Research article

Journal of MASS SPECTROMETRY

Received: 20 January 2014 Revised: 13 March 2014 Accepted: 14 March 2014 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jms.3367

Mass spectrometric behavior of anabolic androgenic steroids using gas chromatography coupled to atmospheric pressure chemical ionization source. Part I: Ionization

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The detection of anabolic androgenic steroids (AAS) is one of the most important topics in doping control analysis. Gas chromatography coupled to (tandem) mass spectrometry (GC–MS(/MS)) with electron ionization and liquid chromatography coupled to tandem mass spectrometry have been traditionally applied for this purpose. However, both approaches still have important limitations, and, therefore, detection of all AAS is currently afforded by the combination of these strategies. Alternative ionization techniques can minimize these drawbacks and help in the implementation of a single method for the detection of AAS. In the present work, a new atmospheric pressure chemical ionization (APCI) source commercialized for gas chromatography coupled to a quadrupole time-of-flight analyzer has been tested to evaluate the ionization of 60 model AAS. Underivatized and trimethylsylil (TMS)-derivatized compounds have been investigated. The use of GC–APCI–MS allowed for the ionization of all AAS assayed irrespective of their structure. The presence of water in the source as modifier promoted the formation of protonated molecules ($[M+H]^+$), becoming the base peak of the spectrum for the majority of studied compounds. Under these conditions, $[M+H]^+$, $[M+H\text{-}H_2O]^+$ and $[M+H\text{-}2\cdot H_2O]^+$ for underivatized AAS and $[M+H]^+$, $[M+H\text{-TMSOH}]^+$ and $[M+H\text{-}2\cdot\text{TMSOH}]^+$ for TMS-derivatized AAS were observed as main ions in the spectra. The formed ions preserve the intact steroid skeleton, and, therefore, they might be used as specific precursors in MS/MS-based methods. Additionally, a relationship between the relative abundance of these ions and the AAS structure has been established. This relationship might be useful in the structural elucidation of unknown metabolites. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: anabolic androgenic steroids (AAS); atmospheric pressure chemical ionization (APCI); gas chromatography (GC); mass spectrometry (MS); doping control analysis

Introduction

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone with a common cyclopentaneperhydrophenanthrene structure (inserted in Table 1). Their use can stimulate the formation of muscle cells, increasing the muscle growth, so they are widely used to improve athletic performance. Since their first prohibition in sports in 1976,[1] AAS are the most frequently detected group of prohibited substances by World Antidoping Agency (WADA) Accredited Laboratories in doping control analyses.[2] Moreover, the number of illegal steroids is constantly increasing; thus, the detection of AAS is a continuous challenge for doping control laboratories.

AAS are prohibited by WADA at all times, in and out of competition. Any trace of these substances or their metabolites is sufficient to report an adverse analytical finding. Therefore, it is important to apply the most sensitive methods available and to keep working on the discovery of new metabolites that can be detected for a longer time after the administration of the drug, the so-called long-term metabolites.

Routine methods for the detection of AAS have been mostly based on GC–MS operating in selected ion monitoring mode.[3] The use of GC–MS/MS methods in selected reaction monitoring (SRM) mode is gradually becoming more popular due to the improvement on sensitivity and selectivity.[4,5] However, the high fragmentation of the compounds in the electron ionization (EI) source can hamper the selection of an adequate precursor ion in these strategies, because quite often, the molecular ion shows a very low relative abundance or it is even absent. For these reason, it cannot be used as precursor ion.

Moreover, the low abundance of the molecular ion when using EI sources also hampers the detection and elucidation of new metabolites. Several approaches have been described for the detection of unknown metabolites based either on the acquisition of open scan methods[6,7] or on the selection of theoretical

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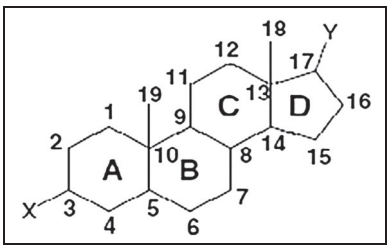
Table 1. Structural information of the groups of steroids investigated

Compound	A ring	B ring	C ring	D ring
General steroid structure				
Group I				
AED	Δ^{4} ^{3}CO	–	–	^{17}CO
1-AED	Δ^{1} ^{3}CO	–	–	^{17}CO
1-T	Δ^{1} ^{3}CO	–	–	^{17}OH
4OH-AED	Δ^{4} ^{3}CO ^{4}OH	–	–	^{17}CO
4OH-NAN	Δ^{4} ^{3}CO ^{4}OH nor ^{19}CH$_3$	–	–	^{17}CO
4OH-T	Δ^{4} ^{3}CO ^{4}OH	–	–	^{17}OH
6OH-AED	Δ^{4} ^{3}CO	^{6}OH	–	^{17}CO
6OXO-AED	Δ^{4} ^{3}CO	^{6}CO	–	^{17}CO
BDmet	Δ^{1} ^{3}CO	–	–	^{17}OH
DANmet	Δ^{4} ^{3}CO	–	–	^{17}OH ^{17}C≡C
E	Δ^{4} ^{3}CO	–	–	^{17}OH
FLU	Δ^{1} ^{3}CO	^{9}F	^{11}OH	^{17}OH ^{17}CH$_3$
FLUmet	Δ^{1} ^{3}CO	^{9}F	^{11}OH nor ^{18}CH$_3$	^{17} CH$_3$ ^{17}CH$_3$
METH	Δ^{1} ^{3}CO ^{1}CH$_3$	–	–	^{17}OH
1-MeT	Δ^{1} ^{3}CO	–	–	^{17}OH ^{17}CH$_3$
MeT	Δ^{4} ^{3}CO	–	–	^{17}OH ^{17}CH$_3$
MIB	Δ^{4} ^{3}CO nor ^{19}CH$_3$	^{7}CH$_3$	–	^{17}OH ^{17}CH$_3$
OXY	Δ^{4} ^{3}CO ^{4}OH	–	–	^{17}OH ^{17}CH$_3$
STEN	Δ^{1} ^{2}CH$_3$ ^{3}CO	–	–	^{17}OH
T	Δ^{4} ^{3}CO	–	–	^{17}OH
Group II				
1,4-AD	$\Delta^{1,4}$ ^{3}CO	–	–	^{17}CO
4,6-AD	Δ^{4} ^{3}CO	Δ^{6}	–	^{17}CO
6OH-4Cl-MTD	$\Delta^{1,4}$ ^{3}CO ^{4}Cl	^{6}OH	–	^{17}OH ^{17}CH$_3$
6-T	Δ^{4} ^{3}CO	Δ^{6}	–	^{17}OH
ATD	$\Delta^{1,4}$ ^{3}CO	Δ^{6}	–	^{17}CO
BD	$\Delta^{1,4}$ ^{3}CO	–	–	^{17}OH
epiMTD	$\Delta^{1,4}$ ^{3}CO	–	–	^{17}OH ^{17}CH$_3$
MTD	$\Delta^{1,4}$ ^{3}CO	–	–	^{17}OH ^{17}CH$_3$
MTDmet1	$\Delta^{1,4}$ ^{3}CO	^{6}OH	–	^{17}OH ^{17}CH$_3$
MDONE	Δ^{4} ^{3}CO nor ^{19}CH$_3$	Δ^{9}	–	^{17}OH ^{17}CH$_3$
THG	Δ^{4} ^{3}CO	Δ^{9}	Δ^{11} ^{13}CH$_2$CH$_3$	^{17}OH ^{17}CH$_2$CH$_3$
TREN	Δ^{4} ^{3}CO nor ^{19}CH$_3$	Δ^{9}	Δ^{11}	^{17}OH
Group III				
11OH-Andros	^{3}OH, 5α-H	–	^{11}OH	^{17}CO
11OH-Etio	^{3}OH, 5β-H	–	^{11}OH	^{17}CO
Andros	^{3}OH, 5α-H	–	–	^{17}CO
CLOSmet1	Δ^{4} ^{3}OH ^{4}Cl	–	–	^{17}CO
DHEA	^{3}OH	Δ^{5}	–	^{17}CO
DHT	^{3}CO, 5α-H	–	–	^{17}OH
DROSmet	^{2}CH$_3$, ^{3}OH, 5α-H	–	–	^{17}CO
Etio	^{3}OH, 5β-H	–	–	^{17}CO

