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Ion trap MS/MS of intact testosterone and epitestosterone conjugates—Adducts, fragile ions and the advantages of derivatisation

David A. Cowan[a,*], Andrew T. Kicman[a], Carlos Kubli-Garfias[b], Helen J. Welchman[a]

[a] Drug Control Centre, King's College London, 150 Stamford Street, London SE1 9NH, United Kingdom
[b] Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico, Coyoacan, Mexico

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ABSTRACT

In ion trap mass spectrometry, fragile ions may fragment under the application of resonance ejection during precursor mass isolation, reducing MS/MS spectral intensity. In this study the steroidal epimers testosterone glucuronide (TG) and epitestosterone glucuronide (EG) have been chosen as a model for exploring whether compound structure is linked to ion trap fragility. Both compounds form multiple adducts by ESI-MS, namely protonation, ammonium and sodium, however, the mass spectrum of EG displays a more intense ammonium adduct peak than TG. $[TG+NH_4]^+$, $[EG+NH_4]^+$ and $[EG+H]^+$ were found to be fragile ions. To explain the differences in adduct formation and fragility, molecular modelling was employed. Ammonium adduction was localised to the glucuronide ring oxygens and while EG has eight possible adduction sites, only seven were located for TG explaining the increased ammonium adduct abundance with EG. In EG the bond between the steroid and the glucuronide was slightly longer and the oxygen in this bond was more basic than TG. This shows that the EG bond is weaker which may contribute to the fact that $[EG+H]^+$ but not $[TG+H]^+$ is fragile. To investigate whether stability could be restored by chemical means, EG was derivatised with tris(trimethoxyphenyl)phosphonium chloride or methylated on the carboxylic acid and Girard P or methoxylamine on the 3-keto group. Derivatisation of the steroid rather than the glucuronide eliminated fragility and using a charged derivative eliminated adduct formation. This work demonstrates the importance of carefully considering the nature of the derivative and the site of derivatisation.

1. Introduction

Chromatography coupled to mass spectrometry is particular valuable for the study of steroid metabolites, which are mainly excreted as phase II glucuronide and sulphate conjugates in urine. These steroid conjugates are excellent markers of endocrine perturbation and are routinely monitored in clinical, forensic and drug control laboratories. Historically this has been performed using GC/MS, however, due to the polarity and thermal lability of such compounds a lengthy sample preparation procedure is required including enzymatic hydrolysis of the glucuronide and derivatisation. More recently, the introduction of LC/MS has enabled the analysis of intact steroid glucuronides and sulphates, reducing the need for sample preparation. Quadrupole ion trap (QIT) mass spectrometers are particularly valuable when used for the analysis

* *Corresponding author.*
E-mail address: david.cowan@kcl.ac.uk (D.A. Cowan).
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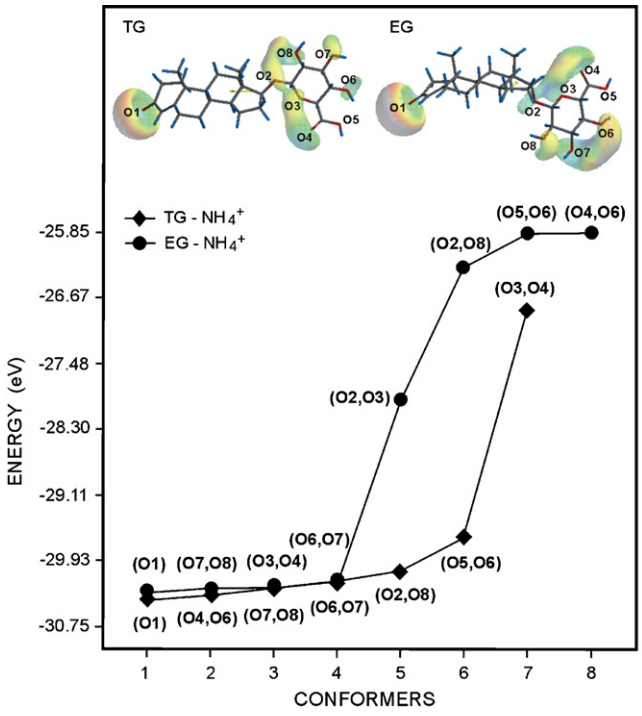

Fig. 1 – (Top) TG and EG with oxygen numbers and the electrostatic potential. (Bottom) Total energy curves of the steroid–ammonium adducts studied. The oxygen atoms linked to the ammonium moiety are in parentheses.

of unknown compounds in complex mixtures due to their ability to provide data dependent MS^n full scan data. Metabolite screening is one area where ion traps have been effectively used in this respect. However, routine use of LC/MS for the quantitative analysis of steroid glucuronides has been hampered by the formation of multiple adducts in the ion source.

