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Improved Steroids Detection and Evidence for Their Regiospecific Decompositions using Anion Attachment Mass Spectrometry

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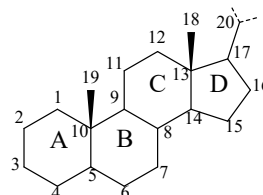
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ABSTRACT: Nonpolar anabolic steroids are doping agents that typically do not provide strong signals by electrospray ionization-mass spectrometry (ESI-MS) owing especially to the low polarity of the functional groups present. We have investigated the addition of anions, in ammonium salt form, to anabolic steroid samples as ionization enhancers and have confirmed that lower instrumental limits of detection (as low as 10 ng/mL for fluoxymesterone-M) are obtained by fluoride anion attachment mass spectrometry, as compared to ESI+/- or APPI+. Moreover, collision-induced decomposition (CID) spectra of precursor fluoride adducts of the bifunctional steroid "reduced pregnenolone" (containing two hydroxyl groups) and its d4-analog provide evidence of regiospecific decompositions after attachment of fluoride anion to a specific hydroxyl group of the steroid. This type of charting of specific CID reaction pathways can offer value to selected reaction monitoring experiments (SRM) as it may result in a gain in selectivity in detection, as well as in improvements in quantification.

Naturally-occurring steroids are endogenous hormones playing essential roles in various physiological pathways, such as muscle and bone growth¹⁻³ or augmenting proteins synthesis.⁴ Dysregulation of the synthesis or metabolism of steroidal hormones has been shown to be linked to many adverse health conditions.⁵⁻⁶ In addition to the endogenous variety, exogenous steroids, especially anabolic androgenic ones, have been illegally used for enhancement of athletic performance for several decades.⁷⁻⁸ Each year, the World Anti-Doping Agency (WADA) publishes a list of prohibited compounds considered to be doping agents. Many steroids can be found on this list,⁹ but their detection at trace levels requires carefully implemented methods.

All steroids share a common structural backbone composed of three six-membered rings and one cyclopentyl ring, referred to as A, B, C and D rings (**Scheme 1**). The low acidities and basicities of many of these compounds, and their low volatilities, have made the development of suitable analytical methods for their trace level detection a difficult task. Over the past decades, mass spectrometry (MS), especially coupled with chromatography, has emerged as the tool of choice to perform determinations of steroids. Gas chromatography (GC)-MS,¹⁰⁻¹² liquid chromatography (LC)-MS¹³⁻¹⁶ and matrix-assisted laser desorption ionization (MALDI)-MS¹⁷⁻¹⁸ are commonly used for detection and characterization of steroid compounds. It is important to note that steroids that are lacking both acidic and

basic functional groups present a great challenge to ionize and detect using electrospray ionization (ESI)-MS alone. In order to address this issue, other ionization sources have been tested, such as atmospheric pressure chemical ionization (APCI)¹⁹⁻²¹ or atmospheric pressure photoionization (APPI).²¹⁻²² However, the most widely used approach remains GC-MS analysis preceded by a chemical derivatization of the targeted steroids.²³⁻²⁴ GC-MS has the advantages of being reliable, sensitive, selective and reproducible, but because derivatization is often required to create volatile and thermally stable analytes, the sample preparation step is both labor-intensive and expensive. Recent efforts have been directed towards developing LC-ESI-MS methods where sample preparation is less complicated, yet performance characteristics are not sacrificed.



Scheme 1. Steroids backbone structure and carbon nomenclature.

To overcome the typical poor ionization efficiency of steroids in ESI, formation of cationic or anionic adducts in the

presence of additives in the analyte solution has been employed. This approach has led to significant signal improvements through the formation of species such as $[M+Na]^+$, $[M+Na+MeOH]^+$, $[M+H+CH_3CN-H_2O]^{+25}$ or $[M+Ag]^{+26}$ in positive ion electrospray and $[M+CH_3COO]^-$ or $[M+F]^-$ adducts in the negative ion mode.²⁷ This anion attachment mass spectrometry approach allows for the detection of steroids without strong acidic functional groups. Adducts of the form $[M+A]^-$, where A^- represents an anion, are thus formed by attachment of an anion to an electron-deficient functional group on the molecule.²⁸ As a first order estimate, the stability of the adduct depends upon an approximate matching of the steroid's attraction for the proton located at a proton-bearing site on the steroid and the attraction of the anion attaching at the same site.²⁹ Choosing an appropriate anion (i.e. by considering the acidity of HA) may thus lead to the withdrawing of the shared proton by the anion A^- which leaves as HA and to the formation of the deprotonated steroid, $[M-H]^-$, either directly in the ESI source mass spectrum or via collision-induced dissociation (CID).³⁰

