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Ion Mobility Spectrometry and Tandem Mass Spectrometry Analysis of Estradiol Glucuronide Isomers

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Abstract

Estradiol is an estrogenic steroid that can undergo glucuronidation at two different sites, which results in two estradiol glucuronide regioisomers. These isomers can be produced by different enzymes and can have different biological activities before being eliminated from the body. Although there have been previous methods that can distinguish the two isomers, these methods often have long acquisition times or high cost per analysis. In this study, traveling wave ion mobility spectrometry (TWIMS) coupled to mass spectrometry (MS) was employed to separate estradiol glucuronides using alkali metal adduction in positive ion mode, where the sodiated dimer adduct provided adequate separation both in single-component standards and in two-component mixtures. Additionally, in negative mode tandem mass spectrometry (MS/MS) was used to quantitatively determine the relative composition of the two isomers. This was possible due to differences in the energetic requirements for loss of the glucuronic acid, which was characterized by energy-resolved collision-induced dissociation (CID). This work demonstrated that the intensity of the glucuronic acid neutral loss product as compared to the intensity of the intact precursor ion can be used to determine the percentage of each isomer present in a mixture. Overall, TWIMS successfully separated estradiol glucuronide isomers in positive ion mode and MS/MS *via* CID enables relative quantitation of each isomer in negative ion mode.

Graphical Abstract.

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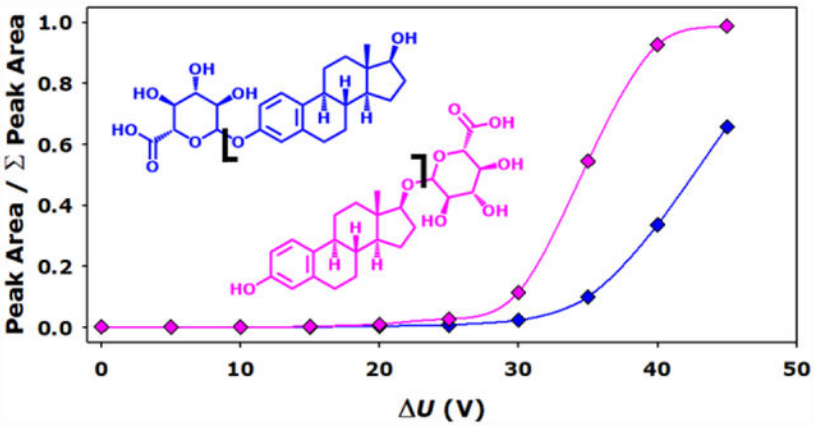
Supplementary Material

The online version of this article contains supplementary material, which is available to authorized users.



Keywords

Steroid Glucuronide Isomers; Traveling Wave Ion Mobility Spectrometry; Tandem Mass Spectrometry; Metal Ion Adduction

Introduction

Glucuronidation is a process that adds a glucuronic acid residue to non-polar substances, such as steroids, which allows them to be more easily transported and eliminated from the body [1]. Estradiol is an estrogenic steroid that can undergo glucuronidation creating estradiol glucuronides (EG) at two sites: the alcohol on carbon 3 near the aromatic ring, resulting in estradiol-3-glucuronide (E3G); or the alcohol on carbon 17 near the five membered ring, resulting in estradiol-17-glucuronide (E17G). These two isomers result from preferential glucuronidation by different enzymes: UDP-glucuronosyltransferases (UGTs) UGT1A3, UGT2B7 (H268), and human liver microsomes HL-15 [1]. Furthermore, E17G and E3G act as substrates for different enzymes resulting in different biological activities [2–5].

Previously, there have been reports of analytical techniques to distinguish isomeric steroids and steroid glucuronides. The primary method to separate various steroids and their respective conjugates has been liquid chromatography (LC); however, LC requires long acquisition times [2, 3, 6]. Additionally, sensitive immunoassays have been used to determine estradiol conjugates. Immunoassays tend to be relatively expensive and are subject to several potential confounding factors in complex matrices [7, 8]. More recently, ion mobility spectrometry (IMS) coupled to mass spectrometry (MS) has been employed to rapidly analyze steroid glucuronides.