In this study, the steroidal epimers testosterone glucuronide (TG) and epitestosterone glucuronide (EG) were chosen as model compounds (Fig. 1) for studying adduct formation with steroid glucuronides. Attempts to detect the intact glucuronides by LC/MS have shown that there are remarkable differences in the ammonium adduct affinities of TG and EG leading to the hypothesis that there may be important structural differences in the molecules which affect adduct formation. Kuuranne et al. [1] reported that the intensity of the ammonium adduct is 80% of the base peak $[EG+H]^+$ intensity by ESI-triple quadrupole. By contrast the epimer TG gave $[TG+H]^+$ as the base peak with a $[TG+NH_4]^+$ intensity of only 20%. Sodium adducts were also prominent. Competing ionisation processes such as addition of H^+, Na^+ or NH_4^+ ions to a single pure substrate are disadvantageous to quantitative analysis because they reduce sensitivity by dividing the analyte signal between several discrete *m/z* values. Also irregular adduct formation increases analytical variability.

The objective of the current investigation was to use structural elucidation to understand firstly, why EG has a higher affinity for ammonium than TG. In order to obtain precise three-dimensional structures and charge localisations on each atom a combination of X-ray crystallography and *ab initio* calculations have been used. Quantum mechanics *ab initio* theory can be used to predict the chemical behaviour, taking into account nuclei and all electrons. This type of modelling has previously been used to explain negative ion [2] and alkali metal adduct [3] formation in ESI-MS. Previously published *ab initio* modelling of epitestosterone and testosterone showed the qualitatively different intermolecular forces present at C_{17} [4]. Building on this knowledge, molecular modelling was applied to the glucuronide conjugates of these steroids to explain adduct formation.

The mass spectrometer used was a 3D QIT, which is more sensitive for obtaining full scan data than typical linear quadrupole mass spectrometers. Multiple mass spectrometry (MS) experiments above MS [2] can only readily be performed by separation in time, making QITs particularly valuable for structural analysis and enhancing the signal-to-noise ratio. QIT mass spectrometers operate by applying isolation and resonance ejection frequencies to ions in the mass analyser. These frequencies increase the internal energies of the ions and can cause some 'fragile' ions to fragment. Previously identified fragile ions include orlistat [5], and some explosive, acylcarnitine and macrolide antibiotic molecules [6].

Derivatisation was used as a complementary approach to investigate whether fragile ions could be made stable by chemical means. The derivatised analyte broadly retains its structure but gains new chemical properties that can be used to gain insights into which structural features account for fragility. In this study, either the 3-oxo (ketone) group of the steroid or the carboxylic acid group of the glucuronide moiety were reacted with uncharged or permanently positive charged reagents to form the structures displayed in Fig. 2.

2. Materials and methods

2.1. *Materials*

TG, EG, testosterone, epitestosterone, testosterone sulphate and epitestosterone sulphate were obtained from Promochem (Teddington, UK). Testosterone benzoate, epitestosterone benzoate, testosterone hemisuccinate and epitestosterone hemisuccinate were obtained from Steraloids (Newport, USA). Ammonium acetate, sodium hydroxide and diethyl ether (all analytical reagent grade) were obtained from Fisher Scientific (Loughborough, UK). Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific (Loughborough, UK). Propranolol, 1-methyl-3-nitrosoguanidine (MNNG), 2-chloro-1-methyl-pyridinium iodide (CMPI), triethylamine (TEA), Girard P reagent, methoxylamine hydrochloride, pyridine, LiCl, NaCl, KCl and RbCl were obtained from Sigma–Aldrich (Poole, UK). Acetic acid and ammonia (both Analar) was obtained from BDH (Poole, UK). Tris(trimethoxyphenyl)phosphonium (TMPP) were prepared in house as described elsewhere [7,8]. Solid phase extraction cartridges (C_8, Isolute) were obtained from Kinesis (St. Neots, UK).

2.2. *Sample preparation*

For infusion the steroid glucuronides (100 μg/mL, 50 μL) were dried under nitrogen (60 °C). They were then redissolved in the positive ion mode infusion solvent (1 mL) consisting of 50:50