A further advantage of anion attachment mass spectrometry is the potential for regioselective attachment of the anion on multifunctional molecules which may be very useful to improve the selectivity in the selected reaction monitoring (SRM) method commonly employed in sports anti-doping detection. Anions may indeed exhibit an increased tendency to bind to a specific electropositive site on a molecule in preference to others.^{27, 31} This feature is evidenced by regiospecific decompositions of $[M+A]^-$ adducts in tandem mass spectrometry experiments and may be of importance to distinguish steroids which have similar or isomeric structures and are thus difficult to separate using conventional analytical techniques. The work presented in this paper is intended to assess the limits of detection obtainable employing anion attachment MS for actual steroids of abuse, while also weighing the factors that affect selectivity and quantification in SRM detection.

EXPERIMENTAL SECTION

Chemicals and Materials. Steroid standards used for experimentation were either commercially bought (Sigma-Aldrich (Saint-Quentin Fallavier, France) or Cambridge Isotopes (Tewksbury, MA) for d4-17,21,21,21-pregnenolone) or chemically synthesized for the Agence Française de Lutte contre le Dopage (AFLD). Reduced pregnenolone was synthesized according to a procedure given in the supplementary material. All solvents used in this study were HPLC grade and were used without further purification. Methanol and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Deionized water (18 M Ω) was obtained from RiOs-Di3 (Millipore, Billerica, MA). Other chemicals (NH₄F, NH₄CH₃COO, NH₄OH and NaBH₄) were analytical grade or better and were from Sigma-Aldrich.

Mass spectrometry. All samples were prepared in a 9:1 v:v MeOH/H₂O solution. Triple quadrupole mass spectrometry data were acquired on a Quattro Premier QqQ (Waters Corp., Milford, MA) equipped with an ESI source employed in the

negative ion mode. Collision-induced dissociation (CID) MS/MS spectra were obtained using nitrogen as collision gas in the collision cell and collision energies (E_{lab}) between 5 and 30 eV. Atmospheric pressure photoionization (APPI) experiments were carried out in an Orbitrap XL high resolution instrument (Thermo Scientific). All experimental details are given in the supplementary material.

Gas phase acidity calculations. Calculations have been performed using the GAUSSIAN 09 suite of programs.³² Geometry optimization was carried out with the B3LYP³³⁻³⁴ functional coupled to the Def2-SVP split valence basis set.³⁵ Single point energies were obtained employing the same functional using the Def2-TZVP basis set. The protocol given by Ho and Coote³⁶ was employed to calculate gas-phase acidities of steroids. The accuracy of the computational approach was probed by comparing calculated gas-phase acidities of some selected molecules with experimental values found in the literature (see supplementary materials for details).

RESULTS AND DISCUSSION

Anion attachment to improve steroid ionization: choice of the anion. Steroids contained in natural matrices (e.g., water, plasma and urine) are often present at only trace levels. The World Anti-Doping Agency (WADA) established a minimum required performance level (MRPL) for analytical laboratories engaged in detection of steroids of abuse of 5 ng/mL for most of exogenous anabolic androgenic steroids. For a method to be validated, the limit of detection (LOD) must not be higher than one-half the value of the MRPL.³⁷ In this work, anion attachment has been tested as an alternative ionization method that may improve the detectability of steroids that are inherently difficult to ionize. The trace amount of compound that the method must be capable of detecting makes it necessary to optimize the amount of anion added in samples in order to achieve the highest signal intensities. The first series of experiments consisted of analyzing solutions of norbolethone-metabolite (M1, structure given in supplementary material, **Scheme S2**) with a fixed steroid concentration (1 μ g/mL, 3.1 μ M in MeOH/H₂O (9:1 v:v)). Varying amounts of NH₄F were added to each sample, with concentrations ranging from 0 to 310 mM, corresponding to a steroid:anion molar ratio varying from 1:0 to 1:500. The experimental abundances of deprotonated $[M1-H]^-$ and anionic adduct $[M1+A]^-$ were recorded in triplicate for each norbolethone-M sample. In the second part of the protocol, ammonium fluoride was replaced with ammonium hydroxide and the process was repeated. As no hydroxide anion adducts were observed with NH₄OH, only $[M1-H]^-$ abundances are reported. All instrumental parameters and experimental protocols were kept identical for the two sets of experiments; results are presented in **Figure 1**.

