Using Ion Mobility Mass Spectrometry in Conjunction With Derivatization Methods to Enhance the Separation of Different Steroid Isomers

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USING ION MOBILITY MASS SPECTROMETRY IN CONJUNCTION WITH DERIVATIZATION METHODS TO ENHANCE THE SEPARATION OF DIFFERENT STEROID ISOMERS

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ABSTRACT

One of the major problems facing analytical chemistry challenge is the isomers separation, due to their closely related molecular structures and physicochemical properties. Therefore, in this thesis, we have performed the separation of steroid isomers through the use of ion mobility mass spectrometry, using various chemical approaches. Ion mobility is an effective high-resolution separation techniques that utilizes the mobility of ions in the gas-phase environment. A useful tool for isomeric analysis due to the ability to separate different isomeric classes of steroids according to their varied collision cross-sections, which is achieved by taking advantage of differences in ion mobility.

Moreover, the derivatization reactions were employed to introduce distinctive functional structures to the steroid molecules, facilitating enhanced separation through ion mobility spectrometry. In this work, a range of derivatization techniques investigated in order to modify the structure, size and charge of steroid isomers and improve their resolution and detectability. These techniques include the use of “shift reagents” such as 1,1-carbonyldiimidazole, Girard’s Reagent P, dansyl chloride, and 4-(bromomethyl)benzenesulfonyl chloride.

To sum up, this thesis provides insight into the continued development and use of advanced techniques for the analysis of steroids, highlighting the role of ion mobility mass spectrometry and the advantage of derivatization reactions in the identification and separation of isomeric forms. The presented methodologies can be a great scientific interest to a large number of fields working with steroids and requiring accurate identification and quantification of isomeric forms, including but not limited to environmental monitoring and pharmaceutical, clinical, and forensic chemistry.

KEYWORDS

Isomers, ion mobility, mass spectrometry, derivatization, collision cross-sections
DEDICATION

This thesis should be dedicated to my family and my friends. A special feeling of gratitude to my parents, Father and my deceased mother, who have profoundly impacted my life. Although my mother is no longer with us, her memories continue to shape my existence. Thanks Mom for your endless love, I will never forget you and always pray for you. I dedicate this thesis to many of friends and church members who have stood by me through-out this journey.

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CHAPTER 1: INTRODUCTION

Steroids have long intrigued scientists. A unique molecular structure unites chemical molecules from the large class of steroids. The word “steroids” form a grammatically accurate description of these complicated molecules that are integral to many biological mechanisms. Steroids most likely derive from the Greek “stereos,” meaning solid and “-oid,” indicating likeness [1]. Steroid discovery can be traced back to the mid-1800s when a critical development occurred, thanks to the German scientist Adolf Butenandt. In 1855, he isolated a crystalline substance from urine and named it “androsterone” shortly after. As a result, the founding of the first steroid chemical gave researchers a remarkable opportunity to study the subject further. Butenandt and his assistant Hanisch did not have another momentous discovery until 1929 when they were able to isolate and synthesize androsterone [2]. This achievement pushed the field of steroid research to new frontiers and opened up new possibilities for researchers to explore the hormones’ biological implications [3].

Steroids were first identified in the adrenal glands above the kidneys. At the time, their numerous actions throughout the body were known and these included their role in maintaining the physiological balance, ranging from immune responses and metabolism to the reproductive axis control [4]. Because cholesterol is the most common precursor of steroids [5], all steroid hormones have a fundamental similarity in their basic structure because cholesterol has a single origin. However, the fundamental difference is that even small structural differences account for profoundly different biological roles and
interactions with the pathway [6], an aspect that makes their analytical separation challenging due to the basic structural similarities.

As the study progressed, scientists discovered a variety of steroid hormones with different functions and modes of action. However, much attention was paid to the role of testosterone, progesterone, and estrogen in the development of the reproductive system and its functioning [7]. Estrogen is the most interesting among these hormones, as it has a significant effect on the reproductive functioning and secondary sexual traits in females. However, the name of the hormone gives an example of the interweaving of ancient languages and modern science: the word estrogen is derived from the ancient Greek word “andr-” , which means man, and “sterone” , meaning steroid [8].

Steroids are not only involved in physiological processes, but hormone derivatives have found their use in pharmacology and medicine. Completely synthetic steroid hormones have acquired the status of indispensable tools in the treatment of various diseases – from inflammatory to hormonal disorders [9]. Steroids perform a variety of functions and treat various abnormalities. Corticosteroids, for example, help reduce inflammation in patients with dermatitis or asthma and arthritis; their anti-inflammatory properties help alleviate symptoms and increase patients’ quality of life [10]. Steroid hormones are also used in hormone replacement therapy for testosterone and estrogen deficiency, as well as to relieve menopausal symptoms, hypogonadism and other hormone-based diseases. Immunosuppressive drugs such as prednisone and dexamethasone are used to regulate immune responses and reduce autoimmunity progression – a condition for which steroids are often used in clinical treatment. Such
steroids as cortisol and prednisone are indispensable in severe allergies and anaphylaxis since they quickly relieve symptoms and prevent a reaction that can be fatal to the patient [11].

However therapeutic their applications might be, steroids have gained infamy as they have long become the object of abuse and misuse in both bodybuilding and sports. The use of synthetic testosterone derivatives, anabolic steroids, began to be linked to improved athletic performance resulting in fierce debates and legislative initiatives [12]. Research continues in the pharmaceutical industry to tailor steroid effects to minimize side effects and pave the way to new therapeutic usage. Whether they are utilized to combat hormonal deficiencies or as an anti-inflammatory means, steroids have a place in modern medicine. Long-term use of corticosteroid could have devastating effects on bone health. It results in interference with bone remodeling processes, such as osteoporosis and a higher chance of fractures [13]. Steroids use is tied to metabolic whims, such as weight increases, excessive hunger, and substantial changes in blood sugar levels [14].

The combination of these factors may expose an individual to the risk of metabolic syndrome and subsequent development of diabetes. In addition, the use of steroids results in a person’s immunosuppression that increases the susceptibility to infections and delays the healing of wounds . Since steroid consumption has been proved to be a major cause of psychological effects such as mood changes and psychiatric morbidity, the person needs to be observed cautiously and receive psychological assistance. Moreover, several steroids, notably anabolic androgenic steroids, present severe health
risks due to their correlation with cardiovascular diseases, including dyslipidemia, cardiomyopathy, and hypertension [15].

**Functional Structure of Steroids**

The steroid structure includes three fused cyclohexane rings and one cyclopentane ring arranged around the molecule’s core, which is characterized by so-called a sterane. This core of steroids is responsible for their strength and robustness, providing a suitable environment for the biological activity of steroids found in nature. Steroids are biochemically distinctive and active in that they contain functional groups such as hydroxyl (-OH), carbonyl (>C=O), and Vol C=C- (Figure 1-1). Functional groups make steroids chemically active chemicals, allowing them to perform functions other than their intrinsic structure [16].

![Figure 1-1: Estrone and Testosterone representing steroids to show the primary chemical functionality which include hydroxyl, carbonyl, methyl, and alkene chemistry.](image)

In the organism, metabolic changes, steroid-protein interactions, and physiological reactions are all mediated by functional groups. For instance, carbonyl groups support
the reactivity and metabolic conversions of steroids, but hydroxyl groups are necessary for their solubility and bioavailability. The biological activity of steroids is attributed to certain chemical interactions that are made possible by these functional groups, which are arranged strategically throughout the molecule [17]. Additionally, side chain changes and variants give steroids a functional variety that further affects their pharmacokinetics and pharmacodynamics. Lipophilicity, receptor binding affinity, and metabolic stability are all regulated by heteroatoms, aromatic rings, and alkyl side chains that are integrated into the steroid structure. The physiological actions and medicinal uses of steroids are further varied by the presence of particular functional groups at different points along the carbon skeleton [18]. The wide range of biological activities and pharmacological effects of steroids are supported by their chemical functionality. Steroids serve essential roles in metabolic control, inflammation, and cell signaling pathways. They can regulate reproductive processes through steroidal hormones, and they can modulate immunological responses through corticosteroids. Steroids' oxidation state, which is determined by the degree of carbon-carbon bond saturation or hydrogenation, affects both their physiological effects and potential therapeutic uses [19].

