# Screening and Confirmation of Anabolic Steroids Using Accurate Mass LC/MS

Application

**Forensic Toxicology**


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## Abstract

**The use of the Agilent LC/MSD TOF, an orthogonal-axis time-of-flight (oa-TOF) mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source, is described for the analysis of four anabolic steroids and one internal standard, in urine extracts at a 1–2 ng/mL concentration. The high degree of mass accuracy (<3 ppm) of the TOF instrument allows for both excellent confirmation by empirical formula determination and quantitation using extracted ion chromatograms (EICs). The accurate mass capability of the LC/MSD TOF produces EICs with sufficiently narrow mass widths to exclude most chemical noise contributions. For increased chromatographic resolution and speed, a C18 column with a 1.8-micron particle size was employed.**


## Introduction

The use of anabolic substances for performance enhancement in sports is receiving increasing attention [1, 3]. The typical doping control analysis for anabolic steroids and related substances in urine includes screening by gas chromatography/mass spectrometry (GC/MS) [2], followed by confirmation of the screening result using high-resolution magnetic sector GC/MS in EI mode [4]. Because of the high initial and operational costs of high-resolution magnetic sector instruments, alternative techniques for the confirmation of screening results are being explored [5].

Tandem GC/MS is an alternative to high resolution GC/MS, because tandem GC/MS is an established technique for drug confirmation, and because selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) acquisitions with ion ratio calculations provide high confidence in the identification. Liquid chromatography/mass spectrometry (LC/MS) has also been used as an alternative confirmation technology with the advantages that it uses a different mode of chromatographic separation and different ionization techniques [6].

Accurate-mass API LC/MS has significant advantages because it provides not only the same spectral information, such as isotope ratios and presence of heteroatoms as previous methods, but also allows for the highly specific detection of the intact molecule and confirmation of the empirical formula. Approximately 40 anabolic substances are currently targeted in doping control analysis, many of which are not easily detected or confirmed using GC/MS, but are amenable to LC/MS [7]. The analysis of many of these compounds is further complicated because they must be detected and confirmed at a 2-ng/mL concentration or lower in urine [the Minimum Required Performance Level (MRPL) for a laboratory, in the World Anti-Doping Agency (WADA) program]. This application note describes the results of using an Agilent LC/MSD TOF instrument for detection and confirmation of a number of analytically challenging anabolic substances at the WADA MRPL.

The anabolic substances analyzed in this work and their structures are shown in Figure 1.

Most of these analytes, including the internal standard (ISTD), showed better sensitivity using APCI than electrospray ionization (ESI). This might be expected for these relatively non-polar analytes that contain no basic groups. APCI is also less susceptible to ion suppression from co-eluting endogenous materials, and tends to give simpler spectra than ESI (free of complicating adducts such as $Na^+$ and $K^+$).

One complication of APCI can be the loss of water from the initially-formed protonated molecule due to either the thermal effect of the heated vaporizer, or to the ionization driven by the corona discharge of APCI. For some compounds, water loss is even observed in the milder ESI mode which involves ionization in the liquid phase and less contact of the analyte with heat. However, the mass

