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Detection and Confirmation of Anabolic Steroids and Metabolites in Urine using Routine Accurate-Mass API-TOF LC/MS

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Introduction

The use of anabolic substances for performance enhancement in sports is receiving increasing attention. Their use is an avowed problem in some professional sports, a growing problem in high school and collegiate athletics, and an area of doping in which new compounds are continually being introduced (e.g. the BALCO/THG situation in the USA and in nutritional supplements). The typical doping control analysis for anabolic steroids and related substances in urine includes screening by derivatization and GC/MS, followed by confirmation of the screening result using high-resolution magnetic sector GC/MS in EI mode. Because of the high purchase and operational costs of high-resolution magnetic sector instruments, alternative techniques for the confirmation of screening results are being explored. Tandem GC/MS is an alternative to high resolution GC/MS, because SRM acquisition and the use of ion ratios provide high confidence in the identification and it is an established technique in drug confirmation. LC/MS is also an alternative confirmation technology with the advantages that it uses a different mode of chromatographic separation and offers different ionization techniques. High-resolution API LC/MS provides not only the usual spectral information such as isotope ratios, but highly specific detection of the intact molecule with confirmation of the empirical formula. Therefore, it was of interest to determine whether API-TOF could be as effective as high-resolution GC/MS for high-confidence confirmation of anabolic steroids at the 1-2 ng/mL concentrations needed for WADA compliance.

Presented below are the results of our initial studies evaluating the use of a benchtop API-TOF LC/MS instrument for steroid confirmation. The study objectives were:

1. To evaluate the sensitivity of an accurate-mass instrument using the same extracts as used in a GC/MS screen, without extract derivatization.

2. To determine the mass accuracy (mass error) for unextracted standards and for urine samples fortified with a representative group of anabolic substances near the WADA MRPL's.
3. To determine if the combination of sensitivity and mass accuracy along with the molecular or other major ion(s) could be used for confirmation.

Experimental

The API-TOF system consisted of an Agilent 1100 LC system (vacuum degasser, binary pump, wellplate autosampler, thermostatted column compartment, and diode array UV-VIS detector), coupled to a G1969A LC/MSD TOF mass spectrometer. The mass spectrometer was operated with either the orthogonal ESI or APCI sources. The instrument was autotuned weekly using the automatic built-in calibrant delivery system and Agilent-developed calibrant compounds. The mass axis was calibrated daily using the same mix and an automatic calibration routine. Spectra were internally mass-corrected in real time using automatically-introduced reference mass solution containing two known compounds bracketing the mass range of interest. The optimized LC, MS and APCI conditions are shown in Table 1.

Table 1. LC/MS Conditions for the Analysis of Anabolic Substances in Urine

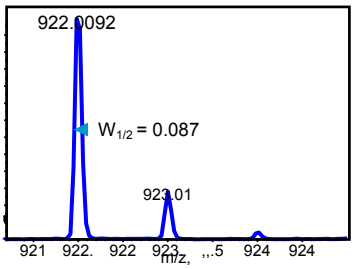
Column:	2.1 x 50mm Zorbax RRHT SB-C18 1.8µ, 0.4 mL/min, 55°C
Gradient:	A = 0.1% formic acid/water
	B = MeOH
	55% B for 5 min to 75%B @ 9 min resulting in a 14 min analysis time
Injection:	4 µL out of 100 µL
Ionization mode:	APCI, positive ion (final method)
Capillary voltage:	3500V
Vaporizer:	450°C
Corona:	4µA
Drying gas:	5 L/min, 350°C
Nebulizer:	60 psi
Fragmentor:	150V (no CID)
Skimmer:	60V (default)
Octopole:	RF 250V (default)
Scan range:	100-1000, 10,000 transients/scan (0.89 sec)
Reference masses:	121 and 922 added post-column at 5 µL /min, 10 µM solution

