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Determination of Acyl-, *O*-, and *N*-Glucuronide using Chemical Derivatization Coupled with Liquid Chromatography – High Resolution Mass Spectrometry

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Running Title: Determination of acyl-, *O*-, and *N*-glucuronide

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Nonstandard Abbreviations: ACN, acetonitrile; Ben-A-Gluc, benazepril acyl-β-D-glucuronide; CID, collision-induced dissociation; DMF, dimethylformamide; ESI, electrospray ionization; Et, ethyl; EtOH, ethanol; HCD, higher-energy C-trap dissociation; HLM, human liver microsomes; HRMS, high resolution mass spectrometry; LC, liquid chromatography; NMR, nuclear magnetic resonance; min, minutes; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Ral-4'-O-Gluc, raloxifene 4'-glucuronide; Ral-6-O-Gluc, raloxifene 6-glucuronide; Sil-O-Gluc, silodosin β-D-glucuronide; $SOCl_2$, thionyl chloride; TMS, trimethylsilyl; TMSI, 1-(trimethylsilyl) imidazole - pyridine mixture; UDPGA, uridine 5′-diphosphoglucuronic acid trisodium salt

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ABSTRACT

Glucuronidation is the most common phase II metabolic pathway to eliminate small molecule drugs from the body. However, determination of glucuronide structure is quite challenging by mass spectrometry due to its inability to generate structure informative fragments about the site of glucuronidation. Herein we described a simple method to differentiate acyl-, *O*-, and *N*-glucuronides using chemical derivatization. The idea is that derivatization of acyl-, *O*- or *N*-glucuronide of a molecule results in predictable and different numbers of derivatized functional groups, which can be determined by the mass shift using mass spectrometry. The following two reactions were applied to specifically derivatize carboxyl and hydroxyl groups that are present on the aglycone and its glucuronide metabolite. Carboxyl groups were activated by thionyl chloride followed by esterification with ethanol. Hydroxyl groups were derivatized via silylation by 1-(trimethylsilyl)imidazole. The mass shift per derivatized carboxyl and hydroxyl group was +28.031 Da and +72.040 Da, respectively. This approach was successfully validated using commercial glucuronide standards including benazepril acyl-glucuronide, raloxifene *O*-glucuronides, and silodosin *O*-glucuronide. In addition, this approach was applied to determine the type of glucuronide metabolites that were isolated from liver microsomal incubation, where alvimopan and diclofenac acyl-glucuronides, darunavir, haloperidol, and propranolol *O*-glucuronides, and darunavir *N*-glucuronide were identified. Lastly, this approach was successfully utilized to elucidate the definitive structure of a clinically observed metabolite, soticlestat *O*-glucuronide. In conclusion, a novel efficient and cost-effective approach was developed to determine acyl-, *O*-, and *N*-glucuronide using chemical derivatization coupled with liquid chromatography-high resolution mass spectrometry.

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SIGNIFICANCE STATEMENT

The method described in this study can differentiate acyl-, *O*-, and *N*-glucuronides and allow for elucidation of glucuronide structures when multiple possibilities of glucuronidation exist. The type of glucuronidation information is particularly useful for a drug candidate containing carboxyl group(s), which can form reactive acyl-glucuronides. Additionally, the method can potentially be used for the definitive structure elucidation for a compound containing a single carboxyl, hydroxyl, or amino group even when multiple types of functional groups are present for glucuronidation.

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Introduction

Metabolite profiling and identification play essential roles in discovery and development of new chemical entities. Identification of metabolites in non-clinical and clinical species is required both in *in vitro* preparations and in *in vivo* biological matrices to understand the metabolic fate of a drug. Its application includes: a) metabolic stability assessment in discovery lead optimization, b) identification of bioactivation potential, c) safety study species selection, d) detection of the presence and determination of coverage of disproportionate metabolites in nonclinical safety evaluation, and e) evaluation of clearance mechanism (circulating and excretory metabolites) of a drug in radiolabeled ADME studies. Structure elucidation of a drug metabolite is usually assisted with the accurate mass to charge ratio obtained in full scan mass spectrometry (MS) analysis and product ion information from subsequent fragmentation analysis of the metabolite using high resolution mass spectrometry (HRMS). HRMS can provide the elemental composition data but generally do not provide sufficient information for the definitive structure elucidation, i.e., the exact location of a given modification of the structure by metabolism. Other tools are hence utilized to assist structure characterization such as nuclear magnetic resonance (NMR) spectroscopy (Subramanian et al., 2010), chemical derivatization (Liu and Hop, 2005), online hydrogen/deuterium exchange technique (Ohashi et al., 1998; Liu and Hop, 2005), and stable isotope labeling (Yan and Caldwell, 2004; Ly et al., 2009). Among them, chemical derivatization can be used to determine a specific functional group of a molecule. For example, one of the most classic chemical derivatizations is to use $TiCl_3$ to identify the presence of *N*-oxide, which can be selectively reduced to amines by $TiCl_3$ in biological matrices such as plasma and urine (Kulanthaivel et al., 2004). Similarly, dansyl chloride can react with

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primary and secondary amine as well as the aromatic hydroxyl group to form sulfonamide and sulfonate, respectively (Dalvie and O'Donnell, 1998).

Glucuronidation is the most common phase II metabolic pathway. The reaction is mediated by uridine 5'-diphosphate-glucuronosyltransferase which is mainly located in the endoplasmic reticulum. Glucuronidation can occur on nucleophilic functional groups such as carboxylic acid, alcohol, and amine leading to the formation of *O*-acyl- (will be called acyl- thereafter), *O*-, and *N*-glucuronide, respectively. These glucuronide metabolites are more polar and hence more water soluble than their aglycones, thus facilitating the elimination of the drugs from the body. *O*- and *N*-glucuronides are generally stable and considered as bioinactivated metabolites. In contrast, acyl-glucuronide is usually unstable at neutral or slightly basic condition and is considered a reactive metabolite although the reactivity is dependent on the structure of the molecule. Reactive acyl-glucuronide can undergo intramolecular rearrangement and intermolecular transacylation (Hyneck et al., 1988), leading to the covalent binding to proteins (van Breemen and Fenselau, 1985; Smith et al., 1990). As a result, formation of acyl-glucuronide can be a safety concern for a drug candidate and may need additional risk assessment (Smith et al., 2018; Walles et al., 2020). It is hence critically important to determine if acyl-glucuronide is formed from a carboxyl bearing drug candidate.