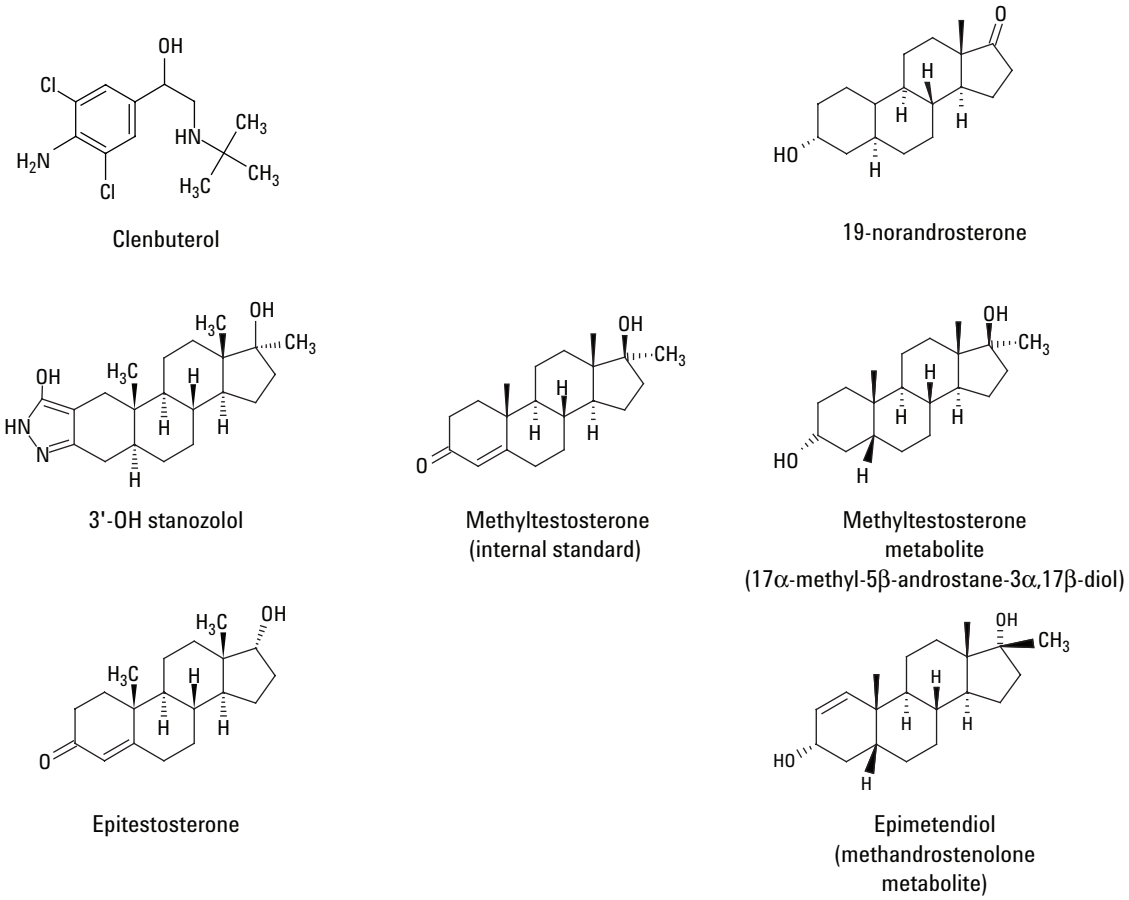


**Figure 1. Compounds analyzed.**

measurement of water-loss ions still retains its mass accuracy and the ion ratios for the $[M+H]^+$ and the subsequent water-loss ions are reproducible with this system. It is interesting to note that more water loss occurred with the later-eluting steroids rather than with the more polar early-eluting compounds.

## Experimental

### Sample Preparation

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 steroids in urine by GC/MS. The same sample preparation was used for the LC/MS analysis, except derivatization was unnecessary. The internal standard (20 µL of 10 ng/µL methyltestosterone) was added to 3 mL of urine, followed by 1 mL of 0.15M sodium acetate, pH 5. This solution was vortexed-mixed and then transferred to an Extrelut-3 column (Merck, VWR catalog number 48219-494, pkg of 100) connected in-line to an amino SPE column (J. T. Baker, VWR catalog number JH7088-3, pkg of 50) also containing 1 g of sodium sulfate. After an 8-minute delay, the columns were eluted with 9 mL of diethyl ether into a 13 × 100 mm silanized conical glass tube. For LC/MS, the final extracts were simply evaporated to dryness with a stream of nitrogen at 40°C. The tubes were capped and sent by overnight courier to the Agilent laboratory (Pleasanton, CA) where they were stored at –10°C while awaiting analysis. The residues were reconstituted in 100 µL of the initial mobile phase just prior to analysis.

### LC/MS Method Details

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), interfaced 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 an automatically-introduced reference mass solution containing two known compounds (purine and calibrant HP-921) bracketing the mass range of interest at *m/z* 121.050873 and 922.009798, respectively. 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**

| | |
|---|---|
| **LC Conditions** | |
| Column: | Agilent ZORBAX RRHT SB-C18<br>2.1 × 50 mm, 1.8 µm<br>(Agilent part number 822700-902) |
| Mobile phase: | A = 0.1% Formic acid/water<br>B = Methanol |
| Flow rate: | 0.4 mL/min |
| Col temp: | 55°C |
| Gradient: | 55% B, hold 5 min<br>55% to 75% B from 5 to 9 minutes |
| Analysis time: | 14 min |
| Post-time: | 5 min |
| Injection volume: | 4 µL |
| **MS Conditions** | |
| Ionization mode: | Positive APCI (final method) |
| Capillary voltage: | 3500 V |
| Vaporizer temp: | 450°C |
| Corona current: | 4 µA |
| Nebulizer: | 60 psig |
| Drying gas flow: | 5 L/min |
| Drying gas temp: | 350°C |
| Scan: | *m/z* 100–1000, 10,000 transients/scan<br>(0.89 sec/scan) |
| Reference masses: | 121 and 922 (added post-column<br>at 5 µL/min, 10 µM solution) |
| Fragmentor: | 150 V [no collision-induced-ionization<br>(CID)] |
| Skimmer: | 60 V (default) |
| Octopole RF: | 250 V (default) |