Functional groups in the organism mediate metabolic changes, steroid-protein interactions, and physiological reactions. For example, although the carbonyl groups enable steroids’ reactivity and metabolic transformations, the hydroxyl groups are required for its solubility and bioavailability. Notably, the steroids’ biological activity is based on certain chemical interactions, that become possible through these functional groups’ strategic placement throughout the molecule[17]. More importantly, the changes in the side chain and the variations make steroids function compact, and thereby further
affect its pharmacokinetics and pharmacodynamics. Heteroatoms, aromatic rings, and alkyl side chains incorporated into the steroid’s structure regulate lipophilicity, receptor binding affinity, and metabolic stability. Furthermore, the presence of certain functional groups at distinct positions along the carbon skeleton not only diversifies steroids’ physiological effects but their medicinal use as well. Chemical functionality sustains steroids’ wide range of biological activities and pharmacological effects [18]. It imperative to indicate that steroids contribute to multiple metabolic pathways, inflammation, and various cell signaling activities. They also regulate reproductive functions through steroidal hormones and modulate immunological responses via corticosteroids. Steroids’ oxidation state, consisting of the level of carbon-carbon bond saturation or hydrogenation, also determines their physiological effects and medicinal application [19].

One of the major constraints in analyzing steroids is the low levels of their presence that is commonly found in biological samples. Steroids are found at low levels in biological samples. Analyses must be very sensitive to detect precisely low levels to address this issue. Furthermore, biological matrices like blood, urine and tissue have sources of numerous endogen compounds that can prevent steroids from being measured. Matrix interferences should be avoided in analytical study of steroids. Matrix interferences may account for inaccurate analytic results, i.e. false-positives or false-negatives. The collection of accurate and dependable analytic results requires study organizers to overcome matrix interferences. The solution to matrix interferences requires study organizers to use sample preparation techniques and instruments [20].
Analysis of Steroids and their Challenges

There are many obstacles to overcome in the analysis of steroids for both analysts and researchers. The complex molecular structures of steroids, which are typified by several fused rings and functional groups, are at the core of these difficulties. Because of this structural complexity, complex analytical methods that can resolve isomeric or stereoisomeric forms and differentiate between closely related compounds are required. Accurate analysis requires the ability to distinguish between structural variants, but doing so is difficult because these molecules have minor variances [21].

The challenges associated with the analysis of steroids have been significantly mitigated by technological improvements. Immunoassays (IAs) has been known to be commonly used for the detection of steroids. In contrast to GC-MS and LC-MS techniques, Immunoassays provide immediate analysis without requiring urine extraction, hydrolysis, or derivatization. Major metabolites and other 17-alkyl-substituted anabolic steroids can be detected simultaneously thanks to the significant cross reactivity of antibodies in testosterone Immunoassays. Immunoassays can be useful as a preliminary test prior to Mass spectrometry confirmation in situations where a rapid, low-cost, and straightforward test can be employed for numerous sample analyses in a short amount of time [22].

One of the many well-known drawbacks of Immunoassays is their high cross-reactivity values. In clinical assays, cross-reactivity values of five percent or more were noted for several anabolic steroids. \([\text{IC}_{50} \text{(interest analyte)}/\text{IC}_{50} \text{(analyte reference)} \times 100]\) is the formula used to compute cross-reactivity values, where \(\text{IC}_{50}\) stands for half-maximal inhibitory concentration [23]. When comparing the results to MS-based approaches, seven out of ten Immunoassays with testosterone values less than 2.30 ng/mL showed
different results [24]. The concentration of testosterone in immunoassays is five times higher than reported in other investigations [25]. Furthermore, reports have indicated compromised accuracy at doses below 55 ng/dL and measurement bias ranging from -14.2 to 63.8% [26]. When detecting substances by immunoassays methods, specificity is reduced due to analytical bias caused by inadequate antibody specificity, non-uniform calibration, and lack of assay linearity [23]. Immunoassays limits of quantification (LOQ) are frequently constrained by non-specific binding (NSB) [27]. LOQs for testosterone can be from 100 pg/mL to 19.9 ng/mL depending on the immunoassays method, but GC-MS can only measure as low as to 0.25 pg/mL [28].

The method based on liquid chromatography mass spectrometry LC-MS provides superior functional sensitivity and selectivity, rendering it an important tool for steroids detection. LC-MS is unrivaled in its capacity to identify steroids in complicated matrices with high precision and is widely used in analytical laboratories. Gas chromatography mass spectrometry is an important tool in the examination of steroids, particularly for volatile and heat-stable compounds. The analysis of steroids using GC-MS has high degree separation and detection ability, but there is such limitations as time-consuming processes such like as derivatization.

For targeted quantification in those with the greatest sensitivity applications, a high duty cycle achieved by triple quadrupole MS [29]. At the moment, triple quadrupole based mass spectrometry (MS) is used in conjunction with complementary LC-MS and GC-MS techniques to test for steroids. To be both better selectivity and sensitivity, triple quadrupole needs to be acquired in selected reaction monitoring SRM mode; however, prior knowledge of the precursor and product ions as well as the collision energies is
required. A precursor ion selected by the first quadrupole (Q1) and fragmented in the collision cell (Q2). Before being detected by the electron multiplier, a vivid and particular fragment/product ion is picked in the next quadrupole (Q3) during SRM [30].

Due to their excellent resolution and throughput, high performance liquid chromatography (HPLC) techniques combined with ultra violet or fluorescence detection are also frequently used for the analysis of steroids. Steroid analysis has broad implications that go well beyond laboratory settings, affecting anti-doping programs, forensic science, and the medical community. Ensuring patient safety, preserving sports integrity, and adhering to regulations all depend on accurate and trustworthy analysis. Future studies should therefore concentrate on strengthening analytical methods, raising detection limits, and tackling new issues with steroids analysis.

**High Resolution/High Accuracy Mass Spectrometry Analysis**

High resolution/high accuracy mass spectrometry systems including time of flight MS, Orbitrap MS, Fourier transform ion cyclotron resonance MS offer increased mass resolution and mass accuracy than conventional mass spectrometers. These facilities allow advanced ionization methods to generate ions from steroids in the gas phase. Examples comprise atmospheric pressure chemical ionization APCI and electrospray ionization ESI. The accurate measurement of molecular masses and the determination of fragmentation patterns are simplified by the analysis of the product via subsequent mass analysis, enabling steroid structural characterisation and identification [31]. The preparation of sample preparation, chromatograph separation, and mass spectrometric detection are all included in HR/HA-MS techniques for steroid analysis. High resolution or high accuracy mass spectrometry systems have revolutionized the steroid analysis in
several areas. In clinical chemistry, pharmaceutical research, and sports doping monitoring, electronic mail, steroids have been detected more precisely using HR/HA-MS imaging. Therefore, it has been necessary to detect endogenous steroids with high precision, such as testosterone and cortisol, hormone problems in clinical samples and monitor therapeutic interventions in clinical settings. In pharmaceutical research, it has made it easier to conduct pharmacokinetic profile and drug metabolism investigations and identify impurities during drug development using HR/HA-MS. Sports doping control contributes to fair competition and protects the integrity of athletes by detecting synthetic steroids and their metabolites in urine and blood samples with meager amounts of selectivity and sensitivity [32].