(Continues)

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Table 1. *(Continued)*

Compound	A ring	B ring	C ring	D ring
MESTmet	$^{1}CH_3$ ^{3}OH, 5α-H	–	–	^{17}CO
METHmet	$^{1}C{=}CH_2$ ^{3}OH, 5α-H	–	–	^{17}CO
NANmet1	^{3}OH, 5α-H nor $^{19}CH_3$	–	–	^{17}CO
NANmet2	^{3}OH, 5β-H nor $^{19}CH_3$	–	–	^{17}CO
OXA	^{3}CO ^{2} –O–, 5α-H	–	–	^{17}OH $^{17}CH_3$
Group IV				
16AOL	^{3}OH, 5α-H	–	–	Δ^{16}
5a3a-DIOL	^{3}OH, 5α-H	–	–	^{17}OH
5a3b-DIOL	^{3}OH, 5α-H	–	–	^{17}OH
5b3a-DIOL	^{3}OH, 5β-H	–	–	^{17}OH
BOLASmet	^{3}OH, 5β-H	$^{7}CH_3$	–	^{17}OH $^{17}CH_3$
CALUSmet	^{3}OH, 5β-H	$^{7}CH_3$	–	^{17}OH $^{17}CH_3$
MADOL	Δ^{2}, 5α-H	–	–	^{17}OH $^{17}CH_3$
MTDmet3	Δ^{1}, ^{3}OH, 5β-H	–	–	^{17}OH $^{17}CH_3$
MTDmet4	Δ^{1}, ^{3}OH, 5β-H	–	Δ^{13} nor$^{18}CH_3$	$^{17}CH_3$ $^{17}CH_3$
METHASmet1	$^{2}CH_3$, ^{3}OH, 5α-H	–	–	^{17}OH $^{17}CH_3$
MeTmet1	^{3}OH, 5α-H	–	–	^{17}OH $^{17}CH_3$
MeTmet2	^{3}OH, 5β-H	–	–	^{17}OH $^{17}CH_3$
NORBOLmet1	^{3}OH, 5α-H nor $^{19}CH_3$	–	$^{13}CH_2CH_3$	^{17}OH $^{17}CH_2CH_3$
NORBOLmet2	^{3}OH, 5β-H nor $^{19}CH_3$	–	$^{13}CH_2CH_3$	^{17}OH $^{17}CH_2CH_3$
NORETHANmet1	^{3}OH, 5β-H nor $^{19}CH_3$	–	–	^{17}OH $^{17}CH_2CH_3$

transitions for expected metabolites.[3,8] GC–EI–MS methods show some limitations for this purpose like (1) the difficulties in establishing the molecular formula for a new metabolite due to the common absence of the molecular ion and (2) the difficulties to predict a theoretical transition for a previously unknown but expected metabolites. For these reasons, the study of AAS ionization and fragmentation using alternatives interfaces can be very useful.

Liquid chromatography coupled to mass spectrometry (LC–MS) with atmospheric pressure interfaces (API) has proved to be an excellent complement to GC–MS in the detection of AAS,[9] particularly for those compounds with low thermal stability that present problems in derivatization, e.g. steroids bearing a 4-9-11-triene nucleus.[9–11] Additionally, the soft ionization of API sources generates adequate/abundant precursor ions, which facilitates the development of sensitive SRM methods for AAS using LC–MS/MS.[12] Together with the generation of adequate precursor ions, the established relationship between API ionization and structure is a useful tool for the elucidation of new metabolites.[13,14] However, AAS ionization by API is not universal, and only those compounds with an ionizable center can be detected. The consequence is that important AAS biomarkers, such as the totally reduced metabolites, are missed by this technique.[13,14] The appearance of commercial atmospheric pressure chemical ionization (APCI) interfaces for GC opened new lines of research for improving the detection of known steroids/metabolites and the discovery of new ones. Since the introduction of the APCI interface to a GC instrument by Horning *et al.* in 1977,[15] this interface has undergone several modifications over the years.

Several works have been carried out using GC–API–MS for the analysis of steroids.[16,17] These articles show the high potential of microchip atmospheric pressure photoionization combined with GC–MS for the detection of AAS. However, up to our knowledge, the relationship between steroid atmospheric pressure ionization and its structure has not been studied.

Recently, a new APCI source using a purge of nitrogen gas has been commercialized.[18,19] This interface promotes ionization with very little fragmentation resulting in the formation of $[M+H]^+$ or $M^{+\bullet}$ ions as the base peaks of the mass spectrum, as occurs in LC–MS. Figure 1a and 1b shows common reactions that take place in the source. Ionization presents two possible mechanisms, charge transfer (in which the principal ion is $M^{+\bullet}$) or protonation (in which the principal ion is $[M+H]^+$). Using modifiers such as water or methanol, protonation can be forced in order to enhance the presence of $[M+H]^+$. Water molecules react with the nitrogen plasma ions generating H_3O^+ which in turn produces analyte ions such as $[M+H]^+$ by proton transfer reactions. The reduced fragmentation observed by using this new source can have a significant impact on target analysis at trace levels.

Since its first application for metabolic profiling in 2009,[20] the usefulness of this interface has been demonstrated in several fields,[21–26] but, up to our knowledge, it has not been applied yet to the doping control analysis.

The main aim of this work is the study of the AAS ionization with the recently commercialized APCI interface by GC–MS using a quadrupole time of flight (QTOF) analyzer in order to establish relationships between the ionization behavior and the AAS structure. For this purpose, 60 AAS with different chemical structures

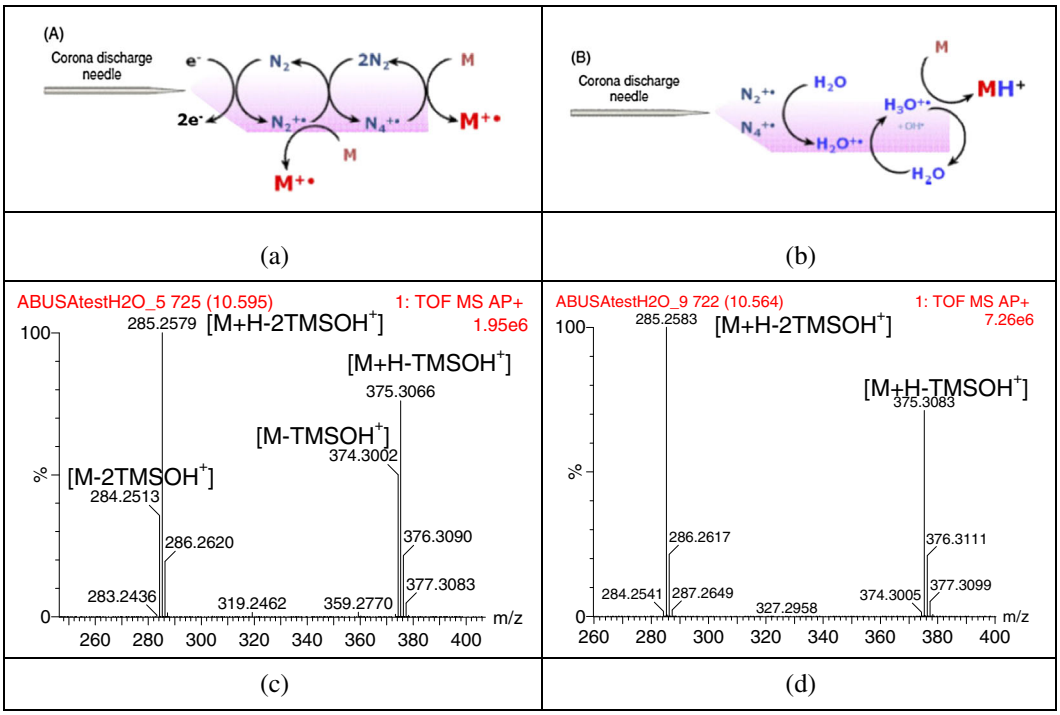

Figure 1. Ionization reactions in APGC source: (a) N_2 transfer conditions, (b) proton transfer conditions, (c) full-scan TOFMS spectra for TMS-derivatized BOLASmet under N_2 transfer conditions and (d) full-scan TOFMS spectra for TMS-derivatized BOLASmet under proton transfer conditions.

and their derivatized compounds have been selected, and their ionization behavior has been studied.