IMS is a rapid gas phase separation method that uses an electric field and a buffer gas to separate molecules by size, shape, and charge [9]. IMS has been coupled to LC to separate testosterone and epitestosterone glucuronides [6]. Additionally, ion mobility has been used solely in conjunction with mass spectrometry to analyze EGs, where their deprotonated ions exhibited a slight difference in their ion-neutral collision cross sections (CCS), a quantitative property of the analyte and drift gas partner [10]. Nevertheless, the arrival time distributions

of the deprotonated EG isomers were nearly overlapped [11]. Chouinard *et al.* and Rister *et al.* previously reported the use of alkali metal adducts and the formation of dimeric species to increase the separation of steroids achievable with IMS-MS [12–15].

Additionally, tandem mass spectrometry (MS/MS) has been used as a means of distinguishing isomers. The most common MS/MS method is collision-induced dissociation (CID), wherein analyte ions are accelerated into neutral gas atoms or molecules. The resulting collisions convert kinetic energy into internal energy, ultimately resulting in fragmentation of the analyte ion. The fragmentation spectra can rapidly provide invaluable structural information, and in some cases can reveal which of a number of possible isomeric structures is most likely [16].

This report illustrates two methods, one in positive ion mode and one in negative ion mode, that allow for the distinction and quantitation of the EG isomers. In positive ion mode, travelling wave IMS (TWIMS) separates dimeric species sufficiently for quantitation by peak height. In negative ion mode, MS/MS by CID is successfully applied to allow for relative quantitation of the EG isomers in a mixture. Overall, this work expands on the currently available methods for EG isomer discrimination by providing very rapid and sensitive IMS-MS and MS/MS approaches.

Methods

Material sources are provided in Table S1 in the Supplementary Material. In short, solutions consisting of one EG isomer or a mixture of the two were mixed with group I metal acetate salts at a concentration of 10 μM total EG to 20 μM of salt in 50% water / methanol. These solutions were then directly infused through a nano-electrospray ionization source into a Waters Synapt G2-S (Milford, MA) undergoing TWIMS separation in positive mode. The TWIMS parameters of wave height, wave velocity, and gas flow was maintained at 40 V, 600 m/s, and 60 mL/min of nitrogen, respectively. CCSs were calibrated using known drift tube values from polyalanine in nitrogen drift gas using a quadratic fit ($t_d' = a\Omega'^2 + b\Omega' + c$) [17]. CID was performed in negative ion mode in the transfer cell using argon as the collision partner at collision energies ranging from 0 V to 45 V. All analyses were performed in four replicates on different days. The peak to peak resolution was calculated based on width at half-height as shown in Equation 1:

$$R_s = \frac{2.35\Delta t}{4w_{\mathrm{FWHM,avg}}}$$

Equation 1

Data was analyzed and visualized through the use of Drift Scope 2.7 and MassLynx 4.0 (Waters), Igor Pro 7.0 (WaveMetrics, Lake Oswego, OR), and SigmaPlot 13.0 (Systat, Chicago, IL).

Results and Discussion

Ion Mobility Spectrometry Separation.

The EG isomers were separated as standards and in a mixture with a resolution value greater than 1.0 as $[2M+Na]^+$ adducts. As seen in Figure 1a, the sodiated dimer adduct arrival time distributions (ATD) as single-component standards showed near-baseline separation, while a mixture of the isomers resulted in a decreased resolution ($R = 1.23$). The apparent decrease in resolution could be the result of heterodimer formation. If so, this could complicate the ability to perform quantitative analyses through this method. We also note that, while both components were infused at equivalent concentrations in the mixture, there were differences in their signal intensities. This could be caused by differing ionization efficiencies and / or potential post ionization fragmentation. CCS values were measured and plotted against the metal in the variety of adduct formations in Figure 1b. The resolution values, CCSs, and comparison of the standard and mixture CCSs are available in Tables S2–S4 in Supplementary Material. Notably, monomers in both positive and negative mode were poorly separated in mixtures, which can be seen in the resolution values provided in Table S2 and Figure S1 in the Supplementary Material. Overall, the separation of E3G and E17G as sodiated dimers was successfully demonstrated.

Tandem Mass Spectrometry.

