Effects of structural characteristics of (un)conjugated steroid metabolites in their collision cross section value

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ABSTRACT

In this work, the collision cross section (CCS) value of 103 steroids (including unconjugated metabolites and phase II metabolites conjugated with sulfate and glucuronide groups) was determined by liquid chromatography coupled to traveling wave ion mobility spectrometry (LC-TWIMS). A time of flight (QTOF) mass analyzer was used to perform the analytes determination at high-resolution mass spectrometry. An electrospray source of ionization (ESI) was used to generate $[M+H]^+$, $[M+NH_4]^+$ and/or $[M-H]^-$ ions. High reproducibility was observed for the CCS determination in both urine and standard solutions, obtaining RSD lower than 0.3% and 0.5% in all cases respectively. CCS determination in matrix was in accordance with the CCS measured in standards solution showing deviations below 2%. In general, CCS values were directly correlated with the ion mass and allowed differentiating between glucuronides, sulfates and free steroid although differences among steroids of the same group were less significant. However, more specific information was obtained for phase II metabolites observing differences in the CCS value of isomeric pairs concerning the conjugation position or the α/β configuration, which could be useful in the structural elucidation of new steroid metabolites in the anti-doping field. Finally, the potential of IMS reducing interferences from the sample matrix was also tested for the analysis of a glucuronide metabolite of bolasterone (5β-androstan-7α,17α-dimethyl-3α,17β-diol-3-glucuronide) in urine samples.

KEYWORDS

Isomeric compounds; ion mobility; anti-doping; urine analysis; collision cross section

1. INTRODUCTION

Anabolic androgenic steroids (AAS) are prohibited in sports by the World Anti-Doping Agency (WADA) [1] due to their potential to enhance performance in a large variety of sports activities, which makes the use of these substances quite common. Their widespread use and demand lead to the continuous synthesis of new compounds. For this reason, this group of substances is the most studied and detected in urine samples during doping control analysis. The misuse of endogenous compounds is especially hard to detect, due to the difficulties differentiating the administration from the natural production [2, 3]. Besides, steroids are extensively metabolized by the human body, so they can be detected in urine in their free form, as phase I metabolites or conjugated with hydrophilic groups like glucuronide and sulfate (and to a lesser extent, with other groups such as cysteine) forming phase II metabolites [4, 5]. Thus, the thorough study of their metabolic pathways is of high importance to improve the confidence in analytical results. In this context, considering the ever-changing wide variety of AAS and their structural similarities, the unequivocal identification of each metabolite

poses an analytical challenge in the anti-doping field.

Anti-doping laboratories traditionally analyze steroids by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS), using sample treatments that involve a hydrolysis step to release the phase I metabolite, followed by the derivatization of the unconjugated form [6-8]. However, the enzymes used to cleave the glucuronide and sulfate groups are sometimes unable to efficiently hydrolyze some metabolites [9]. As glucuronides and sulfates are easily ionized by electrospray ionization (ESI), direct analysis by liquid chromatography-mass spectrometry (LC-MS) seems to be an ideal technique to obtain a complete view of all phase II metabolites that could be present in urine [10-13].

The abovementioned techniques are used to obtain identification parameters such as the retention time and specific mass spectrometric data (*i.e.* characteristic fragments), with the purpose to separate matrix interferences and to characterize the structure of the analytes of interest. In some cases, the selectivity provided by these techniques may not be sufficient for the reliable and complete identification of isomeric compounds or analytes that present unspecific fragmentation pattern. For example, Esquivel et al. [14, 15] described three sulfate metabolites after testosterone administration, by monitoring the transition *m/z* 371→97, that allowed the identification of these metabolites as isomers of androstanediol sulfate. Unfortunately, fragmentation of sulfate metabolites of steroids provides unspecific information and structural features like the conjugation position or the α/β configuration could not be determined. Thus, the discovery of new metabolites by non-targeted analysis [16] often needs further information to perform a tentative structural elucidation and/or to complement the data available.

In the last years, the use of instrumentations based on ion mobility mass spectrometry (IMS) caught the attention of researchers in various analytical fields [17-22], including the analysis of steroids for anti-doping purposes [23-27]. This technique presents the potential to provide an additional separation dimension, as molecules characterized by different mass, charge and shape pass through the IMS cell assuming different mobilities. The dependence of the ion mobility on the three dimensional conformation of the molecule makes also IMS especially powerful to resolve steroid isomeric compounds [23, 28, 29]. In this context, different studies presented the improved separation of isomers and epimers of steroids by IMS, monitoring monomeric or dimeric adduct ions formed with cations of the group I [30-35]. IMS has also been shown to reduce chemical noise arising from biological samples, enhancing sensitivity and selectivity of the methods [23, 24, 30, 36, 37]. Techniques such as drift-tube ion mobility spectrometry (DTIMS) and traveling wave ion mobility spectrometry (TWIMS) also allow to obtain structural information through the determination of the collision cross section (CCS) [38]. The nature of the measurement makes the CCS a matrix-independent parameter, allowing its use as an additional identification point for the characterization of a large variety of analytes in different matrices. The beneficial incorporation of an extra identification point into the criteria for the discovery and detection of anabolic steroids make it pivotal to have extensive and comprehensive databases. In 2018, Hernandez-Mesa et al. [39] presented the first CCS

database for steroids, subsequently cross validated performing inter-laboratory and inter-platform studies [40]. CCS values of various steroids, including some phase II metabolites, were also measured from other authors [23, 26, 37]. However, a wide number of steroids are not yet characterized in terms of CCS and more studies replicating CCS measurement with different instruments and in different matrices are required to guarantee the reliability of reported CCS values. Moreover, the interest using CCS values as structural-characterization parameter to support identification purposes has increased in the last years [18, 19] and attempts have been made using Quantum Chemistry Calculation [41] and computational calculation [27].

In this work, the CCS determination of 103 anabolic steroid metabolites and other related steroids (including 19 sulfate conjugates, 28 glucuronide conjugates and 56 unconjugated metabolites commercially available) was performed using a LC-TWIMS method. As a preliminary study, some structural information was gathered for phase II metabolites that could be useful for the structural elucidation of unknown steroid metabolites in anti-doping. For some compounds, the analysis was performed in both standard and urine samples to evaluate matrix effects and reproducibility of the CCS determination. Finally, the urine analysis of a glucuronide metabolite of bolasterone (5β-androstan-7α,17α-dimethyl-3α,17β-diol-3-glucuronide) was discussed presenting the improved selectivity provided by CCS measurements.

2. MATERIALS AND METHODS

2.1 Standards and reagents

For the database development, 103 endogenous and exogenous steroids were selected, including unconjugated steroids and phase II metabolites conjugated with glucuronide (G) and sulfate (S) groups. All these substances are listed below, and their structures are shown in **Figure S1** (**Supplementary Information**).