The sample preparation method used in this study was developed at the Center for Human Toxicology, Sports Medicine Research and Testing Laboratory, for screening of free steroids by GC/MS. The same sample preparation was used for the LC/MS analysis, omitting the derivitization step. Urine (3 mL) was extracted by passing through an Extrelut-3 column connected in-line to an amino SPE column containing 1 g sodium sulfate, and eluting with 9 mL of diethyl ether after an eight minute delay. For LC/MS, the final extracts were simply evaporated to dryness in conical tubes and the residues sent by overnight to the Agilent laboratory (Pleasanton, CA) where they were stored at –10°C while awaiting analysis. Prior to analysis, the residues were reconstituted in 100uL of the initial mobile phase, mixed and 4 µL were injected onto the LC column.

Results and Discussion

Accurate-mass API-TOF LC/MS is commonly used for empirical formula confirmation of synthetic and naturally-occurring molecules. The instrument used here was specifically designed to be as easy to use as a quadrupole GC/MS or LC/MS, by virtue of features such as automatic tuning and calibration, automatic reference mass correction, and improved instrument stability resulting from its mechanical and electronic design. The instrument has a mass resolution of approximately 7000 at the m/z range of the anabolic steroids, a routine mass accuracy of 3 ppm or less (see Figure 1) and operates in full scan mode. Full scan has the advantage over MRM-MS/MS of not being a target analysis acquisition. Therefore, additional compounds can be detected without modifying the acquisition method or optimizing specific MS/MS parameters.

Resolution = $(m/z)/w_{1/2}$
1000/0.1 = 10,000
922/0.087 = 10,597



Mass accuracy (mass error)

(1000.001-1000.000)/1000)x10^6=1 ppm
Therefore: 1 ppm @ 1000 m/z is 0.001Da
(1 mDa)

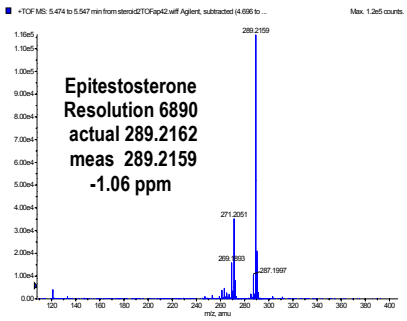


Figure 1. LC/MSD TOF Performance Example

Initial studies of unextracted standards using ESI, demonstrated the ability of the instrument to measure the m/z of the M+H ion to 3 ppm accuracy or better under routine unattended operation (Figure 1 and left side of Figure 2). However, the analytes shown in the right portion of Figure 2, including a proposed internal standard, showed better sensitivity using APCI.

ESI worked well for:	**APCI had significantly better sensitivity than ESI for most of:**
• 17β-Estradiol	• Clenbuterol[1] (non-steroidal anabolic)
• Testosterone	• 3'-OH-stanozolol[1]
• Active 1-AD	• Epitestosterone[1]
• 1-AD-Dione	• Methyl testosterone[1] (IntStd)
	• 19-Norandrosterone[2]
	• Methyltestosterone metabolite[2] (17α-methyl-5β-androstane-3α,17β -diol)
	• Epimetendiol[2]

Figure 2. API Behavior of Analytes

Theoretically, APCI might be predicted as the ionization mode of choice because the analytes are relatively non-polar and often contain no basic groups. Also, APCI is less susceptible to ion suppression from coeluting endogenous materials and tends to give simpler spectra than ESI (free of complicating adducts such as Na and K).

The APCI vaporizer temperature and corona current were optimized for the best sensitivity across the range of analytes. In these studies, no in-source CID was used (in-source CID will be evaluated in future studies as a mechanism of generating fragment ions for ion ratio calculations). Decreasing the number of transients/scan to 10,000 improved the detection limits for the compounds shown in the Figure while maintaining a sufficient number of scans to describe each chromatographic peak.

