Determination of Degradation Pathways and Kinetics of Acyl Glucuronides by NMR Spectroscopy

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Acyl glucuronides have been implicated in the toxicity of many xenobiotics and marketed drugs. These toxicities are hypothesized to be a consequence of covalent binding of the reactive forms of the acyl glucuronide to proteins. Reactive intermediates of the acyl glucuronide arise from the migration of the aglycone leading to other positional and stereoisomers under physiological conditions. In order to screen for the potential liabilities of these metabolites during the early phase of pharmaceutical development, an NMR method based on the disappearance of the anomeric resonance of the *O*-1-acyl glucuronide was used to monitor the degradation kinetics of 11 structurally diverse acyl glucuronides, including those produced from the known nonsteroidal anti-inflammatory drugs (NSAIDs). The acyl glucuronides were either chemically synthesized or were isolated from biological matrices (bile, urine, and liver microsomal extracts). The half-lives attained utilizing this method were found to be comparable to those reported in the literature. NMR analysis also enabled the delineation of the two possible pathways of degradation: acyl migration and hydrolytic cleavage. The previously characterized ^{1}H resonances of acyl migrated products are quite distinguishable from those that arise from hydrolysis. The NMR method described here could be used to rank order acyl glucuronide forming discovery compounds based on the potential reactivity of the conjugates and their routes of decomposition under physiological conditions. Furthermore, we have shown that *in vitro* systems such as liver microsomal preparations can be used to generate sufficient quantities of acyl glucuronides from early discovery compounds for NMR characterization. This is particularly important, as we often have limited supply of early discovery compounds to conduct *in vivo* studies to generate sufficient quantities of acyl glucuronides for further characterization.

Introduction

The glucuronidation of xenobiotics and endobiotics represents one of the major routes of elimination for these compounds in animals. Glucuronidation is catalyzed by a family of UDP-glucuronosyltransferases (UGTs) capable of recognizing and conjugating a wide range of substrates. This conjugation reaction, which depends on the supply of glucuronic acid from the cofactor uridine 5′-diphosphoglucuronic acid (UDPGA), results in the formation of ether, ester, and S-, N-, and C-linked glucuronides of various compounds. The ether conjugates, often formed from free hydroxyl or phenolic groups, lead to detoxification or deactivation of pharmacologically active entities. The S-, N-, and C-linked glucuronides are less common, though they can be important routes of metabolism for some compounds (*1*−*3*). The acyl glucuronides (AG), as the conjugation products of carboxylic acid containing compounds and D-glucuronic acid, have received considerable interest over the last several years because of their ability to mediate hypersensitivity reactions and cellular toxicity (*4*−*6*). These ester glucuronides are electrophilic and show inherent reactivity as compared to other types of glucuronide conjugates (*7*). A subset of ester glucuronides include those that are formed from carbamic acid intermediates of amines (*8*−*10*). These carbamates are considered as being fairly non-consequential, though they can contribute significantly to the overall disposition of some compounds (*8*, *11*, *12*).

Under physiological conditions, the aglycone of the AG may migrate to the 2-, 3-, and/or 4-position of glucuronic acid or the AG may hydrolyze back to the parent compound and glucuronic acid (*13*, *14*). Furthermore, once the aglycone has migrated to the 2-, 3-, or 4-position, these AGs may mutarotate between the β and α configurations. The hydrolysis of AG is considered unidirectional, whereas the products resulting from the rearrangement of AG are thought to be in equilibrium (Figure 1) (*15*). The migration of the aglycone from the 1-position has previously been considered as unidirectional, although recent studies have shown the possibility of low level conversion of the 2α-form to the 1α-isomer (*16*). Because of their chemical reactivity and ability to covalently bind to proteins, AGs are thought to be responsible for liver toxicities exhibited by a number of NSAIDs (*6*, *17*−*20*). The migration of the aglycone can lead to potentially reactive intermediates as described previously (*15* and references therein).