Unconjugated -56-: 17α-estradiol (α-ES), 17β-estradiol (β-ES), estrone (ESO), stanozolol (STAN), 3'-hydroxystanozolol (3-OHSTAN), 4α-hydroxystanozolol (4α-OHSTAN), 4β-hydroxystanozolol (4β-OHSTAN), 16β-hydroxystanozolol (16β-OHSTAN), progesterone (P), 11α-hydroxyprogesterone (11α-OHP), 17α-hydroxyprogesterone (17α-OHP), testosterone (T), epitestosterone (EpiT), 1-testosterone (1-T), 2α-hydroxytestosterone (2α-OHT), 4-hydroxytestosterone (4-OHT), methyl-1-testosterone (m1-T), methyltestosterone (mT), 1-androstenedione (1-AND), androstenedione (AND), 4-hydroxyandrostenedione (4-OHAND), 6α-hydroxyandrostenedione (6α-OHAND), 17β-nandrolone (17β-NAN), 17α-nandrolone (17α-NAN), oxandrolone (OXA), epioxandrolone (EpiOXA), trenbolone (TREN), epitrenbolone (EpiTREN), boldenone (BOLD), epiboldenone (EpiBOLD), bolasterone (BOL), calusterone (CAL), clostebol (CLO), norclostebol (NCLO) stenbolone (STEN), methylstenbolone (mSTEN) gestrinone (G), tetrahydrogestrinone (THG), mibolerone (MIB), norandrostenedione (NAND), methenolone (METH), fluoxymesterone (FLU), 6β-hydroxyfluoxymesterone (6β-

OHFLU), methandienone (MED), epimethandienone (EpiMED), 6β-hydroxymethandienone (6β-OHMED), 4-chloromethandienone (CMED), 6β-hydroxy-4-chloromethandienone (6β-OHCMED), norethandrolone (NTA), norethisterone (NTI), oxymesterone (OXYM), methyldienolone (mDIEN), methyltrienolone (mTRIEN), 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one (FLUm), 5β-androst-1-ene-17β-ol-3-one (BOLDm) and 4-androsten-17α-methyl-11α,17β-diol-3-one (mDiol).

Sulfates (S) -19-: 17α-estradiol 3-sulfate (α-ES-3S), 17β-estradiol 3-sulfate (β-ES-3S), 17β-estradiol 17-sulfate (β-ES-17S), 17α-nandrolone 17-sulfate (17α-NAN-S), 17β-nandrolone 17-sulfate (17β-NAN-S), 17β-boldenone 17-sulfate (BOLD-S), 19-norandrosterone 3-sulfate (NA-S), 19-noretiocholanolone 3-sulfate (NE-S), testosterone sulfate (T-S), epitestosterone sulfate (EpiT-S), androsterone sulfate (A-S), epiandrosterone sulfate (EpiA-S), 6β-hydroxyandrostenedione sulfate (6β-OHAND-S), etiocholanolone sulfate (Etio-S), dihydrotestosterone sulfate (DHT-S), 11-ketoetiocholanolone sulfate (11-KE-S), 5-androsten-3α-ol-17-one 3α-sulfate (DHA-S), 5-androsten-3β-ol-17-one 3β-sulfate (DHEA-S) and 5α-androstan-3β,17β-diol 17-sulfate (5αββ-Diol 17-S).

Glucuronides (G) -28-: 17β-estradiol 3-glucuronide (β-ES-3G), 17β-estradiol 17-glucuronide (β-ES-17G), estrone glucuronide (ESO-G), 17β-nandrolone 17-glucuronide (17β-NAN-G), 17β-boldenone 17-glucuronide (BOLD-G), 19-norandrosterone 3-glucuronide (NA-G), 19-noretiocholanolone 3-glucuronide (NE-G), testosterone glucuronide (T-G), epitestosterone glucuronide (EpiT-G), androsterone glucuronide (A-G), etiocholanolone glucuronide (Etio-G), 11-ketoetiocholanolone glucuronide (11-KE-G), dihydrotestosterone glucuronide (DHT-G), 6-dehydrotestosterone glucuronide (6-DT-G), 5α-androstan-3β,17β-diol 3-glucuronide (5α3β17β-Diol 3-G), 5α-androstan-3β,17β-diol 17-glucuronide (5α3β17β-Diol 17-G), 5α-androstan-3α,17β-diol 3-glucuronide (5α3α17β-Diol 3-G), 5α-androstan-3α,17β-diol 17-glucuronide (5α3α17β-Diol 17-G), 5β-androstan-3α,17β-diol 3-glucuronide (5β3α17β-Diol 3-G), 5β-androstan-3α,17β-diol 17-glucuronide (5β3α17β-Diol 17-G), 3'-hydroxystanozolol 3-glucuronide (3-OHSTAN-G), 5β-androst-1-ene-17β-ol-3-one 17 glucuronide (BOLDm-G), 5β-androstan-7α,17α-dimethyl-3α,17β-diol-3-glucuronide (BOLm-G), 1α-methyl-5α-androstan-3α-ol-17-one 3-glucuronide (MESm1-G), 1α-methyl-5α-androstan-3α,17β-diol 3-glucuronide (MESm2-G), 1-methylen-5α-androstan-3α-ol-17-one 3-glucuronide (MTNm-G), 5β-androstan-7β,17α-dimethyl-3α,17β-diol 3-glucuronide (CALm-G), 2α-methyl-5α-androstan-3α-ol-17-one 3-glucuronide (DROm-G).

Steroid reference standards were purchased from Steraloids (Newport, RI, USA), NMI Australian Government (Pymble, Australia), Toronto Research Chemicals (Toronto, Canada) or Sigma-Aldrich (St. Louis, MO, USA).

Acetonitrile (ACN) (LC gradient grade), methanol (MeOH) (LC grade), formic acid (HFor) (LC/MS grade) and ammonium formate (NH_4For) were purchased from Merck (Darmstadt, Germany). Ultrapure water (H_2O) was obtained

using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Bond Elut C18 (100 mg) cartridges were acquired from Agilent Technologies (Santa Clara, CA, USA)

Stock solution of analytes were prepared in methanol (10 µg mL^{-1}) and kept at -20 °C. Working standard solutions were prepared in ACN:H_2O 20:80 (v/v) (100 ng mL^{-1}). Spiked urine samples were prepared reconstituting standards with blank urine samples (50 ng mL^{-1}).

2.1 Sample treatment

For the CCS characterization in urine samples, the following sample treatment previously developed and validated by Balcells et al. [42] was applied. Spiked urine samples (2 mL) were vortex-mixed and centrifuged 10 min at 3500 rpm. After that, samples were solid phase extracted using a C18 cartridge, previously conditioned with MeOH (2 mL) and then with H_2O (2 mL). After retention, cartridges were washed with 2 mL of H_2O and then analytes were eluted with 2 mL of MeOH. Finally, the samples were evaporated to dryness under a N_2 flow in a water bath at 40 °C and reconstituted with 200 µL of a solution of ACN:H_2O (20:80) (v:v). A sample volume of 5 µL was injected into the LC-IMS-HRMS system.