Experimental

Chemical and reagents

Structure information of the selected steroids is shown in Table 1. Androstenedione (AED), ethisterone (DANmet), testosterone (T), methandienone (MTD), boldenone (BD) and 11β-hydroxy-etiocholanolone (11OH-Etio) were obtained from Sigma (St. Louis, MO, USA). 1-Androstenedione (1-AED), 6α-hydroxy-androstenedione (6OH-AED), fluoxymesterone (FLU), methenolone (METH), mibolerone (MIB), 4,6-androstadien-3,17-dione (4,6-AD), 6-dehydrotestosterone (6-T), androstatrienedione (ATD), trenbolone (TREN), 11β-hydroxy-androsterone (11OH-Andros), oxandrolone (OXA), 16-androstenol (16AOL) were obtained from Steraloids (Newport, RI, USA). 4-hydroxy-androstenedione (4OH-AED), 4-hydroxy-nandrolone (4OH-NAN), 4-hydroxy-testosterone (4OH-T), 17β-hydroxy-5β-androstan-1-ene-3-one (BDmet), 1-testosterone (1-T), epitestosterone (E), 9α-fluoro-11β-hydroxy-17,17-dimethyl-18-norandrosta-4,13-diene-3-one (FLUmet), 17α-metyl-1-testosterone (1-MeT), 1,4-androstadien-3,17-dione (1,4-AD), 6β-hydroxy-4-chloromethandienone (6OH-4Cl-MTD), epimethandienone (epiMTD), 6β-hydroxy-methandienone (6OH-MTD), methyldienolone (MDONE), tetrahydrogestrinone (THG), androsterone (Andros), Etiocholanolone (Etio), 4-chloro-3α-hydroxy-androst-4-en-17-one (CLOSmet), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), 3α-hydroxy-2α-methyl-5α-androstan-17-one (DROSmet), 3α-hydroxy-1α-methyl-5α-androstan-17-one (MESTmet), 3α-hydroxy-1-methylen-5α-androstan-17-one (METHmet), 3α-hydroxy-5α-estran-17-one (NANmet1), 3α-hydroxy-5β-estran-17-one (NANmet2), 5α-androstane-3α,17β-diol (5α3α-DIOL), 5α-androstane-3β,17β-diol (5α3β-DIOL), 5β-androstane-3α,17β-diol (5β3α-DIOL), 5β-androstan-7α,17α-dimethyl-3α,17β-diol (BOLASmet), 5β-androstan-7β,17α-dimethyl-3α,17β-diol (CALUSmet), 17β-methyl-5β-androst-1-en-3α,17α-diol (MTDmet3), 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol (MTDmet4), 5α-androstan-17α-methyl-3α,17β-diol (MeTmet1), 5β-androstan-17α-methyl-3α,17β-diol (MeTmet2), 13β,17α-diethyl-5α-gonane-3α,17β-diol (NORBOLmet1), 13β,17α-diethyl-5β-gonane-3α,17β-diol (NORBOLmet2) and 17α-ethyl-5β-estrane-3α,17β-diol (NORETHANmet1) were purchased from NMI (Pymble, Australia). 6-Oxo-androstenedione (6OXO-AED), oxymesterone (OXY), methyltestosterone (MeT), stenbolone (STEN) and madol (MADOL) were provided by the Toronto Research Chemicals (Toronto, Canada). 5α-Androstan-2α, 17α-dimethyl-3α, 17β-diol (METHASmet1) was a kind gift from the World Association of Anti-Doping Scientists (WAADS).

For underivatized AAS, stock standard solutions at 10 and 100 μg/ml were prepared by dissolving reference standards in methanol and they were stored at −20 °C. Working AAS standard solutions at 1 μg/ml were prepared by dilution of stock solution in acetone. Derivatized compounds were prepared from stock solutions in order to obtain a final concentration of 10 μg/ml.

Reagents, N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA), ammonium iodide (NH_4I) and 2-mercaptoethanol were purchased from Sigma-Aldrich Química SA (Madrid, Spain). Acetone and methanol were purchased form Scharlab (Barcelona, Spain).

Derivatization method

The derivatization protocol employed in this study is based on the use of a mixture of MSTFA/NH_4I/2-mercaptoethanol in order to obtain the enol-trimethylsylil (TMS) derivatives of the analytes. Derivatized standards were prepared by adding 100 μl MSTFA/NH_4I/2-mercaptoethanol (1000/2/6; *v/w/v*) and heating at 60 °C for 20 min.

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Instrumentation

A GC system (Agilent 7890A, Palo Alto, CA, USA) equipped with an Agilent 7693 autosampler and a split/splitless injector port was coupled to a QTOF mass spectrometer Xevo G2 QTOF (Waters Corporation, Manchester, UK) by means of an APCI source, commercialized as APGC source.

For derivatized compounds, an HP Ultra 1 capillary column (length 16.5 m × I.D. 0.25 mm × 0.11 μm of film thickness) was used for the chromatographic separation. The oven program was: 90 °C, 20 °C/min to 300 °C (5 min). Split injections (ratio 1:10) of a 1-μ- sample were carried out having the injector port at 280 °C. For underivatized compounds, an HP Ultra 2 column (length 12 m × I.D. 0.2 mm × 0.33 μm of film thickness) was used. The oven program in this case was: 90 °C (1 min), 20 °C/min to 300 °C (3 min). Splitless injections of 1 μl of the sample were carried out having the injector port at 280 °C. For both, derivatized and underivatized compound analyses, constant pressure mode was used at 25.8 psi.

The temperature in the source was set to 150 °C. Nitrogen was used as auxiliary gas at 150 l/h, make-up gas at 18 l/h and cone gas at 16 l/h. APCI corona pin was fixed at 1 μA for underivatized and 1.6 μA for derivatized compounds, and cone voltage was 30 V, in both cases. APCI positive polarity was selected. The ionization process occurred within an enclosed chamber, which enabled control over the protonation/charge transfer processes.

Xevo G2 QTOF was operated at a scan time of 0.3 s acquiring in a mass range of *m/z* 50–650 using a multi-channel plate voltage of 3350 V. TOF MS resolution mass was approximately 18 000 (FWHM) at *m/z* 614. Heptacosa (Sigma-Aldrich, Madrid, Spain), was used for the daily mass calibration. Internal calibration was performed using a background ion coming from the GC-column bleed as lock mass (protonated molecule of octamethylcyclotetrasiloxane, *m/z* 297.0830).

Results and discussion

Selection of model compounds

Sixty AAS including both parent steroids and metabolites were selected on the basis of their structural differences (Table 1). AAS structures share a common cyclopentaneperhydrophenantrene skeleton with normally between one and three oxygen atoms. These atoms are the only structural features in AAS with lone pairs, and, therefore, they are the most feasible ionization centers. Taking into account the proton affinity of the oxygen containing moieties,[28] the expected order for AAS protonation would be: AAS containing a conjugated carbonyl > AAS with unconjugated carbonyl > AAS with hydroxyl functions. For this reason, and in order to make the study more comprehensive, model AAS was divided into four groups attending to their chemical structures:

- Group I. Compounds containing a mono-conjugated carbonyl. Testosterone and steroids with similar structure are included in this group.
- Group II. Compounds containing a poly-conjugated carbonyl function. Boldenone and trenbolone are included in this group.
- Group III. Compounds containing an unconjugated keto function in their structure. Most of compounds included in this group presented the carbonyl function in C17, with the exceptions of DHT and OXA in which the carbonyl function was in C3.
- Group IV. Compounds with no-keto function in their structure. Androstanediols are included in this group.