It has been previously proposed many times that the rate of degradation of the *O*-1-acyl glucuronide to its isomers can provide an indirect measure of its potential reactivity (*21* and references therein). AGs with short half-lives are considered as being more reactive than those with long half-lives and are therefore more likely to react with proteins through pathways

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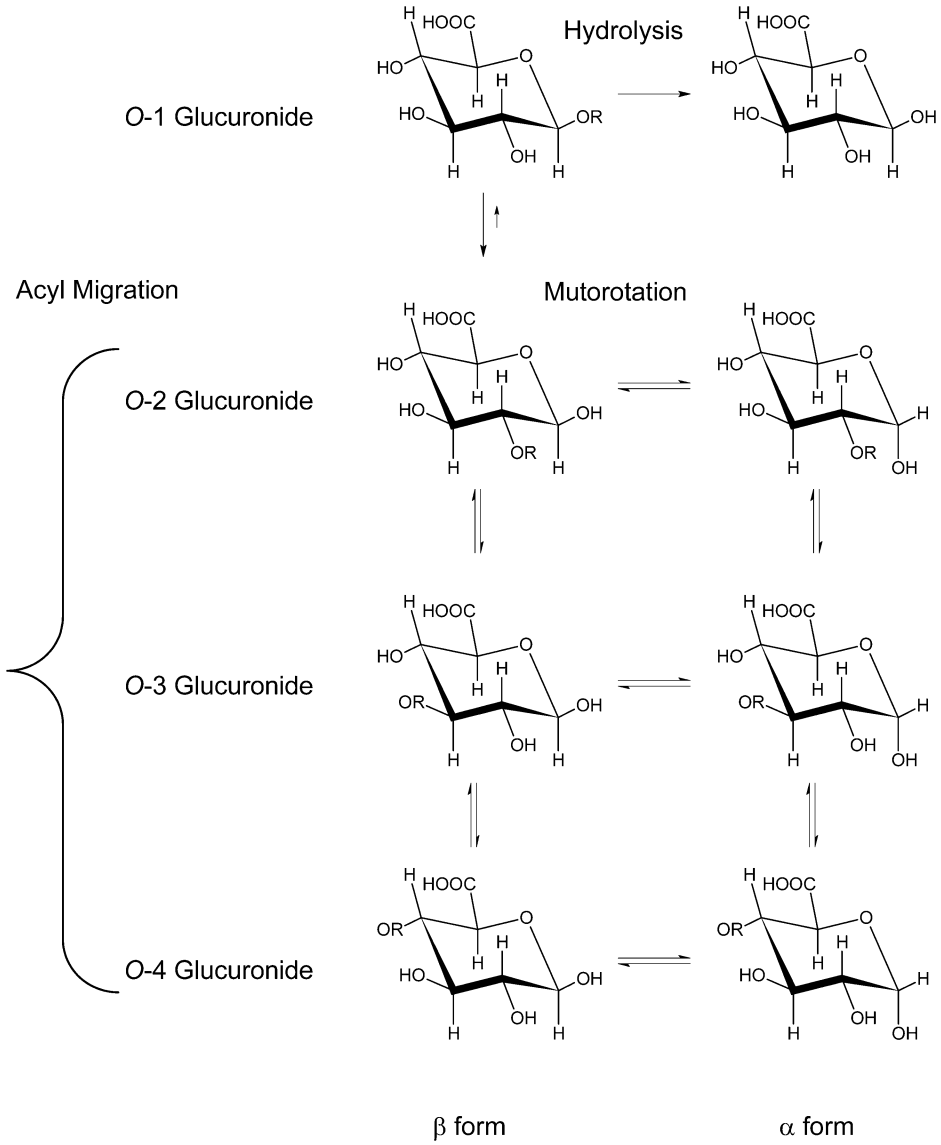

Figure 1. Migration of acyl glucuronides with subsequent mutorotation of the respective isomers.