2.2 LC-TWIMS-QTOF analysis

A waters Acquity I-Class UPLC chromatographic system (Waters, Milford, MA, USA) was coupled with a VION IMS-QTOF mass spectrometer (Waters, Milford, MA, USA), using electrospray (ESI) as ionization technique in positive and negative ionization modes. Nitrogen was used as drying gas as well as nebulizing gas. The desolvation gas flow was set to 1000 L/h. Nitrogen desolvation temperature was set to 550 °C and the source temperature to 120 °C. A capillary voltage of 0.8 kV was used in positive and negative modes and a cone voltage of 40 V was applied. For the chromatographic separation, an Acquity UPLC BEH C18 column (2.1 mm x 100 mm i.d., 1.7 µm particle size) was used. The column temperature was set to 45 °C and the sample temperature was kept at 10 °C. The flow rate was 0.3 mL min^{-1}. Gradient elution was performed using a mobile phase of H_2O with 0.01% HFor and 1 mM NH_4For as solvent A and ACN:H_2O 95:5 v:v with 0.01% HFor and 1 mM NH_4For as solvent B. The percentage of solvent B was linearly changed as follows: 0 min, 20%; 2 min, 20%; 15 min, 40%; 16 min, 70%; 17 min, 95%; 18 min, 95%; 18.5 min, 20%; 20 min, 20%. The injection volume was 5 µL. MS data were acquired in HDMSe mode, in the *m/z* range 50-1000. IMS wave velocity was set to 250 m s^{-1} with a wave height ramp changing between 20-50 V. Nitrogen (≥ 99.999%) was used as collision-induced dissociation (CID) gas and as drift gas. A collision energy of 6 eV for low energy (LE) and a ramp of 28-56 eV for high energy (HE) were applied for the acquisition of two independent scans. In both LE and HE functions, a scan time of 0.3

s was select. For mass correction, leucine enkephalin (*m/z* 556.27658 in ESI positive and *m/z* 554.26202 in ESI negative) was used. All data were explored using the UNIFI platform (version 1.8.2) from Waters Corporation.

3. RESULTS AND DISCUSSION

3.1. CCS determination

This study investigated the CCS characterization of 103 steroids commercially available, including 56 unconjugated steroids, 19 metabolites sulfate (S)-conjugated and 28 metabolites glucuronide (G)-conjugated. The structures of all analytes are shown in **Figure S1** (**Supplementary Information**).

The main ions obtained under ESI conditions depended on the analyte structure. In general, as reported by Balcells et al. in a previous work [42], unconjugated steroids presenting a Δ^n-^{3}CO function were easily ionized in positive mode yielding the protonated molecule $[M+H]^+$. Unconjugated estradiols, presenting a phenol group in their structure, were ionized in negative mode resulting in the deprotonated molecule $[M-H]^-$. All glucuronides (except β-ES-3G and β-ES-17G) were ionized in both positive and negative ionization modes. The formation of $[M+H]^+$ was observed only for glucuronides presenting a Δ^n-^{3}CO function or a pyrazole ring while, metabolites lacking this feature ionized through the formation of the ammonium adduct $[M+NH_4]^+$. In ESI negative mode all sulfates and glucuronides yielded abundant $[M-H]^-$ ions resulting from the deprotonation of the G or S group.

For the CCS measurement, working standard solutions of analytes were injected into the UPLC system in triplicate ($n=3$). The reported CCS values (**Table S1, Supplementary Information**) were obtained from the average of these replicates. For all compounds, the determination showed high reproducibility, with relative standard deviations (RSD) ≤ 0.3 %. Detailed information about measured steroids, including the retention time, mass spectrometric data and ion mobility data are available in the **Table S1 of the Supplementary information** and on the Zenodo online repository [43]. Comparing ions formed in positive and negative modes of the same glucuronide, it was observed that the $[M-H]^-$ showed always a greater CCS than the $[M+H]^+$ and curiously, in some cases (as DROm-G and A-G) even higher than the $[M+NH_4]^+$. The CCS of the $[M+NH_4]^+$.is smaller than expected considering the ion mass, probably because the molecule folds assuming a more compact shape to well interact with the cation. Similar distortions have already been observed previously for the $[M+Na]^+$ adducts of some glucuronides by Hernandez-Mesa et al. [39].

The CCS values observed for the isomeric pair EpiT-G/T-G were also remarkable: in ESI(-) both isomers showed similar CCS values (CCS of the $[M-H]^-$ is 222.95 for EpiT-G and 222.91 for T-G), but in ESI(+) CCS of the $[M+H]^+$ was 204.54 for EpiT-G and 220.89 for T-G. This considerable difference in the CCS value of the $[M+H]^+$ of this isomeric pair has also been observed in a previous work [39]. The difference in CCS is probably due to the α or β conformation of the G

group in C17. When EpiT-G and T-G are ionized in ESI(-),an hydrogen from an OH- group of the glucuronide moiety is lost in both cases, therefore the charge is mainly located in the conjugation group and both presented similar CCS values. When they are ionized in ESI(+) to form the $[M+H]^+$, the site of protonation is located in the C = O conjugated with the double bond of the A ring. Therefore, the $[M+H]^+$ does not present in this part of the molecule the planar symmetry that the $[M-H]^-$ has. By breaking this symmetry, the molecule probably folds and takes a different shape depending on the α or β conformation of the G group, producing a lower CCS value when the G group is in α. Indeed, comparing to EpiT-G, it could be observed that 17β-NAN-G, BOLD-G, BOLDm-G and 6-DT-G showed more similar CCS values for the protonate and deprotonate molecules as they have a β configuration of the G group in C17 (as T-G has). Furthermore, as observed for the isomeric pair EpiT-G/T-G, a similar difference in the CCS values of the protonated and deprotonated molecules were also obtained for the isomeric pair 17β-BOLD-G/17α-BOLD-G by Hernandez-Mesa et al. [37].