Derivatization process

As mentioned in the introduction section, steroid analysis by GC–MS usually requires a previous derivatization step. Although it is a time-consuming procedure, it improves the chromatographic behavior of hydroxylated AAS allowing the profiling of both polar and non-polar AAS.[10,29] The most common derivatization reaction for steroids is the trimethylsilylation, where active hydrogens on hydroxyl or enol groups are replaced with TMS groups.[30] In the doping control field, this derivatization process is based on the methodology developed by Donike in 1969 which consist on the use of N-methyl-N-trimethylsilyltrifluoracetamide in conjunction with NH_4I as a derivatization reagent for those compounds containing hydroxyl and ketone groups.[31] 2-Mercaptoethanol is added as a reducing agent to minimize the formation of iodine; which can degrade the mixture. This approach is the most widely used in the field,[32] and it has shown excellent results in doping control screening, allowing the detection of a large number of steroids, β2 agonists and other substances like endogenous glucocorticosteroids.[27]

However, it is possible to analyze steroids by GC–MS without derivatization at higher concentration, as, for example, in the identification of some endogenous steroids,[33,34] or in the structural elucidation of AAS metabolites.[35,36]

For these reasons, the ionization behavior of both underivatized AAS and TMS AAS derivatives by APCI–GC–MS was investigated. Relationships between APCI ionization and structure were studied separately since complementary information can be obtained when analyzing underivatized and TMS-derivatized analytes.

Optimization of the ionization parameters

The 'soft' ionization behavior of the APCI interface was tested using AAS standards underivatized and TMS derivatized. As mentioned in the introduction (Figs. 1a and 1b), two ionization mechanisms can take place in the source: (1) charge transfer reaction from nitrogen make up gas or proton transfer reaction and (2) promotion of protonation reaction with the addition of water in the source as a modifier. In some occasions, protonation produced by only the water vapor traces present in ambient air of the ion chamber can also be observed.

Some preliminary experiments regarding the analyte ionization were made in order to evaluate the effect of water in the ion chamber. A subset of two analytes belonging to the different groups was selected for analysis with and without the presence of water. Both underivatized and derivatized steroids were tested.

In experiments without water, we observed that, together with the expected molecular ion $M^{+\cdot}/[M\text{-}H_2O]^{+\cdot}$, considerable amounts of the $[M+H]^+/[M+H\text{-}H_2O]^+$ ions in the charge transfer spectrum were also formed. Similar results (showing the $[M+H\text{-}TMSOH]^+$ and the $[M\text{-}TMSOH]^{+\cdot}$ instead the $[M+H\text{-}H_2O]^+$ and the $[M\text{-}H_2O]^{+\cdot}$, respectively) were obtained for derivatized AAS (see Fig. 1c). The simultaneous occurrence of the two mentioned mechanisms, i.e. charge transfer and proton transfer from water vapor traces, seemed to show a tendency of steroids to be protonated. Taking into account this consideration, similar experiments were repeated but adding water as a modifier in the source. In this way, the proton transfer mechanism was

Table 2. Group I. Retention times (RT) and *m/z* ions and relative abundances (in brackets) obtained for underivatized and TMS-derivatized steroids by GC–(APCI)(QTOF)–MS/MS

		Underivatized				TMS-derivatized				
Compound	Formula	RT (min)	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	Derivative	RT (min)	$[M+H]^+$	$[M+H-TMSOH]^+$	$[M+H-2TMSOH]^+$
AED	$C_{19}H_{26}O_2$	11.34	287.2030 (100)	269.1910 (7)	–	bis-O-TMS	8.57	431.2884 (100)	–	–
1-AED	$C_{19}H_{26}O_2$	11.02	287.2012 (100)	269.1905 (30)	–	bis-O-TMS	8.33	431.2811 (100)	341.2310 (5)	–
1-T	$C_{19}H_{28}O_2$	11.10	289.2171 (100)	271.2060 (12)		bis-O-TMS	8.40	433.2953 (100)	343.2457 (50)	–
4OH-AED	$C_{19}H_{26}O_3$	11.35	303.1963 (100)	285.1853 (25)	267.1750 (15)	tris-O-TMS	9.32	519.3167 (100)	–	–
4OH-NAN	$C_{18}H_{26}O_3$	11.23	291.1958 (100)	273.1848 (10)	255.1747 (5)	tris-O-TMS	9.15	507.3155 (100)	417.2650 (50)	–
4OH-T	$C_{19}H_{28}O_3$	11.48	305.2117 (100)	–	269.1904 (18)	tris-O-TMS	9.40	521.3311 (100)	431.2804 (50)	–
6OH-AED	$C_{19}H_{26}O_3$	12.06	303.1957 (100)	285.1852 (22)	–	tris-O-TMS	9.18	519.3194 (100)	–	–
6OXO-AED	$C_{19}H_{24}O_3$	12.00	301.1822 (100)	283.1700 (18)	–	tris-O-TMS	9.08	517.3012 (100)	–	–
BDmet	$C_{19}H_{28}O_2$	11.00	289.2168 (100)	271.2061 (26)	253.1957 (5)	bis-O-TMS	7.76	433.2979 (100)	343.2472 (70)	–
DANmet	$C_{21}H_{28}O_2$	11.70	313.2187 (100)	295.2067 (20)	–	bis-O-TMS	9.07	457.2977 (100)	367.2473 (70)	–
E	$C_{19}H_{28}O_2$	11.37	289.2171 (100)		–	bis-O-TMS	8.45	433.2985 (100)	343.2479 (55)	–
FLU	$C_{20}H_{29}FO_3$	13.21	337.2195 (100)	319.2075 (5)	301.1969 (10)	tris-O-TMS	9.68	553.3389 (100)	463.2884 (45)	353.2300 (3)
FLUmet	$C_{20}H_{27}FO_2$	11.08	319.2081 (100)		–	bis-O-TMS	8.38	463.2930 (100)	373.2373 (5)	–
METH	$C_{20}H_{30}O_2$	12.01	303.2373 (100)	285.2229 (30)	–	bis-O-TMS	8.76	447.3133 (100)	357.2628 (60)	267.2113 (5)
1-MeT	$C_{20}H_{30}O_2$	11.23	303.2335 (100)	285.2221 (12)	–	bis-O-TMS	8.76	447.3143 (100)	357.2635 (60)	–
MeT	$C_{20}H_{30}O_2$	11.40	303.2324 (100)	285.2214 (12)	–	bis-O-TMS	9.01	447.3163 (100)	357.2648 (70)	–
MIB	$C_{20}H_{30}O_2$	11.40	303.2332 (100)	285.2219 (22)	–	bis-O-TMS	8.82	447.3153 (100)	357.2639 (60)	–
OXY	$C_{20}H_{30}O_3$	11.61	319.2276 (100)	301.2168 (6)	283.2064 (15)	tris-O-TMS	9.73	535.3494 (100)	445.2978 (55)	–
STEN	$C_{20}H_{30}O_2$	11.18	303.2336 (100)	285.2219 (12)	–	bis-O-TMS	8.60	447.3102 (100)	357.2606 (35)	–
T	$C_{19}H_{28}O_2$	11.40	289.2215 (100)	271.2072 (18)	253.1955 (5)	bis-O-TMS	8.63	433.2973 (100)	343.2472 (70)	–

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enhanced, causing an increase in the relative abundance of $[M+H]^+$ (see Fig. 1d).