Structure elucidation of glucuronide metabolites is generally not achievable by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The glucuronyl moiety (176.032 Da) can be readily lost upon the collision-induced disassociation (CID) of a glucuronide metabolite. Therefore, the information on the glucuronidation site is typically missed in the MS/MS spectrum of a glucuronide metabolite except in some rare cases. However, there are some liquid chromatography-mass spectrometry (LC-MS) based methods to differentiate different types of

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glucuronides following derivatization. An *O*-glucuronide of an oxidative metabolite *N*-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid from *N*-(3,5-dichlorophenyl)succinimide was identified after chemical derivatization with 3-pyridinylcarbinol in conjunction with LC-MS (Cui and Harvison, 2000). Another study demonstrated that acyl-glucuronide could be identified after derivatization to a hydroxamic acid with hydroxylamine coupled with LC-MS (Vaz et al., 2010). Recently, it has been reported that *O*- and *N*-glucuronides could be differentiated by a tandem mass spectrometric method coupled with gas-phase ion-molecule reactions of deprotonated glucuronide with trichlorosilane (Kong et al., 2018). However, acyl-glucuronide could not be differentiated from *O*- and *N*-glucuronides using this approach. A later report from the same group demonstrated that migrated acyl-, *N*-, and *O*-glucuronide can be differentiated using tandem mass spectrometry based on gas-phase ion-molecule reactions between the glucuronide and BF_3 (Niyonsaba et al., 2019). Nonetheless, unmigrated acyl- and *O*-glucuronide cannot be differentiated using this methodology. More often the structure of the glucuronide metabolite is determined by NMR, which is a time and resource intensive process. NMR analysis typically needs at least micrograms of purified material. Preparation of glucuronide metabolites can be achieved by biosynthesis or organic synthesis and is sometimes quite challenging to find an appropriate biosynthetic system or a synthetic route. Thus, identification of the nature of glucuronidation particularly when multiple possibilities (different functional groups) exist remains a major challenge in drug discovery and development.

At present there is no published methodology to simultaneously characterize acyl-, *O*-, and *N*-glucuronides using chemical derivatization. The objective of the study is to demonstrate a new consolidated approach which uses chemical derivatization toward carboxyl and hydroxyl groups, albeit not novel, coupled with LC-HRMS to efficiently determine the type of acyl-, O-, and N-

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glucuronides simultaneously. Four commercial acyl- and *O*-glucuronide metabolites (Table 1) were first used to validate the method. The approach was then used to determine acyl-, *O*-, or *N*-glucuronide of six compounds (Table 1) generated from liver microsomal incubation. The chemical derivatization reaction and the strategy to differentiate different types of glucuronides are described herein.

Materials and Methods

Chemical and Biologic Reagents:

Alvimopan, benazepril hydrochloride, darunavir, diclofenac sodium salt, haloperidol, propranolol, raloxifene hydrochloride, thionyl chloride ($SOCl_2$), ethanol (EtOH), 1-(trimethylsilyl) imidazole - pyridine mixture (TMSI or Tri-Sil-Z), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), alamethicin, and dimethylformamide (DMF) were obtained from Sigma (St. Louis, MO). Silodosin was purchased from Toronto Research Chemical (Toronto, Canada). Benazepril acyl-β-D-glucuronide (Ben-A-Gluc), diclofenac acyl-β-D-glucuronide, raloxifene 4'-glucuronide (Ral-4'-O-Gluc), raloxifene 6-glucuronide (Ral-6-O-Gluc), and silodosin β-D-glucuronide (Sil-O-Gluc) were purchased from Cayman Chemical (Ann Arbor, MI). Pooled human liver microsomes (HLM) and rat liver microsomes (RLM) were purchased from Xenotech (Kansas City, KS). Soticlestat was synthesized at Takeda Pharmaceutical Company Limited (Fujisawa, Kanagawa, Japan). Soticlestat *O*-glucuronide (Sot-O-Gluc) was prepared by Wuxi AppTec Co., Ltd (Tianjin, China).

Isolation of Glucuronide Metabolites from Microsomal Incubation

A 1-mL of reaction mixture was prepared by addition of 250 µL of 40 µM substrate solution, except darunavir which will be described later, 100 µL of 20 mg/mL pooled HLM, 397.5 µL of

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0.1 M phosphate buffer containing 3 mM $MgCl_2$ (pH 7.4), 2.5 µL of 20 mg/mL alamethicin stock solution prepared in methanol, and 250 µL of 12 mg/mL UDPGA solution. Substrate, alamethicin, and UDPGA were prepared in 0.1 M phosphate buffer containing 3 mM $MgCl_2$ (pH 7.4). Incubation was conducted at 37 °C for 1 hour in ThermoMixer (F1.5, Eppendorf, Enfield, CT), which was subsequently quenched by adding 1 mL of ice-cold acetonitrile (ACN). After brief vortexing and centrifuging at 14,000 *g* for 30 minutes (min), the supernatant was collected. 40 µL of sample was injected into LC-HRMS and the corresponding glucuronide metabolite peak was isolated via fractionation and dried down using SpeedVac vacuum centrifugation (SPD1010, Thermo Fisher Scientific Inc., San Jose, CA).

For darunavir, an approximate 1 mL of reaction mixture was prepared by addition of 5 µL of 5 mM darunavir prepared in DMSO, 100 µL of 20 mg/mL pooled HLM and RLM, 695 µL of 0.1 M phosphate buffer or 0.1 M tris buffer containing 10 mM $MgCl_2$ (pH 7.4), 2.5 µL of 20 mg/mL alamethicin stock solution prepared in methanol, and 200 µL of 25 mM UDPGA solution prepared in 0.1 M phosphate buffer or 0.1 M tris buffer containing 10 mM $MgCl_2$ (pH 7.4). The remaining procedure was the same as above.

Derivatization of Carboxyl Groups

To derivatize carboxyl groups, the isolated glucuronide metabolite from liver microsomal incubation or 10 nmol of substrate if the authentic standard is available was first dried down using SpeedVac (SPD1010, Thermo Fisher Scientific Inc.) and reconstituted in 40 µL EtOH. A reaction was initiated by adding 10 µL ACN that contains 2% $SOCl_2$ (v/v). After incubating at 4 °C in Thermomixer (900 rpm) for 30 min, reactions were stopped by adding 100 µL of water. A 10 µL of the reaction mixture was then injected into LC-HRMS.

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Derivatization of Hydroxyl Groups for Alvimopan, Raloxifene, Silodosin, and Their Glucuronide Metabolites

The derivatization of hydroxyl group followed the published procedure with modified conditions (Watanabe et al., 2003). To derivatize hydroxyl groups, the isolated glucuronide metabolite from liver microsomal incubation or 20 nmol of substrate if the authentic standard is available was dried using SpeedVac (SPD1010, Thermo Fisher Scientific Inc.) and reconstituted in 100 µL of ACN. 100 µL of 1-(Trimethylsilyl) imidazole (TMSI)-Pyridine mixture was then added and incubated at 60 °C for 2 hours. A 10 µL of sample was then directly injected into LC-HRMS.

Derivatization of Hydroxyl Groups for Haloperidol, Propranolol, Soticlestat, and Their Glucuronide Metabolites

After isolating glucuronide metabolites of haloperidol, propranolol or soticlestat as described above, each glucuronide sample was completely dried. Subsequently, 50 µL of DMF and 50 µL of TMSI were added to the dried down mixture. The reaction was incubated overnight for haloperidol and soticlestat glucuronides or 1 hour for propranolol glucuronide at 60℃. Haloperidol and soticlestat glucuronide samples were dried down and reconstituted in 100 µL of 80:20 (v:v) water:ACN before being subjected to LC-HRMS analysis. Propranolol glucuronide was analyzed by LC-HRMS directly. As a positive control, 1 µL of 10 mM haloperidol, propranolol or soticlestat was added to a test tube and diluted with 9 µL of ACN and dried down under N_2 flow. After the sample was completely dried, it was treated the same way as their isolated glucuronide metabolite.