A and B (Figure 2). Even though it is very often difficult to associate the formation of reactive intermediates with specific target organ toxicities, it is important to be able to assess the potential for a compound to form an AG and subsequently determine the ability of the AG to rearrange or hydrolyze to the parent compound. Consequently, determining both the rate and mechanism of AG degradation is very important in assessing the potential metabolic liability of carboxylic acid-containing compounds in a discovery setting.

Historically, acyl glucuronides have been considered potential toxicants in many marketed drugs and a metabolic liability for compounds in discovery (*5*, *22*). Given this, it would be advantageous to have an assay to screen compounds early in the discovery process that have the potential to form highly reactive AGs. The ability to rank order chemical templates capable of forming AGs that undergo facile transacylation using a highly versatile technique such as NMR can potentially eliminate undesirable candidates from further development. It is proposed that an AG of a compound that has a half-life of less than 1.5 h and which degrades essentially through acyl migration may pose too much of a risk to be further advanced in development. This is based on the literature values of NSAID AGs, some of which have never been approved, have been withdrawn from the market, and/or carry a significant black-box warning label to prescribing physicians (Table 1). However, the identification of fast transacylation kinetics for an acyl glucuronide is one of a number of reasons why consideration should be given to removing such an aglycone from further drug development. Compounds that produce AGs with half-lives greater than 1.5 h probably need to be evaluated further through a battery of standard safety assessments. Additionally, compounds that exhibit desirable pharmacology and ADME characteristics and at the same time produce AG should not be hastily terminated from development. Considerations such as the dose, medical condition being treated, duration of therapy, availability of alternate chemical templates, and the intended target population should all be taken into consideration before proceeding with a compound capable of forming the AGs.

The addition of a carboxylic acid moiety to a pharmacophore may produce many desirable characteristics including increased solubility/permeability or increased potency due to better orientation in the active site of a protein. Consequently, the pharmaceutical industry is still pursuing carboxylic acid-possessing chemical templates as potential therapeutic agents to treat a variety of health conditions. Thus, it is highly desirable that we have a robust technique to rapidly evaluate the propensity and reactivity of AGs that may be formed from these compounds. NMR, as an analytical tool, provides the best and