Therefore, we decided to use water as modifier for further experiments. This fact should be taken into account for the following discussion of the results.

Group I: AAS containing mono-conjugated keto function

Table 2 shows the ions for derivatized and underivatized steroids from Group I observed with APCI. A summary on the ionization behavior for these compounds is described below.

When underivatized, all analytes belonging to this group exhibited a prominent $[M+H]^+$ due to the protonation of the keto function. A less abundant $[M+H-H_2O]^+$ was also observed with relative abundances lower than 30%. No relationship could be established between the presence/relative abundance of $[M+H-H_2O]^+$ and additional structural features. Any attempt to determine a relationship between structure and the number of observed losses of water was also unproductive.

Enol-TMS derivatization of AAS from group I followed the expected behavior, and all oxygen atoms of the molecule were derivatized. Similarly to their underivatized counterparts, TMS derivatives from group I showed the $[M+H]^+$ as the base peak of the spectrum. A $[M+H-TMSOH]^+$ ion was also observed in most cases. The relative abundance of this ion (normally above 40%) was higher than the corresponding $[M+H-H_2O]^+$ shown in underivatized AAS. The neutral loss of TMSOH can be generated either after protonation of the TMS ether or by remote loss of TMSOH after protonation of the enol-TMS ether (Fig. 2a). The high proton affinity of the TMS ether compared with the hydroxyl group can explain the differences in relative abundance observed for this loss between TMS derivatives and underivatized analytes.

The occurrence of abundant $[M+H-TMSOH]^+$ could be linked to the structure of the D ring of the AAS. Thus, those analytes with a hydroxyl group in the D ring exhibited an abundant $[M+H-TMSOH]^+$, whereas this ion was absent (or with a relative abundance lower than 10%) in those AAS having a carbonyl (AED, 1-AED, 4OH-AED, 6OH-AED and 6OXO-AED) or a 17,17-dimethyl (FLUmet)

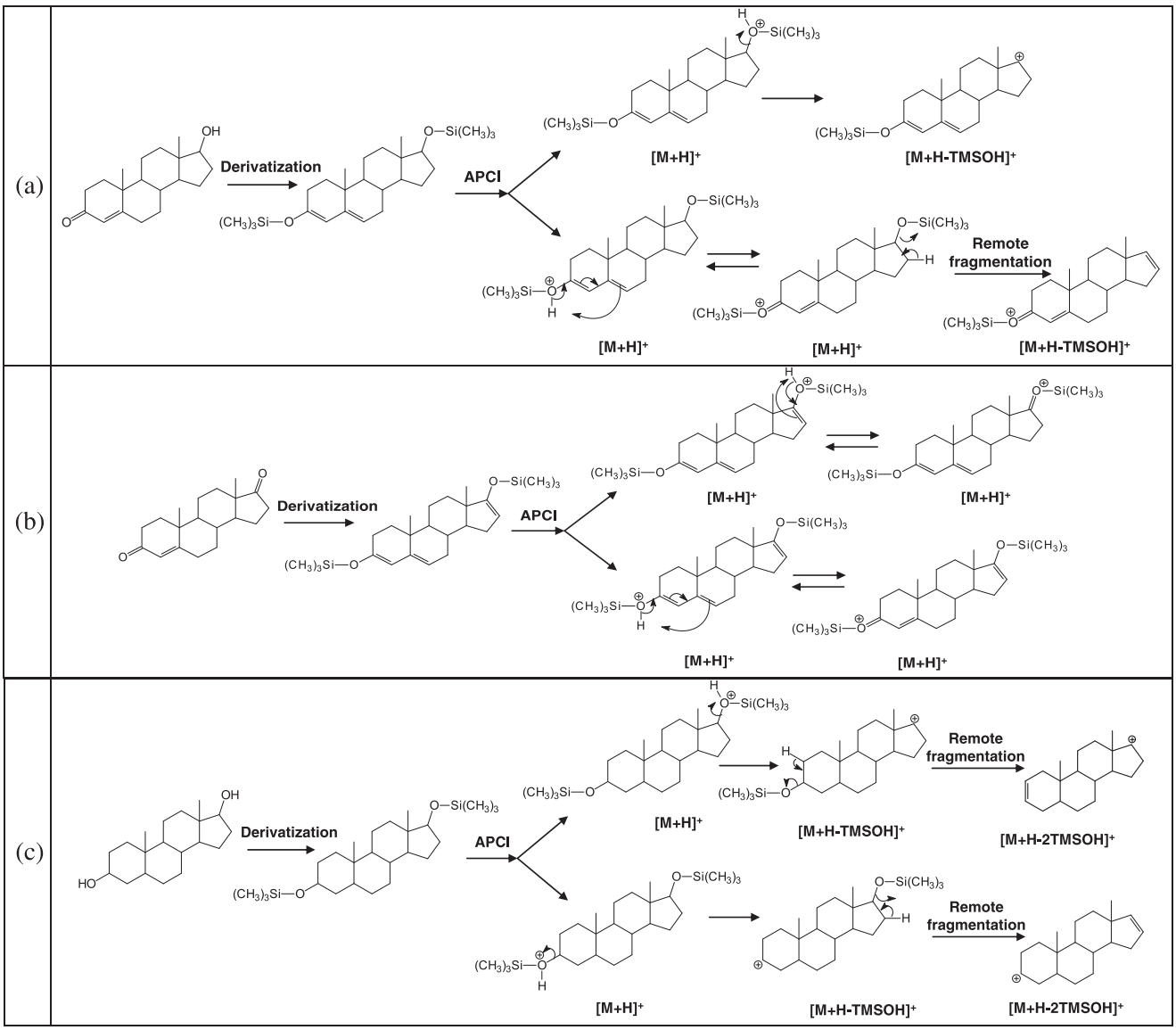

Figure 2. Ionization and in-source fragmentation pathways under proton transfer conditions for a) steroids with carbonyl function in C3 and hydroxyl function in C17, b) steroids with carbonyl functions in both C3 and C17 and c) steroids with hydroxyl functions in both C3 and C17.

functions. A feasible explanation for this observation is the formation of a keto-enol equilibrium when a keto function is derivatized and subsequently protonated (Fig. 2b). The formation of this equilibrium would increase stability and avoid the neutral loss of TMSOH observed after protonation of a derivatized hydroxyl group (Fig. 2a).

4OH-T is shown as an illustrative example of this group in Fig. 3. The spectra of both underivatized (Fig. 3a) and TMS-derivatized 4OH-T (Fig. 3b) showed the $[M+H]^+$ as the base peak. In the case of the TMS derivatized, the $[M+H\text{-}TMSOH]^+$ ion was also relatively abundant.