## Results and Discussion

Accurate-mass API-TOF LC/MS is commonly used for empirical formula determination and confirmation of naturally-occurring and synthetic 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 in the *m/z* range of the anabolic steroids, a routine mass accuracy of 3 ppm or less, 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 developing and optimizing specific MS/MS parameters.

Initial studies using unextracted standards of these and other steroids with ESI demonstrated that the instrument could measure the *m/z* of the $[M+H]^+$ ion to 3 ppm accuracy or less under routine unattended operation. However, the analytes included in this work showed better sensitivity using APCI. Theoretically, APCI might have been predicted to be the ionization mode of choice because the analytes tested are relatively nonpolar, and many contain no readily ionizable functional 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 $K^+$).

Figure 2 shows the base peak chromatogram of an unextracted standard equivalent to 16 ng/mL, if extracted from a 3-mL urine sample. An objective of this method development was to obtain separation of the target analytes in less than 15 minutes. Although this was achieved, it was unexpectedly challenging due to the range of polarities from clenbuterol to epimetendiol. In future work we plan to evaluate additional LC columns to improve the separation between epitestosterone and the internal standard while maintaining a reasonable analysis time.

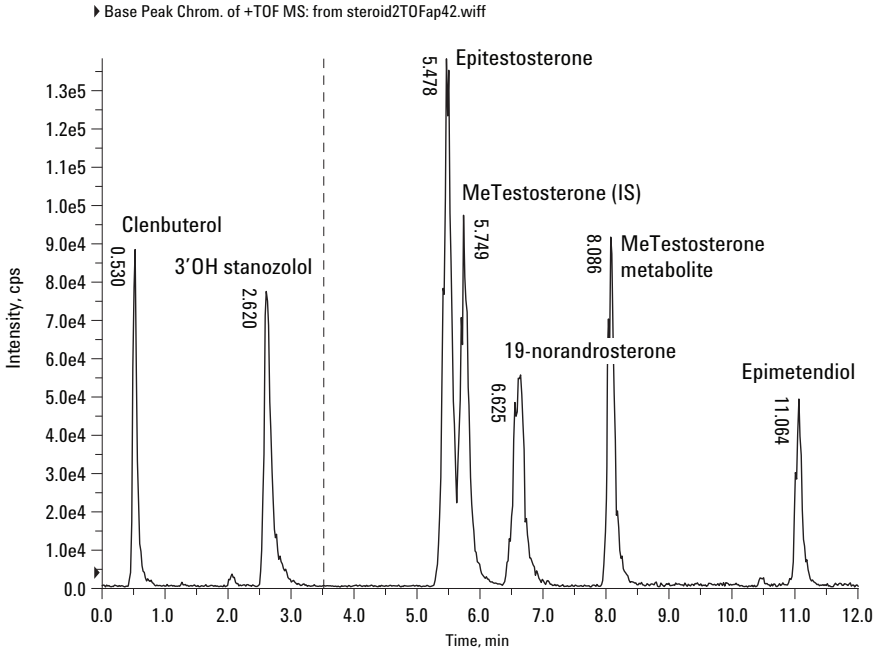


**Figure 2. Base peak chromatogram of anabolics mix (unextracted standard equivalent to 16-ng/mL urine).**

The vaporizer temperature and corona current were optimized for the best sensitivity across the range of analytes. In these studies, no in-source collision induced dissociation (CID) was used but in-source CID will be evaluated in future studies as a mechanism for generating ions for potential ion ratio calculations. The detection limits for these compounds were also improved by optimizing the number of transients/scan while maintaining a sufficient number of scans to accurately describe each chromatographic peak. In addition, increasing the photomultiplier voltage by only 50 V improved the detection limits without a significant increase in background noise.

