Short Communication

A Novel Method for the Determination of the Site of Glucuronidation

by Ion Mobility Spectrometry-Mass Spectrometry (IMS-MS)

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Running title: Identification of Glucuronidation Sites by Ion Mobility

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Nonstandard abbreviations: UGT, UDP-glucuronosyltransferase; CCS, collision cross section; IMS, ion mobility spectrometry; IMS-MS, ion mobility spectrometry-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; LC-IMS-MS, liquid chromatography-ion mobility spectrometry-mass
spectrometry; MS/MS, tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TM, trajectory method.
Abstract

Glucuronidation does not only play a detoxifying role in living body, but also can complicate pharmacological and toxicological profiles of new drug candidates by forming active and reactive conjugated metabolites. The opportunities of structure elucidation of conjugated metabolites have therefore been increased in drug metabolism studies at early development stage. General methodologies for the structure elucidation of glucuronide conjugate(s) include liquid chromatography-tandem mass spectrometry (LC-MS/MS) and NMR spectroscopy. In many cases, LC-MS/MS alone cannot unequivocally identify the site(s) of conjugation in isomeric glucuronidations. In the present study, we established new strategy for the structure elucidation of glucuronide conjugates utilizing ion mobility spectrometry-mass spectrometry (IMS-MS). Linear correlation between calculated collision cross sections (CCS) and actual drift times from ion mobility spectrometry (IMS) was found for each set of parent compound (raloxifen, losartan, telmisartan and estradiol) and the corresponding tandem mass spectrometry (MS/MS) product ions. Thus obtained regression lines accurately and selectively projected the actual drift times of authentic standards of glucuronide conjugate based on the theoretical CCS values. The established method was utilized for the accurate assignment of predominant formation of phenolic glucuronide conjugate (SCH60663) in the isomeric (phenolic and benzylic) glucuronidations of ezetimibe in the incubated sample with cryopreserved human hepatocytes. This application demonstrates the potential to facilitate the structure identification of glucuronide conjugates at early development stage of new drug candidates.
Introduction

Glucuronide conjugation is one of the main phase II metabolic reactions in the living body. The addition of glucuronic acid to the structure of xenobiotics through UGT-dependent glucuronidation makes the compounds more hydrophilic and less active pharmacologically and/or toxicologically, and the resultant conjugated metabolites are eliminated into bile and urine. The glucuronide conjugation had been generally considered to be an important detoxifying pathway in most cases associated with rapid excretion and small volume of distribution, while it has been demonstrated that the glucuronidation can also form active and/or reactive conjugated metabolites which modulate pharmacological, toxicological or pharmacokinetic profiles of parent compounds (Shipkova and Wieland, 2005; Regan, 2010). For example, morphine-6-glucuronide (van Dorp et al., 2006), digitoxin- and digoxin-glucuronides, retinoic acid glucuronide (Kroemer and Klotz, 1992; Ritter, 2000), and ezetimibe glucuronide (Patrick et al., 2002) are known to maintain or increase the pharmacological activities of parent drugs. The formation of acyl glucuronide has been implicated in a wide range of adverse effects, including drug hypersensitivity reactions and cellular toxicity (Ritter, 2000), and the chemical reactivity depends on the stability of acyl glucuronide conjugate (Benet et al., 1993). As an example of pharmacokinetic effect by glucuronide conjugate, it has been demonstrated that the gemfibrozil glucuronide is a mechanism-based CYP2C8 inhibitor in human (Baer et al., 2009; Honkalammi et al., 2011; Jenkins et al., 2011). Therefore, the structure identification of glucuronide conjugates is often required at early development stage of new drug candidates.
Because of its high sensitivity and ability to obtain structural information, the liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become an invaluable tool in drug metabolism studies. LC-MS/MS can readily detect glucuronide conjugates based on an increase of 176 Da over the parent compound and major mass fragments corresponding to the neutral loss of the glucuronyl moiety. However, when multiple sites are potential for the glucuronidation, the determination of exact site requires further experiments such as NMR spectroscopy which needs sufficient amount of isolated and purified sample of conjugated metabolite.