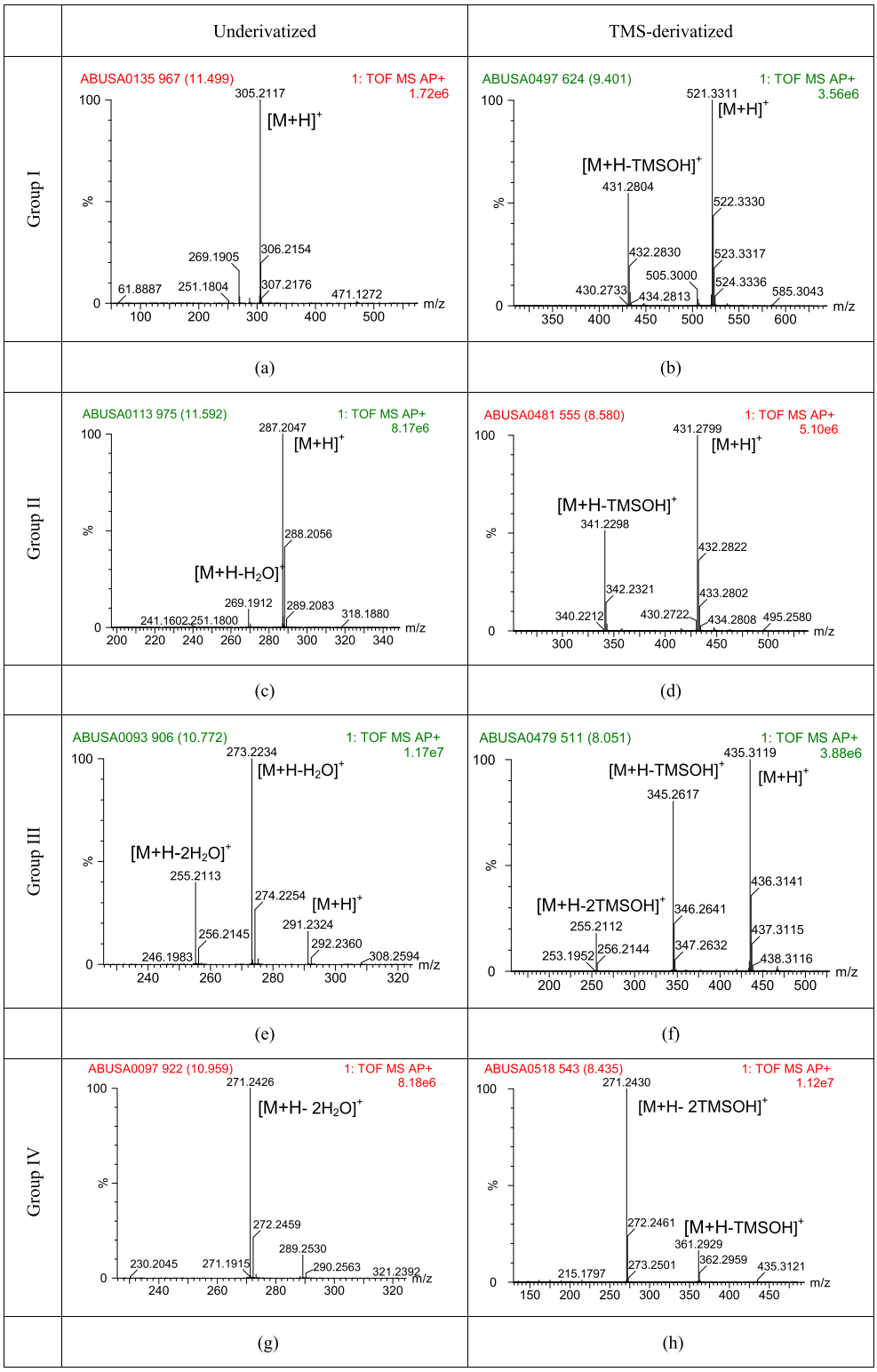

Figure 3. Full-scan TOFMS spectra under proton transfer conditions for selected model compounds belonging to Group I: (a) Underivatized 4OH-T, (b) TMS-derivatized 4OH-T, Group II: (c) Underivatized BD, (d) TMS-derivatized BD, Group III: (e) Underivatized ANDROS, (f) TMS-derivatized ANDROS and Group IV: (g) Underivatized MeTmet1, (h) TMS-derivatized MeTmet1.

Table 3. Group II. Retention times (RT) and *m/z* ions and relative abundances (in brackets) obtained for underivatized and TMS-derivatized steroids by GC–(APCI)(QTOF)–MS/MS

		Underivatized				TMS-derivatized				
Compound	Formula	RT (min)	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	Derivative	RT (min)	$[M+H]^+$	$[M+H-TMSOH]^+$	$[M+H-2TMSOH]^+$
1,4-AD	$C_{19}H_{24}O_2$	11.46	285.1853 (100)	267.1753 (18)	–	bis-O-TMS	8.51	429.2664 (100)		-
4,6-AD	$C_{19}H_{24}O_2$	11.36	285.1853 (100)	267.1749 (8)	–	bis-O-TMS	8.51	429.2676 (100)	–	-
6OH-4Cl-MTD	$C_{20}H_{27}O_3Cl$	14.28	351.1732 (100)	333.1625 (50)	315.1521 (55)	bis-O-TMS	9.95	495.2533 (100)	405.2029 (10)	315.1521 (10)
6-T	$C_{19}H_{26}O_2$	11.55	287.2016 (100)	269.1905 (8)	–	bis-O-TMS	8.60	431.2825 (100)	341.2314 (45)	-
ATD	$C_{19}H_{22}O_2$	11.40	283.1711 (100)	265.1592 (25)		mono-O-TMS	8.50	355.2113 (100)	–	-
BD	$C_{19}H_{26}O_2$	11.59	287.2047 (100)	269.1912 (23)	–	bis-O-TMS	8.58	431.2799 (100)	341.2298 (50)	-
EpiMTD	$C_{20}H_{28}O_2$	11.73	301.2168 (100)	283.2061 (55)	–	bis-O-TMS	8.54	445.2991 (100)	355.2474 (40)	-
MTD	$C_{20}H_{28}O_2$	11.73	301.2209 (100)	283.2073 (5)	–	bis-O-TMS	8.93	445.2976 (100)	355.2469 (55)	-
MTDmet1	$C_{20}H_{28}O_3$	12.41	317.2115 (45)	299.2007 (95)	281.1902(100)	tris-O-TMS	9.50	533.3318 (100)	443.2812 (40)	-
MDONE	$C_{19}H_{26}O_2$	11.75	287.2012 (100)	269.1904 (10)	–	bis-O-TMS	8.96	431.2828 (100)	341.2317 (50)	-
THG	$C_{21}H_{28}O_2$	12.92	313.2168 (100)	295.2061 (12)	–	bis-O-TMS	9.55	[a]455.2823 (100)	[a]365.2319 (35)	-
TREN	$C_{18}H_{22}O_2$	11.67	271.1716 (100)	253.1590 (12)	–	bis-O-TMS	8.72	415.2472 (100)	325.1969 (45)	-

[a]Derivatized THG is detected as $[M+H-2Da]^+$ probably coming from the $[M+H]^+$ of an oxidation product produced during derivatization.

Table 4. Group III. Retention times (RT) and *m/z* ions and relative abundances (in brackets) obtained for underivatized and TMS-derivatized steroids by GC–(APCI)(QTOF)–MS/MS

	Underivatized						TMS derivatized				
Compound	Formula	RT (min)	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	$[M+H-3H_2O]^+$	Derivative	RT (min)	$[M+H]^+$	$[M+H-TMSOH]^+$	$[M+H-2TMSOH]^+$
11OH-Andros	$C_{19}H_{30}O_3$	11.87	307.2276 (20)	289.2174 (65)	271.2076 (100)	253.1968 (30)	tris-O-TMS	8.66	523.3489 (70)	433.2981 (50)	343.24089 (100)
11OH-Etio	$C_{19}H_{30}O_3$	11.63	307.2279 (22)	289.2179 (100)	271.2070 (98)	253.1961 (43)	tris-O-TMS	8.69	523.3468 (20)	433.2982 (100)	343.2453 (70)
Andros	$C_{19}H_{30}O_2$	10.76	291.2324 (20)	273.2234 (100)	255.2113 (50)	–	bis-O-TMS	8.05	435.3119 (100)	345.2617 (80)	255.2112 (20)
CLOSmet	$C_{19}H_{27}ClO_2$	11.53	323.1775 (7)	305.1677 (62)	287.1570 (100)	–	bis-O-TMS	8.75	467.2578 (40)	377.2081 (100)	287.1574 (5)
DHEA	$C_{19}H_{28}O_2$	10.72	289.2177 (20)	271.2111 (100)	253.1974 (65)	–	bis-O-TMS	8.35	433.2980 (65)	343.2485 (100)	253.1962 (10)
DHT	$C_{19}H_{30}O_2$	11.03	291.2353 (100)	273.2219 (30)	255.2113 (22)	–	bis-O-TMS	8.53	435.3166 (100)	345.2656 (85)	–
DROSmet	$C_{20}H_{32}O_2$	10.89	305.2484 (10)	287.2383 (100)	269.2273 (30)	–	bis-O-TMS	8.21	449.3333 (100)	359.2816 (80)	269.2278 (5)
Etio	$C_{19}H_{30}O_2$	10.62	291.2328 (23)	273.2221 (100)	255.2114 (85)	–	bis-O-TMS	8.08	435.3128 (50)	345.2669 (100)	255.2115 (10)
MESTmet1	$C_{20}H_{32}O_2$	11.15	305.2475(7)	287.2375 (100)	269.2265 (20)	–	bis-O-TMS	8.41	449.3315 (100)	359.2800 (65)	269.2281 (10)
METHmet	$C_{20}H_{32}O_2$	11.02	305.2481 (33)	287.2375 (100)	269.2269 (25)	–	bis-O-TMS	8.33	447.3140 (100)	357.2633 (55)	267.2119 (5)
NANmet1	$C_{18}H_{28}O_2$	10.22	277.2168 (22)	259.2061 (100)	241.1955 (35)	–	bis-O-TMS	7.71	421.3008 (100)	331.2498 (80)	241.1968 (20)
NANmet2	$C_{18}H_{28}O_2$	10.35	277.2167 (22)	259.2060 (85)	241.1955 (100)	–	bis-O-TMS	7.92	421.2978 (50)	331.2482 (100)	241.1964 (13)
OXA	$C_{19}H_{30}O_3$	12.05	307.2300 (100)	289.2193 (95)	271.2070 (20)	–	mono-O-TMS	9.13	379.2668 (100)	289.2167 (40)	–