Steroids can be drawn out from biological matrices with the least amount of matrix effects possible by using optimum procedures for sample preparation such as liquid to liquid extraction (LLE) or solid phase extraction (SPE). Using ultra-high-performance liquid chromatography or high performance liquid chromatography for chromatographic separation improves analyte sensitivity and peak resolution. Steroid mass-to-charge ratios (m/z) and isotopic patterns can be precisely quantified by HR/HA-MS detection, providing consistent and repeatable results [33].

High resolution mass spectrometry HRMS has great mass accuracy and resolving power (Rp) while maintaining sensitive and selective during the full-scan mode acquisition. Quadrupole time-of-flight (Q-TOF) offer a mass accuracy of roughly 1-2 ppm and a Rp > 10,000 full width at half maximum (FWHM), whereas orbitraps have slower scanning speeds that are inversely related to resolution, but have mass fidelity in the sub ppm range and a R > 100,000 [34]. Correct peak picking, mass spectrum deconvolution, and
orthogonal chromatography procedures necessitate high spectra per second. Because Q-TOFs have a greater scanning speed of >6000 complete spectra per second, they can be useful when recording numerous acquisitions in parallel or when ion mobility is being used [35]. Additionally, it works well with ultra high performance liquid chromatography, which reduces run times and boosts sensitivity [36]. Fast chromatographic techniques are incompatible with Fourier transform ion cyclotron resonance mass spectrometry since it can only sweep over one scan per second or fewer, despite the fact that it offers greater Rp capabilities of up to 1 million [37].

Compared to Q-TOF mass accuracy of around 1-2 ppm and resolution R = 30000 to 50000, orbitraps have a mass accuracy in the sub-ppm range and Rp > 1,000,000 [38]. In a particular study, the orbitrap MS and Q-TOF MS performance features were compared for the quantification of steroids. For the examined steroid profiles, the LODs for both instruments were much lower than 50% of the sensitivity limit. Even though GC-Q-Orbitrap showed less matrix effects from urine samples, both devices proved to be reliable enough for regular analytical screening and anti-doping testing [39]. Although Orbitrap-MS offered better dynamic range and resolution at slower scanning rates, its primary drawback is the difference in time between the fast IMS separation and the more leisurely Orbitrap MS acquisition [40].

**Analysis of Steroids; Ion Mobility Mass Spectrometry**

While the present GC-MS and LC-MS/MS (HRMS) technology is quite sensitive, it is not suitable for high throughput for most biological sample analysis and is only useful for targeted quantification of known steroid metabolites. Furthermore, volatilized compounds which are typically derivatized with trimethyl silane derivatives following
enzymatic hydrolysis of their metabolites are typically required for the laborious preparation of sample required for steroids analysis by GC-MS [41]. The common stereochemical isomer and steroids constitutional fragmentation patterns that result in comparable chromatographic retentions and fragmentation patterns make separation by GC-LC-MS/MS/MS alone difficult [42].

A new method that has showed promise for better analysis and the ability to more accurately identify unknowns in complicated biological samples is ion mobility spectrometry (IMS) [43]. Targeted quantification of known steroid metabolites and worldwide analysis of untargeted analytes are provided by IM-MS/MS. Additionally, high-throughput steroids analysis is compatible with it. IMS is a promising method for gas-phase separation that separates ions due to variations in their charge state, size, and shape [44].

Unlike chromatographic methods, which can take hours or even longer and may not be able to reliably separate different steroid species in mixtures, IMS operates on a millisecond separation time scale. Nonetheless, IMS can be simply integrated to add an extra layer of separation to the existing GC and LC-MS workflows (LC-IM-MS) because of its quick separation periods [45]. The collision cross section of an ion, or its rotationally averaged area as it tumbles through a buffer gas, can be measured using IM-MS. CCS is often expressed in either nanometers (nm$^2$) or square angstroms (Å$^2$), contingent upon the target analyte’s dimensions. Because it offers a supplementary separation dimension between chromatography-based separation and CCS, knowing an ion's CCS enables more certain identification and characterization even in the presence of changing chromatographic conditions.
In addition to providing structural elucidation of novel or undiscovered steroids and their short and long-term metabolites, IM-MS can differentiate isomeric species. Analytical throughput can be maintained while incorporating IM-MS into current anti-doping procedures to facilitate analyte identification and detection [46].

Types of Ion Mobility Mass Spectrometry

One of the earlier IM-MS techniques is Drift Tube Ion Mobility Mass Spectrometry (DTIMS), (Figure 1-2) which separates ions according to their mobility using a drift tube filled with gas while being subjected to a constant electric field. Ions can be separated due to their size and shape because the length of time it takes them to go through the drift tube is related to their collision cross-section (CCS). DTIMS is an excellent tool for analyzing metabolites and small compounds in complicated mixtures [47]. Given that larger mobility, the lower the CCS, the resulting ion drift time is inversely proportional to the CCS. CCS values can be assigned to small compounds, such as steroids, to increase the confidence in their identification. DTIMS separation is carried out in a device that is normally filled with a homogeneous inert gas under a weak electric field (≤20 V/cm). The ability to get CCS values directly from measured drift times by using empirical techniques. Assuming negligible ion heating between collisions (low-field settings), the Mason-Schamp equation provides the relationship between measured drift time and CCS.

Drift times are measured as a function of experimental parameters, which includes drift tube length, buffer gas pressure and temperature, electric field strength, the drift gas species, and mass of the analyte ion. By using a single-field approach, which has been demonstrated to produce highly repeatable CCS with an average absolute bias of 0.54% in comparison to standardized step field CCS values, DTIMS can be integrated into the
chromatographic timeframe. Additionally, in contrast to scanning equipment, DTIMS permits a thorough analyte collection [48].

![Diagram of IM-MS instrument](image)

**Figure 1-2:** The prototype instrument for IM-MS. (A) An image of the ion mobility component's optical components. (B) A schematic of the equipment used, with key components identified [49].

Another type of ion mobility separator is using a traveling wave ion guide, Traveling Wave Ion Mobility Spectrometry (TWIMS) divides ions into groups according to how easily they move through a buffer gas. Ions of various sizes and shapes move at various speeds along a drift region in TWIMS as a result of a succession of traveling waves. TWIMS is perfect for proteomics and structural biology applications since it provides faster analysis times and higher resolution than DTIMS. The time spent in the device relates to the CCS. TWIMS requires a calibration curve for the purpose of calculating the CCS of unknown
analytes using a sequence of chemicals whose CCS values were previously reported by DTIMS. With TWIMS, a number of research works have effectively verified the profiles and separation of stereoisomeric steroid pairs [50]. By using RF confinement to focus the ion packet as it migrates across the drift region, TWIMS provides reduced ion diffusion. Because the waves are always the same height, it also requires low voltage. The development of instruments that would otherwise require unfeasible voltage requirements that is, a power supply of more than 120,000 V has been made possible by this low voltage demand [51].

Ion mobility can be spatially separated using differential mobility spectroscopy (DMS), which referred to as high-field asymmetric waveform Ion Mobility Spectrometry (FAIMS), which enables ion filtration before mass spectrometry (MS) detection. Ions are driven forward by use of an oscillating asymmetric field. Ions within the device disperse due to this differential field because of the difference between their low-field and high-field mobilities. Ions can be selectively focussed by applying a compensation voltage (CV) to one of the electrodes. This CV can be changed statically or dynamically to allow for more extensive scanning across a range of ion mobilities or more focused separation of ions of interest [52].

Time-dispersive techniques that use a single ion pulse for the ion mobility dispersion process include DTIMS (differential ion mobility spectrometry) and TWIMS (travelling wave ion mobility spectrometry). This requires the use of ion gates in conjunction with a continuous ionization source, such as electrospray ionization (ESI). The entrance gate opens first to let ions build up for about 1–10 ms, and then the exit gate opens for a brief while (100–200 μs). An ion packet can enter the drift region with
this sequence, where ions scatter over time based on their collision cross section (CCS). To avoid spectral overlap, all of the ions in the system must reach the detector before starting the next cycle. Because of this, the exit gate stays closed until the preceding ion packet in the drift region has fully dispersed. Low ion utilization results from signal averaging across numerous pulse events to produce appropriate signal intensity, given the normal ion accumulation durations (~5 ms) [53].

