Potential of atmospheric pressure chemical ionization source in gas chromatography tandem mass spectrometry for the screening of urinary exogenous androgenic anabolic steroids

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Abstract

The atmospheric pressure chemical ionization (APCI) source for gas chromatography-mass spectrometry analysis has been evaluated for the screening of 16 exogenous androgenic anabolic steroids (AAS) in urine. The sample treatment is based on the strategy currently applied in doping control laboratories i.e. enzymatic hydrolysis, liquid-liquid extraction (LLE) and derivatization to form the trimethylsilyl ether-trimethylsilyl *enol ether* (TMS) derivatives. These TMS derivatives are then analyzed by gas chromatography tandem mass spectrometry using a triple quadrupole instrument (GC-QqQ MS/MS) under selected reaction monitoring (SRM) mode. The APCI promotes soft ionization with very little fragmentation resulting, in most cases, in abundant $[M+H]^+$ or $[M+H-2TMSOH]^+$ ions, which can be chosen as precursor ions for the SRM transitions, improving in this way the selectivity and sensitivity of the method. Specificity of the transitions is also of great relevance, as the presence of endogenous compounds can affect the measurements when using the most abundant ions. The method has been qualitatively validated by spiking six different urine samples at two concentration levels each. Precision was generally satisfactory with RSD values below 25 and 15 % at the low and high concentration level, respectively. Most the limits of detection (LOD) were below 0.5 ng mL^{-1}. Validation results were compared with the commonly used method based on the electron ionization (EI) source. EI analysis was found to be slightly more repeatable whereas lower LODs were found for APCI. In addition, the applicability of the developed method has been tested in samples collected after the administration of 4-chloromethandienone. The highest sensitivity of the APCI method for this compound, allowed to increase the period in which its administration can be detected.

Keywords: Anabolic Androgenic Steroids (AAS), Atmospheric Pressure Chemical Ionization (APCI), Gas Chromatography (GC), Tandem Mass Spectrometry (MS/MS), Triple Quadrupole (QqQ), Doping Control Analysis

1. Introduction

Since 2004, the World Anti-Doping Agency (WADA) publishes a list of prohibited substances and methods in sport which is yearly updated [1]. Among the groups of substances included in the list, androgenic anabolic steroids (AAS) are the most frequently reported ones [2]. AAS are mainly used due to their anabolic effects such as muscle and strength growth among others [3].

AAS are prohibited at all times i.e. in and out of competition. This prohibition makes that any evidence of AAS misuse (e.g. the mere presence of traces of the AAS and/or its metabolites) is sufficient for reporting an adverse analytical finding [4]. The detection of AAS misuse is a constant analytical challenge due to their low concentration in urine, the complexity of the matrix and the similarity between endogenous and exogenous AAS. Thus, sensitivity and selectivity of analytical methods are key factors and requirements for AAS detection have evolved hand in hand with instrumental developments.

AAS have been traditionally determined by gas chromatography mass spectrometry (GC-MS) methods working in selected ion monitoring mode (SIM) using electron ionization (EI) sources [5]. After some preparation steps [6] i.e. hydrolysis with *β*-glucuronidase, liquid-liquid extraction and conversion of both hydroxyl and carbonyl function into the corresponding TMS *ether/enol-TMS* ethers, these methods allowed the detection of most of AAS metabolites at concentrations below 10 ng mL $^{-1}$. For this reason, the minimum required performance level (MRPL) for most AAS was set at 10 ng mL $^{-1}$. However, these methods failed for the detection of several AAS at the required MRPL, mainly those with difficulties in the derivatization step. Among them, stanozolol and AAS bearing a 4,9,11-triene nucleus like tetrahydrogestrinone (THG) [7].

The occurrence of high resolution mass spectrometry opened new possibilities for the detection of stanozolol [8], although the scenario drastically changed after the introduction of liquid chromatography tandem mass spectrometry (LC-MS(/MS)) in doping control laboratories [9,10]. Several methods have been developed for the LC-MS/MS detection of AAS with poor derivatization properties like stanozolol and THG [11, 12, 13]. Thus, both GC-MS(/MS) and LC-MS(/MS) have been employed as complementary techniques in doping control laboratories in order to reach the required MRPLs. Qualitative methods for the detection of exogenous AAS in urine by LC-MS/MS with triple quadrupole (QqQ) analyzers and

electrospray ionization source (ESI) [11] have been reported, as well as GC-MS/MS methods with EI [14, 15] or chemical ionization (CI) sources [16].

In the last years, the commercialization of triple quadrupole instruments coupled to GC has allowed for increasing the sensitivity of the previous GC-MS methods. Thus, several GC-EI-MS/MS methods in selected reaction monitoring mode (SRM) have been published either for the detection of target analytes [14, 15] or for metabolic studies [17, 18, 19]. Nowadays, this technique has become the gold-standard in AAS analysis for doping control purposes. Due to the sensitivity improvement, the MRPL for AAS has been recently reduced to 2-5 ng mL^{-1} for most analytes [4]. This fact illustrates the impact of new analytical technologies in the detection of AAS. Therefore, it is valuable to test the performance of emerging analytical tools in this field.

As an alternative to EI, different "soft" ionization sources for GC have been tested for the detection of AAS in doping analysis, i.e. CI [16], heated nebulizer microchip atmospheric pressure photoionization (μAPPI) [20, 21] or atmospheric pressure chemical ionization (APCI) [22, 23]. The recently commercialized APCI source for GC represents an attractive alternative in several application fields [24, 25, 26]. APCI promotes soft ionization for the generation of $[M+H]^+$ or $M^{+\cdot}$ ions as the base peak of the spectrum, by means of protonation or charge transfer mechanisms, deeper explained in literature [22, 27]. This soft ionization presents an advantage in the selection of specific precursor ions in MS/MS based methods.

In the present work, the potential of APCI source using GC-MS/MS was evaluated for the development of a screening method for the detection of selected exogenous AAS in urine. After validation, the performance of the GC-APCI-MS/MS method has been compared with the conventional GC-EI-MS/MS, by analyzing a group of samples prepared under the same conditions. The applicability of the method was also evaluated in a set of samples collected at different times after the administration of 4-chloromethandienone (4Cl-MTD).