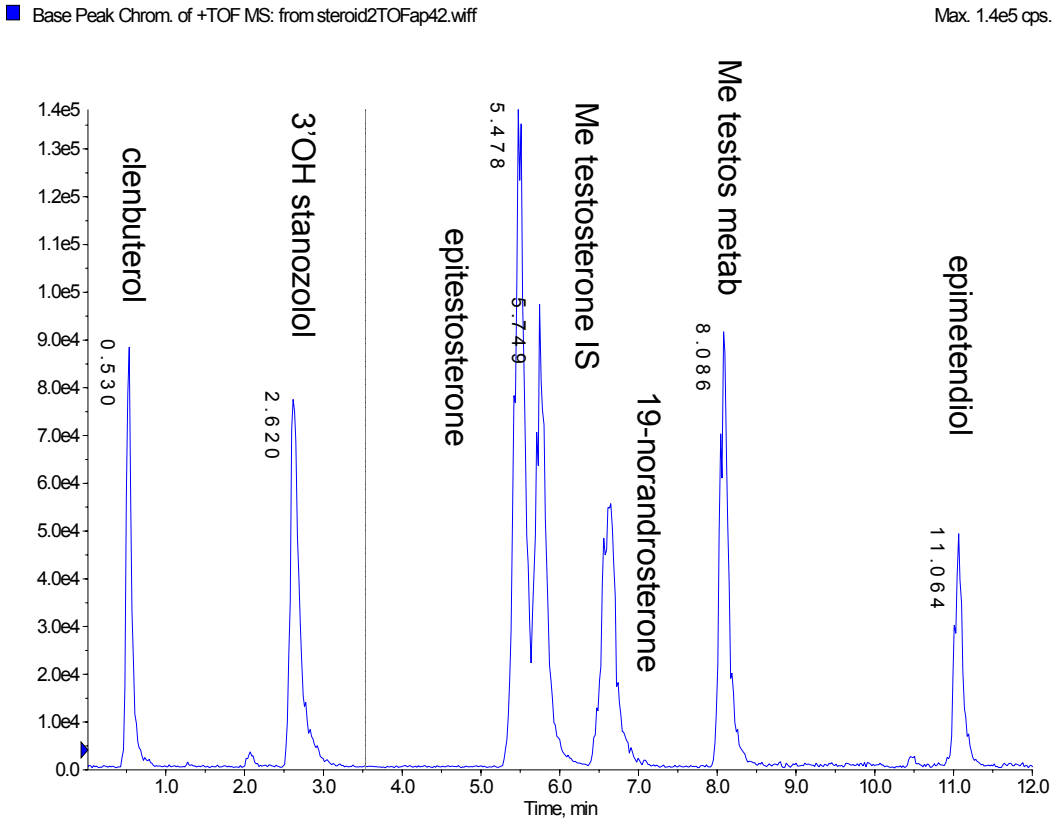


Figure 3. APCI Base Peak Chromatogram of Analyte Mix
Unextracted Standard equivalent to 16 ng/mL/analyte in urine

Figure 3 shows the base peak chromatogram of an unextracted standard equivalent to 16 ng/mL from a 3 mL urine sample. The objective of this experiment was to obtain separation of the target analytes in less than 15 minutes. A column and/or gradient with greater resolution will be used in future studies if methyltestosterone is used as the internal standard.

Figure 4A shows a typical APCI spectrum from these compounds (epitestosterone) as well as the resolution and mass accuracy.