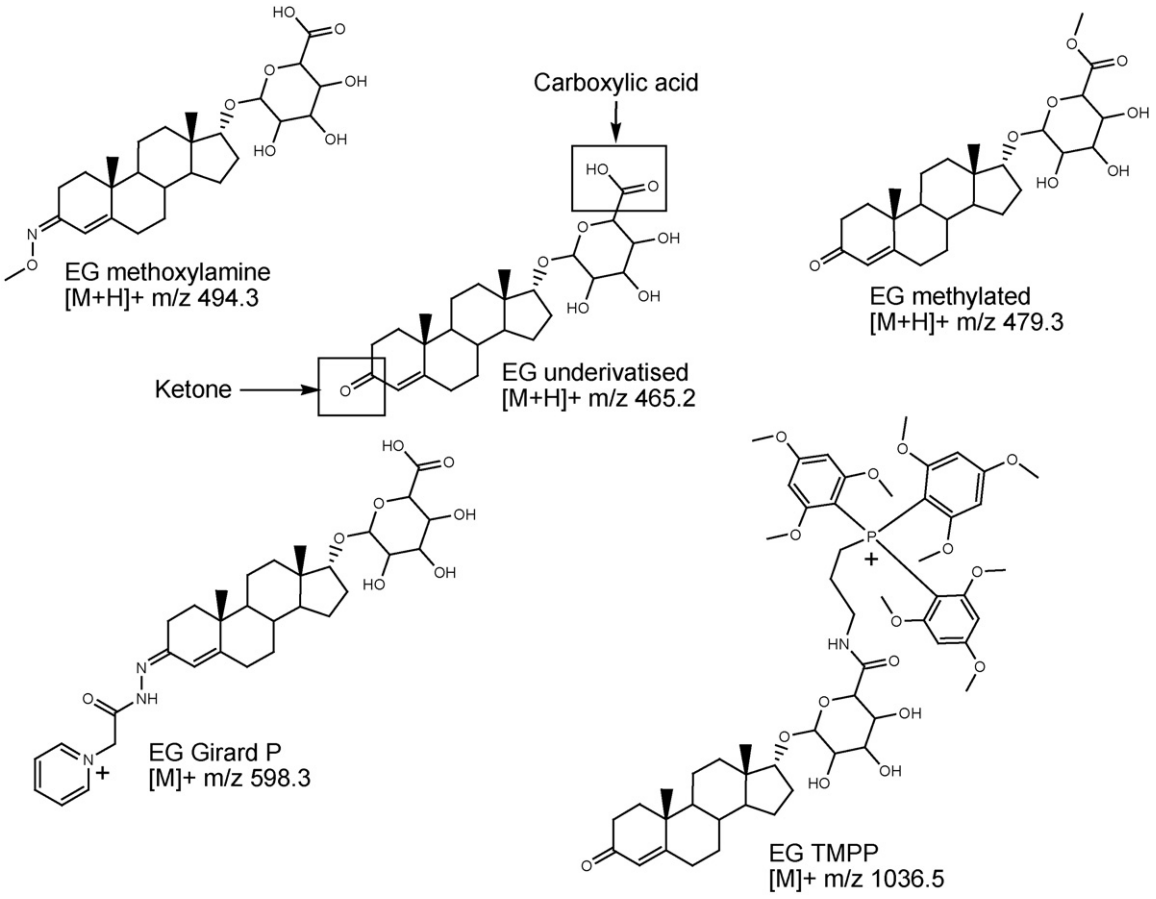

Fig. 2 – The structure of epitestosterone glucuronide (EG, centre) with the ketone and carboxylic acid functional groups labelled. The structures of the EG ketone derivatives (left); methoxyamine (top) and Girard P (bottom) and carboxylic acid derivatives (right); diazomethane (top) and TMPP (bottom) are shown. Theoretical masses shown.

methanol:water containing ammonium acetate (7.5 mM) and acetic acid (0.1%) to give a final concentration of 5 μg/mL. The negative ion mode infusion solvent was 50:50 methanol:water containing ammonia (0.01%). To assess the fragility of lithium, sodium, potassium and rubidium adducts, solutions of EG (5 μg/mL) in 1:1 water:methanol were prepared containing either LiCl, NaCl, KCl or RbCl (0.5 mM).

2.3. *Instrumentation*

The LCQ DECA XP (Thermo Electron, Hemel Hempstead) QIT mass spectrometer equipped with an orthogonal electrospray source was used with the Xcalibur Version 1.3 software. The instrument was tuned using the non-fragile $[M+H]^+$ ion from propranolol for mass, resolution and abundance.

2.4. *Infusion experiments*

Sample solutions were introduced into the electrospray source at a flow rate of 5 μL/min via a metal capillary. The built in syringe pump was used for sample delivery. The sheath gas and auxillary gas flows were set to 10 and 0 'arbitrary units', respectively. The capillary temperature was 260 °C and the spray and capillary voltages were 4 kV and 4 V respectively. Both MS and MS/MS experiments were analysed with automatic gain control using 3 microscans per scan and a maximum injection time of 500 ms, which are the default settings for this instrument. The automatic gain control target values were full MS $2e^7$, SIM $2e^7$, MS^n $2e^7$ and ZoomScan $1e^7$.

2.5. *Derivatisation procedures*

2.5.1. *Derivatisation of the carboxylic acid on the glucuronide moiety*

2.5.1.1. *Methylation*. A diazomethane generator (Sigma–Aldrich, Poole, UK) was used to prepare the diazomethane. Ether (3 mL) was placed in the outer tube and MNNG (147 mg) and water (0.5 mL) were placed in the inner tube. Sodium hydroxide (5 M, 0.6 mL) was added dropwise to the inner tube. The diazomethane co-distills with the ether ready for use.

To methylate the samples, diazomethane in ether (0.5 mL) was added to EG or TG (10 μg/mL, 0.5 mL). The solutions were dried under nitrogen (30 °C) and diluted with the positive ion mode infusion solvent (1 mL) [9].