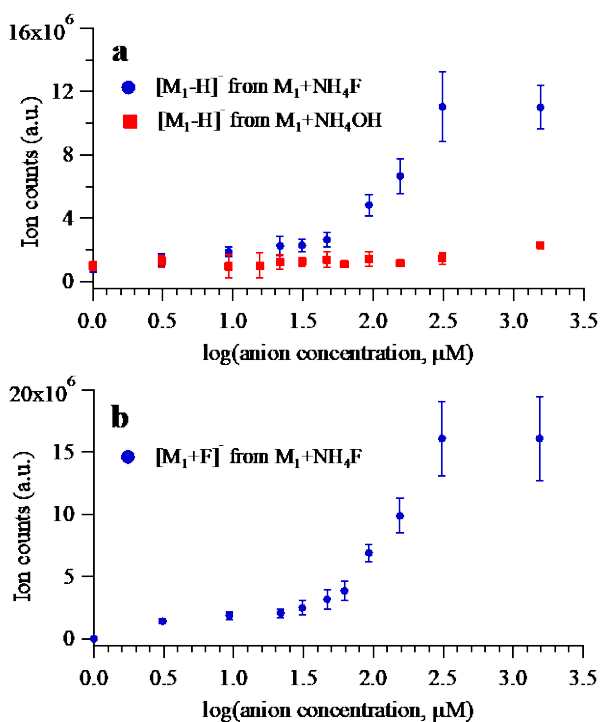


Figure 1. Mean signal intensity of adduct ions and deprotonated molecules of the norbolethone metabolite (M1) formed as a function of anion concentration (logarithmic scale): (a) comparison of $[M1-H]^-$ formed upon addition of NH_4F (blue dots) vs. $[M1-H]^-$ formed upon addition of NH_4OH (red squares). (b) $[M1+F]^-$ ions formed upon addition of NH_4F (blue dots). No hydroxide adducts were observed from NH_4OH addition. Error bars represent the standard deviation of triplicate measurements.

Increasing the concentration of fluoride in the samples leads to signal improvement for both the deprotonated molecules (Figure 1a, blue dots) and the anionic adduct (Figure 1b). Clearly, the addition of anions has an enhancing effect on the ionization efficiency of steroids in negative ion ESI. For the NH_4F solution, the signals of both $[M1-H]^-$ and $[M1+F]^-$ increase roughly proportionally with the fluoride concentration, up to 310 μM (1:100 steroid:anion molar ratio). Above this threshold concentration, adding more anion to the sample does not result in any increase (or decrease) of the signal intensity. This is an interesting feature for practical applications because higher concentrations, i.e., large excesses of anion in samples do not appear to induce signal suppression. In a biological sample, concentrations of various steroids can differ by several orders of magnitude. If an exact steroid:anion molar ratio were required to be used in order to optimize signals, then the procedural optimization becomes much more complicated. By using the anionic solution in large excess, maximal signal intensities can be obtained for a wide variety of analyte concentration levels present in the sample.

In comparing the experiments where F^- or HO^- was used as the attaching anion, it is obvious that the effect of hydroxide is insignificant compared to that of fluoride. At a 1:500 steroid:anion molar ratio, the sample employing F^- undergoes an order of magnitude increase in the $[M1-H]^-$ signal, whereas the

$[M1-H]^-$ signal from the HO^- solution is less than doubled (both compared to respective samples without any anion, Figure 1a). This observation indicates that formation of $[M1-H]^-$ is not occurring primarily in the initial solution when using anion attachment. The pK_a of a hydroxyl proton located on a steroid molecule is indeed expected to be approximately 16 in water vs 15.7 for H_2O and 3.2 for HF .³⁸ An acid-base reaction between the F^- anion and the steroid leading to HF and $[M1-H]^-$ should be completely negligible compared to the acid-base reaction between HO^- and M leading to H_2O and $[M1-H]^-$. But the contrary trend is observed on Figure 1. Furthermore, the similar behavior of the $[M1+F]^-$ and the $[M1-H]^-$ signal intensities when changing the anion concentration supports the argument that the $[M1+F]^-$ adduct plays a role in $[M1-H]^-$ formation. Thus, the improvement of the ionization efficiency observed with the addition of the fluoride anion is not due to a simple acid-base effect, but to the formation of a stable $[M1+F]^-$ intermediate which may subsequently lead to the production of $[M1-H]^-$ in the gas phase. As already outlined in the introduction, the main factor likely affecting the adduct stability is a matching of the gas-phase acidity of HA and of the steroid. In contrast to fluoride ($\Delta_{acid}G^\circ(HF) = 1530$ kJ/mol),³⁹⁻⁴⁰ hydroxide anion has too strong an affinity for protons ($\Delta_{acid}G^\circ(H_2O) = 1635$ kJ/mol)⁴¹ and readily abstracts a proton to exclusively form $[M1-H]^-$ species in the ion source (no hydroxyl adducts were observed). Note that calculations performed on similar hydroxyl-bearing steroids to evaluate gas-phase acidities show that the ($\Delta_{acid}G^\circ$ values generally fall in the 1500 - 1530 kJ/mol range, far below $\Delta_{acid}G^\circ(H_2O)$).