CCS values obtained in spiked urine samples were also determined for some compounds to evaluate the matrix effect on the CCS determination. For this purpose, four blank urine samples (two from female and two from male healthy volunteers) were spiked at 50 ng/mL and analyzed in triplicate. Results are shown in **Table 1.** As it could be observed, the RSD (%) was always ≤ 0.5%, showing high reproducibility between different urine samples. The average CCS value (CCS av.) obtained in urine showed deviations (error) ≤ 1.1%, in comparison to the CCS obtained in standard solutions (CCS st.), except for the deprotonated molecule of EpiT-G that showed an error of about 2.2%, which could potentially be the result of a different structural conformation of EpiT-G in urine matrix due to the distinct pH. Thus, in most cases the CCS determination in urine was in accordance with current studies that accept an error threshold of ± 2% [40, 44]. Values for the measured CCS in standard solutions were also compared with those available in an open database for steroids [39], which was generated using a Synapt TWIMS instrument. 30 coinciding ions were found in positive mode, and in general good correlations were observed, with differences (expressed as relative error) ranging from -1.82% to 0.50%, and an absolute average value of 0.41%. In negative ionization mode, only 11 coinciding ions were found, and a slight bias was observed, with differences ranging from 0.82% and 2.94% (absolute average 2.14%). These increased differences in the negative mode might be due to the lower number of compared ions (30 vs 11), and also because of the different configurations of the instruments used in each work (VION vs Synapt). As for CCS values measured in urine, the obtained values for the 7 analytes for which data were available (all in positive ion mode) fell within the threshold of ± 2% in all cases, with errors ranging between -1.32% and 0.40% and an absolute average value of 0.63%. Overall, observed differences between our results and those from available databases seemed to be more affected by the polarity of the measurement than by the presence of matrix, which indicates a marked influence of factors such as the instrument employed. Different calibration procedures might also affect final results. For all these reasons it is important to increase the availability of open access CCS data measured with different platforms. Thus, adequate references would be provided

for comparison purposes, and the analyst will be able to better understand the role of each factor in CCS measurement, such as instrument configuration, harmonization of calibration mixes, etc. [45].

Steroid	Precursor ion		CCS (A)	CCS (B)	CCS (C)	CCS (D)	RSD	CCS Av.	CCS St.	Error
UNCONJUGATED										
mDIEN	$[M+H]^+$	287.2006	170.19	170.49	171.03	170.47	0.2%	170.55	172.13	0.9%
1-T	$[M+H]^+$	289.2162	171.52	170.86	171.34	171.10	0.2%	171.20	172.28	0.6%
T	$[M+H]^+$	289.2162	171.62	170.95	171.31	171.36	0.2%	171.31	172.12	0.5%
EpiMED	$[M+H]^+$	301.2162	174.04	173.53	174.19	173.82	0.2%	173.89	173.65	-0.1%
mT	$[M+H]^+$	303.2319	176.93	176.87	176.58	176.81	0.1%	176.80	177.41	0.3%
OXA	$[M+H]^+$	307.2268	177.48	177.46	177.23	177.18	0.1%	177.34	177.43	0.0%
EpiOXA	$[M+H]^+$	307.2268	176.82	176.13	176.37	176.69	0.2%	176.50	176.27	-0.1%
P	$[M+H]^+$	315.2319	179.17	179.28	178.88	179.14	0.1%	179.12	180.00	0.5%
FLU	$[M+H]^+$	337.2173	176.51	175.17	176.45	175.61	0.4%	175.94	177.90	1.1%
3-OHSTAN	$[M+H]^+$	345.2537	194.41	194.10	192.84	193.30	0.4%	193.66	193.77	0.1%
SULFATES										
17β-NAN-S	$[M-H]^-$	353.1428	190.17	190.39	189.84	190.10	0.1%	190.13	191.37	0.7%
17α-NAN-S	$[M-H]^-$	353.1428	191.77	192.23	191.53	191.58	0.2%	191.78	192.73	0.5%
BOLD-S	$[M-H]^-$	365.1428	192.63	193.08	192.13	192.32	0.2%	192.54	194.07	0.8%
T-S	$[M-H]^-$	367.1585	193.73	194.33	193.62	193.86	0.2%	193.88	195.28	0.7%
EpiT-S	$[M-H]^-$	367.1585	195.49	195.73	195.28	195.30	0.1%	195.45	197.00	0.8%
DHA-S	$[M-H]^-$	367.1585	198.51	199.18	198.25	197.41	0.4%	198.34	198.39	0.0%
DHT-S	$[M-H]^-$	369.1741	195.44	-	195.90	196.14	0.2%	195.83	196.40	0.3%
5αββ-Diol17-S	$[M-H]^-$	371.1898	197.60	-	197.43	197.13	0.1%	197.39	198.76	0.7%
A-S	$[M-H]^-$	369.1741	198.29	198.69	198.02	197.86	0.2%	198.22	199.98	0.9%
Etio-S	$[M-H]^-$	369.1741	199.03	199.09	199.19	199.24	0.0%	199.14	200.50	0.7%
EpiA-S	$[M-H]^-$	369.1741	199.19	198.75	199.27	198.89	0.1%	199.03	200.97	1.0%
DHEA-S	$[M-H]^-$	367.1585	198.89	199.49	198.52	198.24	0.3%	198.79	199.9	0.6%
GLUCURONIDES										
5α3β17β-Diol3-G	$[M-H]^-$	467.2650	229.71	229.59	229.63	229.70	0.0%	229.66	229.21	-0.2%
5β3α17β-Diol3-G	$[M-H]^-$	467.2650	213.62	212.82	212.09	212.51	0.3%	212.76	212.14	-0.3%
5α3β17β-Diol17-G	$[M-H]^-$	467.2650	227.71	227.28	227.45	227.69	0.1%	227.53	227.74	0.1%
5β3α17β-Diol17-G	$[M-H]^-$	467.2650	226.98	226.88	226.89	226.98	0.0%	226.93	227.03	0.0%
5α3α17β-Diol3-G	$[M-H]^-$	467.2650	220.28	-	220.81	220.55	0.1%	220.55	222.05	0.7%
5α3α17β-Diol17-G	$[M-H]^-$	467.2650	227.75	226.95	227.69	227.69	0.2%	227.52	227.93	0.2%
EpiT-G	$[M+H]^+$	465.2483	205.16	206.21	205.61	205.37	0.2%	205.59	204.54	-0.5%
	$[M-H]^-$	463.2337	218.79	217.86	217.67	217.72	0.2%	**218.01**	**222.95**	2.2%
A-G	$[M+NH_4]^+$	484.2905	215.07	214.03	214.65	214.78	0.2%	214.63	216.02	0.6%
	$[M-H]^-$	465.2494	219.58	220.00	219.16	219.23	0.2%	219.49	219.77	0.1%
Etio-G	$[M+NH_4]^+$	484.2905	211.93	211.81	211.42	211.53	0.1%	211.67	211.09	-0.3%

	$[M-H]^-$	465.2494	210.90	210.87	209.91	210.08	0.2%	210.44	210.85	0.2%
T-G	$[M+H]^+$	465.2483	220.85	221.03	220.32	220.56	0.1%	220.69	220.89	0.1%
	$[M-H]^-$	463.2337	222.93	223.41	-	223.47	0.1%	223.27	222.91	-0.2%
BOLD-G	$[M+H]^+$	463.2326	218.34	218.62	217.88	218.18	0.1%	218.25	217.67	-0.3%
	$[M-H]^-$	461.2181	221.05	221.39	220.52	220.60	0.2%	220.89	221.48	0.3%
BOLDm-G	$[M+H]^+$	465.2483	221.68	-	220.78	220.18	0.3%	220.88	221.76	0.4%
	$[M-H]^-$	463.2337	223.66	221.34	223.23	222.59	0.5%	222.70	223.96	0.6%
BOLm-G	$[M-H]^-$	495.2963	216.68	216.17	216.70	216.60	0.1%	216.54	216.96	0.2%
MESm1-G	$[M-H]^-$	479.2650	222.46	221.79	222.58	222.20	0.2%	222.26	222.91	0.3%

Table 1. CCS values obtained from the analysis of four urine samples in triplicate (a,b,c,d), the average CCS observed in urine (CCS av.) with its RSD(%), the CCS obtained in standard solutions (CCS st.) and the error of the CCS determination in matrix for some steroids in positive and negative ESI mode.