An example of the typical performance of the LC/MSD TOF is shown in Figure 3 for the compound epitestosterone. The APCI spectrum shows both the $[M+H]^+$ protonated molecule and the less abundant $[M+H-18]^+$ water-loss ion. Applying the formula for mass accuracy shown in the Figure demonstrates a mass error of –1.04 ppm for the measurement of the $[M+H]^+$ ion.

The inset in Figure 3 also shows a mass resolution of 6890 measured for the $[M+H]^+$, and the large separation between the $[M+H]^+$ ion and the $^{13}C$ isotope peak at *m/z* 290. The resolution (R) at *m/z* 289.2159 can be calculated by dividing the *m/z* value by the full width at half maximum (FWHM or $w_{1/2}$) in Da. That is, $R = M/w_{1/2}$ where M = 289.2159 and ΔM is the width of the mass peak at half maximum, or half of its intensity. In this case, $w_{1/2}$ = 0.042 Da, so that R = 289.2159/0.042 = 6890. The accurate mass and ion ratio for the $^{13}C$ peak also have to match for the empirical formula calculated from the $[M+H]^+$ to be a correct assignment.

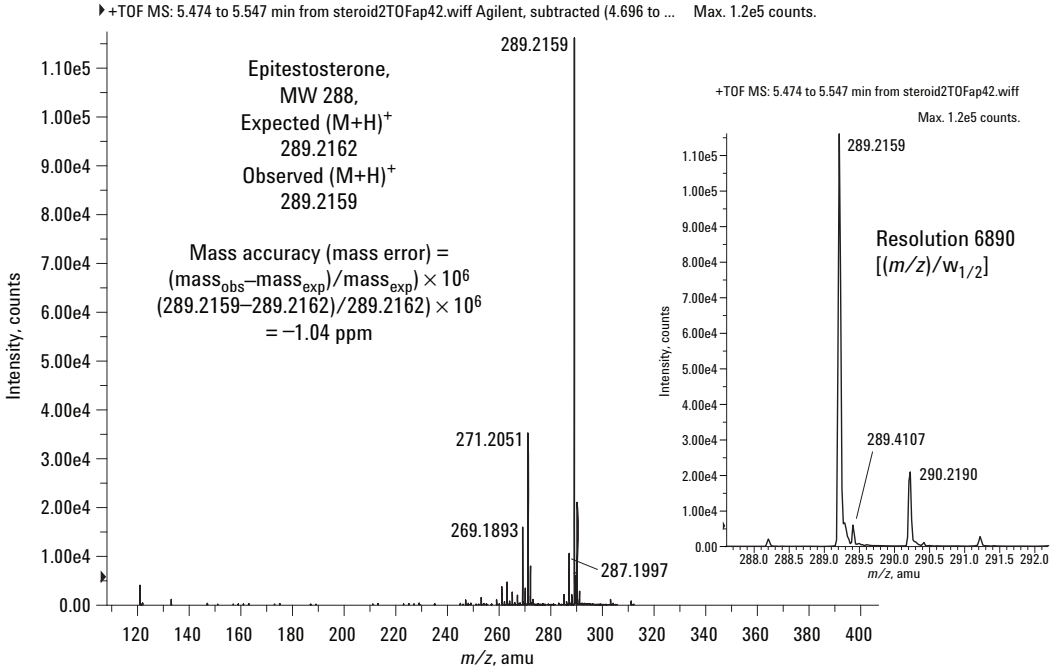


**Figure 3. Typical APCI spectrum and TOF performance for epitestosterone, $[M+H]^+$ = 289.2162. Also shown is a water-loss fragment at *m/z* 271.2051.**

Although there was some water loss observed in the spectrum of epitestosterone (the *m/z* 271 ion), for 19-norandrosterone [Figure 4], the most prominent ions in the spectrum are those at *m/z* 259.2049 and 241.1949. These represent the loss of one and two water molecules, respectively, from the protonated molecule. The loss of water was steroid-dependent and could not be entirely eliminated even by using ESI or by adjusting source conditions such as voltages and solvent-evaporation parameters.

Also note in Figure 4 the presence of the reference mass ions at 121 and 922, used to calibrate the mass axis for every scan in order to maintain mass accuracy throughout the acquisition. These ions are two compounds added automatically post-column as a dilute solution using a separate LC pump and a zero-dead-volume mixing tee at the MS system's inlet filter. In this case, the compounds are purine and HP-921 (a member of the Agilent API calibrant series), which are supplied with the LC/MSD TOF in a reference mass kit. Only a few thousand counts of reference mass are necessary to automatically calibrate each spectrum in real time. The instrument automatically reports any failure to find the reference masses on the screen and in a log file.