The same study was repeated with the anabolic steroid furazabol-M (Scheme S2), which is relatively easy to ionize in ESI, compared to norbolethone-M. These two steroids with divergent behaviors toward ionization were chosen for comparison in order to test whether the effect of fluoride is significant on both difficult and easy-to-ionize compounds. The tests on furazabol-M led to similar results as those obtained with norbolethone-M (results not shown), affirming the efficiency of fluoride addition. Several conclusions can be drawn from these experiments: i) the signal intensity of anionic adducts of steroids increases with the concentration of anion up to a certain level, ii) the $[M-H]^-$ signal increase due to the addition of fluoride is not caused by acid-base reactions in solution but to the formation of a stable $[M+F]^-$ intermediate which decomposes in the gas-phase, iii) the optimum steroid:anion ratio is 1:100 molar (or beyond, i.e., even more anion) and is used for all further experiments, iv) no ion suppression effect was observed, so, even with a large excess of anions, this method can be used in practical applications where the concentration of different steroids can greatly vary (e.g. for anti-doping testing).

Anion attachment to improve steroid ionization: instrumental limits of detection. Our investigation targets specific steroids that have been deemed « problematic » due to their poor ionization behavior in ESI or because of the difficulty to reach the minimum required performance level (MRPL) implemented by the World Anti-Doping Agency (WADA) employing routine methods. In this section, we investigate the instrumental limits of detection (LOD), i.e. those obtained by

direct infusion of reference compounds dissolved in standard ESI solvents. In order to get a broad view of the method performance, steroids displaying various behaviors and ionization responses were chosen for the study; all structures are shown in supplementary material, **Scheme S2**. The metabolite of fluoxymesterone M2 can be analyzed by LC-MS/MS and is readily ionized in positive ESI. Calusterone M3 displays similar characteristics, however its metabolite M4 does not give any signal in positive or negative ESI-MS and is consequently challenging to analyze. The metabolite of norbolethone M1 is not detected in negative ESI-MS. Finally, furazabol M5 gives good signals in all ionization modes. For the QqQ experiments, two sets of solutions were prepared for each compound tested: the first set without any additive and the second set with ammonium fluoride present in a 100:1 steroid:anion molar ratio. Because certain steroids have been shown to offer exceptionally low detection limits by positive-mode APPI^{22, 42-43} obtained results were compared to those obtained by APPI+ on an Orbitrap. Here again, for each compound tested, two sets of solutions were prepared: the first was run by adding 5 % toluene via a T-junction in preparation for APPI whereas the second was prepared by adding ammonium fluoride in a 1:100 steroid:anion molar ratio. Serially-diluted samples of steroids were analyzed in ascending order of concentration in order to evaluate the instrumental limits of detection for the investigated steroids ($S/N < 3$). The results are summarized in **Table 1**.

Table 1. Instrumental limits of detection (LOD) obtained for various steroids ionized by ESI or APPI in the presence or absence of NH₄F.

| Steroid | Limits of detection (ng/mL) | | | | |
|---------|-----------------------------|--------------|------------------|--------------------|-----------------------|
| | ESI+/ QqQ | ESI-/ QqQ | ESI-(F-)/ QqQ | APPI+/ Orbitrap | ESI-(F-)/ Orbitrap |
| M1 | 250 | nd | 25 | nd | 100 |
| M2 | 25 | 25 | 10 | 2500 | 50 |
| M3 | 25 | 50 | 25 | 100 | 100 |
| M4 | 250 | nd | 25 | nd | 100 |
| M5 | 25 | 100 | 25 | 1000 | 100 |

nd = not detected

For the non-polar, difficult-to-ionize compounds, the use of anion attachment systematically results in improved signals. As shown in **Table 1**, LODs obtained in the presence of NH₄F are always equal to, or better than, those obtained in either positive (monitoring MH⁺) or negative (monitoring [M-H]⁻) ESI in the absence of any additive. In the case of the less ionizable compounds (M1, M4), no signal is obtained in conventional negative ESI, but after adding NH₄F, it becomes possible to detect anionic adducts of these compounds at concentrations as low as 25 ng/mL, which represents a 10-fold improvement compared to the LODs obtained in positive ESI. Indeed, M1 and M4 are apolar compounds that tend to stay in neutral form and thus do not readily ionize in ESI. When adding NH₄F to those steroids, the adduct can be observed with a strong signal, while the [M-H]⁻ species is barely detected. This tendency was also observed with other compounds from the list: the less acidic a compound is, the more difficult it is to