LC-HRMS Analysis

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All samples except soticlestat related samples, which will be described later, were analyzed using an Agilent 1290 Infinity ultra-high-performance liquid chromatography (Agilent Technologies Inc., Wilmington, DE) that was composed of a binary pump, an autosampler, a temperature-controlled column compartment, and a diode-array detector. The LC separation was achieved with an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters Corp., Milford, MA) maintained at 40 °C at a flow rate of 0.4 mL/min using mobile phase A (Water, 0.1% formic acid) and mobile phase B (ACN, 0.1% formic acid). The following gradient program was used: 0-1 min (5% B), 1-7 min (5%-95% B), 7-12 min (95% B), 12-12.5 min (95%-5% B), 12.5-16 min (5% B). Samples were analyzed using both a diode-array detector and Orbitrap Elite mass spectrometer (Thermo Fisher Scientific Inc.). For MS analysis, a full MS scan (*m/z* 100-1000, resolution 30000) was first performed in the positive ion mode, followed by MS/MS scan (resolution 15000) of a list of parent masses or the most intense ions if the former were absent. The list contains theoretical *m/z* of a parent compound, corresponding glucuronide metabolite and derivatized products. Other experiment parameters were set as follows: capillary temperature, 350 °C; N_2 sheath gas flow, 60 (arbitrary units); N_2 auxiliary gas flow, 20 (arbitrary units); source voltages, 3.0 kV; S-Lens RF level, 66.2%; normalized collision energy for collision-induced dissociation (CID), 40%; and normalized collision energy for higher-energy collisional dissociation (HCD), 30%. The data was acquired and processed using Xcalibur 2.2 (Thermo Fisher Scientific Inc). For darunavir, 40 µL of darunavir HLM and RLM incubations were injected using the same method described above, except with a change in mobile phases (mobile phase A: 10 mM ammonium formate containing 0.1% formic acid; solvent B: methanol containing 0.1% formic acid) to allow for better separation of *N*- vs *O*-glucuronides.

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Analysis of soticlestat, its glucuronide, and derivatized products were performed by injecting 5 µL of sample into a microLC system (M5 MicroLC system; SCIEX, Framingham, MA) coupled to a TripleTOF 6600 mass spectrometer (SCIEX). Samples were separated using a Kinetex C18 column (0.3 × 50 mm, 2.6 µm; Phenomenex Inc., Torrance, CA) heated to 45℃ at a flow rate of 10 µL/min using mobile phase A (Water, 0.1% formic acid) and mobile phase B (ACN, 0.1% formic acid). The following gradient program was used: 0-1 min (5% B), 1-5 min (5%-95% B), 5-8.5 min (95% B), 8.5-9 min (95%-5% B), 9-10 min (5% B). The HPLC elute was introduced to electrospray ionization (ESI) source using the positive ion mode on a TripleTOF 6600. The ESI parameters were set as follows: declustering potential, 60 V; Gas 1, Gas 2, and curtain gas, 30, 45, and 30 (arbitrary units), respectively; ion spray voltage, 4500 V; and source temperature, 300℃. The samples were analyzed in full scan mode with a mass range of 300 to 1000 Da. MS/MS data were analyzed with a mass range of 100 to 1000 with data dependent acquisition triggered by the mass defect filter list. Collision energy for full scan and MS/MS analysis were 10 and 35 V, respectively. The data was acquired using AnalystTF 1.8 (SCIEX) and processed using PeakView 2.2 (SCIEX).

Results

Method Validation Using Acyl- and *O*-Glucuronide Standards. Four commercial glucuronide metabolite standards, Ben-A-Gluc, Ral-4'-O-Gluc, Ral-6-O-Gluc, and Sil-O-Gluc, were used for the validation of the method. After Ben-A-Gluc was treated with $SOCl_2$ in the presence of EtOH, the only carboxyl group on the glucuronide moiety was converted to the ethyl (Et) ester (Figure 1A). After LC-HRMS analysis, the 1 × Et derivatized Ben-A-Gluc was detected (Figure 1B, Table 1), while the 2 × Et derivatized peak was absent in the carboxyl derivatized sample (Figure 1C).

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After Ral-4'-O-Gluc and Ral-6-O-Gluc were treated with TMSI, the 4 × trimethylsilyl (TMS) derivatized glucuronide metabolite was detected for each of them, while the 5 × TMS derivatized glucuronide metabolite was not detected, suggesting that 4 hydroxyl groups were present on both glucuronides, which was consistent with their structures (Table 1). After Sil-O-Gluc was treated with TMSI, the 3 × TMS derivatized glucuronide metabolite was detected, while the 4 × TMS derivatized glucuronide metabolite was not detected by LC-HRMS, suggesting that 3 hydroxyl groups were present on this metabolite, which was also consistent with its structure (Table 1).

Determination of Acyl-, *O*-, or *N*-Glucuronides Isolated from Liver Microsomal Incubation. After human and/or rat liver microsomal incubation with alvimopan, diclofenac, darunavir, haloperidol, and propranolol, an alvimopan glucuronide, a diclofenac glucuronide, 2 darunavir glucuronides, a haloperidol glucuronide, and a propranolol glucuronide were isolated and the type of glucuronide was determined by carboxyl and hydroxyl derivatization using LC-HRMS. Each of those isolated glucuronide metabolites was treated with $SOCl_2$ in the presence of EtOH and/or TMSI separately. The maximum number of Et and/or TMS derivatized groups was determined by LC-HRMS and their proposed glucuronide type was listed in Table 1.

Alvimopan glucuronide was proposed to be acyl-glucuronide based on both carboxyl and hydroxyl derivatization result. Diclofenac glucuronide was proposed to be acyl-glucuronide based on carboxyl derivatization result. Its structure was confirmed by the comparison of the retention time and MS/MS data to those from the authentic standard (data not shown). Darunavir glucuronides 1 and 2 were proposed to be *N*- and *O*-glucuronides, respectively, based on hydroxyl derivatization result. Both structures were confirmed by the MS/MS spectra (Figure 2). The product ion *m/z* 464.232 from 4 × TMS derivatized glucuronide 1 indicated that the TMS was added to the darunavir moiety, suggesting that the only hydroxyl group on the darunavir

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moiety was silylated and was not conjugated to glucuronide (Figure 2A). Additionally, the product ion *m/z* 332.042 from darunavir glucuronide 1 suggested that glucuronidation occurred on the phenylamine (Figure 2B). Therefore, darunavir glucuronide 1 structure was determined to be the *N*-glucuronide as shown in Table 1. In contrast, the product ion *m/z* 392.199 from 3 × TMS derivatized glucuronide 2 suggested that the hydroxyl group on the darunavir moiety was not silylated and was thus conjugated to glucuronide (Figure 2C), which was consistent with the *O*-glucuronide assignment. Haloperidol glucuronide isolated from HLM incubation was proposed to be *O*-glucuronide based on hydroxyl derivatization result. This was corroborated by the fact that haloperidol *O*-glucuronide has been reported as the major glucuronide metabolite observed in HLM incubation (Kato et al., 2012). Propranolol glucuronide was proposed to be *O*-glucuronide based on hydroxyl derivatization result. Although the structure of the propranolol glucuronide was not confirmed further by other experiment, propranolol *O*-glucuronide has been reported as the major human plasma metabolite (Walle et al., 1979).