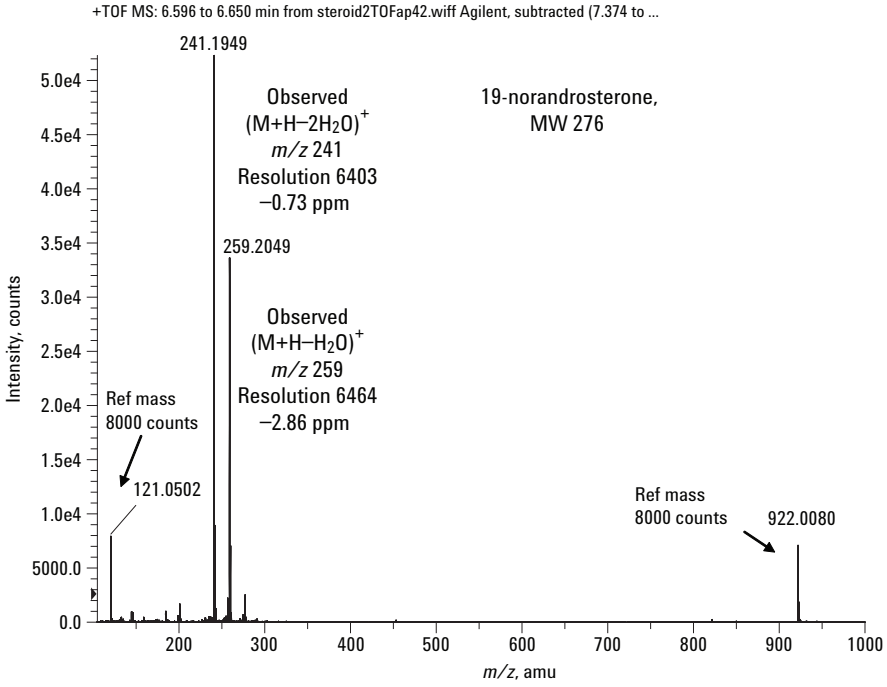


**Figure 4. Typical APCI spectrum for 19-norandrosterone, MW 276, showing two water-loss ions. Reference masses at *m/z* 121 and 922 also shown.**

A base peak chromatogram of an extracted steroid-free control urine fortified with epitestosterone, 19-norandrosterone, methyltestosterone metabolite and epimetendiol at 50 ng/mL, and 66 ng/mL of the methyltestosterone ISTD, is shown in Figure 5. Clenbuterol and 3'-hydroxystanozolol are not shown in the Figure because they were poorly recovered with the extraction procedure used at this stage of the method development. Because of the reduced recovery, these analytes are not shown in the subsequent Figures. Although epitestosterone and the internal standard appear unresolved chromatographically in the base peak chromatogram, they were resolved by their extracted ion chromatograms (EICs).