withdraw one of its protons and the adduct [M+F]⁻ is usually the only steroid form detected. Consequently, the improvement in ionization is achieved owing to the fact that a stable adduct is efficiently formed with the attaching anion, and not because deprotonation is augmented. It should also be noted that, even for compounds giving good results in positive ESI (without any additive), such as M2, the LOD is improved when mixing fluoride with the steroid. M3 is readily ionized in positive ESI and can be detected in concentrations as low as 25 ng/mL; this LOD was also obtained in negative ESI with addition of fluoride. As mentioned above, the use of APPI has been claimed to improve sensitivity of specific compounds that ionize poorly by conventional ESI, including certain steroid analytes. Comparison studies aimed at assessing the relative instrumental limits of detection for anion attachment using ESI vs. APPI were performed on a single instrument, i.e., a high resolution Orbitrap (Thermo Instruments). Please note that this is an older generation Orbitrap that has sensitivity characteristics below those of the Quattro Premier QqQ. Results show that not all of the steroids studied here could be ionized using APPI. M3 ionizes easily by APPI to form MH⁺; this behavior is consistent with the observation of protonated molecules by positive ESI-MS. M5 produces MH⁺ during APPI, but when compared to the adduct formation using anion attachment, we can see that APPI is less sensitive by a factor of 10. M2 produces the radical cation (M^{•+}) when analyzed using APPI, but when compared to the anion attachment analysis, the results are 50 times less sensitive when APPI is used. In summary, none of the tested "difficult to ionize" steroids exhibited better signal responses by APPI than those obtained by anion attachment ESI.

The results obtained from these experiments are promising because they show that the anion attachment approach leads to improved detection of all of the difficult to ionize, apolar steroids, as evidenced by the lower LODs. This method requires very low quantities of analytes. For example, a 30-second acquisition of a 10 ng/mL solution of M2 introduced into the instrument at a 10 μ L/min flow rate consumed 50 pg of sample (0.141 pmol).

Anion attachment on bifunctional steroids. As outlined above, anion attachment leads to stable gas-phase adducts when the involved anion and the functional moiety have close gas-phase acidities $\Delta_{\text{acid}}G^\circ$.²⁹ Two different anions may thus exhibit different sites of preferential attachment to a specific type of bifunctional steroid whose proton-bearing moieties are separated in space, with each steroid site offering distinct $\Delta_{\text{acid}}G^\circ$ values. This feature leads to a regioselective anion attachment that has been already observed in the case of pregnenolone.²⁵ This steroid has two distinct moieties, a hydroxyl group at position C3 and a ketone at position C20 (**Scheme S2**) and it was shown to exhibit regioselective decompositions depending upon the attaching anion. To further advance the understanding of the possibilities for regioselective attachment in an analytical context, we performed reduction of the ketone of pregnenolone and d4-17,21,21,21-pregnenolone in order to obtain a bifunctional steroid with the same functional moiety at each end (**Scheme S2**). The $\Delta_{\text{acid}}G^\circ$ values of the two hydroxyl groups have been evaluated by theory and are close but

distinct (ca. 10 kJ/mol difference). We have chosen this model bifunctional steroid because we would like to test whether regioselective anion attachment is discernible when the two functions are the same, and afterwards, examine the implications of our findings toward SRM analysis.