Determination of Sot-O-Gluc. A soticlestat glucuronide was isolated from HLM incubation and subsequently derivatized with TMSI. Since soticlestat does not contain carboxyl group, the isolated compound is either *O*- or *N*-glucuronide. After TMSI derivatization, the 3 hydroxyl groups on the glucuronide moiety can be derivatized for *O*-glucuronide (Figure 3A). Three potential *N*-glucuronides can be formed for this compound. A total of 4 hydroxyl groups would be derivatized for any of the *N*-glucuronides (Figure 3A). After LC-HRMS analysis, the 3 × TMS derivatized soticlestat glucuronide was detected (Figure 3B), while the 4 × TMS derivatized peak was absent in the hydroxyl derivatized sample (Figure 3C). Therefore, the isolated glucuronide metabolite was proposed to be Sot-O-Gluc (Table 1). The assigned structure

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was later confirmed by comparison of the retention time and MS/MS data to those from the authentic standard (data not shown).

Positive Control Samples. Each of the aglycone compounds was treated the same way as their respective glucuronide metabolites. The maximum number of Et and TMS derivatized groups was determined by LC-HRMS and listed in Table 2. All carboxyl and/or hydroxyl groups on those compounds were successfully derivatized by either $SOCl_2$ in the presence of EtOH or TMSI.

Discussion

Carboxyl, hydroxyl, and amine are common functional groups for marketed drugs, drug candidates under development, and drug metabolites. A carboxyl, hydroxyl, and amine bearing molecule can potentially form acyl-, *O*-, and *N*-glucuronides *in vitro* and *in vivo*. If glucuronidation is a major metabolic pathway for clearance of a drug, identification of the type of glucuronidation can be important for the project team for optimization of clearance. Additionally, the identification of the presence of acyl-glucuronides is particularly important to understand if the reactive metabolite is formed for a drug. If a drug only contains a single type of the functional group such as carboxyl, hydroxyl, or amine that can be glucuronidated, the type of glucuronidation is self-explained and does not need further study to elucidate it. However, if a drug contains multiple types of the functional groups (e.g. carboxyl and hydroxyl; carboxyl and amine; hydroxyl and amine; or carboxyl, hydroxyl, and amine), the method described in this study can be easily and effectively used to differentiate the type of its glucuronide metabolite. If a specific type of glucuronide has only one possible glucuronidation site, the definitive structure is determined for this glucuronide metabolite even though the aglycone contains a combination

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of carboxyl, hydroxyl, and amine. For instance, soticlestat contains a hydroxyl, an amide, and 2 amino groups. The isolated soticlestat glucuronide was determined to be *O*-glucuronide in this study. Consequently, its definitive structure was unambiguously assigned (Table 1).

Different types of glucuronide from an aglycone compound contain predictable and different numbers of carboxyl and/or hydroxyl groups. For example, alvimopan acyl-glucuronide has only 1 carboxyl group (Figure 4A) while alvimopan *O*- or *N*-glucuronide has 2 carboxyl groups (Figures 4B and 4C). On the other hand, alvimopan *O*-glucuronide has 3 hydroxyl groups while alvimopan acyl- or *N*-glucuronide has 4 hydroxyl groups (Figure 4). Therefore, the number of carboxyl and hydroxyl groups on the glucuronide metabolite can inform the type of glucuronidation. The number of carboxyl or hydroxyl groups on a glucuronide metabolite can be determined by the number of corresponding derivatized groups using specific derivatization reactions. In this study, carboxyl group is selectively derivatized with $SOCl_2$ in the presence of EtOH to form the Et ester with a mass shift of +28.031 Da per derivatized group under the described condition. Hydroxyl group is selectively derivatized with TMSI to form trimethylsiloxy group with a mass shift of +72.040 Da per derivatized group. After derivatization, the type of glucuronide can then be determined by the maximum number of derivatized carboxyl or hydroxyl groups calculated from the mass shift detected by LC-HRMS (Table 3). For example, in case of alvimopan glucuronides, detection of 1 × Et derivatized group (i.e., a positive mass shift of 1 × 28.031 Da) after carboxyl derivatization and 4 × TMS derivatized groups (i.e., a positive mass shift of 4 × 72.040 Da) after hydroxyl derivatization means it is an acyl-glucuronide (Figure 4A). Similarly, detection of 2 × Et and 3 × TMS derivatized groups means an *O*-glucuronide (Figure 4B) and detection of 2 × Et and 4 × TMS derivatized groups means an *N*-glucuronide (Figure 4C). It is worth noting that the method is expected to work for the

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migrated acyl-glucuronide as well since the number of the carboxyl and hydroxyl groups is identical between unmigrated and migrated acyl-glucuronides.

Based on this strategy, a decision tree is proposed to determine the type of glucuronide metabolite (Figure 5). The decision tree provides guidance on how to determine acyl-, *O*-, and *N*-glucuronide using chemical derivatization.

In this study, we first used four commercial metabolite standards including Ben-A-Gluc, Ral-4'-O-Gluc, Ral-6-O-Gluc, and Sil-O-Gluc to validate the method. The assigned glucuronide type determined using the above strategy was consistent with the structure of all those commercial standards (Table 1). Subsequently, five commercial compounds, alvimopan, diclofenac, darunavir, haloperidol, and propranolol, known to generate different types of glucuronide metabolites were incubated in HLM or RLM. A total of six glucuronide metabolites were isolated from those liver microsomal samples. The type of glucuronide metabolites was then proposed based on the result from chemical derivatization (Table 1). Among those proposed glucuronide metabolites, the structure of diclofenac acyl-glucuronide was confirmed by the authentic standard, diclofenac acyl-β-D-glucuronide. The structures of darunavir *N*-glucuronide and *O*-glucuronide were confirmed by their respective MS/MS spectra. The assignment of haloperidol *O*-glucuronide and propranolol *O*-glucuronide was supported by the literature data. The assignment of alvimopan acyl-glucuronide was corroborated with the result from both carboxyl and hydroxyl derivatization. Therefore, the method was successfully applied to determine the acyl-, *O*-, and *N*-glucuronides generated *in vitro*. Additionally, the method was also used for the characterization of the structure of a glucuronide metabolite from soticlestat observed in clinical samples. Soticlestat is a potent and highly selective inhibitor of cholesterol 24-hydroxylase and is being tested in clinical trials for the treatment of rare developmental and

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epileptic encephalopathies. A soticlestat glucuronide was formed in HLM incubation, which was also observed in human plasma and urine. The structure of this metabolite was characterized with hydroxyl derivatization using the aforementioned strategy (Figure 5). The derivatization result suggested that it was an *O*-glucuronide. This proposed structure was later targeted for synthesis (as opposed to synthesizing all potential positional isomers) and confirmed to be Sot-O-Gluc. All those data indicates that the type of glucuronide metabolite can be determined by the chemical derivatization method described in this study.