As an alternative and complementary method of distinguishing the EG isomers, negative ion mode MS/MS was performed by applying CID to the deprotonated ions at *m/z* 447. Upon fragmentation of the deprotonated ions, neutral loss of the glucuronic acid resulting in *m/z* 271 was a major product ion for both isomers. However, the fraction of this loss increases with increased collision energy more sharply for E3G than E17G, as illustrated in Figure 2a (the corresponding raw values are presented in Table S5 of the Supplemental Material). The fraction of this loss is distinct between the isomers starting at collision energy 30 V. Additionally, the tandem mass spectrum of both isomers is shown at CE 40 V in Figure 2b, where the ratio of m/z 271 to m/z 447 is clearly higher in E3G's spectrum compared to E17G's spectrum. This difference was proposed to be a result of the aromatic ring of E3G stabilizing the glycosidic bond thus mitigating the loss of glucuronic acid, where E17G does not have access to this stabilization effect. As a result, the distinct energetics for glucuronic acid loss could be a tool for determining steroid glucuronide isomers in mixtures.

Relative Quantitation through Tandem Mass Spectrometry.

The isomer-specific energetics of glucuronic acid neutral loss was exploited for relative quantitation of each isomer in a variety of mixtures where the relative ratio of the isomers was systematically altered. Each mixture was subjected to MS/MS with CID energies of 30 V, 35 V, and 40 V. A scatter plot illustrating the fractional abundance of *m/z* 271 compared to *m/z* 271 + *m/z* 447 as a function of the percentage of E3G in the mixture is shown in Figure 3. From these results, a calibration curve was established to allow measurement of the relative amount of each compound in the mixture with high coefficients of determination ranging from 0.98 to 0.99. Additionally, due to the increasingly distinct dissociation spectra as collision energy increased, the higher collision energies resulted in a more sensitive method (*i.e.*, the plot for 40 V collision energy has a steeper slope than 30 V collision

energy). As a result, the extent of glucuronic acid loss can provide relative quantitation of the E3G and E17G isomers.

Conclusions

TWIMS-MS and MS/MS were shown to separate and relatively quantitate EG isomers, respectively. The sodiated dimeric adduct allowed resolution of $R = 1.23$ in positive ion mode analysis of a mixture. However, shifts in drift time may suggest the formation of a heterodimer that could potentially limit the ability to perform quantitation. In negative ion mode, distinct energy-resolved CID behavior of the EG isomers provided a method to determine the relative quantities of each isomer. We also note that, while both techniques employed here are post-ionization and thus susceptible to signal suppression in complex matrices, these techniques can also be coupled to LC for enhanced analysis. Ultimately, this work demonstrated the ability to apply IMS and MS/MS to the rapid analysis of the E3G and E17G isomers in a mixture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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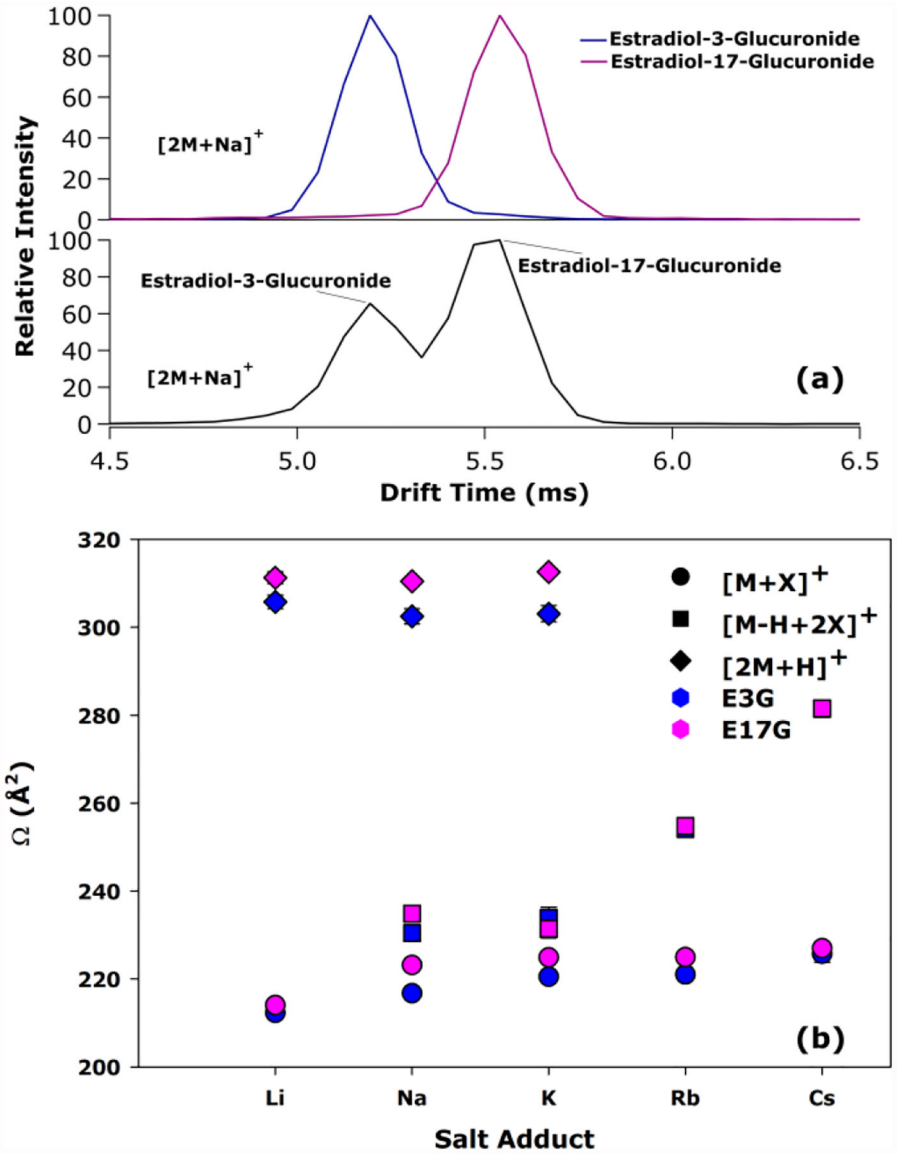