2. Experimental

2.1. Chemical and reagents

The structures of the selected AAS are shown in Figure 1. Boldenone (BD) was obtained from Sigma (St. Louis, MO, USA). 17β-hydroxy-5β-androstan-1-ene-3-one (Boldenone metabolite, BDmet), 17β-methyl-5β-androst-1-en-3α,17α-diol (Methandienone metabolite, MTDmet3), 1-testosterone (1-T), 5α-androstan-17α-methyl-3α,17β-diol (Methyltestosterone metabolite, MeTmet1), 5β-androstan-17α-methyl-3α,17β-diol (Methyltestosterone metabolite, MeTmet2), 5β-androstan-7β,17α-dimethyl-3α,17β-diol (Calusterone metabolite, CALUSmet), 17α- metyl-1-testosterone (Me-1-T), 5β-androstan-7α,17α-dimethyl-3α,17β-diol (Bolasterone metabolite, BOLASmet), 13β,17α-diethyl-5β-gonane-3α,17β-diol (Norbolethone metabolite, NORBOLmet2) 6β-hydroxy-4-chloromethandienone (6OH-4Cl-MTD) and 4-hydroxy-testosterone (4OH-T) were purchased from NMI (Pymble, Australia). Fluoxymesterone (FLU) was obtained from Steraloids (Newport, RI, USA). 5α-Androstan-2α,17α-dimethyl-3α,17β-diol (Methasterone metabolite, METHASmet) was a kind gift from the World Association of Anti-Doping Scientists (WAADS). Oxymesterone (OXY) and madol (MADOL) were provided by the Toronto Research Chemicals (Toronto, Canada).

AAS stock standard solutions at 10 and 100 μg mL^{-1} in methanol were stored at -20 ºC. Working MIX solutions at appropriate concentration levels for validation were prepared in acetone and also stored at -20 ºC, whereas individual standard solutions were employed for the transition optimization step and for potential cross-talk evaluation.

β-glucuronidase solution (*Escherichia coli*, type K12) was purchased from Roche Diagnostics (Mannheim, Germany). Analytical grade potassium carbonate, potassium hydroxide pellets, sodium hydrogen phosphate, di-sodium hydrogen phosphate, *tert*-butyl-methyl ether and ammonium iodide were acquired from Merck (Darmstadt, Germany). The derivatization reagent preparation *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and 2-mercaptoethanol from Sigma-Aldrich (St Louis, MO, USA). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Formic acid and

ammonium formate (LC/MS grade), acetonitrile and methanol (LC gradient grade) were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

2.2.1. GC-APCI-MS/MS

An Agilent 7890A GC system (Palo Alto, CA, USA) equipped with an Agilent 7693 autosampler was coupled to a triple quadrupole mass spectrometer, Xevo TQ-S (Waters Corporation, Manchester, UK), using an APGC source, operating in APCI mode. The GC separation was performed using an HP Ultra 1 capillary column, (length 16 m × I.D. 0.20 mm × film thickness 0.11 μm). For the named as *gradient 1*, the oven was programmed as follows: 185 °C (0.5 min); 25 °C min^{-1} to 230 °C; 10 °C min^{-1} to 290 °C; 70 ºC min^{-1} to 310 ºC (2.5 min), being the total run time 11.6 min. G*radient 2* programme was: 180 ºC (1 min); 3 ºC min^{-1} to 230 ºC; 40 ºC min^{-1} to 310 ºC (3 min), total run time 22.7 min. Split injections (ratio 1:10) of 2 μL using a straight deactivated liner with glass wool were carried out at 280 °C. Helium 99.999% (Carburos Metálicos, Spain) was used as carrier gas at 2 mL min^{-1}.

The interface temperature was set to 300 °C using N_2 as auxiliary gas at 250 L h^{-1}, make up gas at 300 mL min^{-1}, and cone gas at 170 L h^{-1}. The temperature in the source was set at 150 ºC. The APCI corona pin was operated at 1.55 μA and a cone voltage of 20 V was selected. The water used as modifier when working under proton-transfer conditions was placed in an uncapped vial, which was located within a holder placed in the source door. For MS/MS measurement, argon 99.995% (Carburos Metálicos, Spain) was used as collision gas at a pressure of 4.15×10^{-3} mbar in the collision cell (Table 1).

2.2.2. GC-EI-MS/MS

For all EI experiments, a 7890A gas chromatograph equipped with a 7693 autosampler and coupled to a 7000A Series Triple Quadrupole GC/MS (Agilent Technologies) was employed. The same column and chromatographic conditions detailed in the APCI section (gradient 1) were used.

Nitrogen was used as collision gas at a flow rate of 1.5 mL min $^{-1}$, and helium (Abello-Linde) as a quenching gas at a flow rate of 2.25 mL min $^{-1}$. The electron impact source was kept at 230 ºC and the quadrupoles at 150 ºC.

2.3. Sample preparation

Urine samples were treated as previously described in literature [14, 15]. Briefly, 25 µL of internal standard solution (methyltestosterone, 10 µg mL $^{-1}$) was added to 2.5 mL of urine. Then, the solution was hydrolyzed by the addition of 1 mL phosphate buffer (pH 7) and 30 µL of *β*-glucuronidase solution (55 ºC, 1h). After cooling at room temperature, 200 mg of $NaHCO_3/Na_2CO_3$ buffer (1:2; w/w) was added (pH 9.5). A LLE step was carried out by adding 6 mL of methyl tert-butyl ether (MTBE). After centrifugation (4350 rpm, 5 min.), the organic phase was separated and evaporated to dryness (45 ºC). Finally, in order to obtain the *enol-trimethylsylil* (TMS) derivatives of the analyte, 50 µL of a mixture of MSTFA/NH_4I/2-mercaptoethanol (1000/2/6; v/w/v) was added to the dry extract and then kept at 60 °C for 20 min.

2.4. Validation

Following the WADA criteria [4], the validation of the screening method was designed in order to confirm the suitability of the method to detect half the MRPL of the compound. The method validation was performed using spot urine samples collected from six volunteers (three male and three female which did not take any steroid). Two spiking levels were selected taking into account the current MRPL for the compounds (Table 2). Low concentration levels (LCL) of 1 ng mL $^{-1}$ and 2 ng mL $^{-1}$, and high concentration levels (HCL) of 10 ng mL $^{-1}$ and 20 ng mL $^{-1}$ were selected for AAS with MRPLs of 2 ng mL $^{-1}$ and 5 ng mL $^{-1}$, respectively. In this sense, around 0.5xMRPL and 5xMRPL levels were assayed in both cases.

For the evaluation of the extraction recoveries of each analyte, six blank samples were spiked at the high concentration level and extracted. The same samples were extracted and spiked after the extraction. The extraction recovery was calculated by comparing peak areas for each analyte in both cases.

Relative standard deviation (RSD) of the ratio between the peak areas of each compound and the internal standard were calculated. Repeatability (expressed as RSD) for each analyte was evaluated at the two concentration levels tested.

Based on WADA suggestions [4], the limit of detection (LOD) for each analyte was estimated as the concentration that produced a peak signal of three times the background noise in the chromatogram at the lowest fortification level.

Selectivity was tested by analyzing 10 different blank urines and monitoring the absence of interferences with signal to noise (S/N) ratios above 3.