3.2. General correlation between CCS and *m/z* for steroids

Figure 1 shows the correlation between the *m/z* and the CCS, measured for the main precursor ion of each analyte (103 steroids in total). The CCS of the $[M-H]^-$ was reported for sulfates and glucuronides while the CCS of the $[M+H]^+$ was considered for unconjugated steroids. Generally, it could be observed that data distribution were well fitted to a lineal model (**Figure 1a**), presenting a R^2 value of 0.9459, and allowed a clear differentiation between the three groups of steroids (**Figure 1b**). For unconjugated steroids, presenting *m/z* ranges between 269 and 353, CCS values ranged from 166 to 194 $Å^2$ ($\Delta CCS = 28\ Å^2$). The greater values of this range (CCS values between 191 - 194 $Å^2$) were observed for stanozolol metabolites (**Figure 1b**). Compared to the other steroids selected for this study, these compounds present an additional pyrazole ring bound to the steroidal skeleton that could explain the higher CCS values observed. In fact, excluding these metabolites, the CCS range for unconjugated steroids is greatly reduced to 166 - 184 $Å^2$ ($\Delta CCS = 18\ Å^2$) with the same *m/z* range.

S-conjugated steroids covering a *m/z* range of 351 - 383 showed CCS values between 189 and 201 $Å^2$ ($\Delta CCS = 12\ Å^2$) while glucuronides, with *m/z* values between 445 and 519 showed a CCS range of 208 - 234 $Å^2$ ($\Delta CCS = 26\ Å^2$).

Considering the ionization of glucuronides in positive modes ($[M+NH_4]^+$ and of the $[M+H]^+$ ions), G-conjugated steroids presented a *m/z* range of 451 - 521 with CCS values between 205 and 238 $Å^2$ ($\Delta CCS = 33\ Å^2$). The general data distribution is still fitted by a lineal model ($R^2 = 0.9435$).

Thus, the range of variability of CCS obtained for glucuronide metabolites was larger than for the other groups. The CCS value for the stanozolol metabolite glucuronide, which presents an additional pyrazole ring in its structure and the largest ion mass measured, was coherent with the other glucuronide metabolites of steroids, showing that the higher variability was probably due to the greater distortion of the molecular shape caused by the voluminous G group. In fact, although the ion mass strongly affects the CCS value, this latter parameter depends also on the three-dimensional conformation of

the molecule, and the greater the mass, the greater the molecule could change its shape. Hence, this result could indicate that some glucuronides might be more compact or elongated than expected if only the ion mass is considered, as different orientation of this group could produce a great differentiation in the overall molecular shape and CCS value. It is therefore reasonable to think that the group S can also cause similar molecular distortions, although to a lesser extent, as it is a less voluminous group.

To try to understand which structural features affected the most the molecular shape, especially for phase II metabolites, a deeper discussion of the CCS values is included in the following sections.

3.3. General correlation between CCS and conjugation characteristics for phase II metabolites

One of the main reasons why the determination of CCS is of high interest is its potential to help in the characterization of isomers, a topic of paramount importance in the analysis of steroids. Therefore, the most interesting structural characteristics to evaluate in this study are those that do not involve a mass change but explain differences in the CCS value of isomeric compounds.

Useful features for the structural elucidation of phase II metabolites are the conjugation position and the orientation of the bond that links the S or G group to the steroid skeleton. Most of steroids and phase I metabolites present hydroxyl groups in position C3 and C17 that could be subjected to glucuronidation and/or sulfation during further steps of metabolism [5]. The substitution could take place below (α configuration) or above (β configuration) the steroid skeleton depending on the configuration of the hydroxyl group subjected to conjugation. Therefore, the formation of the α isomer or the β isomer in position C3 or C17 are among the most common conjugation sites of S and G groups. Even though α/β isomers can be chromatographically separated in most cases, identification of the specific configuration on the basis of retention time alone without any other reference, for instance if only one of the isomers is present, cannot be achieved as RT shifting might occur due to matrix effects.

Figure 2a depicts the CCS values of metabolites conjugated with S and G according to their conjugation position. As already previously stated in section 3.2, it could be observed that sulfates and glucuronides can be clearly distinguished based on their CCS value, while a generic differentiation between steroids of the same group cannot be achieved, since the conjugation in C3 and C17 provided similar CCS values. The same trends have been observed representing the CCS in function of the α and β configurations (**Figure 2b**). Thus, unfortunately, the change of a single structural feature, especially if did not involve a change in the ion mass, is not able to sufficiently affect the CCS value such as to make possible a clear and general differentiation between positional isomers.

3.4. Variations in CCS observed for phase II metabolites isomeric couples

CCS value by itself might not be able to help for the identification of a specific isomer in the case of isomeric steroids with slight changes in their structure. Therefore, the effect of a single structural change might be better understood considering differences in the CCS (ΔCCS) observed between isomeric compounds differentiated by the conjugation site and/or the bond configuration. Considering that some analytes were ionized in both positive and negative ESI modes, the comparison among isomers has been performed selecting the same precursor ion to avoid any contribution caused by the different charge and, therefore, different physical properties shift to accommodate the charge into the molecular structure. Taking into account the great reproducibility observed in the CCS determination, as calculated between replicates obtained for the same analysis (on the same day and with the same instrumentation), a ΔCCS of about 1 $Å^2$ for sulfates and 2 $Å^2$ for glucuronides was considered the minimum difference significant enough to consider a different isomer.
ΔCCS discussed below were obtained from the analysis of steroids in standard solutions. However, although ΔCCS magnitudes could be different, the same conclusions could be derived comparing the CCSs observed in urine samples.