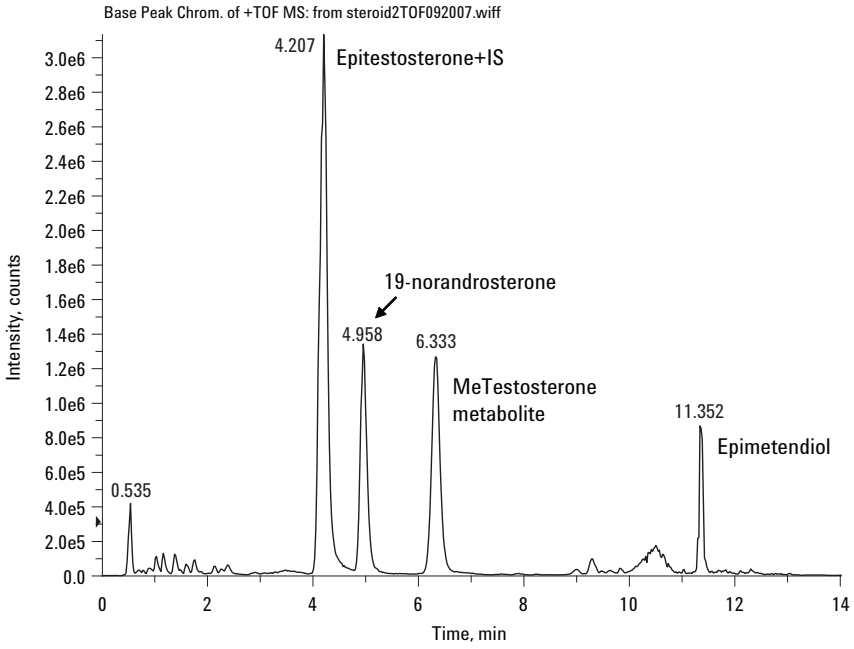


**Figure 5. Extract of 50 ng/mL urine. Analytes are visible in base peak chromatogram.**

Figure 6 shows the EICs from an extract of urine that was fortified with 2 ng/mL of epitestosterone, methyltestosterone metabolite and epimetendiol, and 1 ng/mL (the MRPL) of 19-norandrosterone. As shown, the EICs had 50,000–100,000 count intensities and excellent signal-to-noise, which permitted easy peak detection and location of spectra. For quantitation, one can take advantage of the high degree of mass accuracy with the TOF by designating narrow mass widths for the EICs. In this study, we used mass widths of 1 mDa (~3 ppm) for the EICs. For example, for 19-norandrosterone detection, the $[M+H - 2H_2O]^+$ ion at 241.1949 was selected, and the EIC mass window was (241.1944 to 241.1954). By using the TOF's resolving power for selectivity, nearly all of the chemical noise was eliminated and the S/N vastly improved.

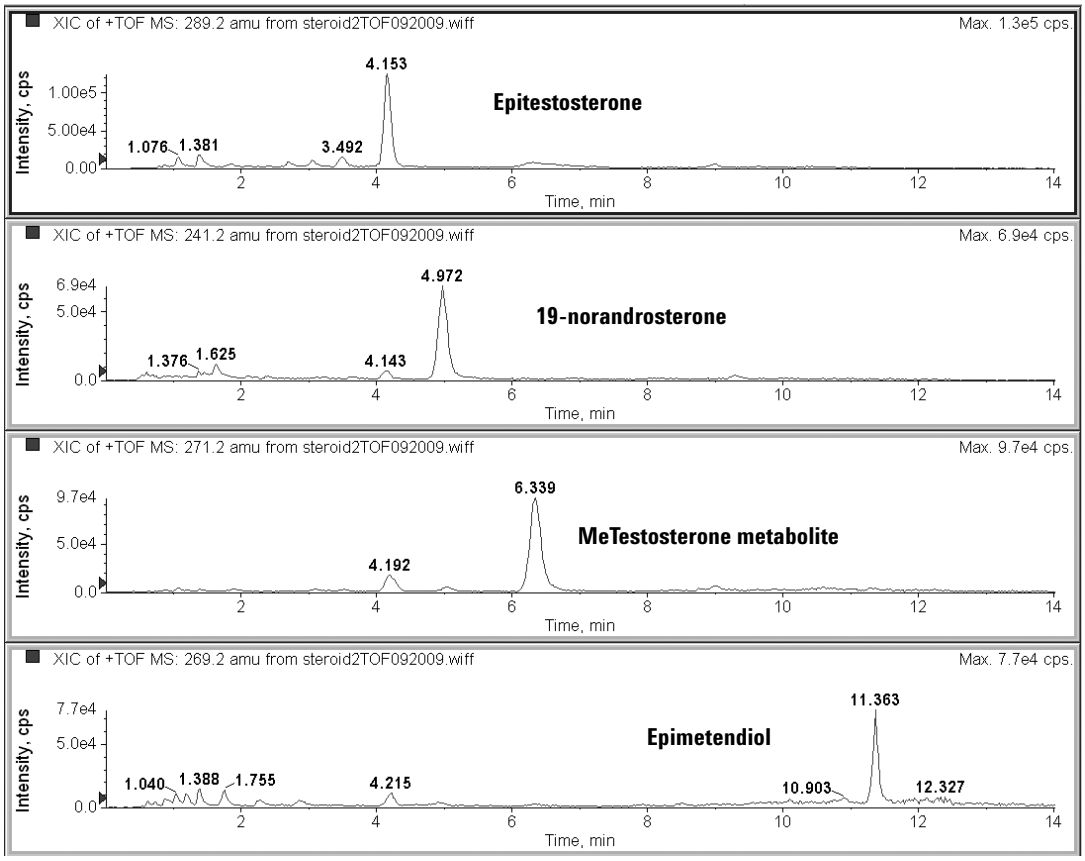


**Figure 6. Extract of 2 ng/mL urine (1 ng/mL 19-norandrosterone). Analytes detected using accurate mass EICs (1 mDa width).**