2.5. Application to real samples

To study the applicability of the validated method, samples from an excretion study of 4-chloromethandienone (4Cl-MTD) were analyzed. A single dose of 20 mg of 4Cl-MTD (oral Turinabol) was administrated to a male volunteer (49 years, 85 kg) and different urine samples at intervals of 0-4 h, 4-8 h, 8-12 h, 12-24 h, 24-36 h, 48-56 h and 72-84 h were collected.

The study was conducted in accordance with the Declaration of Helsinki. Subject signed an informed consent before participation. Treatment was well tolerated by the subjects and no serious adverse events were observed.

3. Results and discussion

3.1. Transition optimization

Two transitions were optimized for each compound to improve the reliability of the method. The first step was the acquisition of a full scan spectrum for each individual TMS-derivative standard. Once the main precursor ions were selected, $[M+H]^+$, $[M+H\text{-}TMSOH]^+$ or $[M+H\text{-}2TMSOH]^+$, depending on the structure of the steroid [21], product ion spectra were obtained at different collision energies (10, 20, 30 and 40 eV) (Figure S1, supplementary information). Based on this information, the largest number of possible optimized SRM transitions was preselected. Then, ten blank urine extracts and ten extracts spiked at the LCL were tested in order to choose the best transitions for each analyte in terms of sensitivity and specificity. Transitions showing the maximum S/N and the minimum influence of the background of the matrix interferences were selected.

For most analytes, the most sensitive transition was found to be specific enough since matrix interferences were not observed. Therefore, it was selected for detection of the compound in the screening. However, in some cases such as OXY, (Figure S2, supplementary information), the selected transition was not the most

abundant (535.2>269.2), because of the presence of matrix interferences. Thus, a less sensitive but more specific transition was selected (535.2>389.5). In the case of NORBOLmet2 and BD in APCI, it was not possible to select any specific transition because of the presence of endogenous steroids with the same transitions at the same retention times under the selected conditions.

A list of the selected SRM transitions used in APCI, facing EI ones, is summarized in Table 1.

3.2. Method validation

Table 2 summarizes the results obtained for extraction recovery, repeatability and LOD by using GC-APCI-MS/MS. Suitable extraction recovery values between 67 and 89% were obtained in all cases except for Me-1-T (47%) and BDmet (53%).

Repeatability was evaluated by the RSD at both LCL and HCL (n=6 for each level). Values between 3% and 30% were obtained confirming the satisfactory precision of the method. As expected, better repeatability was observed at the HCL, being in most cases below 15%, except for 6OH-4Cl-MTD (RSD 22%).

In terms of selectivity, no interferences were detected in the ten blank samples for the transitions selected for each compound. Regarding LOD, most of them were lower than 0.5 ng mL $^{-1}$ and always below the established MRPL (Table 2). As stated in the previous section the main exceptions for this behaviour were NORBOLmet2 and BD, which were interfered by the presence of matrix components irrespective of the selected transition. Chromatographic separation was found to be critical for the proper validation of these compounds. The use of a longer gradient (gradient 2 in the experimental section) allowed for the discrimination between analytes and the matrix interferences (Figure S3, supplementary information). Using this gradient all analytes were adequately validated. In order to isolate as much as possible the effect of the interface, results using gradient 1 will be discussed. Only in the case of NORBOLmet2 and BD results for gradient 2 are discussed.

It is well-known that, differently to EI, atmospheric pressure ionization is more affected by matrix constituents that lead to possible matrix-induced suppression/enhancement of the analytes ionization. Since the main goal of the developed method was not quantification of the analytes but the detection/identification of all selected AAS at the LCL,, this effect was not

evaluated as that qualitative objective was satisfactorily reached independently on the matrix effects that might affect to ionization. However, matrix effect may be behind the higher RSD observed in APCI and it should be evaluated if the purpose of the analyses was quantification of analytes.

Figure 2 shows typical chromatograms obtained for a blank urine sample compared with those of a sample spiked at the LCL.

3.3. Comparison APCI vs EI

In order to evaluate the performance of the developed GC-APCI-MS/MS method, the validation results were compared with those obtained by GC-EI-MS/MS. All the factors involving the detection (urine used, extraction, derivatization, column and gradient of temperatures) were controlled in order to isolate as much as possible the effect of the ionization source. Ideally, both sources should be coupled to the same analyzer. Unfortunately, this ideal situation is currently not affordable, i.e. both interfaces are not interchangeable, and, therefore, every source was coupled to a different QqQ analyzer. Thus, although the discussion will be focused on the effect of the interface, a potential influence of the specific analyzer on the results cannot be discarded.

Validation results for both methodologies are shown in Table 2. Regarding repeatability, in general, RSD values were lower for EI. At LCL, RSDs ranged from 0.6% to 14% in EI whereas in APCI increased up to 3.2-18%. At HCL, RSDs in the range 0.3-5% and 3-14% were obtained for EI and APCI, respectively. Some values higher than 20 were punctually obtained. Thus, although RSDs from both studies can be considered acceptable, a slightly better repeatability of the EI source was found in this study. The lower repeatability of APCI might be due either to factors affecting the ionization process such as the amount of water in the interface or to the potential matrix effect suffered by APCI.

Regarding sensitivity, the results largely depended on the MS behaviour of the steroid. Thus, analytes with an abundant $[M+H]^+$ in APCI (BDmet, 1-T, Me-1-T, 4OH-T, FLU, 6OH-4Cl-MTD, BD and OXY exhibited LODs in the sub-ng/mL range (below 0.4 ng/mL) i.e. more than 10 times lower than the current MRPL for most AAS. For these AAS, LODs estimated for APCI were between 5 and 20 times lower than for EI (Figure 3a), even in those cases in which an abundant $M^{+\cdot}$ was also present in EI.

Worse LODs (typically between 0.3 and 1 ng/mL) were obtained for AAS showing an abundant $[M+H-nTMSOH]^+$ in APCI (MADOL, MTDmet3, MeTmet1, CALUSmet, MeTmet2, METHASmet, NORBOLmet2 and BOLASmet). For these compounds, LODs using APCI were commonly in the same range as those obtained by EI (Table 2, Figure 3b). The low specificity of the product ions can be behind this fact. After the in-source neutral loss of all TMS present in the molecule (lost as TMSOH), the remained hydrocarbon skeleton was selected as precursor ion. Under these conditions the selection of a specific product ion was troublesome. Thus, most of the product ions obtained was not specific and both matrix interferences and high background decreased the sensitivity of the method. Only for BOLASmet, a specific product ion (*m/z* 175) could be obtained. In this case, the sensitivity was similar to those AAS exhibiting a $[M+H]^+$.

The selection of abundant and specific precursor ions was a key factor when aiming at the maximum sensitivity. In the light of obtained results, it is noteworthy to mention that the presence of $[M+H]^+$ in APCI led to the best results in terms of sensitivity. Future work in the search of diverse derivatizing agents that maximize the protonated molecule in APCI would be valuable.

