20% B 2.5 min- 75% B 2.8 min- 90% B 3.0 min- 90% B 3.1 min- 20% B 4.0 min- 20% B
GW501516	Acquity BEH C18	35 °C	0.1% formic acid	Acetonitrile	0.5 mL/min 0.0 min- 10% B 4.0 min- 95% B 5.0 min- 95% B 5.1 min- 10% B 6.0 min- 10% B
Ibutamoren	Acquity CSH C18	50 °C	0.1% formic acid	Acetonitrile	0.4 mL/min 0.0 min- 2% B 6.0 min- 40% B 6.1 min- 90% B 7.0 min- 90% B 7.1 min- 2% B 9.0 min- 2% B
LGD-4033	Acquity BEH C18	35 °C	0.1% formic acid	Acetonitrile	0.5 mL/min 0.0 min- 20% B 4.0 min- 90% B 4.1 min- 20% B 5.0 min- 20% B
Ostarine	Acquity BEH C18	40 °C	0.1% formic acid	Acetonitrile	0.3 mL/min 0.0 min- 20% B 5.0 min- 90% B 5.1 min- 20% B 6.0 min- 20% B
Tamoxifen	Acquity BEH C18	35 °C	0.1% formic acid	Acetonitrile	0.5 mL/min 0.0 min- 10% B 4.0 min- 90% B 4.1 min- 10% B 5.0 min- 10% B

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eTable 2. Mass spectrometric parameters for confirmatory analyses[a]

Analyte	Instrument	Polarity	Transitions Precursor- Product (CE)	Internal Standard Transitions
Andarine	Quantiva	Negative	440- 150 (24) 440- 205 (29) 440- 261 (19) 440- 289 (20)	Clopamide 344- 189 (30)
Androstatrienedione	Quantiva	Positive	283- 147 (22) 283- 161 (19) 283- 173 (18) 283- 187 (14)	Methyltestosterone 303- 97 (25)
GW501516	Quantiva	Positive	454- 85 (60) 454- 172 (65) 454- 188 (44) 454- 256 (50) 454- 257 (30)	Methyltestosterone 303- 97 (25)
Ibutamoren	Xevo	Positive	529- 148 (20) 529- 235 (20) 529- 263 (20) 529- 267 (20) 529- 444 (20)	*des*-alaninyl[3]-GHRP- 2 374- 241 (12) 374- 269 (12) 374- 479 (12)
LGD-4033	Quantiva	Positive	339- 199 (25) 339- 220 (25) 339- 240 (25) 339- 319 (25)	Methyltestosterone 303- 97 (25)
Ostarine	Quantiva	Negative	388- 118 (20) 388- 185 (32) 388- 211 (28) 388- 241 (21) 388- 269 (16)	Clopamide 344- 189 (30)
Tamoxifen	Quantiva	Positive	372- 72 (10) 372- 129 (26) 372- 207 (20) 372- 327 (20)	Methyltestosterone 303- 97 (25)

[a] Abbreviations used: Collision Energy (CE)

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