Figure 7 shows the spectra corresponding to the extracted analytes shown in Figure 6. Note the mass accuracy results that were calculated automatically by the TOF's data analysis method. These spectra were obtained from an estimated 240 pg of each analyte on-column, assuming 100% recovery (120 pg for 19-norandrosterone). Even at such a low concentration and in a urine extract, the mass accuracy was better than 2 ppm for each analyte. This demonstrates that there were no matrix interferences with the mass measurements even with simple and fast chromatography.

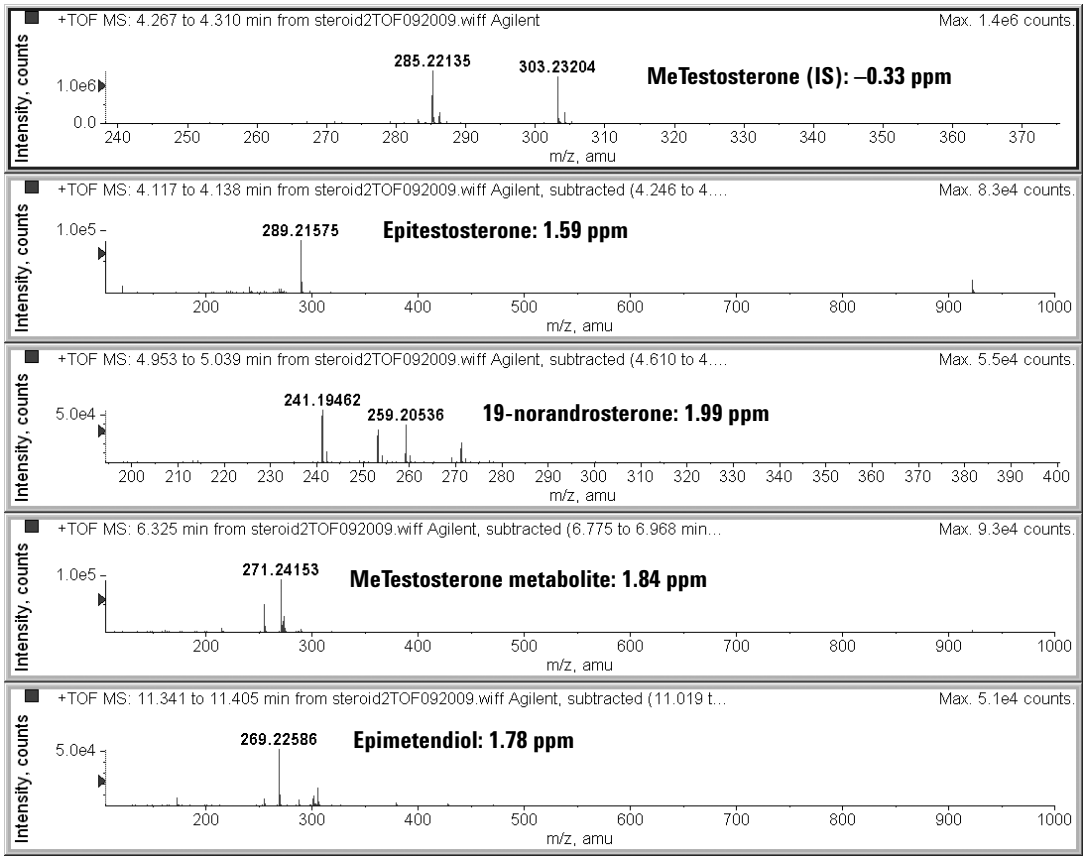


**Figure 7. Spectra from 2 ng/mL urine extract (1 ng/mL 19-norandrosterone) (240/120 pg on-column) [see Figure 6].**

One final experiment was performed to estimate the detection limit for the internal standard, epitestosterone, 19-norandrosterone, methyltestosterone metabolite, and epimetendiol using the developed LC/MSD TOF method. Serial dilutions of an unextracted standard were prepared and analyzed down to the concentration corresponding to 0.16 ng/mL extracted from 3 mL of urine. A 4-µL injection corresponding to 20 pg on-column was analyzed. The accurate-mass (1 mDa width) EICs are shown in Figure 8 and still exhibit excellent signal-to-noise.