3.4. Application to real samples

To check the applicability of the developed methodology, samples collected after 4Cl-MTD administration were analyzed by GC-APCI-MS/MS and the results were compared with those obtained by GC-EI-MS/MS.

As expected, the main metabolite of 4-chloromethandienone (6OH-4Cl-MTD) was detected by both methods in the urines collected during the first hours after administration (Figure 4a). Owing the better sensitivity provided by APCI, the misuse of 4Cl-MTD could be detected in samples in which the metabolite was undetectable by the commonly used GC-EI-MS/MS methods (Figure 4b). Therefore, the period of time in which the misuse can be detectable increased from 56 h to 84 h (the last sample collected) by using APCI. This fact illustrates the potential and future of this source in the detection of AAS misuse.

4. Conclusions

The suitability of GC-APCI-MS/MS for sensitive detection of AAS has been demonstrated by the validation of a method for the detection of 16 exogenous AAS in urine.

The present work illustrates the potential of the new APCI source as an adequate alternative to the traditional EI source in GC-MS methodologies. Due to the endogenous steroids present in urine, the selection of a specific transition has been found to be a key factor in the method development. Optimization of the chromatography was found to be critical for the correct detection of two of the analytes (BD and NORBOLmet2). Although suitable precision (RSD values below 25 and 15% at LCL and HCL, respectively) was obtained with the APCI method, it was slightly higher than the one obtained with EI.

Sensitivity was found to be higher with APCI for the majority of compounds tested, with LODs commonly lower than 0.5 ng mL $^{-1}$. These LODs are similar to the obtained with other soft ionization sources like CI [16]. The higher sensitivity obtained can be related with the abundance of a specific product ion. This, in around 50% of the analytes, the soft ionization of provided by the APCI source allowed for the selection of the $[M+H]^+$ as precursor ion. In the rest of analytes, ions resulting from one or two losses of TMSOH from the derivatizing reagent were selected as precursor. These ions keep still the steroidal skeleton helping in the selection of specific product ions.

Anyway, the presence of an abundant $[M+H]^+$ in the mass spectra and its selection as precursor ion was found to be related with a higher sensitivity. Since the selected derivative (TMS) does not favor the protonation, the use of derivatives with higher proton affinity would theoretically improve the sensitivity of the method. Further research in order to investigate the applicability of other derivatizing agents able to generate specific fragments would be desirable.

The notable improvement in sensitivity provided by the use of APCI source in GC-MS/MS methods is of great relevance in doping control field, as revealed in the application of the method for the detection of 4-chloromethandienone misuse. Therefore, the use of GC-(APCI) MS/MS based methods could increase the period of time in which the misuse of the AAS can be detected, and opens interesting possibilities in the near future.

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FIGURE CAPTIONS

Figure 1.- Structures of the selected analytes.

Figure 2.- APCI optimized transitions of selected AAS in (a) blank urine sample and (b) urine sample spiked at LCL.

Figure 3.- Comparison between APCI and EI for selected compounds: (a) BDmet and (b) MADOL.

Figure 4.- Comparison between APCI and EI in urine samples collected after administration of 4Cl-MTD. Chromatograms of samples collected between (a) 24-36 h after administration and (b) 72-84 h after administration.

526 **Figure 1.**

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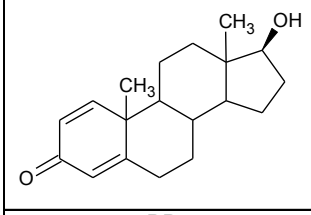	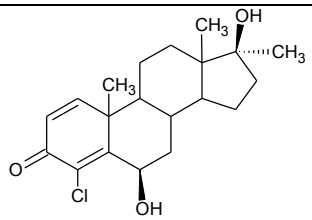	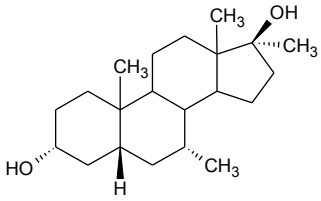
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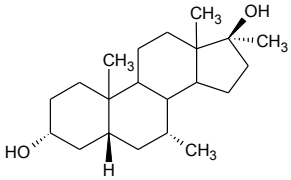	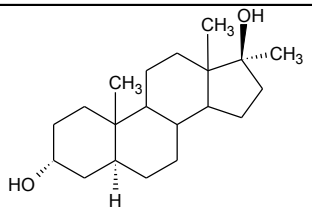	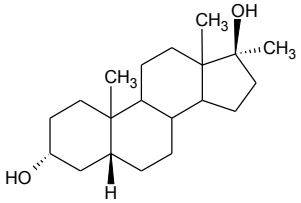
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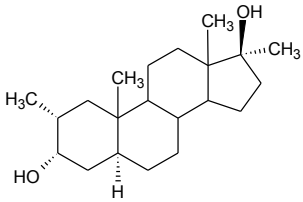	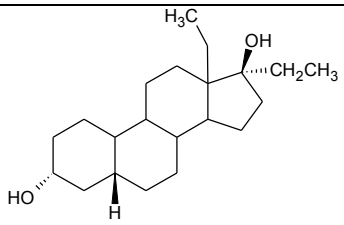	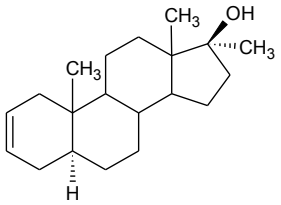
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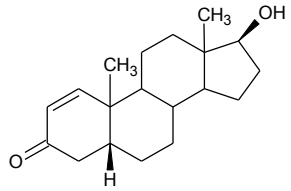	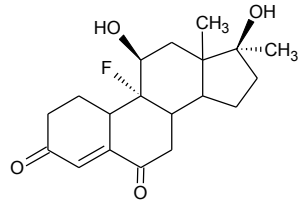	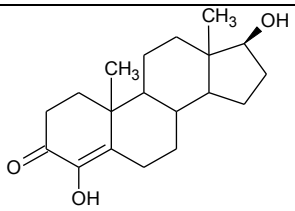
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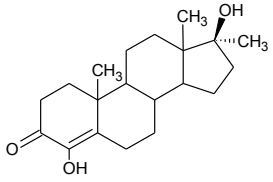	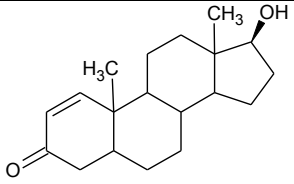	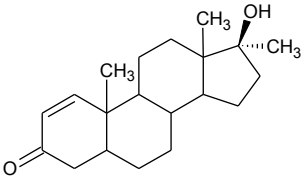
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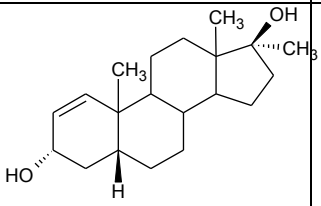		
MTDmet3		