The duty cycle in conventional DTIMS configurations usually stays below 10%. Reducing the length of time the gate is pulsed open in order to achieve better resolutions further constricts the ion packet and lowers the duty cycle. Average repetitions are often used to make up for the low duty cycle’s effect on signal intensity, but at the expense of slower data collecting rates. Fortunately, tailored analysis performance is not impaired due to the high selectivity of IMS devices. In order to effectively analyze complicated mixtures with the necessary sensitivity, a duty cycle that is more efficient is necessary. In order to satisfy the needs of evaluating complex sample compositions, this innovation would enable faster data collecting without reducing signal quality [54].

An alternate technique to raise ion utilization efficiencies to roughly 50% without compromising separating power is ion multiplexing. Several ion pulses are injected into the IMS during each data duty cycle as part of this method. The Hadamard binary sequence is used to modulate the ion packets. When the proper hadamard transform (HT) is applied to the data, the IMS deconvoluted spectrum is recreated [55]. As opposed to pulsed mode, IMS duty cycle in HT mode has demonstrated a 200-fold increase and a 10-fold drop in LOD [56]. The signal obtained from an HT-IMS experiment consists of drift spectrum superposition. As a result, multiplexing raises instrument sensitivity and S/N.
It has been demonstrated that fourier transform (FT) approaches can improve IMS duty cycle by multiplexing; however, they necessitate the functioning of a second ion gate, which can be accomplished physically or through software [57]. No physical instrument adjustments are needed to adapt an IMS system for hadamard-type multiplexing, and implementing the Hadamard algorithm takes less computing power than the Fourier approach [58].

Other compounds with comparable structural characteristics that can be the focus of additional research can be found with the help of structural clarification and mobility-mass correlations. Furthermore, it has been shown that the structure (and accompanying CCS) for steroids may be determined by computer modeling based on density functional theory, with predicted CCS values within <2% of the experimental values [59]. Complexes, various ion adducts (such as transition metals, alkali, and alkaline earth), and steroids examined with various buffer gases have all been skillfully modeled in our group. A comprehensive methodology that combines computational modeling, machine learning algorithms, and experimental LC-IM-MS technologies may be able to simplify the identification of substances that will be detected in the future and allow for targeted quantitation of known steroids [60].

Reference:


https://doi.org/10.1093/clinchem/42.7.1001


https://doi.org/10.1093/icb/icp015


https://doi.org/10.3390/stresses3020033


http://doi.org/10.1007/s43032-022-00932-z


https://doi.org/10.1152/advan.00086.2006


https://doi.org/10.1007/s00535-018-1439-1


https://doi.org/10.52711/0974-4150.2021.00078


https://doi.org/10.3390/medicina59081439


https://doi.org/10.1542/peds.2007-3381


https://doi.org/10.1016/j.siny.2019.05.005

21. Viljanto, M.; Gray, B.; Scarth, J., Presence, and detection of endogenous steroids in the horse—A review. *Drug Testing and Analysis* *n/a* (*n/a*). 
https://doi.org/10.1002/dta.3624

https://doi.org/10.1016/j.aca.2006.03.108.


CHAPTER 2: BREAKING BOUNDARIES IN STEROID ISOMERS SEPARATION: ION
MOBILITY MASS SPECTROMETRY AND ADVANCED DERIVATIZATION

Abstract

Ion mobility-mass spectrometry (IM-MS) has been used a lot more recently, mostly because reliable equipment from several vendors is now widely available. Nevertheless, the routine separation of constitutional and stereochemical isomers has been hampered by the low resolving power of many platforms, which is usually between 40 and 60. While equipment improvements have occasionally allowed resolving power to exceed 150, chemical techniques offer an other way to improve resolution with current IM-MS systems.

Here, we investigate how well the ion mobility separation of challenging steroid isomers, including Testosterone, 1-testosterone and Epitestosterone, 4-androstenediol Androsterone, Etiocholanolone, Epiandrosterone and 5-androstenediol using parallel derivatization with 1,1-carbonyldiimidazole and Girard's reagents. On other hand estrogen isomers can be enhanced by two derivatization reactions: dansyl chloride derivatization and 4-(bromomethyl)benzenesulfonyl chloride derivatization. These reactions provide simple (only minimal laboratory equipment and knowledge are needed), quick (less than 30 minutes), and cost-effective chemicals. Interestingly, these reactions target hydroxyl groups, which are common in naturally occurring steroids, exhibiting structural selectivity. These reactions can highlight small structural differences and improve IM resolution since the quantity, position, and stereochemistry of these functional groups varies in many steroid isomers.
Our findings show notable improvements in resolution between the isomer groups. Additionally, the use of typical collision cross section ($^{\text{DT}}\text{CCS}_{\text{N}_2}$) measurements might increase the level of assurance when recognizing these substances in intricate complex mixtures. In conclusion, our findings show that the combination of selective derivatization processes and IM-MS is a powerful approach for significantly enhancing the resolution of challenging isomer groups, enabling more accurate and effective studies of complex mixtures.

**Introduction**

Steroids are crucial for biomolecular analysis as biomarkers in many diseases. In endocrinology, reliable and timely testing of the steroid hormones is vital simply because, for many conditions, the clinic’s findings do not clarify the diagnosis: too many conditions carry similar symptoms, e.g., pain or headache [1]. Therefore, proper analytical testing is important in ensuring the correctness of the final diagnosis and the plans for accurate medical treatment. Furthermore, it should be mentioned that cholesterol is the source of all endogenous steroid hormones [2]. The steroid molecules show various constitutional and stereochemical isomers, which result from the complex and interconnected biosynthetic pathways of steroids and results from synthetic steroids’ structural analogs. All endogenous steroids have a common structural foundation; they comprise three six-member rings and one five-member ring (Figure 2-1) [3].
Figure 2-1: Structures of androgeneous Estrogens as representative structures for steroids composed of three six-member rings (red) and one five-member ring (blue).

A wide range of analytical techniques is currently employed in the field of endocrine hormone analysis. These include immunoassays, chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy. Among them, IA and GC or LC-MS/MS remain the most popular methods for steroid analysis [4]. IA is commonly used due to its high-throughput, cost effectiveness, and user friendliness. However, it does have its drawbacks: performing IA is time- and money-consuming; more frequently, IA loses specificity after manufacture and storage due to brief lifespan of immunoreagents, and there is noticeable lack of intralaboratory reproducibility [5]. Thus, LC–MS/MS became an undisputed gold standard, with high precision, ability to analyze numerous analytes in one sample simultaneously, and much less interference from the substantial structural similarities between steroid hormones [6].

Ion mobility (IM) spectrometry is a method that measure the mobility of ions in the gas phase under the influence of an electric field while crossing a buffer gas [7]. Mobility
of ion (K) is dependent on its charge, size, and form. The Mason-Schamp equation can be used to determine an ion's collision cross section (CCS) by measuring its mobility (K) and a number of experimental parameters [8]. The remarkable repeatability of these CCS values and their ability to fill experimental databases increase identification confidence in complex sample sets [9]. IM measurements can be completed within tens of milliseconds, enabling the technique to seamlessly integrate between liquid chromatography and mass spectrometry LC-IM-MS without prolonging analysis time [10]. The ability to filter data based on ion mobility/CCS can reduce chemical noise from co-eluting isobaric or isomeric species by adding a further dimension of separation into the study. The signal-to-noise ratio and detection limits are improved by this method [11].