2.5.1.2. *TMPP*. The steroid glucuronides (100 μg/mL, 50 μL) were dried under nitrogen (60 °C). Solutions of the catalysts CMPI (15 mM in acetonitrile, 10 μL) and TEA (30 mM in acetonitrile, 10 μL) were added and the samples were sonicated (20 °C, 15 min). TMPP solution was then added (20 mM in ace-

tonitrile, 20 μL) and the samples again sonicated (20 °C, 15 min) and then made up to 1 mL with the positive ion mode infusion solvent [8].

2.5.2. *Derivatisation of the ketone on the steroid moiety*

2.5.2.1. *Girard P.* The steroid glucuronides (100 μg/mL, 50 μL) were dried under nitrogen (60 °C). A solution of Girard P (10 mg/mL, 1 mL) prepared in methanol:water:acetic acid (7:2:1) was added to each sample. The samples were incubated (70 °C, 30 min), dried and the steroid hydrazone derivative reconstituted in the positive ion mode infusion solvent (1 mL) [10].

2.5.2.2. *Methoximation.* The steroid glucuronides (100 μg/mL, 50 μL) were dried under nitrogen. Methoxylamine hydrochloride (2% in pyridine, 50 μL) was added to each sample and the samples were incubated at 70 °C for 1 h [11]. The samples were then dried under nitrogen (60 °C) and the residue was resuspended in water (0.5 mL). The steroid methyloxime derivative was extracted from the excess methoxylamine hydrochloride by passing the solution through a C8 solid phase extraction cartridge pre-conditioned with methanol (3 mL) and water (3 mL). The sample was washed with water (3 mL) and the derivatised steroid glucuronides were eluted with methanol (1 mL). The solution was dried under nitrogen (60 °C) and reconstituted in the positive ion mode infusion solvent (1 mL).

2.5.2.3. *Fragile ion experiments.* Using the standard tune conditions the resolution of the instrument was fixed with the non-fragile $[M+H]^+$ ion from propranolol. The test solutions were infused into the QIT mass spectrometer (5 μL/min) using the operating parameters described above. ZoomScan (10 u mass range) data was acquired for 30 s centred on the mass of interest ($[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ in MS mode) and the data averaged so that the width at 10% ($W_{10\%}$) could be determined. The instrument was then set to MS/MS mode using each appropriate centroid mass (to one decimal place). To optimise isolation of ions, rather than fragmentation, no collision energy was applied. The isolation width was then increased from 1 to 10 u in increments of 0.25 u. Each isolation width was monitored for 20 s and the average spectrum over this period was used to determine the peak intensity.

2.5.2.4. *TG and EG modelling from X-ray conformers and computational chemistry procedures.* TG was modelled from the testosterone *ab initio* conformer reported previously [4] and the glucuronide moiety was added from data of the X-ray crystal of estriol 17β-monoglucuronide [12]. Co-ordinates of EG were acquired from its crystal co-ordinated with potassium (TG-K) [13]. Regardless of the intrinsic lower energy conformation of the crystals and *ab initio* conformers, both molecules were submitted to further energy minimization. An initial geometry was obtained by the semi-empirical method MP3, and refined at *ab initio* Hartree–Fock theory using sequentially the 3-21G and 6-31G(*) basis set levels, to unveil the electronic properties of the models.

The lower energy conformers of TG and EG were used to study the steroid ammonium adduct formation and its ion fragility. The NH_4^+ moiety was joined to TG and EG based on the co-ordinates of the potassium atom from TG-K [13] which is co-ordinated with the oxygen atoms of the steroid–glucuronide molecule.

The NH_4^+ ion was placed close to the oxygen atoms looking for spontaneous hydrogen bond formation. Thus the NH_4^+ ion was linked to the glucuronide by one or two hydrogen bonds forming the adduct. For each adduct after the spontaneous hydrogen bond formation, a molecular mechanics energy minimization was carried out allowing the NH_4^+ ion to find its best place. However, in some cases some constraints were used (e.g., fixing a neighbouring torsional angle) to obtain the conformer. After that, each steroid–ion complex was submitted to single point energy calculations with *ab initio* levels similar to those applied to TG and EG. The following properties were measured: total energy, free energy of the steroid and ion components, dipole moment, electrostatic charges, highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) location and energies, electron density encoded electrostatic potential and hydrogen bond distances. Calculations were carried out using Spartan software (Wavefunction Inc., Irvine, CA, 2004).

3. Results and discussion

3.1. *Analysis of EG and TG by mass spectrometry*

Comparing the results from a triple quadrupole [1] to those from a QIT, similar abundances of protonation to ammonium adduction were obtained for TG and EG when infused into the mass spectrometer via an ESI source in positive ion mode. The same solvents were used in the two experiments. In the QIT, the ratio of protonation to ammonium adduction was 5:1 for TG and 3:1 for EG. This indicates that the pattern of adduction is unaffected by the type of mass analyser used, being determined mainly in the ion source. Varying the concentration of ammonium acetate (0, 7.5, and 15 mM) caused a proportionate increase in ammonium adducts. Of course, chromatography is also affected by this change in concentration of the buffer. Furthermore, attempts to control adduct formation were hampered by sodium contamination in solvents and the LC/MS equipment. This contamination could not be eradicated.