Figure 1.

Arrival time distributions for estradiol glucuronides standards (top) and mixtures (bottom) as their respective sodium adduct dimer in positive ion mode ***(a)***. Scatter plot of nitrogen collision cross sections for E3G (blue) and E17G (pink) as a function of the salt adduct (X = Li, Na, *etc.*) for [M+X] (circle), [M-H+2X] (square), and [2M+X] (diamond) in positive ion mode ***(b)***.

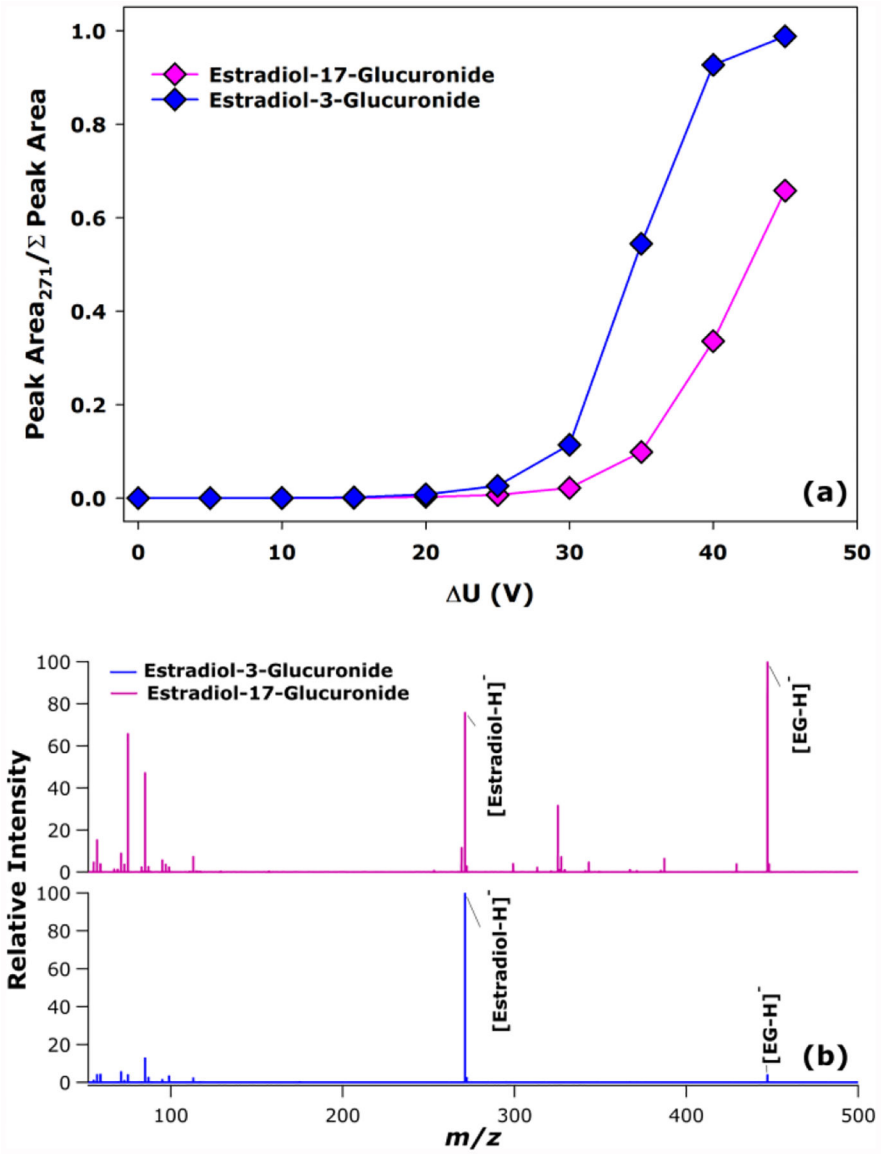

Figure 2.