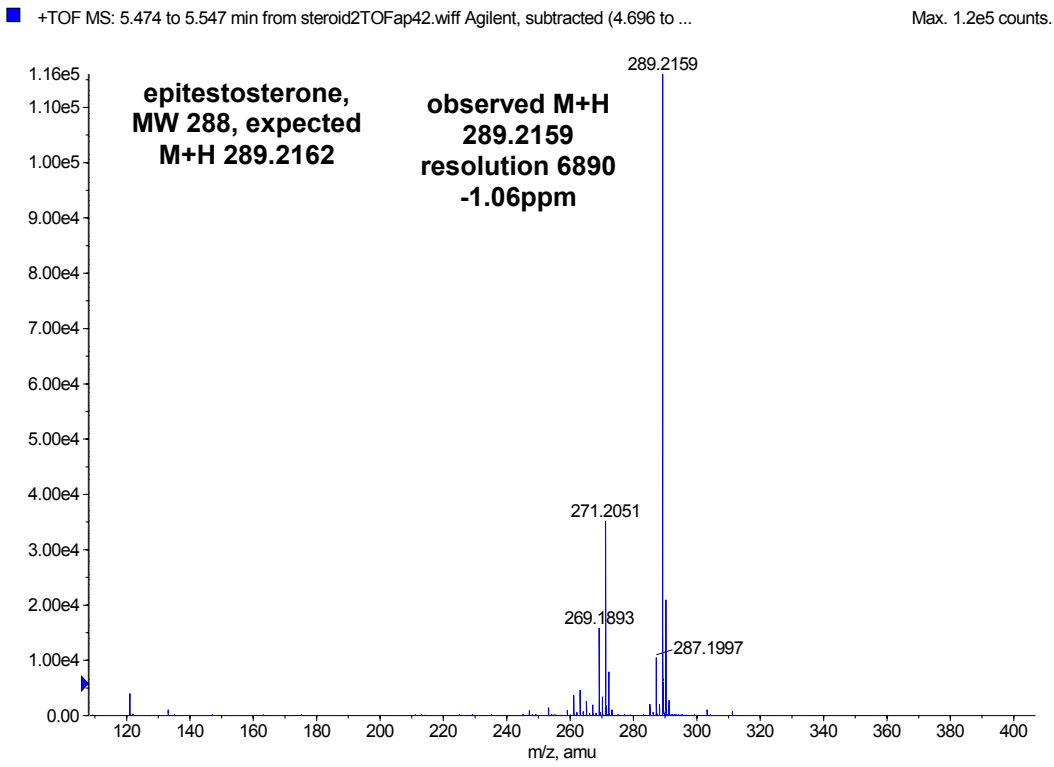


Figure 4A. Typical APCI spectrum with M+H base peak

APCI can result in a loss of water from the initially-formed quasi-molecular ion due to either thermal effects of the heated vaporizer, or by the ionization that is driven by the corona discharge of APCI (Figure 4B). However, the ions observed following water loss maintained accurate masses and the ion ratios for the M+H and subsequent water loss ion were reproducible. Figure 4B also shows the two reference masses (generally bracketing the mass range of interest) used by the system to calibrate each spectrum as it is acquired. The reference compounds (purine at m/z 121 and a unique perfluorinated compound m/z 922) are added post-column in µM concentrations using a dual-sprayer source (which helps prevent reduction of analyte signal during ionization in solution droplets) by the built-in Calibrant Delivery System in ESI, or by an auxiliary pump in APCI.