ESI of unconjugated testosterone (T) and epitestosterone (E) and sulphated T and E using the same infusion solvent did not generate ammonium adducts, nor did hemisuccinate or benzoate moieties, substituents with structural features similar to those of glucuronic acid. Hence, ammonium adduct formation appears to be specific to glucuronide conjugates.

The MS/MS spectra of each of the TG and EG adducts were determined using a QIT instrument. It was observed that an isolation width >2.5 u was required to achieve sensitive MS/MS spectra. This occurs with "fragile ions" or ions which (unusually) fragment under the application of the isolation waveform, leaving little or no precursor ion available for CID and hence generating weak MS/MS spectra [6]. Increasing the isolation width, reduces the amount of energy imparted to the isolated precursor and hence reduces fragmentation. McClellan et al. proposed criteria for fragile ions, those requiring an isolation width greater than 2.5 u and giving a peak width greater than 0.31 u in ZoomScan mode. Using these criteria, the $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions of EG and

Table 1 – Minimum isolation width for the different adducts of EG and TG

Ion	*m/z*	Min i.w. (u)[a]	$W_{10\%}$ (u)
$[EG-H]^-$	463	1.5	0.22
$[TG-H]^-$	463	1.5	0.25
$[EG+H]^+$	465	7.0	0.56
$[TG+H]^+$	465	2.5	0.25
$[EG+NH_4]^+$	482	6.5	0.61
$[TG+NH_4]^+$	482	8.0	0.89
$[EG+Na]^+$	487	2.5	0.17
$[TG+Na]^+$	487	2.5	0.25

[a] The minimum isolation width (min i.w.), defined as the isolation waveform notch width that preserves 90% of the maximum isolated ion intensity, is presented for each ion. For comparison with peak width data, the $W_{10\%}$ data for each ion are presented.

TG in positive ion mode and the $[M-H]^-$ ion in negative ion mode were tested for fragility in the QIT. The results are shown in Table 1. TG was found to be stable in the protonated form with a peak width of 0.25 u in ZoomScan mode and requiring an isolation width in MS/MS mode of 2.5 u. By contrast, the ammonium adduct of TG was very fragile with a peak width of 0.89 u (i.e. $\gg$0.31 u) and requiring isolation widths of 8.0 u (Table 1) because of the dissociation of the adducts caused by energy imparted by the notch frequency isolation waveform. Unlike TG, protonated EG was fragile ($W_{10\%}$ = 0.56 u, i.w. = 7.0 u) as was its ammonium adduct ($W_{10\%}$ = 0.61 u, i.w. = 6.5 u). Comparatively, $[TG+NH_4]^+$ is less stable than $[EG+NH_4]^+$ as evidenced by their ZoomScan peak widths and this is also indicated by the relative proportion of protonation to ammonium adduction of TG (5:1) and EG (3:1). This difference in stability indicates the importance of the 17-configuration of these epimers causing their different behaviour in the mass spectrometer. A comparison of the extent of fragmentation in normal full MS mode shows that EG fragments more than TG. Of note is the greater formation of the aglycone (at *m/z* 289) suggesting that the fragility stems from the glucuronide breaking away from the steroid molecule as discussed later.

We tested whether the size of the adducting ion or molecule might be important to determining its stability. All the sodium adducts in positive ion mode were non-fragile. Sodium ions exhibit strong affinity to oxygenated centres (ether, ester, carboxylic or alcohol groups) and hence are also likely to adduct at the same site as the ammonium. The fact that the ammonium adducts are fragile but the sodium adducts are not can be explained in terms of the specific interactions of the cation with the glucuronide. Since Na^+ and NH_4^+ have different ionic radii (Na^+: 0.98 Å, NH_4^+: 1.43 Å) their co-ordination with the glucuronide moiety depends on the spatial availability which is determined by the constitution and configuration of the glucuronic acid. To test this hypothesis the fragility of adducts of increasing ionic radii were evaluated (lithium, 0.68 Å; sodium, 0.98 Å; potassium, 1.33 Å; rubidium 1.48 Å) to encompass that of ammonium. Metal cation adduction compared with protonation was approximately four times greater for lithium and sodium than for potassium and rubidium. Rubidium was the only metal cation adduct that was fragile ($W_{10\%}$ values: Li^+ 0.31; Na^+ 0.28; K^+ 0.28; Rb^+ 0.56 u respectively). The rubidium adduct and ammonium adducts whose ionic radii differ by just 0.05 Å demonstrated the same $W_{10\%}$ providing strong evidence that the ammonium and alkali metal cations adduct at the same site. The degree of fragility of these adducts is likely therefore to be based on the dissociation of the adduct ion from the adduction site during the isolation process because cations of the alkali metals with a higher charge density and therefore stronger interaction with the adduction site were not fragile. However, the limitation of this explanation is that metallic cations have their own charge, which is completely available to accept electrons from the oxygen lone pairs (ionic bonding) whereas the NH_4^+ ion has its charge delocalised among its atoms leaving only sufficient charge to form weaker H-bonds.