AS recently reviewed by Dodds et al. , a widespread analysis of steroid isomers has been accomplished in various uses of IM-MS [3]. Earlier works applied the same to separate and evaluate testosterone and epitestosterone glucuronides using both the IM-MS and UHPLC separations [12]. Furthermore, Ahonen et al. [13] demonstrated that p -toluenesulfonyl isocyanate derivatization improves separation of three isomer pairings α and β -estradiol, 3α and 3β -androsterone, and 17α and 17β -testosterone [14]. Additional DMS, an altered IM method, has shown that five endogenous hormonal steroids and steroids like progesterone and corticosterone can be accurately measured in this method. In 2018, Hernandez-Mesa et al. [15] also built a database of 300 steroids, including free steroids, conjugates, and esters. These authors also completed additional validations for steroids on several platforms and laboratories [16]. Furthermore, other work conducted by our group involved further endogenous steroid isomers [17], glucocorticoids [18],
calciferol metabolites [19], fentanyl [20], cannabinoid [21] collectively and anabolic steroids as anti-doping control [22].

Despite the potential offered by IM-MS workflows, traditional IM techniques still face challenges in routine separation. Because steroid hormone isomers have similar molecular sizes and shapes, the low resolving power (~50–60) frequently proves insufficient for differentiating between them. But in the last few years, more advanced IM systems designed specifically for steroid analysis have emerged. These include cyclic IMS (cIMS) [23], trapped IMS (TIMS) [24] and Structures for Lossless Ion Manipulations (SLIM) [25], and all of which have resolving powers more than 150. In the absence of new instrumentation, alternative strategies are being explored to address the IM "isomer problem." These include post-processing procedures for data and chemical approaches like functionalization or derivatization. Our group has shown different chemical approaches targeting alkene, hydroxyl, and carbonyl chemistry. This has improved quantification and structural elucidation as well as significantly improved IM resolution of steroid isomers [26].

Our group has focused on derivatizing androgenic steroids with 1,1-carbonyldiimidazole and Girard’s reagents, which target hydroxyl and carbonyl groups, respectively [27]. This paper explores different steroid classes possessing multiple carbonyl and hydroxyl groups situated in various locations on the steroid molecule, as we have investigated earlier with different derivatization reagents. These reactions have an established place in LC–MS work-flows as they can enhance ionization efficiencies, alter retention times in chromatography, increase and lower chromatographic polarity
depending on the mode, and/or create unique MS/MS fragmentation patterns based on overall molecular structure and bond energies [28]. These reactions are fully used in many analytical laboratories, and their necessity is evident due to the carbonyl and/or hydroxyl groups present in every naturally occurring steroid molecule. Because many isomers differ exclusively in the number and location of these functional groups, their utility becomes abundantly apparent. Consequently, such reactions are ideal for identifying small structural distentions and refining IM separation.

**Experimental methods: Materials and reagents**

All estrogen standards purchased from cayman chemical in (Ann Arbor, MI) as powders and prepared as 1 mgmL⁻¹ stock solutions in acetonitrile. Similarly, 1-testosterone, testosterone, and epitestosterone standards purchased from cerilliant corporation in (Round Rock, TX), 1 mgmL⁻¹ as solutions in acetonitrile. The rest of steroids standards were also purchased from cayman chemical (ann Arbor, MI) as powders and prepared a 1 mgmL⁻¹ as solutions in acetonitrile: androsterone, etiocholanolone, 4-androstenediol, epiandrosterone, and 5-androstenediol. 1,1-carbonyldiimidazole, Girard’s Reagent P, Dansyl Chloride, and Bicarbonate buffer were purchased from Fisher Scientific in Bridgewater, NJ. Other solvents water w/0.1% formic acid, methanol, acetic acid, acetonitrile were Fisher Scientific Optima LC-MS grade in (Pittsburgh, PA).

**Preparation for Sample**

Steroid stock solutions prepared by dissolving 1 mg of steroid powders in methanol to achieve a concentration of 1 mg/mL. Each solution was then stored in vials at a temperature of -20 °C. These standard solutions remained stable for at least two months.
In the analysis, only stock solutions within these two months’ time frame were utilised; any older solutions were excluded. Before each analysis, the stock solutions were tested to ensure that the estrogen standards provided consistent results, similar to those obtained when freshly prepared.

For the study of testosterone, epitestosterone, androsterone, etiocholanolone, 4-androstenediol, epiandrosterone and 5-androstenediol we employed a parallel derivatization approach by adapting existing methodologies for 1,1-carbonyldiimidazole and Girard’s reagent P. [27] The CDI reaction involved combining 100 μL of an 8 mgmL⁻¹ CDI solution in acetonitrile with 895 μL of additional acetonitrile and 5 μL of a 1 mg/mL steroid stock solution. The resulting mixture was incubated at 60 °C for 30 minutes and then dried down under a nitrogen stream at room temperature. Subsequently, the dried sample underwent the Girard’s Reagent P reaction, which entailed combining 100 μL of a 1 mgmL⁻¹ Girard’s Reagent P solution in methanol with 250 μL of methanol containing 10% acetic acid. The mixture was sonicated at room temperature for 15 minutes, dried down under nitrogen at room temperature, and finally reconstituted in 1 mL of mobile phase A.

The derivatization of estrogen with dansyl chloride, 4-(bromomethyl)benzenesulfonyl chloride was performed according to the previous method [29], Briefly, To the 5μL of sample, 100 μL of 0.1 M sodium bicarbonate buffer (pH at 9.0) and 100 μL of dansyl chloride solution (1 mgmL⁻¹ in acetone) were added. After vortexing, the sample was heated at 60 °C for 5 min to form the dansyl derivatives. The mixture was
dried under nitrogen gas at room temperature, and finally reconstituted in 1 mL of mobile phase A.

**Instrumentation and Tuning**

All experiments were carried out using an Agilent 6560 IM-QTOF coupled with a 1290 Infinity II UHPLC system (Santa Clara, CA). Samples (10 μL) were directly injected into an Agilent ZORBAX Extend-C18 column (2.1 × 50 mm, 1.8 μm) at a temperature of 40°C. The mobile phase consisted of two components: (A) water containing 0.1% formic acid, and (B) methanol. The flow rate set at 0.400 mL min⁻¹ with a total runtime of 10 minutes. The mass spectrometry data was collected in full scan mode for the range of m/z 100 to 3200. The ion mobility (IM) drift tube was maintained using nitrogen gas at an approximate pressure of 4 Torr and a temperature of 25°C. Ion mobility measurements were acquired using a single-plexed acquisition method. The ion funnel trap was filled for 3900 μs and released after 150 μs.

**Data processing**

All CCSs were measured using the established single-field method, which involves measurement of Agilent Tune Mix ions to determine β (slope) and tfix (y-intercept) [30]. For quantitative analysis, all peak areas were integrated using the Agilent MassHunter Qualitative analysis software B.08.00.

**Results and discussions**

This study demonstrating the significance of including the Ion Mobility for separation, as most steroid isomers remained unresolved within the chromatographic region. This observation may lead to shorter retention durations in the chromatographic
area, which would enable faster analysis. This study suggest that Ion Mobility is a potentially very useful tool for solving steroid isomers that are difficult to separate in the chromatographic region. Furthermore, Ion Mobility may improve overall analytical efficiency by reducing the need for extended retention durations in chromatography.

**Steroid isomer group selection**

The chosen compounds for analysis were purposefully selected to exemplify the efficacy of the described methods. Their structural similarities were taken into careful consideration, as most of these compounds have a historical record of posing intricate challenges in ion mobility (IM) separations.