Scatter plot of the fraction of the peak area of the fragment *m/z* 271 as a function of transfer collision energy ***(a)***. Tandem mass spectra of deprotonated E17G (pink, top) and E3G (blue, bottom) at collision energy 40 V ***(b)***.

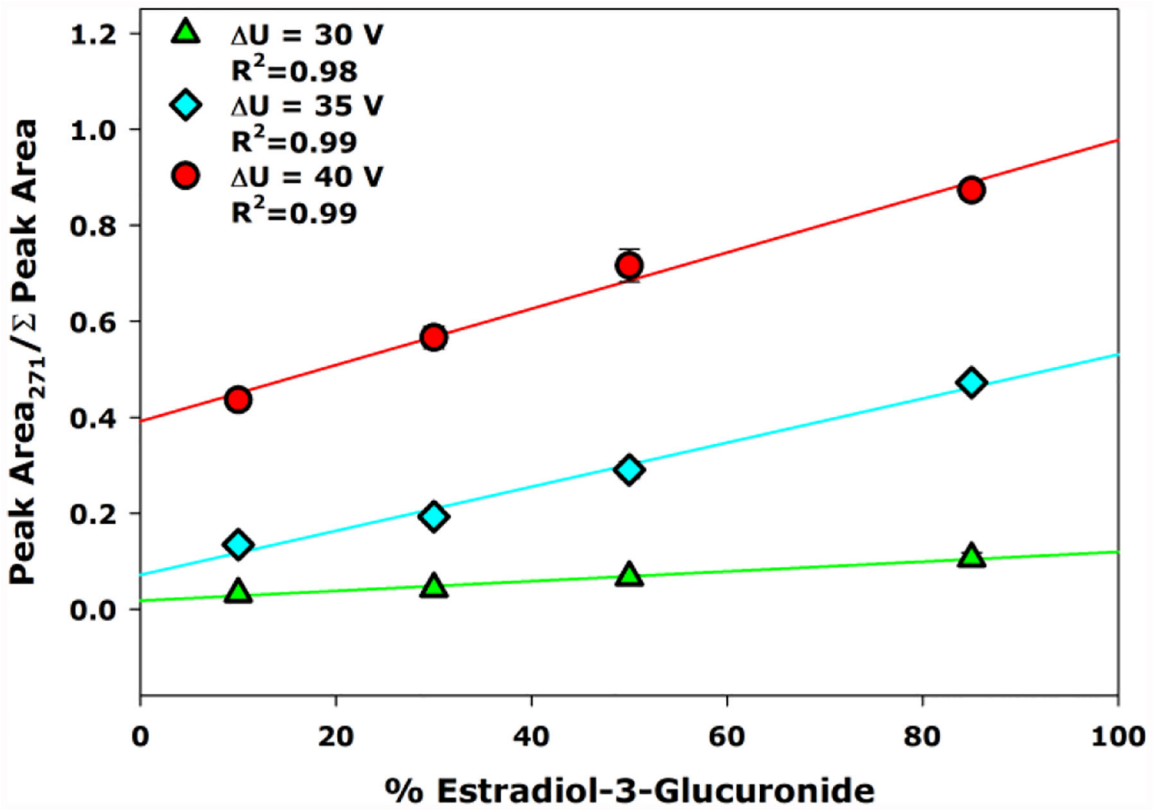

Figure 3.

Scatter plots with regression lines for the fraction of the peak area of *m/z* 271 as a function of the percentage of E3G compared to E17G in the solution at the collision energies of 30 V (green triangle), 35 V (cyan diamond), and 40 V (red circle).