In negative ion mode $[EG-H]^-$ and $[TG-H]^-$ are non-fragile ions. The abstraction of a hydrogen atom is most likely to occur at the carboxylic acid of the glucuronic moiety as this is the most acidic hydrogen in the molecule. However, previous investigators have found that using negative ion mode for the analysis of TG and EG is not as sensitive as positive ion mode. MS/MS spectra from triple quadrupoles (which do not suffer from a low mass cut-off) also show that fragments are mainly derived from the glucuronide giving poorer specificity and signal to noise which is especially important if quantifying the analyte in a complex biological matrix [1]. MS/MS analysis in the QIT gave very weak spectra as any fragments derived from the glucuronide were below the low mass cut-off and could not be stored in the trap.

Even though TG and EG are epimers, the $[EG+H]^+$ was fragile but $[TG+H]^+$ was not. Since both the EG and TG ammonium adducts were also fragile, complete conversion of these analytes to ammonium adducts using a high ammonium acetate concentration confers no benefit for reliable quantification. Given the apparent structural similarity of the two glucuronides it was possible that structural analysis of the two compounds may uncover important but subtle differences that would account for the fragility of one but not the other. This would add to the scientific knowledge of fragile ions since no publication was identified that explained which structural features made an analyte more likely to be fragile.

3.2. *Theoretical assessment of EG and TG ammonium adduct abundance and fragility*

Structural features may be responsible for the different behaviour of TG and EG in MS. To explore this further, structural analyses involving *ab initio* molecular modelling of TG and EG were performed. The intention was to better understand the structural features that favour adduct formation and fragility so that strategies could be developed to overcome these problems.

Although ammonium adduction to steroid glucuronides is a well known phenomenon, a publication mapping the site of adduction was not found. Therefore the adduction of ammonium to EG and TG was studied using molecular models based on X-ray crystallographic data. The EG X-ray crystallography results contained a potassium ion and co-ordination of this ion by oxygens of the glucuronide and the oxygen of the C3 keto group was observed. Based on that, and similar features observed in potassium and ammonium picrate crystals [14],

it was clear that oxygen atoms readily accept the ammonium ion through hydrogen bonds, forming the ammonium adduct. Only one NH_4^+ ion is allowed at once in both TG and EG because the NH_4^+ ion modifies the glucuronide electrostatic environment changing it from negative to positive and generating a huge dipole moment. This concurs with the mass spectral results where no di-ammonium adduct was observed. Several sites of ammonium adduction were possible utilising the oxygens of the glucuronide ring to co-ordinate the adduct molecule. In total, molecular modelling identified seven possible ammonium adduct conformers for TG and eight for EG (Fig. 1). Interestingly, the two ether oxygens labelled O2 and O3 of TG do not partake in adduct formation together because of steric hindrance by the steroidal C_{18} methyl group and the two hydrogens of C_{16}. These findings show that TG has some restrictions in NH_4^+ adduct formation giving fewer possible conformers than EG and this may explain why ammonium adduction is more favourable with EG than with TG.

Since the fragility of the ammonium adduct is likely to be caused by displacement of the adduct molecule during the isolation step of MS/MS in the QIT, the binding energy of the ammonium to the glucuronide for each conformer was calculated. This showed that although EG ammonium adducts are more abundant, they have higher energy than TG adducts as shown in Fig. 1. The energy difference is more marked in four of the eight EG adducts suggesting lower stability. Although all these conformers are possible theoretically it is unknown which ones actually form in the source. For example, one of the most favourable of the theoretical conformers was ammonium adducted to the C3 ketone group (O1 in Fig. 1). However, this conformer could also form with unconjugated or sulpho conjugated T and E by MS but ammonium adducts are not observed with these molecules. This suggests that formation of this conformer is not favourable in the ESI with T, E and their conjugates presumably because protonation at this site is much more energetically favourable.

The proposed adduction sites agree well with the finding that only glucuronide and not sulphate, benzoate or hemisuccinate conjugates of T or E form ammonium adducts. Also, previous investigations have shown that steroid glucuronides without the 4-ene-3-one structure form the ammonium adduct almost exclusively [1]. These workers conclude that

Table 2 – Minimum isolation width for the derivatives of EG and TG

Derivative	m/z	Min i.w. (u)[a]	$W_{10\%}$ (u)
EG diazomethane	479.0	10.0	0.78
TG diazomethane	479.0	2.5	0.29
EG TMPP	1036.7	8.5	0.33
TG TMPP	1036.7	2.2	0.28
EG Girard P	598.5	1.0	0.28
TG Girard P	598.5	1.0	0.28
EG methoxyamine	494.3	1.0	0.28
TG methoxyamine	494.3	1.0	0.28

[a] The minimum isolation width (min i.w.), defined as the isolation waveform notch width that preserves 90% of the maximum isolated ion intensity, is presented for each ion. For comparison with peak width data, the $W_{10\%}$ data for each ion are presented.