**Parallel derivatization using CDI and GP reagents**

The derivatization with 1,1'-carbonyldiimidazole involves a series of reactions targeting the hydroxyl groups present in these steroids. Steroids such as testosterone and epitestosterone. Testosterone, contains three hydroxyl groups, while epitestosterone, its epimer, shares a similar hydroxyl group distribution. The reaction happens through the nucleophilic attack of CDI on these hydroxyl groups, resulting in the formation of stable carbamate derivatives. Upon reaction with CDI, each hydroxyl group undergoes derivatization, leading to the formation of carbamate derivatives at the respective positions. At the C-3 position, CDI reacts with the hydroxyl group to form a carbamate derivative. Similarly, CDI reacts with the hydroxyl groups at the C-17 positions, resulting in the formation of carbamate derivatives at these sites as well.

These derivatization reactions play a critical role in enhancing the stability and detectability of testosterone and epitestosterone in analytical procedures. The
introduction of carbamate groups through CDI derivatization improves the properties of these steroids, allowing for more efficient separation and detection in complex biological or environmental samples.

On other hand, Girard's reagent P, is a versatile compound commonly used in analytical chemistry for the derivatization of functional groups, particularly carbamate groups. This reagent is named after its inventor, Louis N. Girard reagent contains two functional groups: a hydrazine moiety and a quaternary ammonium group. Its chemical structure allows it to react specifically with carbamate groups, forming stable derivatives amenable to detection and analysis by various analytical techniques. The reaction between GP reagent and carbamate groups proceeds through nucleophilic attack by the hydrazine moiety on the carbonyl carbon of the carbamate functional group. This results in the formation of a hydrazone derivative, which is characterized by its stability and compatibility with analytical methods such as liquid chromatography, gas chromatography, and mass spectrometry. One of the key advantages of GP reagent is its high specificity towards carbamate groups. This selectivity enables the derivatization of carbamate-containing compounds without interference from other functional groups present in the sample matrix. The derivatives formed through the reaction between GP reagent and carbamate groups exhibit enhanced detectability and chromatographic properties compared to the parent compounds. The introduction of the hydrazone moiety imparts unique physicochemical properties to the derivatives, resulting in improved retention, resolution, and sensitivity during analysis.

In the pursuit of advancing ion mobility (IM) resolution and ionization efficiency, individual derivatization reactions, specifically employing 1,1-carbonyldiimidazole,
Girard’s Reagent P methodologies, were systematically investigated. A selection of challenging steroid isomers, including Testosterone, 1-testosterone and epitestosterone. Other group of steroid isomers namely 4-androstenediol androsterone, etiocholanolone, epiandrosterone and 5-androstenediol served as model compounds for this study. The individual CDI and GP derivatization reactions exhibited discernible enhancements in both IM resolution and abundance for the aforementioned steroid compounds.

The testosterone isomer group provided an additional avenue to investigate the impact of CDI derivatization on gas phase structural measurements via ion mobility. Testosterone and epitestosterone, while structurally similarity apart from their C17 hydroxyl stereochemistry, offered distinct insights. The enhancements in IM resolution for the testosterone group are shown in figure 2-2B.

Furthermore, when the mixture included 1-testosterone, a noticeable reduction in the CDI product of testosterone was observed, as illustrated in (Figure 2-2C). The second reaction explored involved the use of Girard’s Reagent P for derivatization, a process in which its group of hydrazino selectively reacts with carbonyl groups (Figure 2-3A). Girard’s reagents are commonly favored for derivatization due to their inclusion of a fixed-charge quaternary amine on their pyridinium moiety, enhancing ionization efficiency, sensitivity, and limits of detection. As anticipated, derivatization led to a prominent base peak, with the [M+GP]^+ ion detected at m/z 422.280 for both testosterone and epitestosterone isomers.

In contrast to the CDI reaction, which was expected to introduce increased structural diversity through targeting the stereochemistry of the hydroxyl group, it was predicted that the planar nature of the carbonyl group relative to the steroid ring might not
yield significant benefits in ion mobility separation. This hypothesis was validated by the results, which indicated no improvement in mobility resolution. However, the primary advantage of Girard's Reagent P derivatization lay in its enhanced ionization efficiency. This was immediately apparent, even with an approximate yield of 80% (based on a comparison of steroid abundance before and after derivatization), as the integrated peak area of the product ion [M+GP]⁺ at m/z 422.280 exhibited a nearly tenfold increase in abundance. Such enhanced abundance was consistently observed across nearly all measured steroids.

![Reaction conditions showing the successful 1,1-carbonyldiimidazole derivatization of (i) Testosterone and (ii) epitestosterone with major protonated products at m/z 405.215. (B) The [M+CDI]⁺ species shows baseline separation between testosterone and epitestosterone based on the CCS 187.9 Å² and 206.2 Å². (C) [M+CDI]⁺ species of products showing low yield of epitestosterone in a mixture.](image)

Figure 2-2: (A) Reaction conditions showing the successful 1,1-carbonyldiimidazole derivatization of (i) Testosterone and (ii) epitestosterone with major protonated products at m/z 405.215. (B) The [M+CDI]⁺ species shows baseline separation between testosterone and epitestosterone based on the CCS 187.9 Å² and 206.2 Å². (C) [M+CDI]⁺ species of products showing low yield of epitestosterone in a mixture.
Figure 2-3: (A) Reaction conditions showing the successful Girard's reagent P derivatization of (i) Testosterone and (ii) epitestosterone with major protonated products at m/z 422.280. (B) The [M+GP]+ species with no separation (C) [M+GP]+ species of products in a mixture with no separation.

In a strategic endeavour to synergistically harness the benefits of these individual reactions, parallel derivatization reactions were executed. This concerted approach yielded a prominent product ion, [M+CDI+GP]+, at m/z 516.297 for Testosterone and Epitestosterone isomers. Furthermore, the parallel reaction product demonstrated a sustained level of IM separation, successfully resolving the intricate isomer set (Figure 2-4B). We employed parallel derivatization on Androsterone and Etiocholanolone which resulted in major protonated products at m/z 518.313. The [M+CDI+GP]+ species shown
a baseline separation between Androsterone and Etiocholanolone based on the CCS 243.5 Å² and 223.4 Å² (Figure 2-4).

**Figure 2-4:** (A) Reaction conditions showing the successful parallel derivation using 1,1-carbonyldiimidazole and Girard’s reagent P derivatization of (i) testosterone and (ii) epitestosterone with major protonated products at m/z 516.297. (B) The [M+CDI+GP]+ species shows baseline separation between Testosterone and Epitestosterone based on the CCS 249.8 Å² and 223.9 Å². (C) The separation of [M+CDI+GP]+ species of products in a mixture.
Figure 2-5: (A) Reaction conditions showing the successful parallel derivatization using 1,1-carbonyldiimidazole and Girard’s reagent P derivatization of (i) androsterone and (ii) etiocholanolone with major protonated products at m/z 518.313. (B) The [M+CDI+GP]^+ species shows baseline separation between androsterone and etiocholanolone based on the CCS 243.5 Å² and 223.4 Å². (C) The separation of [M+CDI+GP]^+ species of products in a mixture.

The reaction conditions for the parallel derivatization using 1,1-carbonyldiimidazole (CDI) and Girard’s Reagent P were carefully maintained to ensure successful derivatization of another set of steroid compounds, including etiocholanolone, epiandrosterone, and androsterone. The major protonated products for these compounds were observed at m/z 518.313.
The derivatization process involved specific reaction conditions tailored for each reagent to ensure efficient and selective derivatization of the target compounds. For CDI derivatization, appropriate concentrations of CDI and reaction buffers were employed to facilitate the reaction with the carbonyl groups present in the steroid molecules. Similarly, for Girard's Reagent P derivatization, the reaction conditions were optimized to promote the hydrazino group's selective reaction with the carbonyl groups.