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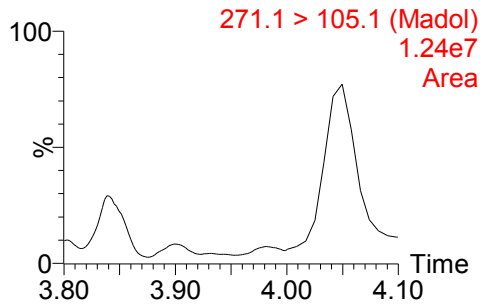
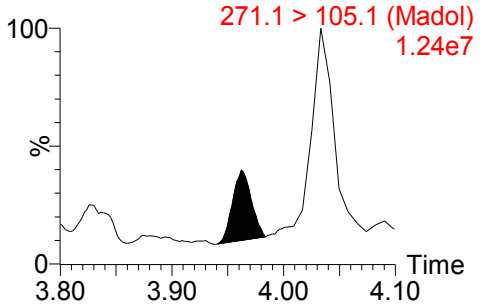
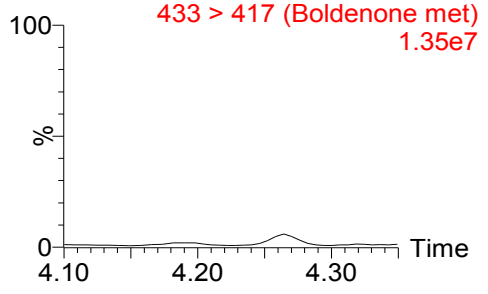
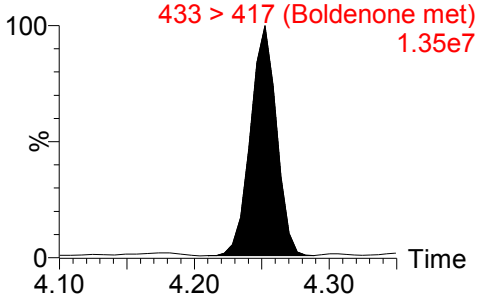
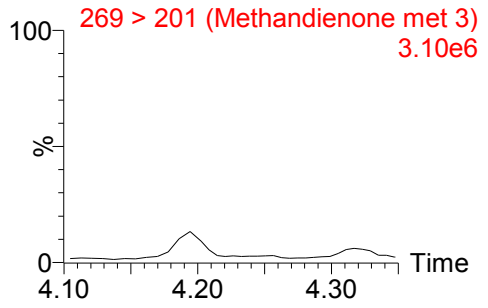
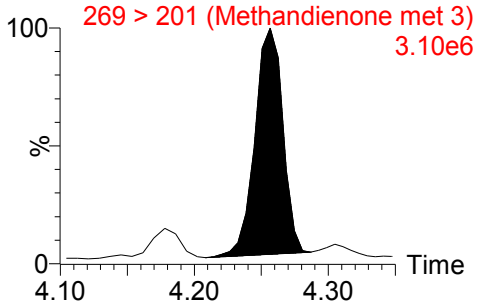
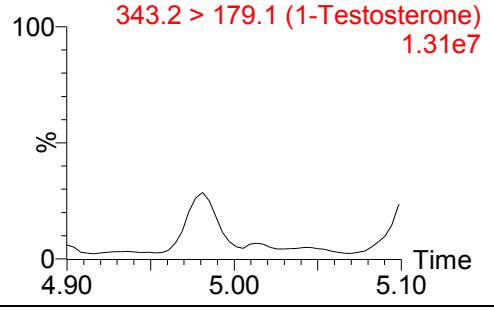
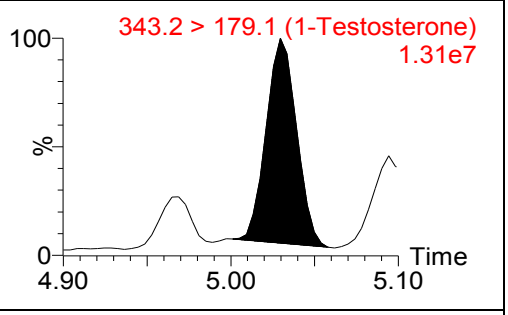
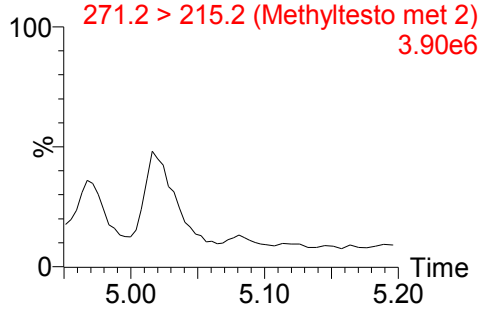
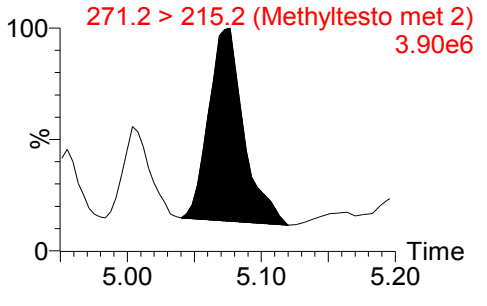