It is worth noting that when the chemical derivatization experiment is conducted, it is critically important to run the derivatization reaction under the same conditions on the aglycone compound simultaneously as the positive control. This method only works when the derivatization is successful on all carboxyl or hydroxyl group(s) on the aglycone. If any carboxyl or hydroxyl group(s) on the aglycone cannot be derivatized, the maximum number of the derivatized groups may be identical for acyl-, *O*-, and *N*-glucuronides, which may lead to an incorrect assignment of glucuronide type. For example, compound A contains an inert hydroxyl group, which cannot be derivatized by TMSI. Although *N*-glucuronide of compound A has 4 hydroxyl groups, only the 3 on the glucuronide moiety can be derivatized. As a result, a maximum of 3 × TMS derivatized groups will be detected for the *N*-glucuronide of compound A. That observation will result in a misleading conclusion that this is an *O*-glucuronide of compound A according to the decision tree (Figure 5). In this study, the chemical derivatization on the aglycone compounds listed in Table 2 were all successful. However, the chemical derivatization on some other compounds was unsuccessful (data not shown). As a result, determination of the type of their glucuronide metabolites for the latter compounds was not pursued. To efficiently utilize this method, a good strategy is to first run the method development

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of the desired chemical derivatization on the aglycone compound. The derivatization conditions described in this study have been successfully applied for multiple compounds, which can be a good starting point for the method development. If carboxyl or hydroxyl derivatization is effective on the aglycone compound, the method can then be used for the determination of the type of its glucuronide metabolite. The carboxyl and hydroxyl groups on the glucuronide moiety are relatively reactive and their reactivity should be similar for any glucuronide metabolite regardless of the aglycone structure. Therefore, method development of the chemical derivatization is not needed for the glucuronide metabolite. Additionally, the derivatization reaction was selective on either carboxyl or hydroxyl group under the conditions used in this study. The method will not work if the derivatization reaction is not selective on a specific functional group.

In summary, the type of 11 glucuronide metabolites from 9 compounds was successfully determined by chemical derivatization coupled with LC-HRMS. Among them, the definitive structures of 8 glucuronide metabolites were unambiguously assigned as there was only one possible glucuronidation site for the type of glucuronide. The method utilizes the maximum number of derivatized carboxyl and/or hydroxyl groups to differentiate acyl-, *O*-, and *N*-glucuronide. Carboxyl and hydroxyl groups were derivatized by $SOCl_2$ in the presence of EtOH and TMSI, respectively, in this study. However, the method can be extended to any other chemical derivatization as long as the reaction is specific towards carboxyl or hydroxyl group.

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Authorship Contributions

Participated in research design: Guo, Shah, Zhu.

Conducted experiments: Guo, Shah, Oh.

Contributed new reagents or analytic tools: Guo, Shah, Zhu.

Performed data analysis: Guo, Shah, Zhu.

Wrote or contributed to the writing of the manuscript: Guo, Shah, Oh, Chowdhury, Zhu.

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References

Cui D and Harvison PJ (2000) Determination of the site of glucuronidation in an N-(3,5-dichlorophenyl)succinimide metabolite by electrospray ionization tandem mass spectrometry following derivatization to picolinyl esters. *Rapid Communications in Mass Spectrometry* **14:**1985-1990.

Dalvie DK and O'Donnell JP (1998) Characterization of polar urinary metabolites by ionspray tandem mass spectrometry following dansylation. *Rapid Communications in Mass Spectrometry* **12:**419-422.

Hyneck ML, Munafo A, and Benet LZ (1988) Effect of pH on acyl migration and hydrolysis of tolmetin glucuronide. *Drug Metabolism and Disposition* **16:**322-324.

Kato Y, Nakajima M, Oda S, Fukami T, and Yokoi T (2012) Human UDP-Glucuronosyltransferase Isoforms Involved in Haloperidol Glucuronidation and Quantitative Estimation of Their Contribution. *Drug Metabolism and Disposition* **40:**240-248.

Kong JY, Yu Z, Easton MW, Niyonsaba E, Ma X, Yerabolu R, Sheng H, Jarrell TM, Zhang Z, Ghosh AK, and Kenttämaa HI (2018) Differentiating Isomeric Deprotonated Glucuronide Drug Metabolites via Ion/Molecule Reactions in Tandem Mass Spectrometry. *Analytical Chemistry* **90:**9426-9433.

Kulanthaivel P, Barbuch RJ, Davidson RS, Yi P, Rener GA, Mattiuz EL, Hadden CE, Goodwin LA, and Ehlhardt WJ (2004) Selective Reduction of N-Oxides to Amines: Application to Drug Metabolism. *Drug Metabolism and Disposition* **32:**966-972.

Liu DQ and Hop CECA (2005) Strategies for characterization of drug metabolites using liquid chromatography–tandem mass spectrometry in conjunction with chemical derivatization and on-line H/D exchange approaches. *Journal of Pharmaceutical and Biomedical Analysis* **37:**1-18.

Ly VT, Caceres-Cortes J, Zhang D, Humphreys WG, Ekhato IV, Everett D, and Çömezoğlu SN (2009) Metabolism and Excretion of an Oral Taxane Analog, [14C]3'-tert-Butyl-3'-N-tert-butyloxycarbonyl-4-deacetyl-3'-dephenyl-3'-Ndebenzoyl-4-O-methoxy-paclitaxel (BMS-275183), in Rats and Dogs. *Drug Metabolism and Disposition* **37:**1115-1128.

Niyonsaba E, Easton MW, Feng E, Yu Z, Zhang Z, Sheng H, Kong J, Easterling LF, Milton J, Chobanian HR, Deprez NR, Cancilla MT, Kilaz G, and Kenttämaa HI (2019) Differentiation of Deprotonated Acyl-, N-, and O-Glucuronide Drug Metabolites by Using Tandem Mass Spectrometry Based on Gas-Phase Ion–Molecule Reactions Followed by Collision-Activated Dissociation. *Analytical Chemistry* **91:**11388-11396.

Ohashi N, Furuuchi S, and Yoshikawa M (1998) Usefulness of the hydrogen–deuterium exchange method in the study of drug metabolism using liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **18:**325-334.

Smith DA, Hammond T, and Baillie TA (2018) Safety Assessment of Acyl Glucuronides—A Simplified Paradigm. *Drug Metabolism and Disposition* **46:**908-912.