Table 5. Group IV. Retention times (RT) and *m/z* ions and relative abundances (in brackets) obtained for underivatized and TMS-derivatized steroids by GC–(APCI)(QTOF)–MS/MS

		Underivatized				TMS-derivatized				
Compound	Formula	RT (min)	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	Derivative	RT (min)	$[M+H]^+$	$[M+H-TMSOH]^+$	$[M+H-2TMSOH]^+$
16AOL	$C_{19}H_{30}O$	9.32	–	257.2223 (100)	–	mono-O-TMS	6.60	–	257.2334 (100)	–
5a3aDIOL	$C_{19}H_{32}O_2$	10.81	–	275.2391 (60)	257.2300 (100)	bis-O-TMS	8.13	–	347.2773 (20)	257.2272 (100)
5a3bDIOL	$C_{19}H_{32}O_2$	10.87	–	275.2369 (90)	257.2284 (100)	bis-O-TMS	8.47	–	347.2776 (50)	257.2279 (100)
5b3aDIOL	$C_{19}H_{32}O_2$	10.67	–	275.2376 (60)	257.2274 (100)	bis-O-TMS	8.16	–	347.2771 (70)	257.2268 (100)
BOLASmet	$C_{21}H_{36}O_2$	11.17	–	303.2690 (20)	285.2585 (100)	bis-O-TMS	8.75	–	375.3083 (70)	285.2583 (100)
CALUSmet	$C_{21}H_{36}O_2$	11.04	–	303.2697 (25)	285.2590 (100)	bis-O-TMS	8.65	–	375.3093 (15)	285.2629 (100)
MADOL	$C_{20}H_{32}O$	9.72	289.2531 (5)	271.2492 (100)	–	mono-O-TMS	7.47	361.2943 (5)	271.2440 (100)	–
MTDmet3	$C_{20}H_{32}O_2$	10.7	–	–	269.2271 (100)	bis-O-TMS	7.78	–	359.2781 (5)	269.2334 (100)
MTDmet4	$C_{20}H_{30}O$	9.36	–	269.2270 (100)	–	mono-O-TMS	6.83	–	269.2328 (100)	–
METHASmet1	$C_{21}H_{36}O_2$	11.09	–	303.2685 (18)	285.2583 (100)	bis-O-TMS	8.58	–	375.3116 (45)	285.2653 (100)
MeTmet1	$C_{20}H_{34}O_2$	10.97	–	289.2530 (12)	271.2430 (100)	bis-O-TMS	8.43	–	361.2929 (15)	271.2430 (100)
MeTmet2	$C_{20}H_{34}O_2$	10.75	–	289.2531 (20)	271.2429 (100)	bis-O-TMS	8.45	–	361.2962 (50)	271.2521 (100)
NORBOLmet1	$C_{21}H_{36}O_2$	11.43	–	303.2689 (15)	285.2584 (100)	bis-O-TMS	9.10	–	375.3097 (20)	285.2605 (100)
NORBOLmet2	$C_{21}H_{36}O_2$	11.51	–	303.2690 (20)	285.2590 (100)	bis-O-TMS	8.99	–	375.3102 (10)	285.2601 (100)
NORETHANmet1	$C_{20}H_{34}O_2$	11.09	–	289.2528 (20)	271.2423 (100)	bis-O-TMS	8.76	–	361.2946 (40)	271.2460 (100)

Group II: AAS containing poly-conjugated keto function

Similar to Group I, the ionization of underivatized steroids from Group II in APCI source generated mainly $[M+H]^+$ being $[M+H-H_2O]^+$ also present with relative abundances normally lower than 30% (see Table 3). The main exception for this common behavior was 6OH-4Cl-MTD and MTDmet1 in which the $[M+H-H_2O]^+$ had 50% and 95% relative abundance, respectively. The presence of the hydroxyl group in C6 can be the driving force for this behavior. However, this abundant $[M+H-H_2O]^+$ was not observed in 6-hydroxy analytes belonging to group I like 6OH-AED. This fact suggests that either the extra conjugation of the keto function or the configuration of the hydroxyl group in C6 can also play an important role in this behavior.

The number of water losses observed for underivatized analytes from group II was always one less than the sum of hydroxyl and carbonyl functions of the molecule. This result can be due to the extra conjugation of the keto function, which might difficult the loss of the CO oxygen as water.

Enol-TMS derivatization is problematic for polyconjugated carbonyl functions.[10,11] This limiting factor was also observed in our study. On one hand, incomplete derivatization was obtained for some compounds as ATD and 6OH-4Cl-MTD in which the most abundant peaks corresponded to species containing one underivatized oxygen (Table 3). On the other hand, compounds with the polyconjugation extended to A, B and C rings showed several peaks in the chromatogram, some of them coming from derivatization artefacts. This was the case of THG where the most abundant peak corresponded to $[M+H-2Da]^+$ probably arising from the protonated molecule of an oxidation product formed during derivatization.

Steroids with satisfactory derivatization properties followed the ionization behavior described for Group I and summarized in Fig. 2a and 2b. Thus, those analytes with a carbonyl moiety in C17 did not show an abundant $[M+H-TMSOH]^+$, whereas those with a hydroxyl function in C17 commonly presented a relative abundance for this ion higher than 40%.

BD is shown as an illustrative example for AAS from Group II (Fig. 3). Similar to Group I, $[M+H]^+$ was found to be the main peak in the underivatized spectra (Fig. 3c), whereas both $[M+H]^+$ and $[M+H-TMSOH]^+$ were observed in the TMS-derivatized one (Fig. 3d).

Group III: AAS containing unconjugated keto function

Compounds included in this group (Table 4) contain an unconjugated keto function in their structures. This keto function is normally present in C17 although two compounds (DHT and OXA) have it in C3. The position of the keto function seems to guide the ionization behavior of underivatized analytes from Group III. Similar to Groups I and II, the $[M+H]^+$ was the base peak for analytes with the carbonyl in C3, whereas the $[M+H]^+$ relative abundance was less than 30% for 17-oxo compounds (Table 4). Analytes with the keto function in C17 showed a prominent $[M+H-H_2O]^+$ (relative abundances higher than 60%) being in most cases the base peak of the spectrum.