3.4.1. Sulfates

Among the 19 sulfate steroids analyzed in this study, 11 sulfate metabolites, differing for the conjugation position of the S group and for its the α/β configuration were selected. Considering all their possible combinations, 7 isomeric pairs could be gathered, as showed in **Table 2**. The isomerism and the highest CCS value of each pair are highlighted in bold. The pair formed by A-S and DHT-S, that differ in both position and configuration of conjugation, showed a ΔCCS of about 3.6 $Å^2$ in standard solutions, with a higher CCS value for the 3α isomer (A-S). However, the pair EpiA-S/DHT-S, presenting a β configuration and differing solely for the conjugation position, showed a ΔCCS of 4.6 $Å^2$ with a higher value for the 3β isomer (EpiA-S). Although, their ΔCCS are slightly different due to the differences in the α/β configurations, both cases showed higher CCS values for the isomer conjugated in position C3. The structures of the isomers forming the pair EpiA-S/DHT-S were shown in **Figure 3.** As it could be observed, the highest CCS value corresponds to a more elongated structure producing a bigger rotational sphere in the gas phase, while the lowest CCS correspond to a slightly more compact molecule resulting in a smaller globe. The third pair of **Table 2**, formed by β-ES-3S and β-ES-17S also showed a higher CCS value for the sulfate conjugated in C3 with a ΔCCS value similar to that observed for abovementioned sulfates pairs.

The other four pairs reported in **Table 2** were characterized by differences in α/β configuration on the same conjugation position. Pairs formed by DHA-S/DHEA-S and A-S/EpiA-S, presenting both the S group conjugated in position C3, showed similar ΔCCS and the same behavior as, in both cases, the isomer 3β was characterized by a higher CCS value. Although this difference was not as high as when comparing conjugation sites, it might help in applications where

complete chromatographic separation is critical, since the difference in retention times is very small, especially for DHA-S/DHEA-S (10.3 vs 10.1 min). Pairs 17α-NAN-S/17β-NAN-S and EpiT-S/T-S, presenting the S group linked in C17, also provided the same information with comparable ΔCCSs and higher CCS values for the isomer 17α.

Thus, the conjugation position for sulfates can be hinted by CCS values since the highest CCS can be observed when the sulfate is linked in position C3, with a ΔCCS of up to 4 $Å^2$ could be obtained. Moreover, considering also the α/β configuration**,** an order of CCS values could be defined (3β > 3α > 17α > 17β), in agreement with the magnitude of the ΔCCS showed in **Table 2**.

Isomer	**Mass (*m/z*)**	**Ion**	**Sulphation**		**CCS ($Å^2$)**	**ΔCCS ($Å^2$)**	**Observations**
			Pos.	**Conf**			
A-S	369.1741	$[M-H]^-$	**C3**	**α**	**200.0**	3.6	CCS (3α) > CCS (17β)
DHT-S	369.1741	$[M-H]^-$	**C17**	**β**	196.4		
EpiA-S	369.1741	$[M-H]^-$	**C3**	β	**201.0**	4.6	CCS (3β) > CCS (17β)
DHT-S	369.1741	$[M-H]^-$	**C17**	β	196.4		
β-ES-3S	351.1272	$[M-H]^-$	**C3**	**plane**	**193.2**	4.1	CCS (3) > CCS (17β)
β-ES-17S	351.1272	$[M-H]^-$	**C17**	**β**	189.1		
DHA-S	367.1585	$[M-H]^-$	C3	**α**	198.4	1.5	CCS (3β) > CCS (3α)
DHEA-S	367.1585	$[M-H]^-$	C3	**β**	**199.9**		
A-S	369.1741	$[M-H]^-$	C3	**α**	200.0	1.0	
EpiA-S	369.1741	$[M-H]^-$	C3	**β**	**201.0**		
17α-NAN-S	353.1428	$[M-H]^-$	C17	**α**	**192.7**	1.3	CCS (17α) > CCS (17β)
17β-NAN-S	353.1428	$[M-H]^-$	C17	**β**	191.4		
EpiT-S	367.1585	$[M-H]^-$	C17	**α**	**197.0**	1.7	
T-S	367.1585	$[M-H]^-$	C17	**β**	195.3		

Table 2: Isomeric pairs of sulfates metabolites

Two additional pairs were also selected considering the α/β configuration of the hydrogen atom in position C5. As most steroids present a hydrogen or a methyl group in position C10 with a β configuration, different α/β configuration of the H in C5 also means a different *cis/trans* isomerism of the A ring, and that could produce a significant effect on the three-dimensional conformation of the steroidal skeleton (**Figure S2**). Two examples of pairs of compounds characterized by this isomerism were found: A-S/Etio-S and NA-S/NE-S (**Table 3**)**.** Although a greater value was obtained for the isomers

presenting a β configuration, unexpectedly, this isomerism did not show a great effect on the CCS value of these sulfates. In this case, probably improvements in the IMS resolution should be necessary to efficiently distinguish these isomers.

Isomer	Mass (*m/z*)	Ion	H in C5	Sulphation		CCS ($Å^2$)	ΔCCS ($Å^2$)	Observations
			Conf.	Pos.	Conf			
A-S	369.1741	$[M-H]^-$	**α (*trans*)**	C3	α	200.0	0.5	No great effect
Etio-S	369.1741	$[M-H]^-$	**β (*cis*)**	C3	α	**200.5**		
NA-S	355.1585	$[M-H]^-$	**α (*trans*)**	C3	α	197.0	0.3	
NE-S	355.1585	$[M-H]^-$	**β (*cis*)**	C3	α	**197.3**		

Table 3: *Cis/trans* isomerism for sulfate pairs

3.4.2. Glucuronides

Among the 28 glucuronide metabolites analyzed in this study, the attention was focused on glucuronide metabolites of isomeric androstanediols. For the monoglucuronide metabolites, 16 different isomers could exist considering all the possible combinations between conjugation position in C3 or C17, α/β configuration of the bonds linking the G group and the OH group and α/β configuration of the hydrogen in position C5. Thus, any relationship between differences in CSS and differences in the structure for these analytes could be useful for the structural interpretation of other isomers.

As abovementioned, glucuronides can be ionized both in negative mode forming the deprotonated molecule and in positive mode mainly through the ammonium adduct $[M+NH_4]^+$. Generally, ions generated in positive mode showed greater molecular distortions and thus greater ΔCCS that could provide higher significance towards structural information. However, data observed for this same set of compounds in in negative mode yielded the same conclusions for both standards and spiked urine samples.

Six isomers commercially available were included in this work (5α3β17β-Diol 3-G, 5α3β17β-Diol 17-G, 5α3α17β-Diol 3-G, 5α3α17β-Diol 17-G, 5β3α17β-Diol 3-G and 5β3α17β-Diol 17-G) and combined to form the eight isomeric pairs (A-H) showed in **Table 4.**

Pairs A to D were formed by isomers differing for the α/β configuration of the hydrogen in position C5. As explained before, this feature defines the *cis/trans* isomerism of the A ring (**section 3.4.1.**). Pair A, showed in **Figure 4a**, was formed by isomers that present the G group linked in C3 with α configuration. In this case a large ΔCCS was observed (of about 11.6 $Å^2$) with a higher value for the *trans* isomer. In fact, as could be observed in **Figure 4a**, the *cis* isomer is more folded and compact while the *trans* isomer showed a more extended structure. Isomers of pair B (**Figure 4b**), linking the G group in C3 but differing also for its α/β configuration, also showed a higher value of CCS for the *trans* isomer. In this case, ΔCCS was greater than that of pair A (18.0 $Å^2$) due to the different α/β configuration of the G group. As shown in **Figure 4b**, the β configuration provides a further extension of the molecule that might explain the larger ΔCCS observed.