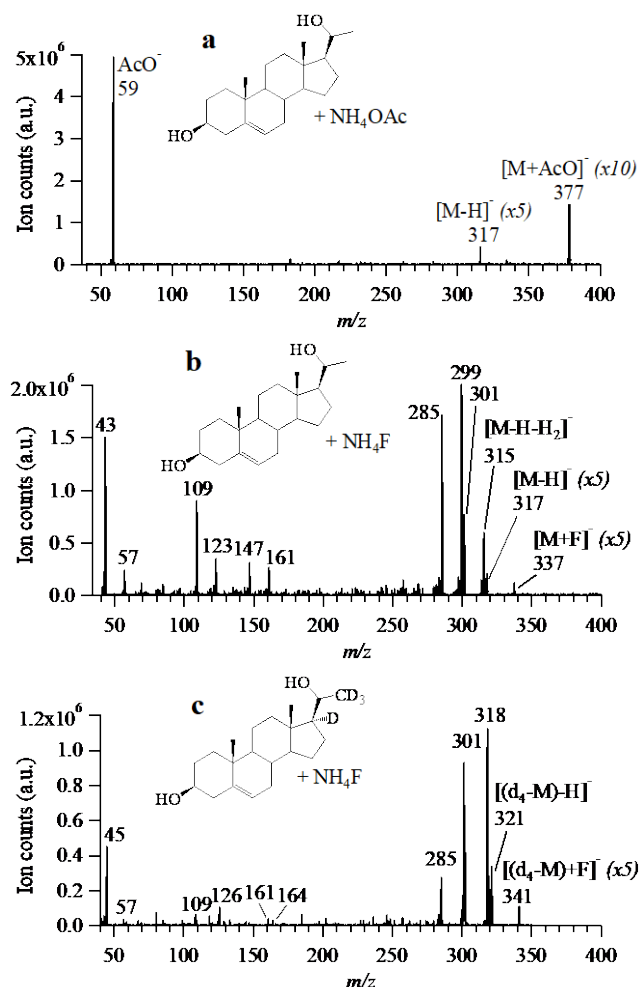
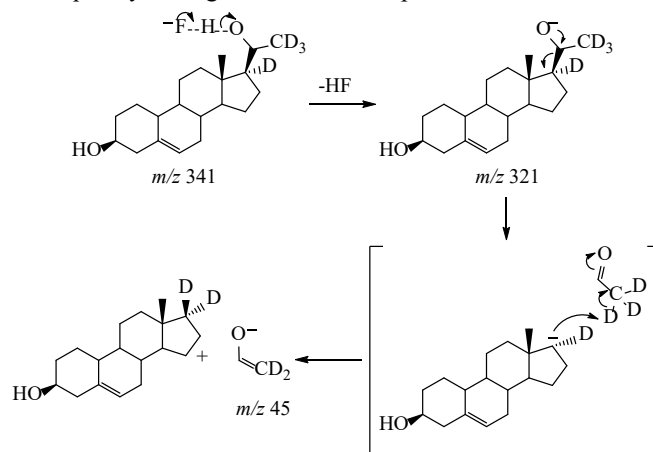


Figure 2. Mass-selected CID spectra of (a) [reduced pregnenolone + CH₃COO]⁻, (b) [reduced pregnenolone + F]⁻ and (c) [reduced d₄-17,21,21,21-pregnenolone + F]⁻. Collision energy is (a) 5 eV and (b), (c) 30 eV. Some ion signals have been increased: the multiplication factor is indicated in italics.

MS experiments on reduced pregnenolone revealed that from the pool of tested anions, good negative ion signals could be obtained for both [M+F]⁻ and [M+CH₃COO]⁻ species (Figure S1). The $\Delta_{\text{acid}}G^\circ$ values of both anions ($\Delta_{\text{acid}}G^\circ(\text{CH}_3\text{COOH}) = 1429 \text{ kJ/mol}^{44,40}$ and $\Delta_{\text{acid}}G^\circ(\text{HF}) = 1530 \text{ kJ/mol}^{40,39}$) are different enough to have the possibility of each anion attaching to, and forming stable adducts at, distinct functional groups on the steroid.²⁵ Fragmentation of the mass-selected acetate and fluoride adduct precursors led to the MS/MS spectra shown in Figure 2a and 2b, respectively. Fragmentation profiles are noticeably different for the two species. The CID spectrum of the acetate adduct displays only

low abundance fragments with low global signal intensity and the base peak at m/z 59 (departure of acetate anion). For both [M+A]⁻ adducts (where A⁻ is the employed anion), loss of HA led to the formation of [M-H]⁻. Although the yield is poor for acetate adducts, both anions demonstrate their capability of capturing a proton from the steroid.