Recently, the high resolution mass spectrometer equipped with ion mobility spectrometry (IMS) has emerged as an alternative way of structure analysis. IMS separates ionic species as they drift through a gas under the influence of an electric field. The rate of the drift depends on the multiple factors such as the mass and charge state of the ion, and the collision cross section (CCS) of the ion in the gas phase: therefore, it is possible to separate ions with the same m/z value if they have different CCS values (Smith et al., 2009). In addition, the theoretical drift times from IMS are linearly proportional to the values of CCS when the masses of ions are much larger than the buffer gas under the same IMS condition (Kanu et al., 2008; Smith et al., 2009). There are many examples of structure analysis using ion mobility spectrometry-mass spectrometry (IMS-MS), in most cases for the macromolecules such as protein complexes or 3 dimensional structure of polypeptide (for review, Jarrold, 2000, 2007). In contrast, there have been only a few examples of structure analyses of metabolites of small molecule drugs: the structure analysis of hydroxylated metabolite of ondansetron (Dear et al., 2010) and

Despite promising technical innovation of IMS-MS for the structure determination of isomeric metabolites (Dear et al., 2010), the application of technology at early development stage of new drug candidates has been so far very limited: the calculated 3D structures under vacuum phase by the energy-minimizing modeling vary with the adopted 2D-3D conversion methods; the theoretical CCS value is significantly affected by error ratios in the modeling especially for small molecule metabolites; and both prevent the application of this novel technology from the routine analysis without authentic standards at the early development stage.

In this communication, we present a novel approach for the IMS-MS technology to the identification of exact site(s) of glucuronide conjugation: the linear relationships were found in the present study between theoretically calculated CCS values and experimentally determined drift times by liquid chromatography-ion mobility spectrometry-mass spectrometry (LC-IMS-MS) for the parent compound and its tandem mass spectrometry (MS/MS) fragments; and the regression line was used for the projection of drift times of the potential isomeric conjugated metabolites based on their theoretical CCS values. Therefore, the present method can omit validation processes for the absolute values of CCS calculated by the particular molecular modeling method with authentic standards, and consequently, it facilitates the routine application of IMS-MS to the structure identification of small molecule metabolites at early development stage of new drugs.
Materials and Methods

Materials: Raloxifene, β-estradiol 3-sulfate sodium salt, β-estradiol 3-(β-D-glucuronide) sodium salt and β-estradiol 17-(β-D-glucuronide) sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Raloxifene-4’-glucuronide, raloxifene-6-glucuronide, ezetimibe, ezetimibe phenoxy β-D-glucuronide and ezetimibe hydroxyl β-D-glucuronide were purchased from Toronto Research Chemicals Inc. (North York, ON). Losartan potassium was purchased from LKT Laboratories, Inc. (St. Paul, MN). Losartan tetrazole-N2-glucuronide was purchased from Santa cruz biotechnology, Inc. (Santa cruz, CA). All other reagents were of analytical grade. Cryopreserved human hepatocytes were purchased from Tissue Transformation Technologies (Rockville, MD). Williams’ medium E was purchased from Invitrogen (Carlsbad, CA).

Analytical Procedure: The LC-IMS-MS was conducted on an Acquity UPLC system equipped with a binary pump, autosampler, thermostat, and column compartment (Waters Corp., Milford, MA), coupled with Synapt G2 HDMS (Waters Corp., Manchester, UK) including ion mobility spectrometer and time-of-flight type high resolution mass spectrometry. Solvent A was 0.1% formic acid. Solvent B was 0.1% formic acid in acetonitrile. Chromatographic separations were performed on an Acquity UPLC BEH C18, 1.7 μm, 2.1×50 mm (Waters Corp., Ireland) utilizing a 5 min gradient at a flow rate of 0.5 ml/min, starting at 10% solvent B, linearly increasing to 90% solvent B over 3 min, followed by 90% solvent B for 1 min and re-equilibration for 1 min. Eluents from UPLC during pre- and post-analysis (0.3 and 3 min, respectively) were discarded through
the diversion valve. IMS analyses were performed on a Synapt G2 HDMS under the conditions described as follows: Capillary voltage, 3kV; Cone voltage, 30V; Trap CE, 0V or 10-40V ramping; Transfer CE, 0V; Trap/Transfer gas, argon; IMS gas, nitrogen; IMS T-wave speed, 900 m/sec; IMS T-wave height, 40V; IMS gas flow, 20 mL/min; IMS-MS acquisition time, 3 min after samples injected onto column.