Isomers of pairs C and D, presenting the glucuronidation in position C17 with a β configuration, showed the same behavior, with *trans* isomers presenting higher CCS values, but in this case, the ΔCCS was significantly lower, of about 4 $Å^2$ in both cases. As an example, couple D was shown in **Figure 4c**.

Therefore, for the glucuronide metabolites of androstanediols included in this study, the CCS was highly influenced by the *cis/trans* isomerism of the A ring, resulting always in greater CCS values for *trans* isomers due to their more elongated structure. Clearly, the glucuronidation position plays an important role, especially when the G group is linked to the position C3 that is directly situated in the A ring. In this position also the α/β configuration has a relevant effect on the molecular shape, as it could be observed when comparing *trans* isomers of **Figure 4a** and **Figure 4b**, and as revealed observing the ΔCCS of couple G in **Table 4.** Conversely, when the G group is linked in position C17 (couples C and D) that is situated in the D ring, the *cis/trans* isomerism of the A ring has a minor effect on the molecular shape that in fact provides smaller ΔCCSs, as shown for couple D in **Figure 4c**.

Pair	**Isomeric diol**	**Mass (*m/z*)**	**Ion**	**H in C5**	**Glucuronidation**		**CCS ($Å^2$)**	**ΔCCS ($Å^2$)**	**Observations**
				Conf.	**Pos.**	**Conf**			
A	5α3α17β-Diol 3-G	486.3061	$[M+NH_4]^+$	**α (*trans*)**	C3	α	**227.6**	11.6	CCS (trans) > CCS (cis)
	5β3α17β-Diol 3-G	486.3061	$[M+NH_4]^+$	**β (*cis*)**	C3	α	216.0		
B	5α3β17β-Diol 3-G	486.3061	$[M+NH_4]^+$	**α (*trans*)**	C3	**β**	**234.0**	18.0	
	5β3α17β-Diol 3-G	486.3061	$[M+NH_4]^+$	**β (*cis*)**	C3	**α**	216.0		
C	5α3α17β-Diol 17-G	486.3061	$[M+NH_4]^+$	**α (*trans*)**	C17	β	**230.0**	3.8	
	5β3α17β-Diol 17-G	486.3061	$[M+NH_4]^+$	**β (*cis*)**	C17	β	226.2		
D	5α3β17β-Diol 17-G	486.3061	$[M+NH_4]^+$	**α (*trans*)**	C17	β	**229.8**	3.6	
	5β3α17β-Diol 17-G	486.3061	$[M+NH_4]^+$	**β (*cis*)**	C17	β	226.2		
E	5α3α17β-Diol 3-G	486.3061	$[M+NH_4]^+$	α (*trans*)	**C3**	**α**	227.6	2.4	CCS (17β) > CCS (3α)
	5α3α17β-Diol 17-G	486.3061	$[M+NH_4]^+$	α (*trans*)	**C17**	**β**	**230.0**		
F	5α3β17β-Diol 3-G	486.3061	$[M+NH_4]^+$	α (*trans*)	**C3**	β	**234.0**	4.2	CCS (3β) > CCS (17β)
	5α3β17β-Diol 17-G	486.3061	$[M+NH_4]^+$	α (*trans*)	**C17**	β	229.8		
G	5α3α17β-Diol 3-G	486.3061	$[M+NH_4]^+$	α (*trans*)	C3	**α**	227.6	6.4	CCS (3β) > CCS (3α)
	5α3β17β-Diol 3-G	486.3061	$[M+NH_4]^+$	α (*trans*)	C3	**β**	**234.0**		
H	5β3α17β-Diol 3-G	486.3061	$[M+NH_4]^+$	β (*cis*)	**C3**	**α**	216.0	10.2	CCS (17β) > CCS (3α)
	5β3α17β-Diol 17-G	486.3061	$[M+NH_4]^+$	β (*cis*)	**C17**	**β**	**226.2**		

Table 4: Isomeric pairs of glucuronide metabolites

Taking into account that the *cis/trans* isomerism is an important feature for these compounds, *trans* isomers should be considered separately from *cis* isomers as the effect of the conjugation position in C3 or C17 of the G group and its α/β configuration could be different. For *trans* (5α) isomers, additional comparisons of the other structural features could be performed (pairs E, F and G). In pair E, the effect of the location of the glucuronide group is compared in a 3α,17β structure, while in pair F the same effect is compared in a 3β,17β structure. These pairs presented ΔCCS of about 2 and 4 $Å^2$ respectively, meaning that the difference is small, yet significant. In the case of pair G the effect of the configuration (α/β) of the hydroxyl in C3, where the glucuronide is linked, is considered, and a ΔCCS of about 6 $Å^2$ was observed. So, in the case of *trans* isomers of androstanediols, the following order for CCS values is observed: 3β > 17β > 3α. Considering this result, we can conclude that a differentiation based only on the conjugation position could not be done as the α/β configuration of the glucuronide group showed a significant influence on the CCS value of *trans* compounds.

For *cis* (5β) isomers, unfortunately, only one pair was available (couple H), where both isomers (5β,3α,17β) differed in the location and configuration of the glucuronide moiety. In this case, a large ΔCCS of about 10 $Å^2$ was observed with a higher CCS value for the isomer conjugated in C17. As it could be observed from the representation of this pair in **Figure 4d**, the conjugation in C3 of a *cis* isomer generates a folded structure while the conjugation in C17 produces a more planar and elongated molecule. This significant effect diverged from what happened with Couple E (equivalent feature in a *trans* pair), in which the same modification produced a ΔCCS of only 2 $Å^2$ (**Table 4**). Considering that, the possibility to differentiate the conjugation position in *cis* pairs would seem to be promising but further information is required.

To compare with the *cis/trans* isomerism of S-conjugated pairs (**Table 3, section 3.4.1**), the G-conjugated pairs of the same compounds are shown in **Table 5**. As previously observed, no relevant ΔCCS were obtained for $[M-H]^-$ of S-conjugated pairs, while in this case, both A-G/Etio-G and NA-G/NE-G showed significant differences (ΔCCS of about 8.9 $Å^2$ and 10.6 $Å^2$ respectively)**,** demonstrating that greater effects were provided by groups that are more voluminous. Also for these couples, *trans* isomer showed higher CCS values as already observed for the other glucuronide pairs discussed above.