It is possible that the method could be further improved by using 3.5-µm columns, rather than the 1.8-µm particle column used in this study, which would allow for larger injection volumes. The choice of columns would depend on the need for more sensitivity with larger injections, but might sacrifice the speed and resolution achieved with the 1.8-µm column.

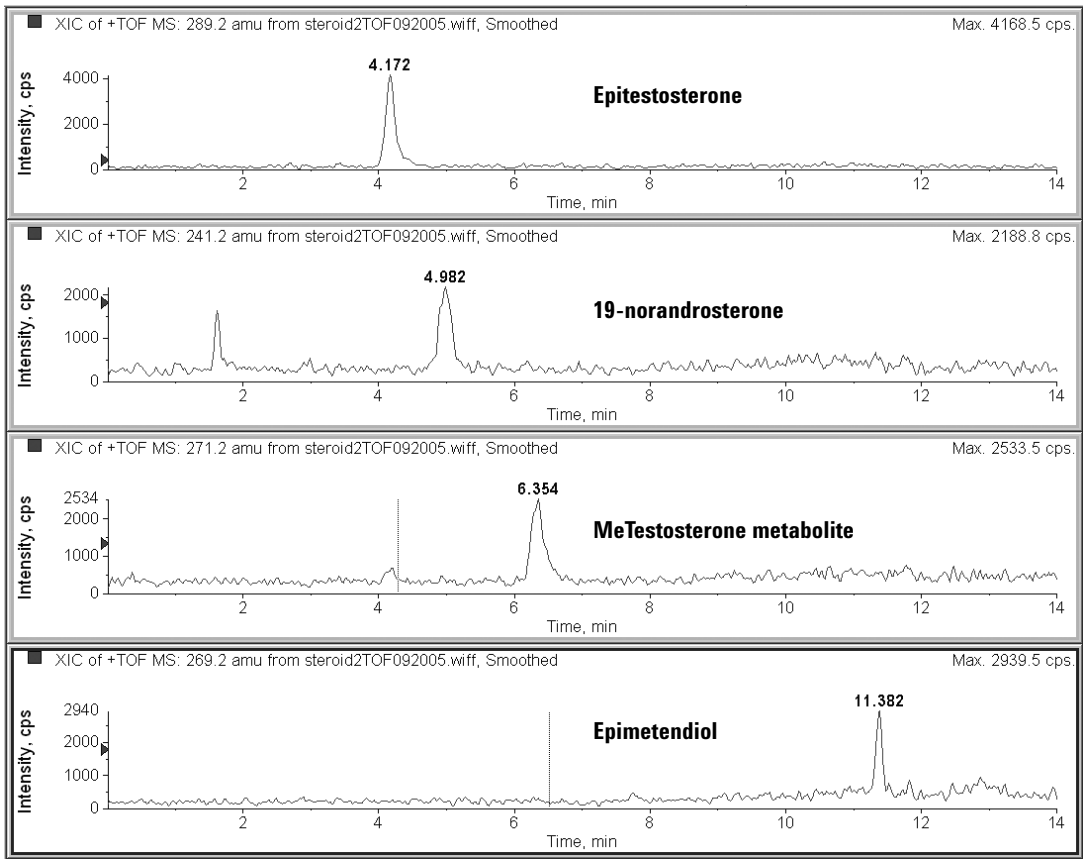


**Figure 8. Accurate-mass EICs (1 mDA width) from 0.16-ng/mL unextracted standard (20 pg on-column).**

## Conclusions

Using a standard sample preparation method developed for GC/MS screening, but without derivatization, an LC/MS method for an easy-to-use, bench top API-TOF instrument readily detected a representative group of anabolic substances at 1–2 ng/mL concentrations in urine. This sensitivity was achieved by capitalizing on the resolution of the LC/MSD TOF that allowed for accurate mass analysis, EICs with extremely narrow mass windows, and enhanced S/N. The analysis consumed only 4 µL of the 100 µL of reconstituted extract, which allowed for re-analysis, analysis of replicates, or different analyses from a single extraction. The use of "accurate-mass EICs" (that is, EICs with an *m/z* width of 1 mDa) allowed for specific detection of the target steroids in a complex sample. The spectra obtained at these low ng/mL concentrations all showed mass errors of less than 2 ppm.

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