**Calculations of Collision Cross Section (CCS):** Theoretical values of CCS were calculated using the method suggested by Dear et al. (2010). Briefly, the conformation for each molecule was energy-minimized with the MMFF94 forcefield followed by the extraction of 3-dimensional coordinate sets of each atom. Theoretical CCS values were then calculated by the open source software, MOBCAL (Mesleh et al., 1996) with 3D coordinate data sets. The MOBCAL output is based on three different models/algorithms including Projection Approximation, Exact Hard Sphere Scattering, and the Trajectory Method (TM). Among three outputs, the MOBCAL TM has been demonstrated to provide most accurate projections of CCS values with %differences of < 4.5% for isomeric organoruthenium anticancer complexes (Williams et al., 2009b) and of < 1% for ondansetron and its hydroxylated metabolites (Dear et al., 2010). Therefore, the outputs of MOBCAL TM were used for the projections in this study.

**Regression Line for CCS and IMS Drift Time:** Although the trajectory method (TM) is generally accepted as the most accurate method of calculating the theoretical CCS values among multiple MOBCAL outputs, the relationship between CCS and IMS drift times can not serve as an absolute basis for the exact identification of unknown structure of target analyte. Therefore, in addition to the TM-based CCS value for the parent
compound, those values for its MS/MS fragments were plotted against corresponding actual IMS drift times for the generation of regression line. The generated regression line facilitates accurate projection of IMS drift time of unknown analyte (glucuronide conjugate) by the comparison with (multiple) theoretical CCS value(s) of potential structure(s).

**Incubation of Ezetimibe with Human Hepatocytes:** Ezetimibe (1mM in DMSO) was incubated at the final concentration of 10 μM for 2 h at 37°C in 250 μl of Williams’ E incubation medium with L-glutamine containing cryopreserved human hepatocytes (1.0×10^6 cells/ml) under the condition of CO2/O2 (5/95) saturated humidity. Reactions were terminated by the addition of 500 μl of acetonitrile/methanol solution (2:1, v/v) and the supernatant (600 μl) was separated by the centrifugation (13800 g, 10 min, 4°C) for the injection onto LC-IMS-MS. Solvent A was 0.1% formic acid. Solvent B was 0.1% formic acid in acetonitrile. Chromatographic separations were performed on an Acquity UPLC BEH C18, 1.7 μm, 2.1×50 mm (Waters) utilizing a 10 min gradient at a flow rate of 0.4 ml/min, starting at 0% solvent B for 0.3 min, linearly increasing to 70% solvent B over 6.7 min, followed by 90% solvent B for 1.5 min and re-equilibration for 1.5 min. Eluents during pre- and post-analysis (0.5 min and after 7.0 min, respectively) were discarded through the diversion valve. IMS analyses were performed on a Synapt G2 HDMS. Instrument conditions are the same as described above.
Results and Discussion

A novel approach in the present method to the identification of the site of isomeric glucuronide conjugates is to generate a regression line between theoretically calculated TM-based CCS values and drift times determined by LC-IMS-MS analysis: the product ion scan of the parent ion was firstly carried out and then the drift time of each fragment ion as well as the parent ion was determined by IMS; secondly, the CCS values for energy-minimized conformation of the respective ions were calculated by MOBCAL; and finally, the regression line was generated by the comparison between theoretical CCS and observed drift times for the parent and its MS/MS fragments. Based on the generated regression line, the most likely isomer of glucuronide conjugate was identified from the comparison between actual IMS drift time(s) of target analyte (conjugated metabolite) and the theoretical CCS values of potential candidates of conjugated metabolite calculated by MOBCAL.