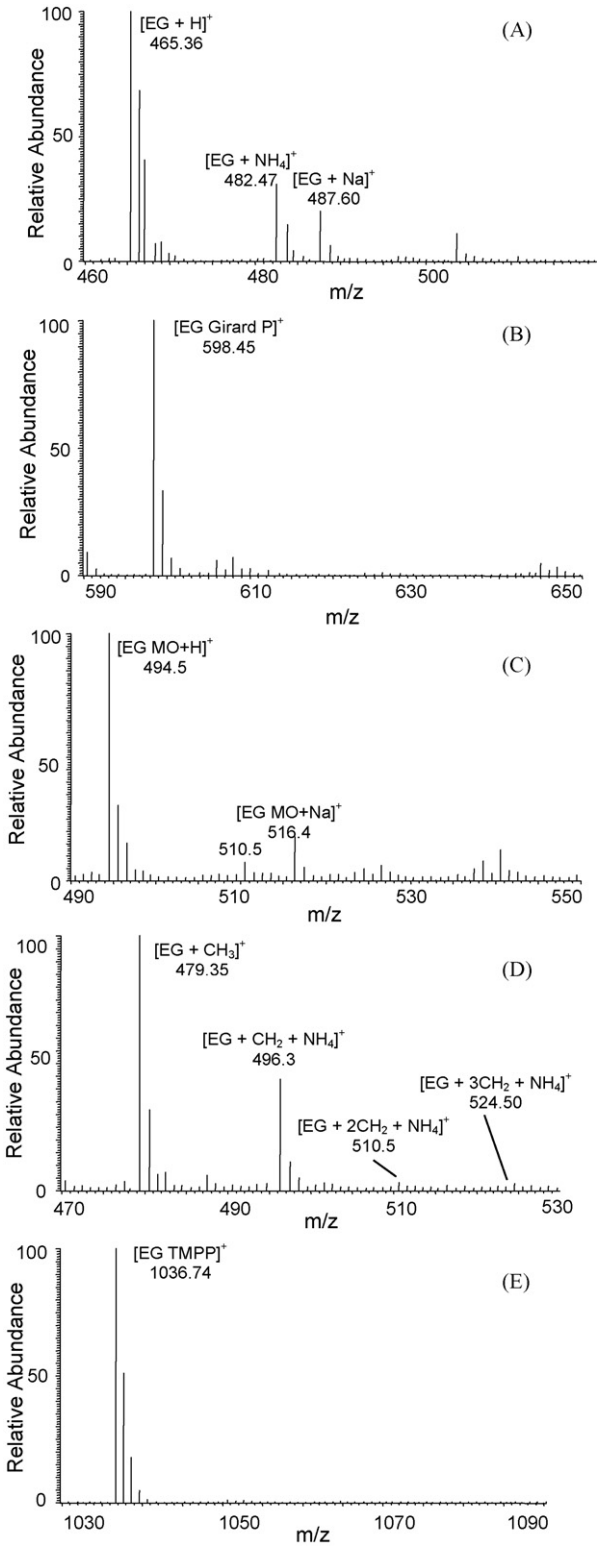

Fig. 3 – Spectra of EG (EG was derivatised and infused into the ion trap at 5 μL/min, and the samples analysed in MS mode): EG underivatised (A); EG Girard P (B); EG methoxyamine (C); EG methylated (D); EG TMPP (E). For structures see Fig. 2.

protonation occurs on the 4-ene-3-one structure but do not comment on the location of the ammonium adduct. Thus, since they are epimers both with 4-ene-3-one structures, the fact that $[EG+H]^+$ was fragile but $[TG+H]^+$ was not is paradoxical. Molecular modelling was employed to investigate whether any particular structural characteristic could explain this. Firstly the adduction sites were differentiated by their position on the analyte. There appears to be two major adduction sites, one located at the A-ring and one located around the glucuronide oxygens. Ammonium adduct formation elicits a large dipole moment which makes the glucuronide moiety quite positive and the A–B ring region very negative. At the A–B ring region the highly resonant 4-ene-3-one system is very important to this polarity because it is the site of both the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) in TG and EG. This concurs with previously published molecular models of unconjugated T and E [4]. This negative system in the A-ring, is the site of protonation while the cation adducts such as sodium adducts attach in a similar way to the ammonium adducts at the glucuronide.