Smith PC, Benet LZ, and McDonagh AF (1990) Covalent binding of zomepirac glucuronide to proteins: evidence for a Schiff base mechanism. *Drug Metabolism and Disposition* **18:**639-644.

Subramanian R, Lee MR, Allen JG, Bourbeau MP, Fotsch C, Hong F-T, Tadesse S, Yao G, Yuan CC, Surapaneni S, Skiles GL, Wang X, Wohlhieter GE, Zeng Q, Zhou Y, Zhu X, and Li C (2010) Cytochrome P450-Mediated Epoxidation of 2-Aminothiazole-Based AKT Inhibitors: Identification of Novel GSH Adducts and Reduction of Metabolic Activation through Structural Changes Guided by in Silico and in Vitro Screening. *Chemical Research in Toxicology* **23:**653-663.

van Breemen RB and Fenselau C (1985) Acylation of albumin by 1-O-acyl glucuronides. *Drug Metabolism and Disposition* **13:**318-320.

Vaz ADN, Wang WW, Bessire AJ, Sharma R, and Hagen AE (2010) A rapid and specific derivatization procedure to identify acyl-glucuronides by mass spectrometry. *Rapid Communications in Mass Spectrometry* **24:**2109-2121.

Walle T, Fagan TC, Conradi EC, Walle UK, and Gaffney TE (1979) Presystemic and systemic glucuronidation of propranolol. *Clinical Pharmacology & Therapeutics* **26:**167-172.

Walles M, Brown AP, Zimmerlin A, and End P (2020) New Perspectives on Drug-Induced Liver Injury Risk Assessment of Acyl Glucuronides. *Chemical Research in Toxicology* **33:**1551-1560.

Watanabe Y, Nakajima M, Ohashi N, Kume T, and Yokoi T (2003) Glucuronidation of Etoposide in Human Liver Microsomes Is Specifically Catalyzed by UDP-Glucuronosyltransferase 1A1. *Drug Metabolism and Disposition* **31:**589-595.

Yan Z and Caldwell GW (2004) Stable-Isotope Trapping and High-Throughput Screenings of Reactive Metabolites Using the Isotope MS Signature. *Analytical Chemistry* **76:**6835-6847.

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Figures

Figure 1. Carboxyl derivatization of Ben-A-Gluc and XIC of the carboxyl derivatized sample. (A). Proposed carboxyl derivatization of Ben-A-Gluc by $SOCl_2$ in the presence of EtOH; (B). XIC of the precursor ion *m/z* 629.2705 of 1 × Et derivatized Ben-A-Gluc; (C). XIC of the precursor ion *m/z* 657.3018 of 2 × Et derivatized Ben-A-Gluc.

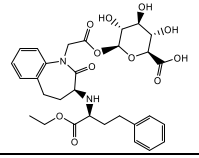
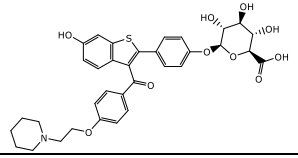
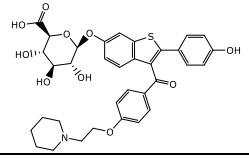
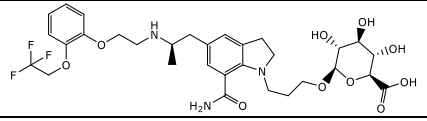
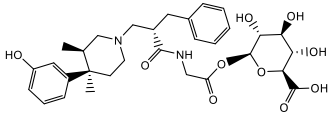
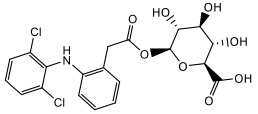
Figure 2. MS/MS spectra for the TMS derivatized darunavir glucuronides and darunavir *N*-glucuronide. (A). MS/MS of *m/z* 1012.43@CID40 for 4 × TMS derivatized darunavir *N*-glucuronide; (B). MS/MS of *m/z* 724.27@HCD35 for darunavir *N*-glucuronide; (C). MS/MS of *m/z* 940.39@CID40 for 3 × TMS derivatized darunavir *O*-glucuronide.

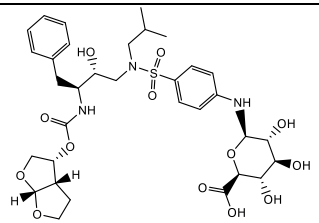
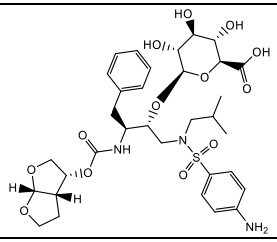
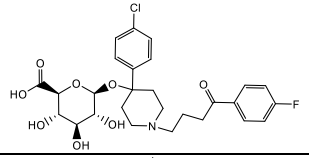
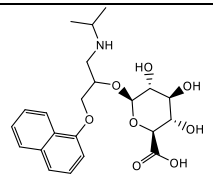
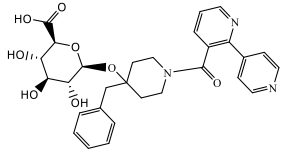
Figure 3. Hydroxyl derivatization of the soticlestat glucuronide isolated from HLM incubation by TMSI. (A). Proposed hydroxyl derivatization of Sot-O-Gluc and one of the potential soticlestat *N*-glucuronides; (B). XIC of the precursor ion *m/z* 766.3370 of 3 × TMS derivatized Sot-O-Gluc; (C). XIC of the precursor ion *m/z* 838.3765 of 4 × TMS derivatized Sot-O-Gluc.

Figure 4. Carboxyl and hydroxyl derivatization by $SOCl_2$ in the presence of EtOH and TMSI, respectively, of the alvimopan glucuronides. (A). Proposed carboxyl and hydroxyl derivatization of alvimopan acyl-glucuronide; (B). Proposed carboxyl and hydroxyl derivatization of alvimopan *O*-glucuronide; (C). Proposed carboxyl and hydroxyl derivatization of alvimopan *N*-glucuronide.

Figure 5. Decision tree on how to determine acyl-, *O*-, and *N*-glucuronide.

Table 1. Maximum number of Et and TMS derivatized groups observed by LC-HRMS for four commercial glucuronide metabolites and seven glucuronide metabolites isolated from liver microsomal incubation after carboxyl and hydroxyl derivatization, respectively. The carboxyl and hydroxyl groups of interest are highlighted in red and blue, respectively.