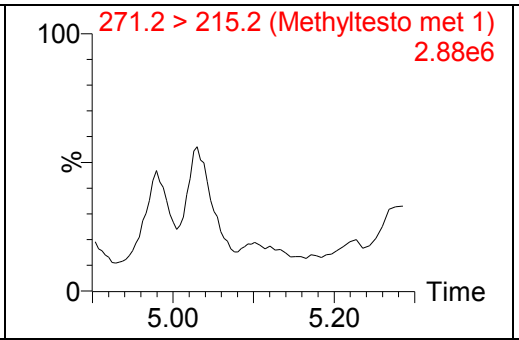
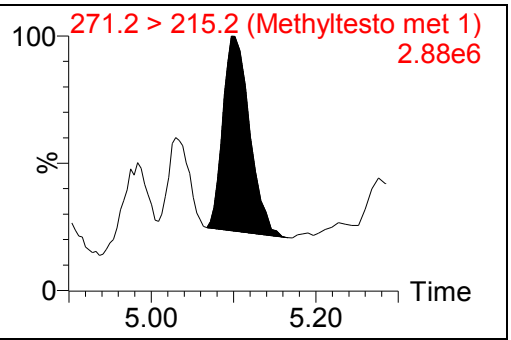
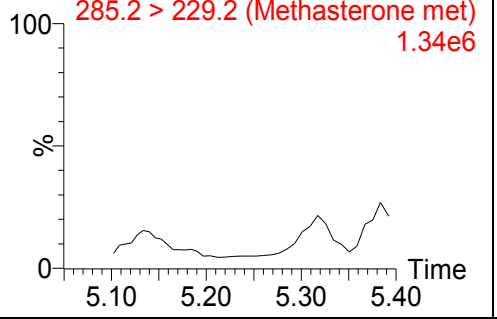
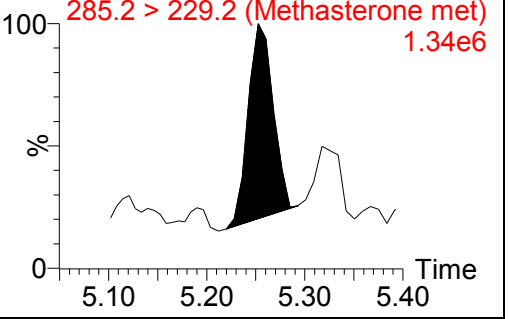
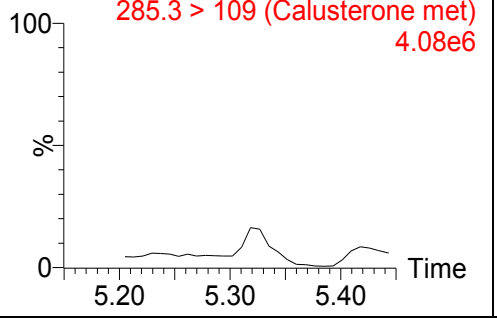
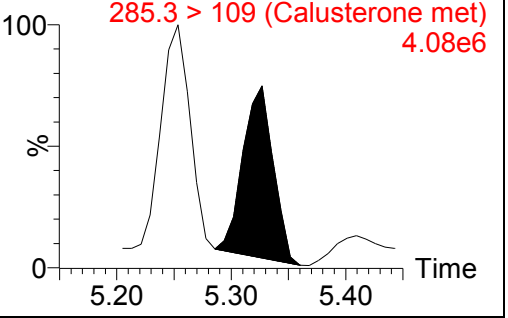
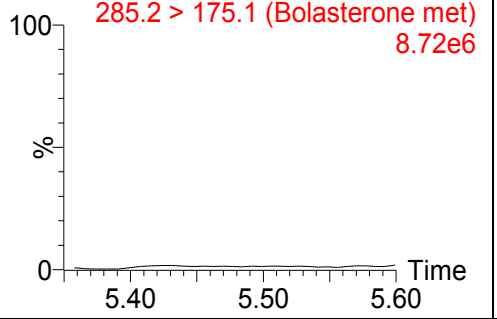
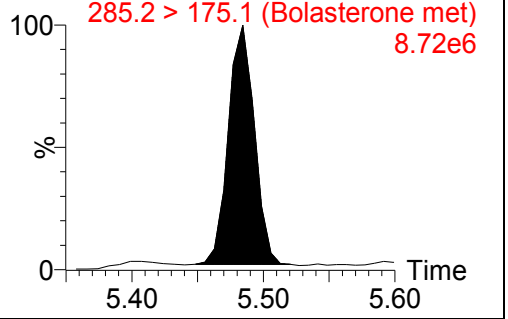
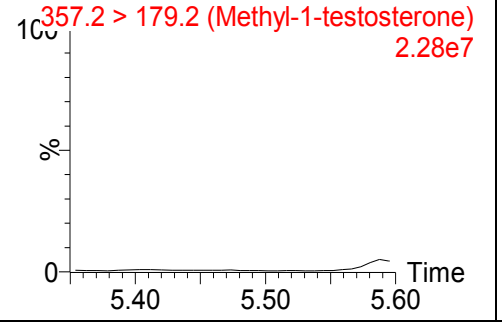
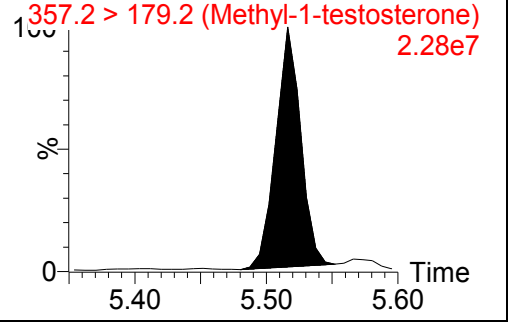
531 **Figure 2.**