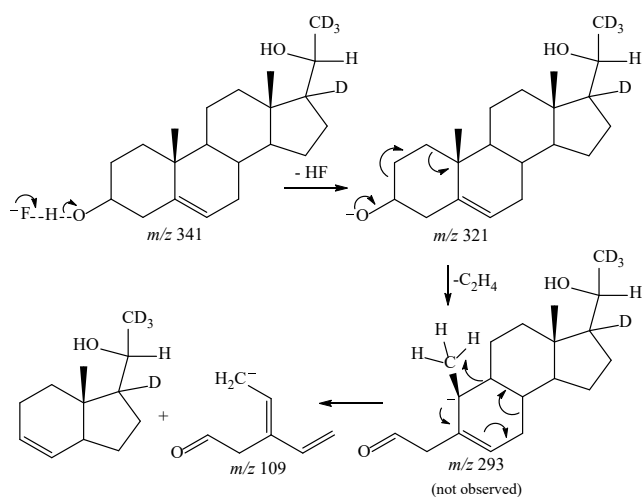
Taking a closer look at decompositions of the precursor acetate adduct shown in Fig. 2a, the appearance of m/z 59 (departure of acetate anion) as the base peak indicates that acetic acid is more acidic than the most acidic steroid site of attachment and that acetate anion is barely capable of abstracting a proton from the steroid. This observation agrees well with the calculated gas-phase acidities values: the most acidic site of reduced pregnenolone is located on the C20 hydroxyl group and is about 77 kJ/mol less acidic than acetic acid. This contrasts sharply with the behavior of the fluoride adduct precursor whose MS/MS spectrum shows [M-H]⁻ in relatively high yield. This observation is rationalized by comparing the calculated $\Delta_{\text{acid}}G^\circ$ values of the two hydroxyl sites of the steroid with that of HF. HF has a slightly higher $\Delta_{\text{acid}}G^\circ$, but the difference is less than 15 kJ/mol, which is coherent with the formation of stable [M+F]⁻ adducts. Importantly, because of the acidity difference, CID activation of this complex (Fig. 2b) leads mainly to [M-H]⁻ (m/z 317) and not to F⁻. Note also the presence of the m/z 315 fragment ion, which likely corresponds largely to formation of deprotonated pregnenolone (non-reduced form). CID of [M+F]⁻ adducts of non-reduced pregnenolone has already been investigated, and among its main product ions, one can cite m/z 299, 161, 123 and 57.²⁷ Fragment ions appear at these same m/z values in the CID spectrum of [M+F]⁻ adducts of reduced pregnenolone (Fig. 2b) of deprotonated reduced pregnenolone fluoride adduct and it is thereby plausible that their formation pathways involve, in part, initial formation of deprotonated pregnenolone which subsequently undergoes further decompositions.



Scheme 2. Proposed mechanism for the formation of the m/z 45 fragment obtained from decomposition of [reduced d₄-17,21,21,21-pregnenolone + F]⁻.

Despite these similarities with non-reduced pregnenolone, abundant product ions *specific to reduced pregnenolone* are also observed in the CID spectrum of its [M+F]⁻ m/z 337 precursor: m/z 301, 285, 109 and 43 (Figure 2b). The CID

spectrum of the deuterated analogue [d4-M+F]⁻ *m/z* 341 precursor (**Figure 2c**) shows either no shift for these ions (*m/z* 301, 285 and 109) or a quantitative *m/z* shift as for *m/z* 43 → 45. Considering this last product ion, the formula C₂H₃O⁺, implicating one of the hydroxyl groups, seems more reasonable than C₃H₇⁺ which would typically involve a terminal propyl or isopropyl group (not present on the molecule). In principle, however, a C₂H₃O⁺ fragment, could originate from either end of the molecule. But, because of the quantitative shift observed with the quadruply-deuterated analog [CD₂CHO]⁻, it is clear that the incorporation of two deuterium atoms in the *m/z* 45 fragment provides unambiguous evidence that the ion was formed uniquely by a fragmentation pathway originating from the D-ring of the steroid. This decomposition can be explained by the mechanism given in **Scheme 2**, involving the formation of an ion-dipole complex as an intermediate.



Scheme 3. Proposed mechanism for the formation of the *m/z* 109 fragment produced from decomposition of [reduced d₄-17,21,21-pregnenolone+F]⁻.

Another intense product ion of the MS/MS spectrum appearing in both **Figure 2b** and **2c** (no shift) is the *m/z* 301 ion. This resonance-stabilized fragment is formed via charge-driven CD₄ loss (-20 amu) from the [d₄-M+F]⁻ *m/z* 321 precursor after deprotonation of the C₂₀ hydroxyl of reduced pregnenolone (**Scheme S3**). Formation of *m/z* 285, observed in **Figure 2b** and **2c** (no shift), is then attributed to a consecutive loss of CH₄, implicating the methyl group at C₁₉ and a nearby H atom.

The fact that the product ion observed at *m/z* 109 in **Figure 2b** is not at all shifted when the deuterated analog is employed (this product ion appears again (quantitatively) at *m/z* 109 in **Figure 2c**) allows us to attribute some clarity to its mechanism of formation. In this case, we propose initial attachment of the fluoride anion at the C₃ hydroxyl group (**Scheme 3**), followed by abstraction of the hydroxyl proton, provoking first, charge-driven decomposition of the A-ring, and ultimately formation of the resonance stabilized *m/z* 109 product ion after a series of charge-driven steps. The analogous mechanism is applicable to the non-deuterated starting compound. Interestingly, this