In order to ensure the precision and accuracy of the regression line-based projection for unknown analytes, the linearity of regression line between CCS values and IMS drift times was confirmed for multiple sets of parent compound, MS/MS fragments and authentic glucuronide conjugate(s). Fig. 1 shows the relationships and linear regression lines between CSS values and IMS drift times for the sets of parent compounds of glucuronide conjugation [raloxifene (A), losartan (B), telmisartan (C) and estradiol (D)], the corresponding MS/MS fragments, and authentic standards of conjugated metabolite. The drift times (scans) of raloxifene (3 in Fig. 1A) and its MS/MS fragments (4, 5, 6 and 7) were 121, 102, 81, 76 and 57, respectively,
and the corresponding CCS values (Å²) were 158.28, 119.25, 97.174, 89.817 and 58.608, respectively. The relationship generated a linear regression line (drift time = 0.6621 CCS + 18.126) with high correlation coefficient (R² = 0.9866) which allowed for an accurate identification of two potential isomers of glucuronide conjugate at 6- and 4’-positions (1; 150 scans/201.53 Å² and 2; 143 scans/186.49 Å², respectively) with predicted drift times of 152 and 142 scans, respectively, from regression line. The regression line thus generated can identify the specific formation of 4’-glucuronide conjugate 2 by UGT1A10 (Kemp et al., 2002) with sufficient selectivity between isomeric glucuronide conjugates, which otherwise only elucidated by the NMR spectroscopy (Dodge et al., 1997). The linear regression line (drift time = 0.6896 CCS + 12.991, R² = 0.9974) based on the drift times and the corresponding calculated CCS values of losartan 10 (in Fig. 1B) and its MS/MS fragment 11, 12, 13 and 14 (drift time, 110, 108, 107, 77 and 71 scans, respectively; CCS value, 141.55, 138.17, 135.11, 91.133 and 85.767 Å², respectively) projected the potential O-glucuronide 8 (136 scans/179.01 Å²) and tetrazole-N2-glucuronide 9 (134 scans/172.27 Å²) (Fig. 1B) with predicted drift times of 136 and 132 scans, respectively. Similarly, the regression line (drift time = 0.6254 CCS + 16.385; R² = 0.9845) based on the drift times and the corresponding calculated CCS values of telmisartan 16 (Fig. 1C), and its two MS/MS fragments 17 and 18 (drift time, 126, 94 and 68, respectively; CCS value, 177.49, 117.53 and 86.877 Å², respectively) accurately projected actual drift time (147 scans) of telmisartan glucuronide 15 at the theoretical CCS value (207.93 Å²) and predicted drift time of 146 scans. Estradiol 22 in Fig. 1D) forms not only two isomers of glucuronide conjugate at 3-O- and 17-O-positions (19 and 20, respectively), but also
3-\textit{O}-sulfate conjugate 21. Estradiol and its MS/MS fragments 22-27 (Biancotto et al., 2002) gave a linear regression line (drift time $= 0.7969$ CCS + 8.6183; $R^2 = 0.9921$) based on the data set of drift times/CCS values for 22, 23, 24, 25, 26 and 27 [drift times (scans), 89, 85, 82, 80, 65 and 60, respectively; CSS values ($\AA^2$), 99.380, 95.689, 91.920, 91.938, 70.045 and 64.613, respectively], which selectively projected the potential conjugated metabolites 19 (121 scans/141.14 $\AA^2$), 20 (118 scans/138.85 $\AA^2$) and 21 (102 scans/116.91 $\AA^2$) (Fig. 1D) with predicted drift times of 121, 119 and 102 scans, respectively.