Analyte	(a) Blank	(b) LCL
MADOL		
BDmet		
MTD met3		
1-T		
MeTmet2		

MeTmet1		
METHAS met		
CALUS met		
BOLAS met		
Me-1-T		

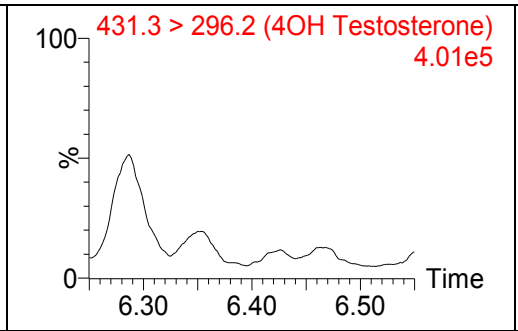
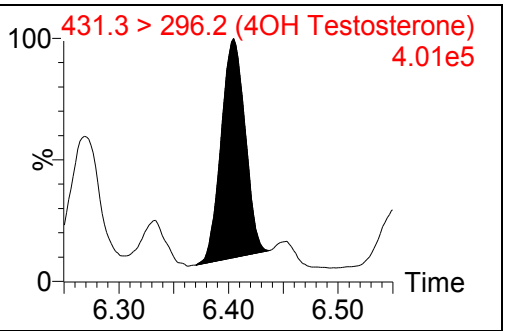
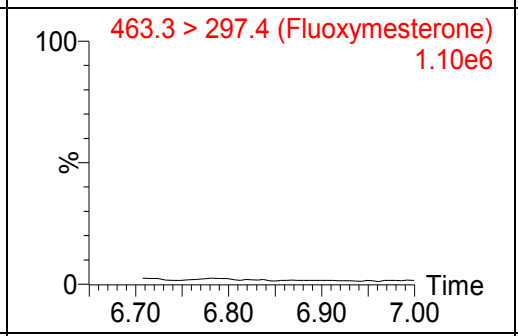
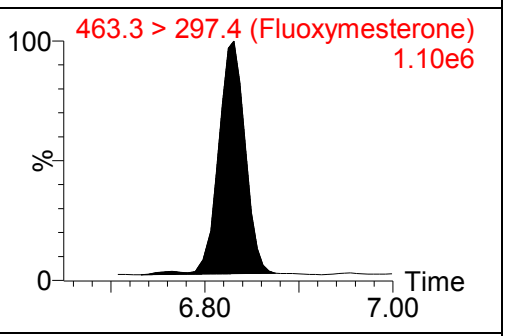
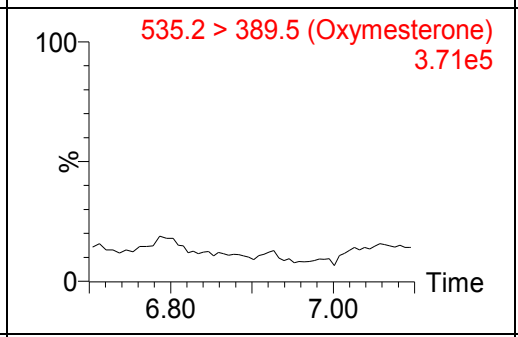
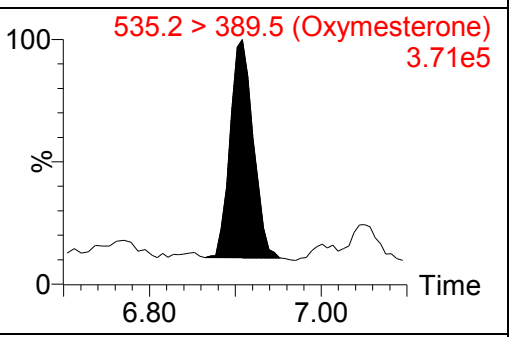
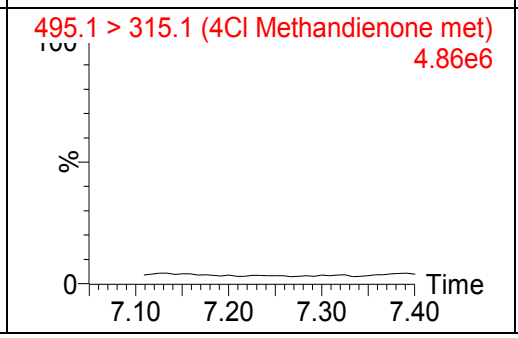
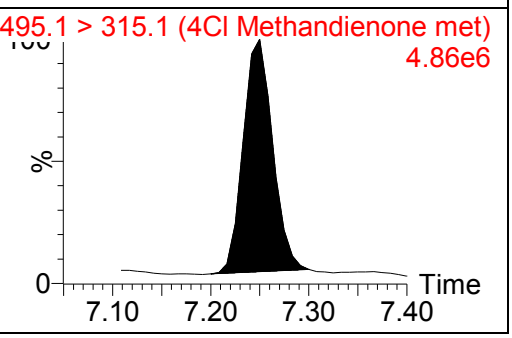
4OH-T		
FLU		
OXY		
6OH-4Cl -MTD		

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Figure 3.

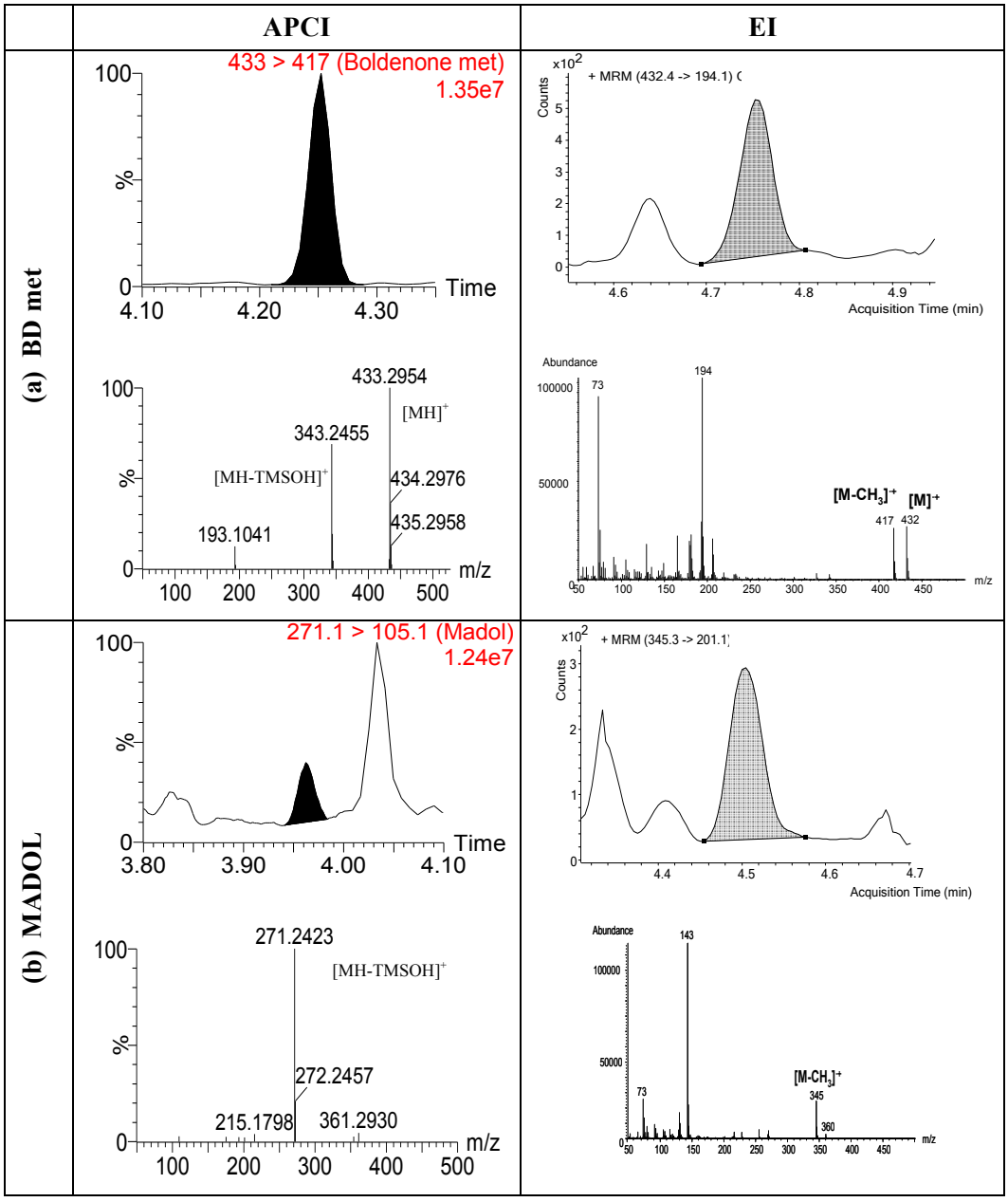
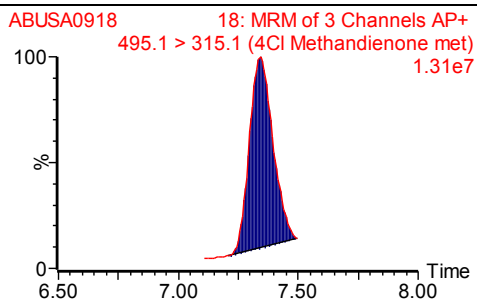
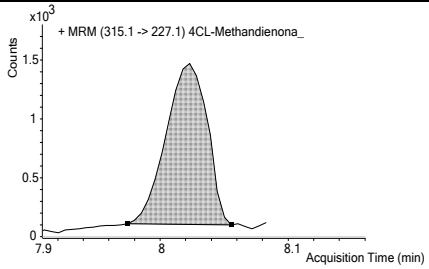
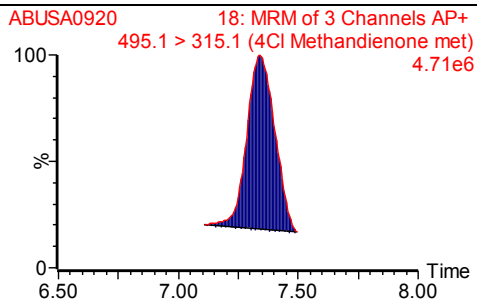
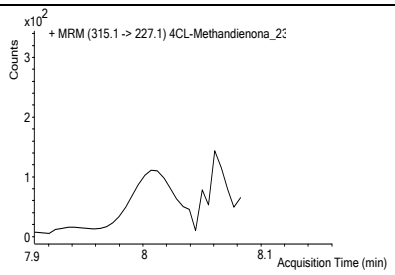