Glucuronide	Known or proposed glucuronide structure*	Maximum number of Et derivatized groups	Maximum number of TMS derivatized groups	Proposed glucuronide type based on the derivatization	Notes
Glucuronide standards for method validation					
Ben-A-Gluc		1	-	Acyl-	Commercial compound
Ral-4'-O-Gluc		-	4	*O*-	Commercial compound
Ral-6-O-Gluc		-	4	*O*-	Commercial compound
Sil-O-Gluc		-	3	*O*-	Commercial compound
Glucuronides isolated from liver microsomal incubation					
alvimopan glucuronide		1	4	Acyl-	Both carboxyl and hydroxyl derivatization suggests that it was an acyl-glucuronide.
diclofenac glucuronide		1	-	Acyl-	Structure confirmed by the authentic standard

darunavir glucuronide 1		-	4	*N*-	Structure confirmed by MS/MS
darunavir glucuronide 2		-	3	*O*-	Structure confirmed by MS/MS
haloperidol glucuronide		-	3	*O*-	Haloperidol *O*-glucuronide has been reported as the major glucuronide observed in HLM incubation (Kato et al., 2012).
propranolol glucuronide		-	3	*O*-	Propranolol *O*-glucuronide has been reported as the major human plasma metabolite (Walle et al., 1979).
soticlestat glucuronide		-	3	*O*-	Structure confirmed by the authentic standard

*, the structure for glucuronides separated from liver microsomal incubation was proposed based on the derivatization result; -, data not available

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Table 2. Number of carboxyl and hydroxyl groups as well as maximum number of Et and TMS derivatized groups observed by LC-HRMS for aglycone compounds after carboxyl and hydroxyl derivatization, respectively

Compounds	Number of carboxyl groups	Maximum number of Et derivatized groups	Number of hydroxyl groups	Maximum number of TMS derivatized groups
benazepril	1	1	0	-
raloxifene	0	-	2	2
silodosin	0	-	1	1
alvimopan	1	1	1	1
diclofenac	1	1	0	-
darunavir	0	-	1	1
haloperidol	0	-	1	1
propranolol	0	-	1	1
soticlestat	0	-	1	1

-, data not available

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Table 3. Maximum number of carboxyl and hydroxyl derivatized groups for acyl-, *O*-, or *N*-glucuronide after derivatization

Glucuronide type	Acyl-glucuronide	*O*-glucuronide	*N*-glucuronide
Maximum number of carboxyl derivatized groups	N (1)	N+1 (2)	N+1 (2)
Maximum number of hydroxyl derivatized groups	M+3 (4)	M+2 (3)	M+3 (4)

N: the number of carboxyl group on the aglycone (N >= 0)
M: the number of hydroxyl group on the aglycone (M >= 0)
The number in the parentheses is from an aglycone compound with a single carboxyl and/or a single hydroxyl group and any number of amino groups.

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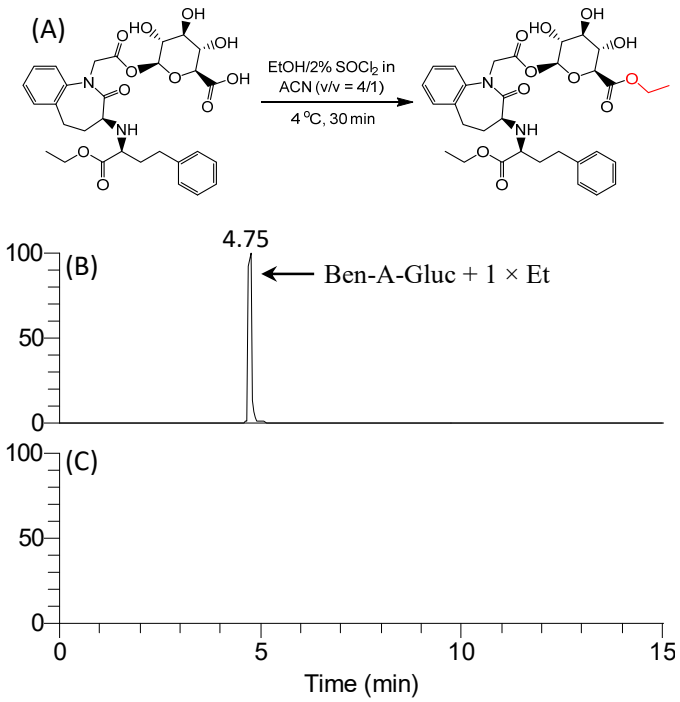

Figure 1

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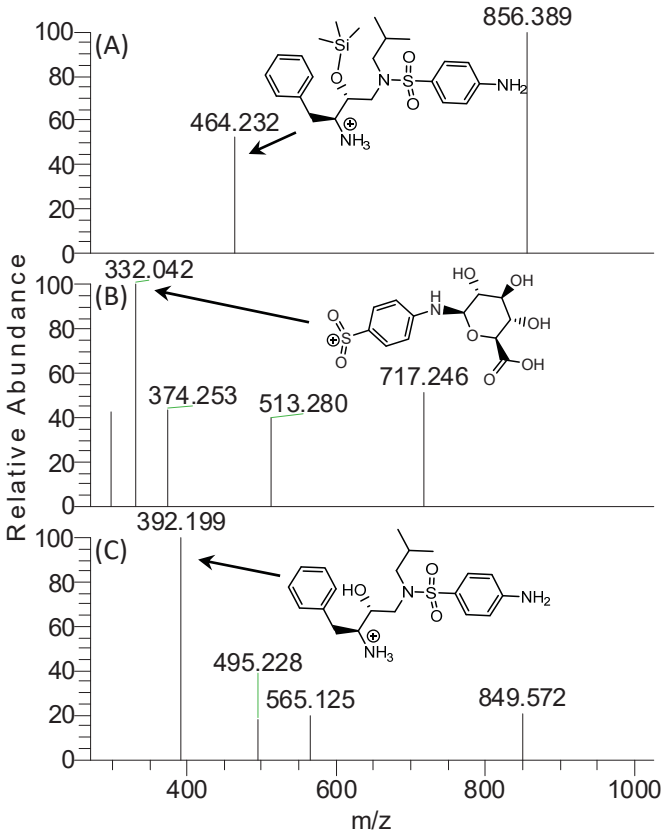

Figure 2

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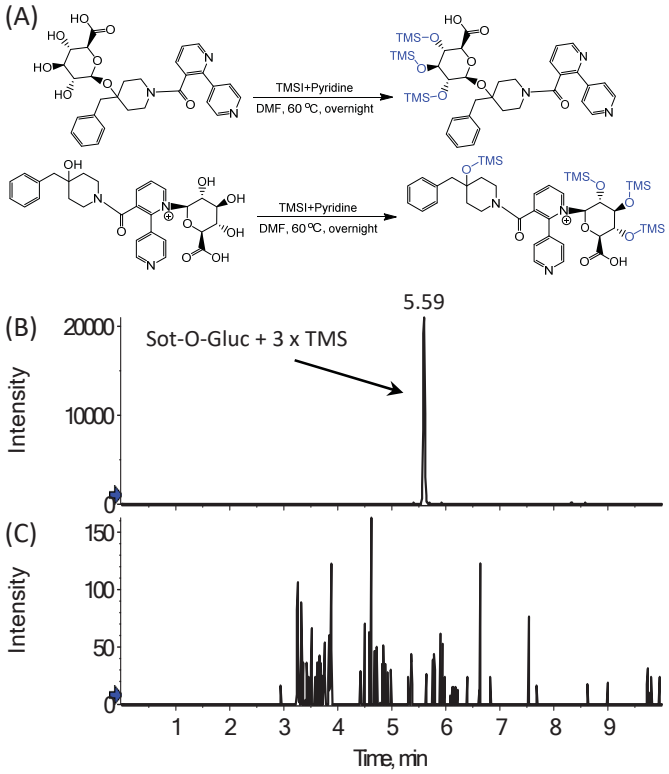