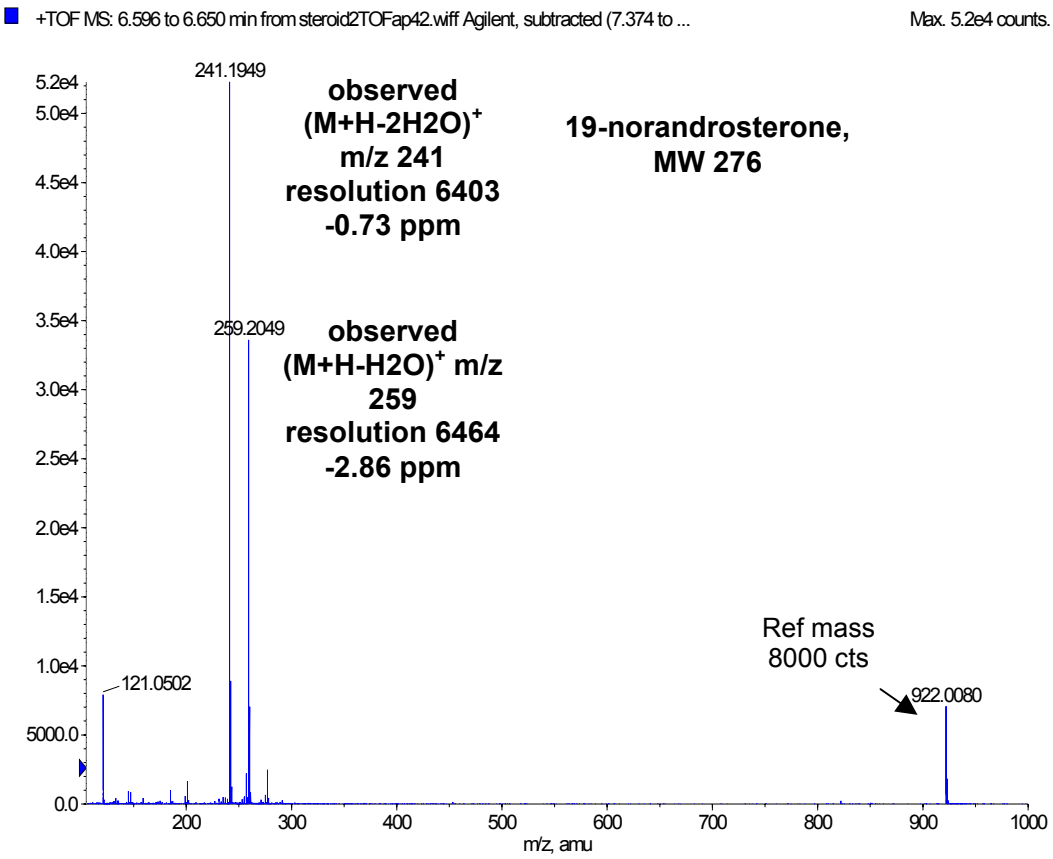


Figure 4B. APCI Spectrum with Water Loss and Reference Masses

The automatic system reports were used to determine the mass accuracy for all analyses. Table 2 shows the results for the standard shown in Figure 3. Expected mass accuracy for the system is less than 3 ppm. However, in this experiment the average mass error was less than 2 ppm.

Table 2. Mass Accuracy for 16 ng/mL unextracted standard
(* water loss)

Analyte	Empirical Formula	Exact Mass	Target Mass	Measured Mass	Mass Error (Da)	Mass Error (ppm)]	
clen	$C_{12}H_{18}Cl_2N_2O$	276.07962	277.08690	277.08636	-0.00053	-1.93	
3'-OH-stan	$C_{21}H_{32}N_2O_2$	344.24638	345.25365	345.25292	-0.00074	-2.14	
epitest	$C_{19}H_{28}O_2$	288.20893	289.21621	289.21590	-0.00031	-1.07	
MeTes (IS)	$C_{20}H_{30}O_2$	302.22458	303.23186	303.23158	-0.00028	-0.92	
19-nor andros	*$C_{18}H_{24}$	240.18780	241.19508	241.19459	-0.00048	-2.01	
MeTes Metab	*$C_{20}H_{30}$	270.23475	271.24203	271.24139	-0.00063	-2.34	Avg Mass Acc (ppm)
epimeten	*$C_{20}H_{28}$	268.21910	269.22638	269.22608	-0.00030	-1.10	-1.64

As an evaluation of the MS performance, a drug-free urine extract was fortified at 33 ng/mL with all six analytes plus IS. The average mass accuracy in detecting these analytes was -0.95, with < 1ppm for 3 analytes and none greater than 1.83 ppm.

Serial dilutions of unextracted standards were analyzed to determine the instrumental limit of detection. For an unextracted standard equivalent to 1.6 ng/mL in urine (400pg on-column) and approximating the WADA steroid MRPL, peak heights of extracted ion chromatograms were in the range of 20,000-40,000 counts.