m/z 109 ion is *not* observed in the CID spectrum of [pregnenolone+F]⁻ (non-reduced form) although it could be expected, as both reduced and non-reduced forms of the steroid possess the same C₃ moiety where the dissociation takes place. This feature is attributed to the regioselective attachment of fluoride onto the C₂₀ site of non-reduced pregnenolone²⁷; from this adduct conformation, the formation of *m/z* 109 is not possible. The Δ_{acid}G° values of both functional groups of pregnenolone have also been evaluated and results show, as expected, a much larger difference between the two most acid sites of pregnenolone (hydroxyl on C₃ and ketone on C₂₀, >20 kJ/mol) than between those of reduced pregnenolone (hydroxyl on C₃ and C₂₀, <10 kJ/mol). Hence, only the C₂₀ site of pregnenolone is subjected to a deprotonation whereas reduced pregnenolone may undergo a deprotonation on both C₃ and C₂₀ sites. This result demonstrates the great potential of the anion attachment method as it can block fragmentation pathways on some molecules and, at the same time, confer specific dissociations to others that were not initially inherent to the latter.

For reduced pregnenolone, we offer evidence that the product ion at *m/z* 43 is formed by decompositions occurring after specific F⁻ attachment at the C₂₀ hydroxyl group, whereas *m/z* 109 is formed after specific F⁻ attachment at the C₃ hydroxyl. The fact that the decomposition pathways leading to these product ions require anion attachment at a particular site on the molecule augments the selectivity of the decomposition process, which is an important consideration in selected reaction monitoring (SRM) experiments. An ambiguity in the decomposition pathway will diminish the selectivity of a SRM transition. Ideally, a transition of the highest selectivity will involve only a single decomposition pathway, that is, only one mechanism is applicable to its formation. In addition, quantification using SRM can also be affected when there is an ambiguity regarding decomposition pathways involved in a monitored transition. This is because when two competing pathways lead to product ions of the same *m/z* value, the yield of the monitored product ion by the two different mechanisms will likely be different, and the yield of each will change independently of one another as collision conditions change. In this sense, the product ions at *m/z* 43 and 109 arise from decomposition pathways that are regioselective. In a previous publication on anion attachment to pregnenolone, we could demonstrate that, owing to the significant differences in the acidities of the two functional groups, regioselective attachment of fluoride anion was occurring at only one end of the bifunctional molecule.²⁷ The reduced pregnenolone example in the current paper shows that when the two functional groups of the bifunctional steroid are the same (hydroxyl at both C₃ and C₂₀), then attachment is no longer regioselective. But, because of the regioselectivity in anion attachment observed with pregnenolone, a decomposition pathway (formation of *m/z* 109) becomes specific to reduced pregnenolone despite the fact that this same functional group is available on the former.

CONCLUSION

In this work, we have been considering two aspects of the anion attachment mass spectrometry method as it concerns the analysis of steroids lacking polar functional groups. First, the ionization efficiency of this technique in terms of limits of detection has been tested and compared to conventional ionization methods (ESI+/- and APPI+). Results show a dramatic improvement for weakly polar to apolar steroids, whereas they are mostly similar for steroids which are easily ionizable. Secondly, we have tried to gain more insight into the mechanisms of the anion attachment itself, as well as into the fragmentation pathways resulting from the formed anionic adducts. We were able to confirm with the help of theoretical $\Delta_{\text{acid}}G^\circ$ evaluations of different steroid sites that a close matching of gas-phase acidities of HA and M (A^- is the anion and M, the steroid) results in the most stable $[M+A]^-$ adducts. Moreover, it has been shown that regioselectivity of anion attachment can occur when a bifunctional steroid possesses acidic groups with significantly different $\Delta_{\text{acid}}G^\circ$ values, such as pregnenolone. Here we have investigated the decomposition pathways observed for a bifunctional steroid, i.e., reduced pregnenolone, bearing hydroxyl groups at opposite ends of the molecule. Fluoride attachment was deduced to occur at either of the two hydroxyl groups, but deuterium labeling enabled the identification of specific product ions that were shown to originate from attachment at a specific hydroxyl site. Knowledge of a precise reaction pathway leading to a product ion of a specific m/z value that is not formed by any other pathway can lead to both improved selectivity and more reliable quantification in SRM experiments. Quite interestingly, the lack of regioselectivity in anion attachment for reduced pregnenolone enables accessibility to $[M+F]^-$ adduct dissociation pathways from both functional groups. This contrasts with the behavior of pregnenolone for which the observed regioselectivity excluded an entire portion of the conceivable dissociation pathways.

The investigation reported here lays the foundation for a future protocol for analyses of "difficult" steroids, but it has to be further developed in order to become even more efficient. The addition of liquid chromatography separation prior to the addition of anion to the steroids could have a potential to provide a viable alternative for the compounds that display poor responses with the existing methods.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Reduction of pregnenolone-d4 hydroxyl-19; Experimental details; Structures of steroids under study; Validation of the theoretical method; MS spectra

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