Figure 3

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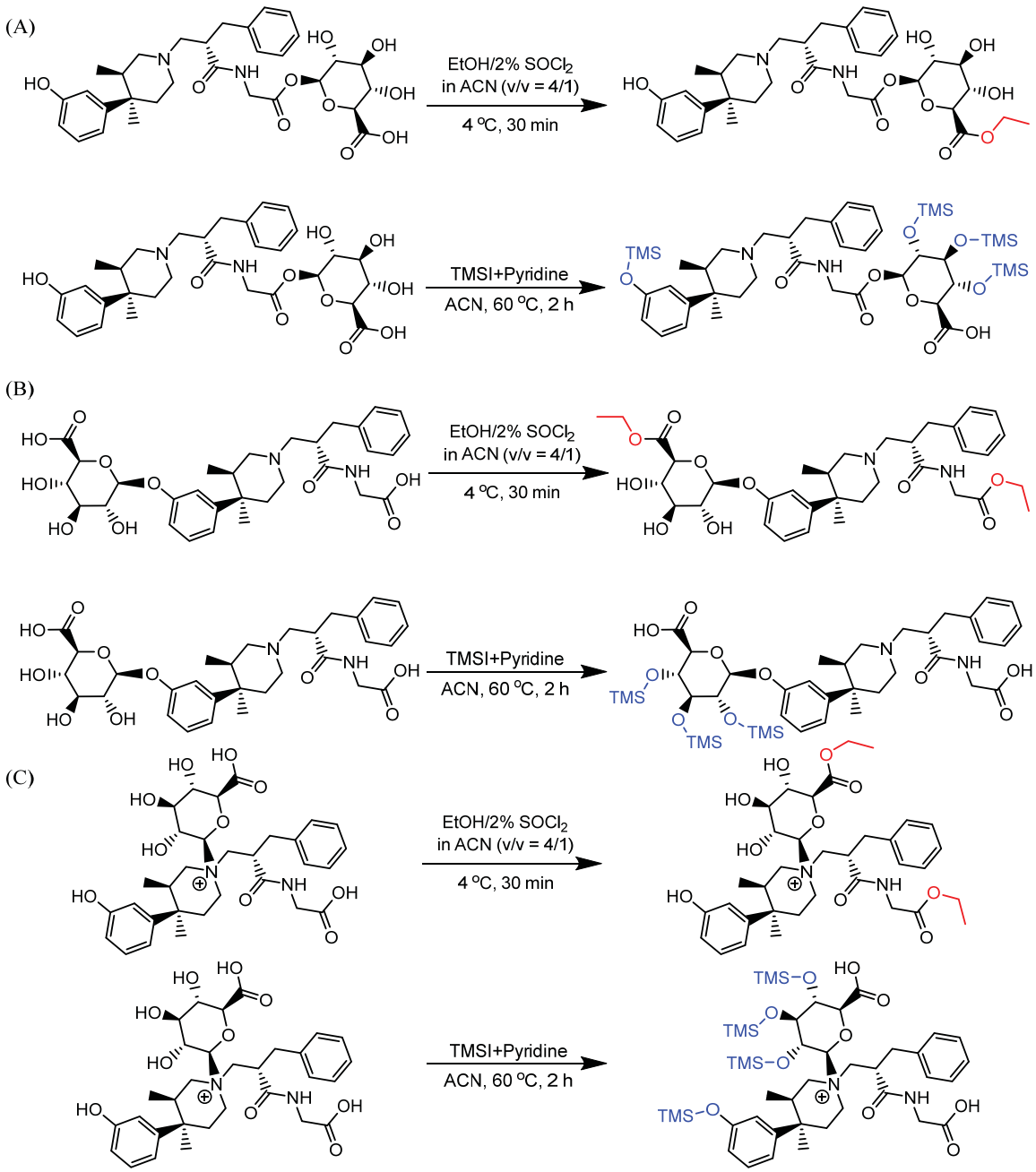

Figure 4

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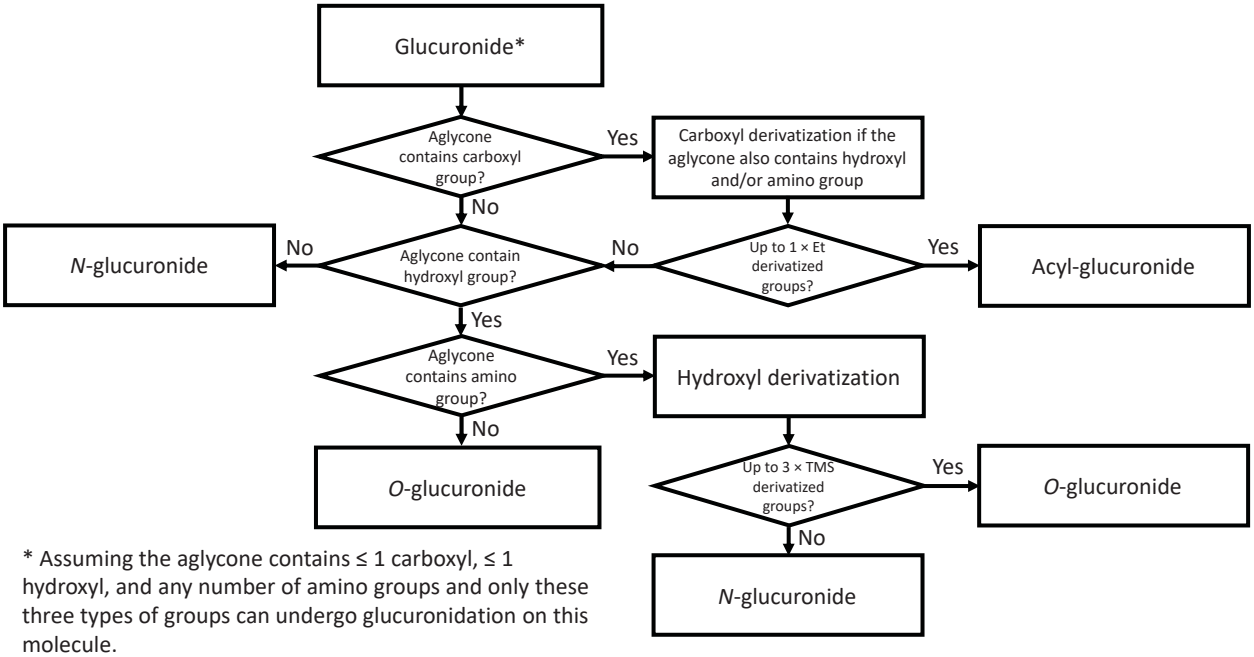

* Assuming the aglycone contains ≤ 1 carboxyl, ≤ 1 hydroxyl, and any number of amino groups and only these three types of groups can undergo glucuronidation on this molecule.

Figure 5