The analysis of an extracted urine containing 2 ng/mL of epitestosterone, the methyl testosterone metabolite, and epimentendiol, and 1 ng/mL of 19-nor-androsterone, is shown in Figure 5. In the Figure, the 1mDa-EIC's for the four tested analytes have peak heights ranging from 75,000 to 100,000 counts with excellent signal-to-noise. Therefore, this concentration is readily detectable using LC-TOF method. The analyte spectra shown in Figure 6 had mass errors of less than 2 ppm, even with these levels of nominally 200-400 pg on-column, demonstrating high confidence in the identification.

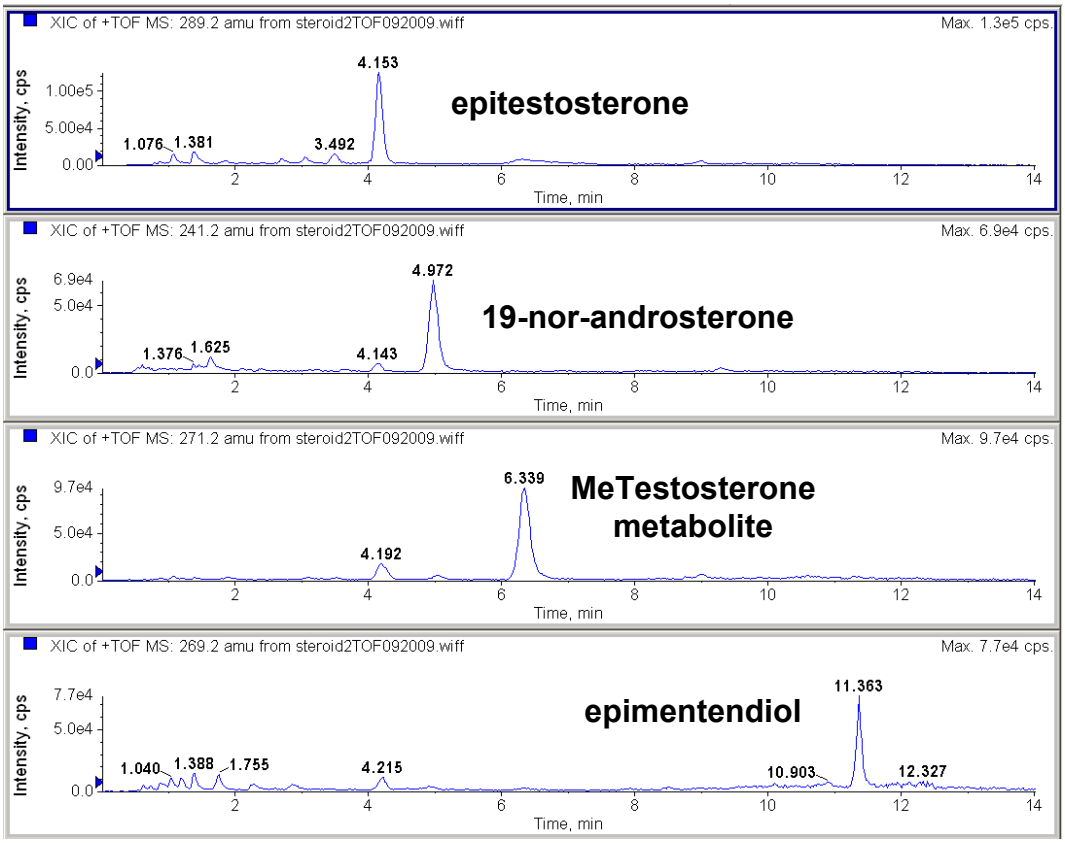


Figure 5. Extracted Ion Chromatograms (1 mDa mass width) for 2 ng/mL urine extract (1 ng/mL 19-nor-androsterone)