The success of the derivatization reactions was confirmed by the observation of prominent protonated products at the specified mass-to-charge ratios (m/z) for each compound, indicating the formation of the desired derivatives. These derivatization reactions enabled the enhancement of ionization efficiency and facilitated subsequent analysis by ion mobility spectrometry. The [M+CDI+GP]+ species exhibited notable separation between isomers based on their collision cross section (CCS) values. Collision cross section is a measure of the gas-phase ion's size and shape and is influenced by the ion's mass, charge, and conformation. By derivatizing the steroid compounds with both CDI and Girard's Reagent P, the resulting derivatives displayed distinct CCS values, allowing for effective separation and characterization of isomeric compounds.

The separation based on CCS values provided valuable structural information about the isomeric compounds, enabling the identification and differentiation of closely related steroid species. This enhanced separation capability facilitated more accurate and comprehensive analysis of complex mixtures containing multiple steroid isomers.

The separation of [M+CDI+GP]+ species of products in a mixture was demonstrated, illustrating the effectiveness of the derivatization strategy for resolving complex mixtures.
of steroid compounds. By subjecting the mixture to ion mobility spectrometry analysis after derivatization with both CDI and Girard's Reagent P, distinct peaks corresponding to different derivatized species were observed in the ion mobility spectra.

Additionally, 5-androstenediol yielded a protonated product at m/z 478.258, while 4-androstenediol yielded a protonated product at m/z 385.249. The discrepancy in the mass-to-charge ratios (m/z) of the protonated products derived from 5-androstenediol and 4-androstenediol during parallel derivatization with 1,1-carbonyldiimidazole (CDI) and Girard's Reagent P (GP) can be explained by the absence of a carbonyl group and presence of alkene group in 4-androstenediol. As we anticipated that the derivatization with GP reagent would not happen to both 5-androstenediol and 4-androstenediol lack carbonyl group, we observed only the compound reacting with CDI (figure 2-4A (iv) and (v). 5-Androstenediol, lacking an alkene or aromatic structure, contain a hydroxyl group susceptible to reaction with both CDI. In the derivatization process. Consequently, the protonated product formed from 5-androstenediol exhibits a higher m/z value, observed at m/z 478.258.

Conversely, 4-androstenediol possesses an alkene group. Only CDI, which reacts with hydroxyl group on five membrane ring, can effectively derivatize 4-androstenediol. The derivatization with CDI results in the attachment of the derivative to specific functional groups unique to 4-androstenediol, leading to a modified molecular structure with a different mass compared to 5-androstenediol. This yields a distinct protonated product with a lower m/z value, observed at m/z 385.249.
Figure 2-6: (A) Reaction conditions showing the successful parallel derivatization using 1,1-carbonyldiimidazole and Girard’s reagent P derivatization of (i) etiocholanolone (ii) epiandrosterone, (iii) Androsterone with major protonated products at m/z 518.313. (iv) 5-androstenediol and (v) 4-androstenediol yielded protonated products at m/z 478.258 and m/z 385.249 respectively. (B) The [M+CDI+GP]* species shows separation between isomers based on the CCS values. (C) The separtion of [M+CDI+GP]* spicies of products in a mixture.
Derivatization with Dansyl Chloride

In this study, two isomer groups; each consists of three isomers were selected because their structural differences are in the position arrangement of the functional groups such as hydroxyl, carbonyl, and alkene groups. Groups A containing (i) 17-epiestriol isomer $C_{18}H_{24}O_3$, MW. 288.173 Da; (ii) 2-Hydroxyestradiol isomer $C_{18}H_{24}O_3$, M.W. 288.173 Da; and (iii) 4-Hydroxyestradiol isomer $C_{18}H_{24}O_3$, MW. 288.173 Da each isomer with its molecular weight 288.173 Da because the positions of the hydroxyl groups in them are different. Group B containing ; (i) 16α-hydroxy Estrone isomer $C_{18}H_{22}O_3$, M.W. 286.157 Da; (ii) 2-hydroxy Estrone isomer $C_{18}H_{22}O_3$, MW 286.157 Da; and (iii) 4-hydroxy Estrone isomer $C_{18}H_{22}O_3$, MW. 286.157 Da. such as these isomers differ in the position of the hydroxyl group as shown in (Figure 2-7)

![Figure 2-7: Estrogen isomers which differ in the position of hydroxyl group.](image-url)
Since the aim of this study is to enhance the separation for those challenging isomers using derivatization reactions, dansyl chloride used as a first set of our derivatization reactions. These simple reactions are commonly used to improve ionization and chromatographic retention in many GC-/LC-MS techniques and improving resolution. Dansyl chloride, has been demonstrated to selectively react with phenolic alcohols such as those found in several of these estrogen isomers. This derivatization reaction was applied to these isomers, for which the reaction happened all of these isomers because of the presence of phenolic alcohol group. The reaction products were clearly identified as its dominant protonated [M+ H]⁺ ions at m/z 522.231 for 17-epiestriol, both dansylated products of 2-Hydroxyestradiol and 4-Hydroxyestradiol identified as its protonated [M+ H]⁺ ions at m/z 755.282. While, the dansylated product for 16α-hydroxy Estrone identified as its protonated [M+ H]⁺ ions at m/z 520.216. Then, the dansylated products of 2-hydroxy Estrone and 4-hydroxy Estrone identified as its protonated [M+ H]⁺ ions at m/z 753.267 (Table 2-1).

Table 2-1: Theoretical Exact m/z and Experimentally Measured CCS for the Dansylated Products.

<table>
<thead>
<tr>
<th>Isomers</th>
<th>Formula</th>
<th>m/z[M+H]⁺</th>
<th>DTCCSN₂ (Å²) [M+H]⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-epiestriol</td>
<td>C₁₈H₂₄O₃</td>
<td>522.231</td>
<td>240.2±01</td>
</tr>
<tr>
<td>2-Hydroxyestradiol</td>
<td></td>
<td>755.282</td>
<td>273.4±01</td>
</tr>
<tr>
<td>4-Hydroxyestradiol</td>
<td></td>
<td>755.282</td>
<td>273.5±01</td>
</tr>
<tr>
<td>16α-hydroxy Estrone</td>
<td>C₁₈H₂₂O₃</td>
<td>521.216</td>
<td>240.2±01</td>
</tr>
<tr>
<td>2-hydroxy Estrone</td>
<td></td>
<td>753.267</td>
<td>273.5±01</td>
</tr>
</tbody>
</table>
To investigate the capabilities of the importance of derivatization in separation of these isomers, investigation of individual isomers and mixtures was performed (Figure 2-8). The dansylated compounds were best separated as protonated, with the 17-epiestriol isomer baseline resolved from the others; however, the similarity in dansylated product structure of the 2-hydroxyestradiol and 4-hydroxyestradiol isomers precluded their baseline separation (Figure 2-8B). Investigation of the protonated products allowed separation of the 17-epiestriol isomer, but significant overlap was observed 2-hydroxyestradiol and 4-hydroxyestradiol isomers, whose $^{DT}\text{CCS}_{N2}$ values of 273.4 and 273.5 Å$^2$ differed by 0.04%; as such, neither dansylated product resulted in definitive resolution of all three isomers. Noteworthy observations included the emergence of multiple mobility peaks for certain species such as dansylated product of 17-epiestriol and this phenomenon, characterized by multiple peaks with similar mass spectra, is postulated to arise from the conformational flexibility inherent in the derivatized steroids. When run in mixtures, the derivatized product shows mobility peaks indicating 17-epiestriol isomer well separated from 2-hydroxyestradiol and 4-hydroxyestradiol isomers and this confirmed by looking on the peaks pattern and CCS values which corresponded to those when run individually.