The regression line-based approach was applied to the identification of positions of glucuronide conjugates formed from ezetimibe in cryopreserved human hepatocytes. Fig. 2A shows a representative liquid chromatography-mass spectrometry (LC-MS) chromatogram. Both peaks at retention times of 4.65 and 4.85 min gave same molecular ions [M-H] at m/z 584, which corresponded to the glucuronide conjugates of ezetimibe. Due to two potential positions of glucuronidation on ezetimibe (i.e. phenolic and benzylic hydroxyl groups), the structural information by MS/MS did not afford unequivocal identification of isomeric substituted metabolites. As shown in Fig. 2B, ezetimibe 30 and its MS/MS fragments (31 and 32) generated a regression line (drift time $= 0.6499$ CCS + 19.158; $R^2 = 0.9981$) based on the relationship between drift times (114, 85 and 70 scans, respectively) and CCS values (145.28, 103.03 and 77.164 $\AA^2$, respectively). The theoretical CCS values of phenolic glucuronide 28 and benzylic glucuronide 29 were 188.76 and 178.26 $\AA^2$, respectively, and predicted IMS drift times were 142 and 135 scans, respectively, by the regression line. The actual drift time of the dominant peak at 4.65 min (Fig. 2A) was 141 scans, suggesting that the major
metabolite of ezetimibe in the incubated sample with human hepatocytes was the phenolic glucuronide 28 which is consistent with clinical data that the main circulating metabolite of ezetimibe is a phenolic glucuronide conjugate (SCH 60663) in human plasma (Patrick et al., 2002). Further analyses with authentic glucuronide conjugates of ezetimibe confirmed the regression line-based metabolite identification by the actual drift times of 141 and 135 scans for 28 and 29, respectively, both in excellent agreement with the corresponding calculated values.

In conclusion, applications of the present method to the structure elucidation of glucuronide conjugates demonstrated that the regression line selectively and accurately projected drift time for each potential glucuronide conjugate based on the theoretical CCS value calculated by the same molecular modeling method as that used for the regression line. The present regression line-based LC-IMS-MS analysis with theoretical modeling for CSS calculation has proven to be a powerful tool for the preliminary elucidation of isomeric substituted conjugate metabolites in the biological matrices especially at early development stage without synthesis of authentic metabolites or isolation of metabolites for NMR spectroscopy. Further studies with structurally diverse sets of parent compound/metabolites are warranted to evaluate potential and versatility of this new technique.
Authorship Contributions

Participated in research design: Shimizu, Ohe and Chiba

Conducted experiments: Shimizu

Contributed new reagents or analytical tools: Shimizu

Performed data analysis: Shimizu

Wrote or contributed to the writing of the manuscript: Shimizu, Ohe and Chiba
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References


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Legends for figures

**Figure 1.** Plot of the drift time values (scans) against calculated CCS values (Å²) for the singly charged ions of glucuronide conjugates, parent ion and its MS/MS product ions of raloxifene (A), losartan (B), telmisartan (C) and estradiol (D). Linear regression for raloxifene 3 and its MS/MS fragments (4-7) gave drift time = 0.6621 CCS + 18.126 (R²=0.9866). Two glucuronide conjugates (1 and 2) are shown by open circles (A). Linear regression for losartan (10) and its MS/MS fragments (11-14) gave drift time = 0.6896 CCS + 12.991 (R²=0.9974). Two glucuronides of losartan (8 and 9) are shown by open circle (B). Linear regression for telmisartan (16) and its MS/MS fragments (17 and 18) gave drift time = 0.6254 CCS + 16.385 (R²=0.9845). The glucuronides of telmisartan (15) is shown by open circles (C). Linear regression for estradiol (22) and its MS/MS fragments (23-27) gave drift time = 0.7969 CCS + 8.6183 (R²=0.9921). Two glucuronides (19 and 20) and 3-sulfate (21) of estradiol are shown by open circles (D).

**Figure 2.** LC-MS chromatogram of ezetimibe incubated in cryopreserved human hepatocytes (A). Plot of the drift time values (scans) against calculated CCS values (Å²) for the singly charged ions of glucuronide conjugates, parent ion and its MS/MS product ions of ezetimibe. Linear regression for ezetimibe (30) and its MS/MS fragments (31 and 32) gave drift time = 0.6499 CCS + 19.158 (R²=0.9981). Two glucuronides of ezetimibe (28 and 29) are shown by open circles (B).
Figure 1

A

B

C

D

Calculated CCS (Å²)

Drift time (scans)
Figure 2