APCI
EI
(a) BD met
433 > 417 (Boldenone met)
1.35e7
Time
433.2954
[MH]+
343.2455
[MH-TMSOH]+
434.2976
435.2958
193.1041
m/z
+ MRM (432.4 -> 194.1)
Counts
Acquisition Time (min)
Abundance
194
73
[M-CH3]+
[M]+
417
432
(b) MADOL
271.1 > 105.1 (Madol)
1.24e7
271.2423
[MH-TMSOH]+
272.2457
215.1798
361.2930
+ MRM (345.3 -> 201.1)
143
345
360

Figure 4.

	APCI	EI
(a)		
(b)		

TABLES

Table 1.- Selected acquisition conditions for the SRM method for both GC-APCI-MS/MS and GC-EI-MS/MS.

Analyte	Derivative	Mw $[M+H]^+$	APCI			EI		
			RT (min)	Transition	CE (eV)	RT (min)	Transition	CE (eV)
MADOL	mono-O-TMS	361	3.96	271.1>105.1*	30	4.49	345.3>201.1	15
				271.1>90.9	30		345.3>255.1	15
BDmet	bis-O-TMS	433	4.27	433>417*	10	4.73	432.4>194.1	14
				433>187	20		432.4>206.1	14
MTDmet3	bis-O-TMS	449	4.27	269>201*	10	4.74	358.3>301.3	12
				269>105	30		358.3>196.1	12
1-T	bis-O-TMS	433	5.03	343.2>179.1*	20	5.61	432.4>194.1	15
				433.3>417	20		432.4>207.2	15
MeTmet2	bis-O-TMS	451	5.07	271.2>215.2*	10	5.64	270.2>213.2	15
				361.2>271.1	10		270.2>199.1	15
MeTmet1	bis-O-TMS	451	5.08	271.2>215.2*	20	5.64	255.2>199.1	25
				361.3>255.2	20		255.2>159.1	25
METHASmet	bis-O-TMS	465	5.25	285.2>229.2*	20	5.84	449.4>269.2	19
				375.3>245.2	20		449.4>213.2	19
BD	bis-O-TMS	431	5.29	341.2>193.1*	20	5.86	430.4>206.2	18
				431.3>193.1	20		430.4>191.2	30
CALUSmet	bis-O-TMS	465	5.32	285.3>109*	20	5.91	284.2>227.2	15
				285.3>175	20		374.3>269.2	13
BOLASmet	bis-O-TMS	465	5.49	285.2>175.1*	20	6.08	284.2>227.2	15
				375.2>245.2	20		284.2>269.2	15
Me-1-T	bis-O-TMS	447	5.52	357.2>179.2*	20	6.14	446.4>194.1	20
				447.3>431.3	20		446.4>143.1	20
NORBOLmet2	bis-O-TMS	465	5.97	375.3>285.2*	10	6.61	435.4>255.2	12
				375.3>231.2	20		435.4>345.3	12
4OH-T	tris-O-TMS	521	6.40	431.3>296.2*	30	7.08	520.4>147.1	33
				521.3>405.3	40		505.4>147.1	10
FLU	tris-O-TMS	553	6.82	463.3>297.4*	30	7.52	552.4>462.4	20
				553.3>353.4	20		552.4>319.3	20
OXY	tris-O-TMS	535	6.91	535.2>389.5*	20	7.62	534.4>429.4	30
				535.2>269.2	30		389.3286.2	30
6OH-4Cl-MTD	bis-O-TMS	495	7.25	495.1>315.1*	10	7.99	315.1>227.1	20
				495.1>155	40		315.1>241.1	15

* Most specific transition

549 **Table 2.-** Validation parameters obtained for extraction recovery (n=6),
550 repeatability and LOD for APCI and EI analysis.

Analyte	Current MRPL (ng mL^{-1})	Extraction recovery (%)	LCL (ng mL^{-1})	Repeatability (%)		HCL (ng mL^{-1})	Repeatability (%)		LOD (ng mL^{-1})	
				APCI	EI		APCI	EI	APCI	EI
MADOL	5	75	2	10	1	20	6	0.3	1	1
BDmet	5	53	2	30	14	20	4.8	5.3	0.1	0.5
MTDmet3	2	70	1	5.3	1.2	10	4.6	0.5	0.3	0.3
1-T	5	79	2	4.2	2.5	20	14	0.5	0.3	2
MeTmet2	2	85	1	4.8	2.3	10	3.9	0.4	0.3	0.5
MeTmet1	2	83	1	12	2.7	10	6.3	1	0.5	0.5
METHASmet	5	78	2	24	8.5	20	9.1	2.3	1	0.3
BD*	5	89	2	3.8	2.5	20	5.9	2	0.1	1
CALUSmet	5	76	2	18	2.3	20	3.5	2	1	2
BOLASmet	5	80	2	23	5.2	20	6.8	1.6	0.1	1
Me-1-T	5	47	2	9.7	5.4	20	4.9	5.1	0.3	1
NORBOLmet2*	5	71	2	13	0.6	20	7.7	1.3	0.4	2
4OH-T	5	68	2	9.4	2.4	20	4.6	1.1	0.4	2
FLU	5	73	2	25	21	20	4	2.3	0.1	1
OXY	5	71	2	13	27	20	3	1.4	0.5	1
6OH-4Cl-MTD	2	86	1	3.2	4.2	10	22	0.3	0.2	1

551 * APCI values for BD and NORBOLmet2 were calculated using gradient 2 (see experimental section)

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