On other hand, the 16α-hydroxy estrone isomer exhibited complete baseline resolution from the others, showcasing the efficacy of this technique. However, the structural similarities in the dansylated products of the 2-hydroxy estrone and 4-hydroxy
estrone isomers posed a challenge, impeding their baseline separation (as depicted in Figure 2-9B). While the analysis of protonated species allowed for successful separation of the 16α-hydroxy estrone isomer, a notable issue arose with the 2-hydroxy estrone and 4-hydroxy estrone isomers. Despite efforts, no clear separation was observed between these two isomers. Their close similarity was evident in their respective $^{DT}\text{CCS}_N$ 273.5 and 274.5 Å$^2$ values for 2-hydroxy estrone and 4-hydroxy estrone isomers respectively, with only a marginal difference of 0.36%. Upon examining mixtures, however, a more nuanced picture emerged. Here, the derivatized products displayed mobility peaks that suggested the 16α-hydroxy estrone isomer's distinct separation from the 2-hydroxy estrone and 4-hydroxy estrone isomers. This observation a detailed analysis of the peak patterns and CCS values, which consistently mirrored those obtained when the compounds were analyzed individually.
Figure 2-8: (A) Reaction conditions showing the successful dansyl chloride derivatization of (i) 17-Epiestriol, (ii) 2-Hydroxyestradiol, and (iii) 4-Hydroxyestradiol with major protonated products at m/z 522.231, 755.282, 755.282 respectively. (B) The [M+H]+ species shows baseline separation between 17-Epiestriol and the others based on the m/z 522.231 and the CCS 240.2 Å². (C) [M+H]+ species of dansylated products showing the separation in a mixture.
**Figure 2-9:** (A) Reaction conditions showing the successful dansyl chloride derivatization of (i) 16α-hydroxy Estrone, (ii) 2-Hydroxy Estrone, and (iii) 4-Hydroxy Estrone with major protonated products at m/z 521.216, 753.267, 753.267 respectively. (B) The [M+H]+ species shows baseline separation between 16α-hydroxy Estrone and the others based on the m/z 521.216 and the CCS 240.2 Å² and also a slight separation between 2-Hydroxy Estrone, and 4-Hydroxy Estrone based on their CCS 273.5 Å² and 274.5 Å². (C) [M+H]+ spcies of dansylated products showing the separation in a mixture.
Derivatization with 4-(bromomethyl)benzene sulfonyl chloride

To optimize separation, we employed 4-(bromomethyl)benzene sulfonyl chloride as a derivatizing agent, resulting in the formation of sodiated species. Both individual isomers and mixtures underwent thorough analysis to assess the effectiveness of this approach, as depicted in (Figure 2-10).

Upon derivatization, notable improvements in separation were observed between isomers, including 17-Epiestriol, 2-Hydroxyestradiol, and 4-Hydroxyestradiol. Each isomer exhibited distinct resolution from the others, highlighting the precision of the chosen derivatizing agent in facilitating clear differentiation between them with $^{\text{DTCCS}}_{\text{N2}}$ 237.9, 267.6 and 262.3 Å$^2$ respectively (Figure 2-10B).

Despite the structural similarities between the 2-hydroxyestradiol, and 4-hydroxyestradiol isomers, their derivatized forms displayed remarkable separation, overcoming previous challenges with dansyl chloride reaction. This enhanced separation was evident in the discernible mobility peaks observed in mixtures, unequivocally indicating the individual presence of each isomer. The use of 4-(bromomethyl)benzene sulfonyl chloride as a derivatizing agent proved highly effective in improving the separation of the 17-epiestriol, 2-Hydroxyestradiol, and 4-hydroxyestradiol isomers. Again, 16α-hydroxy estrone, 2-hydroxy estrone, and 4-hydroxy isomers exhibited distinct resolution from the others, for a clear separation between them with $^{\text{DTCCS}}_{\text{N2}}$ 237.9, 267.6 and 262.3 Å$^2$ respectively (Figure 2-11B).
Figure 2-10: (A) Reaction conditions showing the successful 4-(bromomethyl)benzene sulfanyl chloride derivatization of (i) 17-Epiestriol, (ii) 2-Hydroxyestradiol, and (iii) 4-Hydroxyestradiol with major sodiated products at m/z 543.082, 775.001, 775.001 respectively. (B) The [M+Na]+ species shows baseline separation between 17-Epiestriol and the others based on the m/z 543.082 and the CCS 237.9 Å² and also a separation between 2-Hydroxyestradiol and 4-Hydroxyestradiol based on their CCS 267.6 Å² and 262.3 Å². (C) [M+Na]+ species of products showing the separation in a mixture.
Table 2-2: Theoretical Exact m/z and Experimentally Measured CCS for products derivatized with 4-(bromomethyl)benzene sulfonyl chloride.

<table>
<thead>
<tr>
<th>Isomers</th>
<th>Formula</th>
<th>m/z[M+Na]+</th>
<th>m/z[M+Na]+ $^{DT}\text{CCS}_{N_2}$ (Å$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-epiestriol</td>
<td>C$<em>{18}$H$</em>{24}$O$_3$</td>
<td>543.082</td>
<td>237.9 ±01</td>
</tr>
<tr>
<td>2-Hydroxyestradiol</td>
<td></td>
<td>775.001</td>
<td>267.6 ±01</td>
</tr>
<tr>
<td>4-Hydroxyestradiol</td>
<td></td>
<td>775.001</td>
<td>262.3 ±01</td>
</tr>
<tr>
<td>16α-hydroxy Estrone</td>
<td>C$<em>{18}$H$</em>{22}$O$_3$</td>
<td>541.066</td>
<td>237.9 ±01</td>
</tr>
<tr>
<td>2-hydroxy Estrone</td>
<td></td>
<td>772.985</td>
<td>267.6 ±01</td>
</tr>
<tr>
<td>4-hydroxy Estrone</td>
<td></td>
<td>772.985</td>
<td>262.3 ±01</td>
</tr>
</tbody>
</table>
**Figure 2-11:** (A) Reaction conditions showing the successful 4-(bromomethyl)benzene sulfonyl chloride derivatization of (i) 16α-hydroxy estrone, (ii) 2-hydroxy estrone, and (iii) 4-hydroxy estrone with major protonated products at m/z 541.066, 772.985, and 772.985 respectively. (B) The [M+Na]+ species shows baseline separation between 16α-hydroxy estrone and the others based on the m/z 541.066 and the CCS 237.9 Å² and also a slight separation between 2-hydroxy estrone, and 4-hydroxy estrone based on their CCS 267.6 Å² and 262.3 Å². (C) [M+Na]+ species of products showing the separation in a mixture.
CHAPTER 3: CONCLUSION AND FUTURE DISCUSSION

This thesis addresses the challenge of separating and characterizing steroid isomers in analytical chemistry. By leveraging ion mobility spectrometry (IMS) and various chemical approaches, the research explores innovative strategies to enhance separation efficiency. IMS emerges as a promising technique, capitalizing on the distinct mobility of ions in gas-phase environments to achieve high-resolution separation. Through exploiting differences in collision cross-sections, IMS effectively discriminates between different classes of steroid isomers. Additionally, derivatization reactions play a crucial role in introducing unique functional groups to steroid molecules, thereby improving their resolution and detectability in IMS. Various derivatization techniques, including the use of reagents such as 1,1-carbonyldiimidazole, Girard's reagent P, dansyl chloride, and 4-(bromomethyl)benzenesulfonyl chloride, are explored to modify the physicochemical properties of steroid isomers. Overall, this research contributes to advancing analytical methodologies for steroid analysis, offering valuable insights into the synergistic integration of IMS and derivatization reactions. The methods developed hold promise for diverse applications in the fields such as environmental monitoring, pharmaceutical analysis, and clinical diagnostics, where accurate identification and measurement of steroid isomers are crucial.

Reference:


Endocrinology & Metabolism 2013, 98 (10), 3971-3973. https://doi.org/10.1210/jc.2013-3375


20. Aderorho, R.; Chouinard, C. D., Improved separation of fentanyl isomers using metal cation adducts and high-resolution ion mobility-mass spectrometry. Drug Testing and Analysis n/a (n/a). https://doi.org/10.1002/dta.3550