Isomer	**Mass (*m/z*)**	**Ion**	**H in C5**	**Sulphation**		**CCS ($Å^2$)**	**ΔCCS ($Å^2$)**	**Observations**
			Conf.	**Pos.**	**Conf**			
A-G	465.2494	$[M-H]^-$	**α (*trans*)**	C3	α	**219.8**	8.9	CCS (trans) > CCS (cis)
Etio-G	465.2494	$[M-H]^-$	**β (*cis*)**	C3	α	210.9		
NA-G	451.2337	$[M-H]^-$	**α (*trans*)**	C3	α	**218.5**	10.6	
NE-G	451.2337	$[M-H]^-$	**β (*cis*)**	C3	α	207.9		

Table 5: *Cis/trans* isomerism for other glucuronide pairs

3.5. Variations in CCS observed for other isomeric pairs

Considering all the 103 steroids included in this work, other isomeric features were also studied for better understanding their effect on the CCS value. Among all compounds, some isomeric pairs differing in the position and/or the α/β configuration of hydroxyl groups linked to the steroid skeleton have been found and are listed in **Table S2 of Supplementary Information**. Although in most cases CCS of the α isomer is slightly higher, the magnitude of the ΔCCS did not show a relevant effect. These results are coherent with what was observed in the previous sections considering the ΔCCS magnitudes for glucuronides and sulfates metabolites as a whole. Thus, it is not surprising that isomeric steroids differing in the position and/or configuration of small groups presented similar CCS values. Slightly larger ΔCCS were observed for the epimers OXA/EpiOXA and MED/EpiMED, differing in the configuration of an OH group linked in C17. In these cases, a methyl group is also linked in C17 and thus, the change in the configuration of the two substituents might explain the greater variation. The same could be observed for the couple 11α-OHP/17α-OHP due to the presence of an acyl group in C17.

Similar CCS values were also generally obtained for isomers differing for the position of a double bond in the steroid skeleton as these changes only provided different configurations of hydrogen atoms (**Table S3 of Supplementary Information**). Considering this feature, four further isomeric pairs of androgen sulfates could be evaluated. These isomeric pairs, differing for the conjugation position of the S group in C3 and C17 and also for the position of a double bond in C5 and C4, are shown in **Table 6**. As the position of the double bond in the steroid skeleton has a negligible effect, ΔCCS were explained only considering the S position. For these sulfates pairs the same CCS order previously observed was obtained (3β > 3α > 17α > 17β), supporting the confidence in the previous results.

Isomer	Mass (*m/z*)	Ion	Double bond position	Sulphation		CCS ($Å^2$)	ΔCCS ($Å^2$)	Observations
				Pos.	Conf			
DHEA-S	367.1585	$[M-H]^-$	**C5**	**C3**	β	**199.9**	4.6	CCS (3β) > CCS (17β)
T-S	367.1585	$[M-H]^-$	**C4**	**C17**	β	195.3		
DHA-S	367.1585	$[M-H]^-$	**C5**	**C3**	α	**198.4**	1.4	CCS (3α) > CCS (17α)
EpiT-S	367.1585	$[M-H]^-$	**C4**	**C17**	α	197.0		
DHA-S	367.1585	$[M-H]^-$	**C5**	**C3**	**α**	**198.4**	3.1	CCS (3α) > CCS (17β)
T-S	367.1585	$[M-H]^-$	**C4**	**C17**	**β**	195.3		
DHEA-S	367.1585	$[M-H]^-$	**C5**	**C3**	**β**	**199.9**	2.9	CCS (3β) > CCS (17α)
EpiT-S	367.1585	$[M-H]^-$	**C4**	**C17**	**α**	197.0		

Table 6: Additional isomeric pairs of sulfate metabolites

3.6. Use of IMS to improve selectivity for the analysis of phase II metabolites of steroids in urine

In parallel to its use for the determination of CCS as characterization parameter [19], the integration of TWIMS in the LC-MS technique also provides an additional spatial separation which benefits the removal of interferents [18], which is of great importance in the field of steroid analysis [23]. Thus, the separation power of IMS helps to improve method selectivity, allowing the separation of isomeric compounds (as abovementioned shown) as well as the reduction of isobaric interferences arising from the sample matrix by means of drift time alignment without increasing the overall analysis time.

The determination of BOLm-G in urine samples by LC-HRMS has been hampered by the presence of an almost co-eluting isobaric interferent which is endogenous for urine matrix. However, the utilization of TWIMS-HRMS technique might contribute for a better determination of BOLm-G. With this example, we aim to show the benefits for a facilitated identification one can harvest when using IMS-HRMS. **Figure 5a** compares the extracted ion chromatograms as well as the driftogram (ion mobility separation) for BOLm-G in both spiked and blank urine samples. The expected RT for BOLm-G is 10.79 min. However, when monitoring the deprotonated molecule of BOLm-G (*m/z* 495.2963 ± 5mDa), an unknown isobaric can be observed in the blank urine sample at RT 10.76 min. By the use of ion mobility, a further separation of ions can be achieved. This extra separation is plotted in the driftogram (greyed color line) which show only one ion being present in the blank urine sample and two ions in the spiked sample. With the combination of RT and drift time values, two regions can be observed (region 1 with RT 10.79 min and DT 6.48 ms; and region 2 with RT 10.76 min and DT 7.12 ms). Since compound eluting in region 2 is the one observed both in blank and spiked samples, this can be assigned as the interferent, and therefore enables the identification of BOLm-G as the compound eluting at region 1. Then, the inclusion of CCS values (by means of drift time alignment) in the data processing workflow for the analysis of steroids in urine samples permits the removal of interferences that difficult reliable identifications. In this sense, **Figure 5b** shows the drift-time aligned extracted ion chromatograms for BOLm-G (*m/z* 495.2963 ± 5mDa; DT 6.48 ± 0.37 ms) in which the interferent is satisfactorily removed from both blank and spiked urine samples.

4. CONCLUSIONS

In this work, The CCS characterization of 103 steroids was performed and its potential as identification parameter for sulfate and glucuronide metabolites was investigated. For phase II metabolites some structural information were gathered comparing ΔCCS of isomeric couples. Sulfates conjugated in position C3 or C17 were distinguished as higher CCS values

were observed when the sulfate is linked in position C3. Moreover, considering also the α/β configuration, this CCS order was obtained: 3β > 3α > 17α > 17β. For isomeric glucuronide androstanediols, *cis/trans* isomers were differentiated as *trans* isomers always showed greater values of CCS due to their extended molecular shape. Comparing *trans* isomeric couples, the differentiation based on α/β configuration was possible when G group is conjugated in position C3 while the differentiation based on the conjugation position was difficult as this CCS order was observed: 3β > 17β > 3α. For *cis* isomers, although only one couple was available, the possibility to differentiate isomers based on their conjugation position it would seem more promising considered the folded structure provided by the A ring and the great ΔCCS observed. However, a greater number of isomeric couples under study can be of great help to support the observations herein presented. Nevertheless, the coupling of the CCS information with chromatographic and mass spectrometric data, provided an improved tool, potentially useful for the structural elucidation of new steroid metabolites in urine. Moreover, the capability of IMS reducing matrix interferences was also tested for the analyte Bolasterone met G as a case study, obtaining improved selectivity in the analysis of urine samples.

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