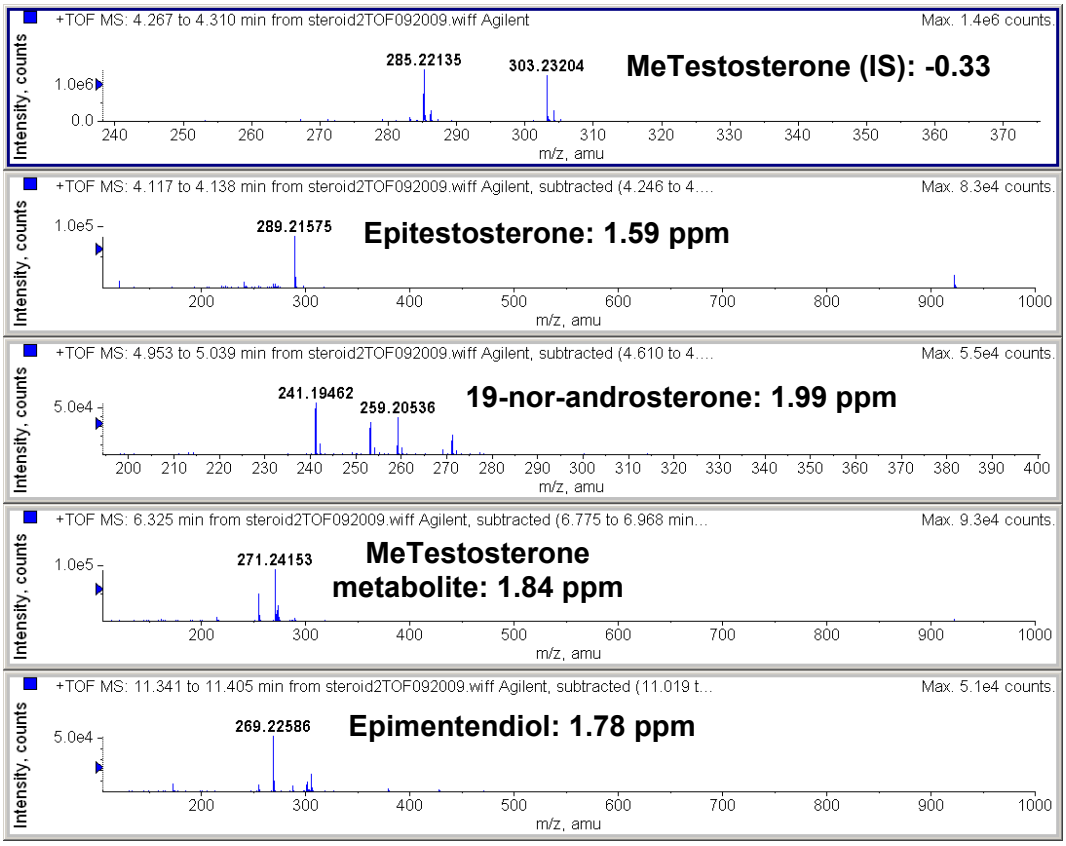


Figure 6. Spectra and mass errors for 2 ng/mL urine extract (1 ng/mL 19-nor-androsterone) – Figure 5

Conclusions

Using a standard sample preparation method developed for GC/MS screening, but without derivatization, a LC/MS method using an easy-to-use, benchtop API-TOF instrument readily detected a representative group of anabolic substances at 1-2 ng/mL concentrations in urine. The analysis consumed only 4 µL of the 100 µL of reconstituted extract, which allows for replicate, re-analysis or additional analyses from a single extraction. The use of "accurate-mass extracted ion chromatograms" (i.e. EIC's with an m/z width of 1mDa) allowed for specific detection of the target steroids in a potentially complex sample. The detection of 1-2 ng/mL of four steroids with good S/N was also demonstrated. The spectra obtained at these low ng/mL concentrations all showed mass errors of less than 2 ppm.

The LC/MSD TOF is a continuously-scanning instrument and, therefore, has the advantage over tandem MS-MRM of being able to detect non-target compounds without method modification. Additional work is planned to further evaluate this method with an expanded list of analytes, with in-source CID for additional confidence from ion ratios, and to compare

the new Agilent multimode source (simultaneous ESI and APCI ionization) to the dedicated APCI source.

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