Contrary to Groups I and II, an abundant $[M+H-2H_2O]^+$ was found in the spectra of underivatized compounds with unconjugated keto functions. The relative abundance of the $[M+H-2H_2O]^+$ was found to be dependent of the structural properties of C5. Thus, 5α-steroids exhibited $[M+H-2H_2O]^+$ ions with relative abundances lower than 50%. On the other hand, 5β-steroids and steroids having a double bond in C5 (either Δ^5 or Δ^4)

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Table 6. Summary of the relationships found between ionization behavior and structure for the different groups of compounds

Underivatized						
Group	Primary structural feature	Secondary structural feature	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	Water losses from $C_xH_yO_z$
I	Mono-conjugated CO	–	100%	0–30%	0–15%	Variable
II	Poly-conjugated CO	^{6}H	100%	5–25%	*n.d.*	z – 1
		^{6}OH	45–100%	50–95%	55–100%	z – 1
III	Unconjugated CO	^{3}CO	100%	30–95%	~20%	z
		^{17}CO, 5α	7–33%	100%	20–50%	z
		^{17}CO, 5β/5ene	7–23%	62–100%	65–100%	z
IV	Hydroxylated	Secondary ^{17}OH	*n.d.*	60–90%	100%	z
		Tertiary ^{17}OH	0–5%	12–25%	100%	z
TMS derivatized						
Group	Primary structural feature	Secondary structural feature	$[M+H]^+$	$[M+H-TMSOH]^+$	$[M+H-2TMSOH]^+$	TMSOH losses from $C_xH_yO_z$
I	Mono-conjugated CO	^{17}OH	100%	35–70%	0–5%	Variable (normally z – 1)
		^{17}CO/^{17,17}diCH$_3$	100%	0–5%	*n.d.*	0
II	Poly-conjugated CO[a]	^{17}OH	100%	10–55%	*n.d.*	z – 1
		^{17}CO	100%	*n.d.*	*n.d.*	0
III	Unconjugated CO	^{3}CO	100%	40–85%	*n.d.*	z – 1
		^{17}CO, 5α	70–100%	50–80%	5–20%[b]	z
		^{17}CO, 5β/5ene	20–65%	100%	5–20%[b]	z
IV	Hydroxylated	Secondary ^{17}OH	*n.d.*	20–70%[c]	100%	z
		Tertiary ^{17}OH	0–5%	5–70%[c]	100%	z

[a]Results obtained for properly derivatized analytes
[b]Larger abundances for analytes with an extra hydroxyl group
[c]100% for compounds with only one oxygen
n.d. not detected

showed relative abundances for $[M+H-2H_2O]^+$ higher than 65%, being in some cases the base peak of the spectrum.

Finally, the number of water losses observed for this group was always the sum of hydroxyl and carbonyl functions present in the molecule.

The absence of any conjugated carbonyl moiety facilitated the derivatization of this group of analytes, and all hydroxyl and keto functions were converted to TMS groups. Similarly to the behavior observed for underivatized analytes, TMS-derivatives of compounds with a carbonyl group in C3 exhibited a $[M+H]^+$ as base peak of the spectrum. Regarding those analytes with a keto function in C17, different ionization behavior was observed depending on the structure at C5. Thus, 5α-analytes showed an abundant $[M+H]^+$ which was the base peak of the spectrum in all cases except for 11OH-Andros (70%). The relative abundance of the $[M+H]^+$ for TMS-derivatives of 5β-analytes and compounds with a double bond in C5 (either Δ^5 or Δ^4) was found to be approximately 50% with the main exception of 11OH-Etio (20%). In these analytes, the base peak of the spectrum was $[M+H-TMSOH]^+$.

An additional relationship between structure and ionization for TMS-derivatives was found in the study of the number of neutral losses of TMSOH. Similar to the behavior observed for Groups I and II, analytes with the carbonyl in C3 only exhibited the TMSOH loss coming from the hydroxyl group. However, steroids with a keto function in C17 showed as many TMSOH losses as oxygen atoms present in the molecule. This fact indicates that, although with low relative abundance (5–20%), the derivatizated keto function in C17 can be also lost as TMSOH.

In Fig. 3, an example of this group is displayed. Andros shows a minor peak at $[M+H]^+$ being the $[M+H-H_2O]^+$ the base peak of the mass spectrum. A $[M+H-2H_2O]^+$ was also observed (Fig. 3e). In the TMS derivatized, the $[M+H]^+$ becomes the base peak with an abundant $[M+H-TMSOH]^+$ also present in the spectrum (Fig. 3f).

Group IV: AAS with no-keto function in their structure

The last group contains compounds with no keto function in their structures, like hydroxyandrostanes and hydroxyandrostenes (Table 5). The main characteristic of the APCI spectrum for underivatized analytes from this group was the total absence of the $[M+H]^+$ (only a small $[M+H]^+$ signal representing a 5% was found for MADOL). This might be explained because protonation of the hydroxyl groups would facilitate the loss of water. The maximum possible number of losses of water was observed, i.e. a $[M+H-zH_2O]^+$ was detected for $C_xH_yO_z$ steroids. This $[M+H-zH_2O]^+$ ion was found to be the base peak of the spectrum.

No differences were observed in the spectra obtained for 5α and 5β-metabolites. However, a relationship was found between ionization behavior and the structural characteristics of the hydroxyl at C17. Analytes with a tertiary hydroxyl in C17 (17-alkyl-17-hydroxy steroids) exhibited lower relative abundances of $[M+H-H_2O]^+$ (<30%) than those containing a secondary alcohol in C17 ($[M+H-H_2O]^+$ relative abundances >60%).

Due to the absence of any keto function, the TMS derivatization of the analytes belonging to this group provided suitable derivatives. Contrary to the behavior observed for other groups, ionization of TMS derivatives from Group IV did not substantially differ from their underivatized counterparts (Table 5). Thus, similar structural considerations could be extracted from this study, for instance, the absence of $[M+H]^+$ which can be explained by the easy loss of TMSOH after the protonation of the TMS-ether function (Fig. 2c).

In Fig. 3, the MS spectrum for MeTmet1 is shown as an example. In this case, $[M+H-2H_2O]^+$ appears as the base peak of the spectrum for the underivatized compound (Fig. 3g), whereas a prominent $[M+H-2TMSOH]^+$ was observed for the enol-TMS derivative (Fig. 3h). In both cases, minor ions corresponding to single losses of water or TMSOH were also observed.

Conclusions

The use of a new APCI interface coupled to GC–QTOF has been investigated for the ionization of model AAS. The addition of water in the interface promoted the proton transfer reaction producing mainly protonated species, i.e. $[M+H]^+$ and $[M+H-H_2O]^+$ (or $[M+H-TMSOH]^+$ for TMS derivatives). A relationship between ionization behavior and structure has been established. The main conclusions are summarized in Table 6.

The low fragmentation observed in the APCI spectra (all ions contained the intact steroid skeleton) would facilitate the selection of abundant and/or more specific precursor ions in tandem MS experiments. Theoretically, this fact would lead to a neat sensitivity and selectivity improvement in SRM-based methods. The effect of this improvement in the control of the abuse of steroids in sports should be investigated.

Furthermore, the knowledge gained regarding the behavior of AAS ionization in the APCI source might be helpful in the identification and elucidation of unknown metabolites of steroids in urine. Thus, based on the information depicted in Table 6, a theoretical ion for a predicted metabolite can be postulated. This can help in the target detection of potential metabolites by GC–APCI–MS. Additionally, if an unknown steroid is detected by untargeted methods, some valuable structural information can be obtained based on the results showed in this work.

Acknowledgements

The authors acknowledge the financial support of the Ministry of Education and Science, Spain, in the project DEP2011-28573-C02-01/02. The authors from University Jaume I also acknowledge the support from *Generalitat Valenciana* (Research Group of Excellence Prometeo/2009/054). The authors from IMIM acknowledge the support from *Generalitat de Catalunya* (*Consell Català de l'Esport* and DIUE 2009SGR4929).

M. Raro is also grateful to the Ministry of Education and Science for her predoctoral grant.

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