The stability of Li^+, Na^+ and K^+ adducts in the ion trap mass spectrometer and the fragility of Rb^+ demonstrates that the size of the adduct is important in terms of the strength of its interactions with the analyte. By modelling these adducts with EG it was possible to determine the bond distances between the oxygens of the analyte and the cation. The average bond distance increases steadily as we descend the alkali metal adducts: Li^+ (1.793 Å), Na^+ (2.149 Å), K^+ (2.470 Å) and Rb^+ (2.626 Å). Since K^+ adducts are stable but Rb^+ adducts are not, the critical oxygen to cation bond length required to retain the adduct during isolation in the ion trap is likely to be between 2.470 and 2.626 Å. This explains why the association between the Rb^+ and the analyte is weaker than the other cations resulting in this adduct being a fragile ion.

Analysis of the two molecules shows that the A–B ring is stable and very similar in EG and TG, therefore the main differences exist at the glucuronide end of the molecule. Examination of the MS spectrum of EG showed that the aglycone was also present in the spectrum indicating that the bond between the C17 oxygen and the glucuronide is very labile. EG appears to be more labile than TG since the aglycone is not observed in the MS spectrum of TG. Given this information, the bonds C17 to O2 and O2 to C1′ of the glucuronide were examined in more detail since these connect the glucuronide moiety to the steroid. The lengths of these bonds were found to be 1.422 and 1.369 Å respectively in EG compared to the slightly shorter bonds (1.411 and 1.360 Å) in TG. In this respect the shorter bonds of TG may be considered to be slightly stronger. However, greater differences were observed when the point charges were calculated on each atom of these bonds. Both the O2 atoms were slightly basic but the charge on the O2 atom of EG is −0.708 eV compared to −0.497 eV with TG. Hence protonation of the O2 atom of EG is more favourable than in TG. Protonation is a common precursor to hydrolysis reactions in chemistry by stabilising the charge on the leaving group. In a similar way, protonation at the O2 of EG may promote bond rupture under the stresses of the ion trap environment explaining its observed fragile ion status. This scenario would release E (with associated proton at O2) which would explain the observed peak of *m/z* 289 in the MS spectrum.

3.3. *Derivatisation*

Since structural features could account for some of the differences in the behaviour of TG and EG by MS, it was possible that by derivatising these molecules fragility and multiple adduct formation would be eliminated. Derivatives have been used in mass spectrometry to improve sensitivity [8]. The analyte broadly retains its chemical structure and gains new chemical properties that can be used for quantification and separation such as a positive charge to enhance ionisation. The *ab initio* calculations demonstrated that the addition of a second ammonium adduct to an already charged analyte is highly unfavourable. Therefore it was likely that the use of charged derivatives on these molecules would eliminate multiple adduct formation. Positively charged and neutral derivatives of the keto function on the steroid moiety and the carboxylic acid of the glucuronide moiety were tested for fragility in the QIT to investigate whether stability could be restored by chemical means.

The keto structure was derivatised using Girard P hydrazine or methoxylamine hydrochloride. These derivatives convert the steroid ketone group to a hydrazone or oxime, respectively, thus removing the 4-ene-3-one functionality. The carboxylic acid was derivatised using diazomethane or TMPP. Diazomethane methylates the carboxylic acid leaving it less acidic whilst TMPP is a bulky group with a permanent positive charge. The structures of these molecules are shown in Fig. 2.

When EG was derivatised using either of the charged derivatives (Girard P or TMPP) no adduct was observed in the MS spectra. However, when non-charged derivatives were used (methoxylamine and diazomethane) adducts were still formed, for example in the spectrum of methylated EG, intense ions for $[EG+CH_2+NH_4]^+$, $[EG+(CH_2)_2+NH_4]^+$ and $[EG+(CH_2)_3+NH_4]^+$ were observed and in the methoxyamine (MO) spectrum $[EG\,MO+Na]^+$ was observed (Fig. 3). This was predicted since it is unfavourable for a charged adduct to associate with a previously permanently charged analyte.

Derivatisation of the carboxylic acid on the glucuronide with TMPP led to partial stabilisation of EG but not enough to bring it within the non-fragile range. The fragility of EG remained after methylation of the carboxylic acid group with diazomethane. The fragility experiments showed that derivatisation of the '4-ene-3-one' structure totally stabilised both protonated EG and TG so that they could be analysed by ion trap MS/MS with an isolation width of 1 u (Table 2).

4. Conclusions

Adduct formation occurs in the source of ESI mass spectrometers and is therefore a problem with all types of analyser such as triple quadrupole and QIT. In the current study the sites of ammonium and sodium adduction in steroid glucuronides were mapped which can help to develop strategies to minimise adduct formation in the future. One such strategy is to derivatise the molecules.

Molecular modelling has proved a useful tool to assist with the design of chemistries to avoid adduction and fragility of labile steroids. Our results show that derivatisation can eliminate both adduct formation and fragility from TG and EG. To eliminate adduct formation the derivative should be charged thus making the addition of a second charge by adduction highly unfavourable. However, to eliminate fragility, the site of derivatisation should be on the steroid rather than the glucuronide. Hence in the current example Girard P was the optimum derivative to use. Nevertheless, for other analytes, it is important to consider carefully which derivative to use and the site of derivatisation.

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