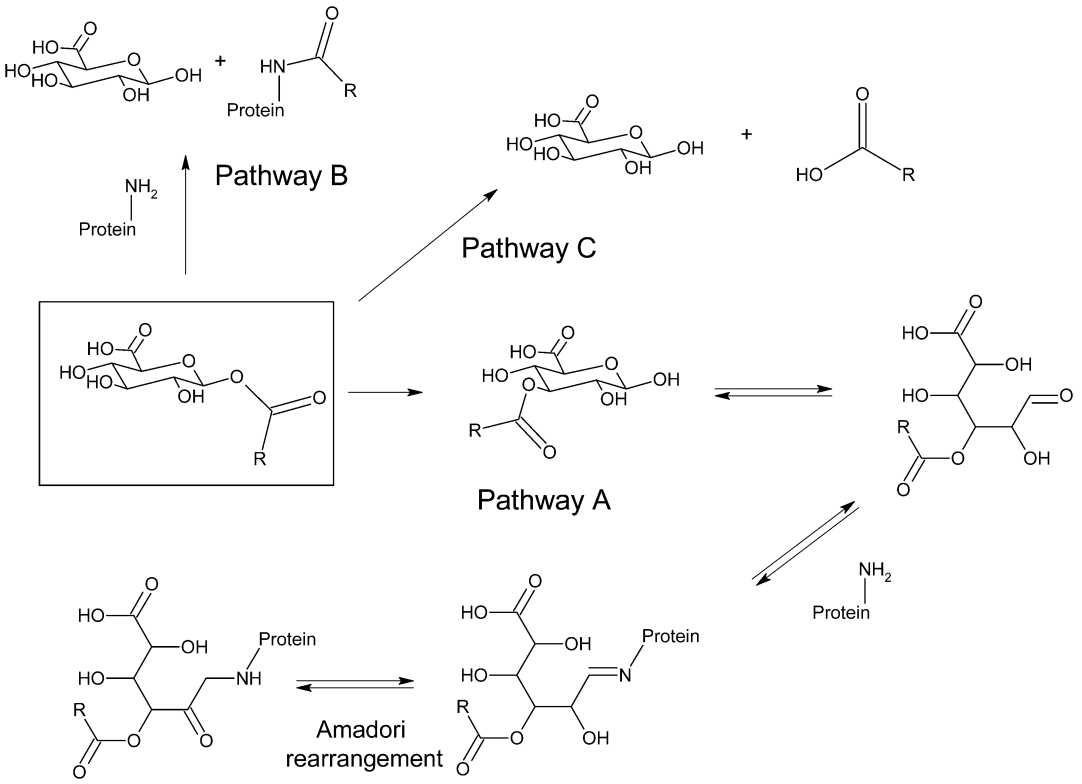

Figure 2. Mechanisms of acyl glucuronide degradation and potential mechanisms of interaction with protein.

Table 1. Comparison of Acyl Glucuronide Half-Lives Determined by NMR with Those Published in the Literature[a]

	literature half-life (h)	NMR determined half-life (h)	reference
Compound B	0.2	0.2	unpublished data
diclofenac	0.5	0.6	(*23*)
zomepirac	0.6	0.5	(*4*)
ibufenac	1.1	1.1	(*18*)
indomethacin	1.5	1.4	(*25*)
ibuprofen	3.3	4.6	(*18*)
furosemide	3.6	5.3	(*38*)
flufenamic acid	7.0	10	(*25*)
mefenamic acid	22	17	(*23*)
Compound C	52	58	unpublished data
Compound A	< 500	< 500	unpublished data

[a] Half-lives for Compounds A, B, and C were determined by HPLC analysis and are published in internal corporate reports.

most efficient way of evaluating these properties of carboxylic acid-containing compounds.

The migration and mutarotations of the AGs make the monitoring of the kinetics of *O*-1- acyl glucuronide degradation by traditional HPLC methods problematic (*15*). Chromatographic analysis is confounded by the fact that all of these compounds are isomeric and have very similar LC retention and mass spectral properties. This makes the identification of the initial *O*-1-acyl glucuronide and its subsequent monitoring difficult in many cases. Additionally, for AGs with short half-lives, sample analysis time may limit the number of potential data points available to describe the degradation curve of an *O*-1-AG. Monitoring the disappearance of the anomeric hydrogen of the *O*-1-AG via NMR analysis of crude isolates from *in vivo* or *in vitro* sources is a far superior alternative to chromatographic assays. The unique chemical shift (approximately 4.9−6.0 ppm), coupling pattern (doublet), and coupling constant (6−9 Hz) of the anomeric hydrogen of AGs make it readily identifiable and free from the interference from either endogenous or AG-related materials. This gives the desired specificity needed to monitor the disappearance of *O*-1-AG, even in the presence of other components in the sample. Furthermore, the degradation of AG can be monitored using only one sample during NMR analysis. In contrast, numerous injections of AG have to be made if the kinetics of AG is studied by the traditional HPLC methods.

In order to screen compounds for this potential liability during the early phase of pharmaceutical development, an NMR assay was developed on the basis of the disappearance of the anomeric resonance of *O*-1-acyl glucuronide. This assay uses H_2O/D_2O (90/10) phosphate buffer (100 mM, pH 7.4) and simple 1-D NMR experiments (^1H) making it amenable to automation. NMR data can also be used to distinguish between the two pathways of degradation, hydrolysis and acyl migration. The ^1H resonances characteristic of acyl migration are quite distinguishable from those that arise from chemical hydrolysis. This NMR technique could be used to rank order discovery compounds whose acyl glucuronides exhibit different degrees of stability under physiological conditions. In this article, we describe the application of this technique in confirming the previously published half-lives of AGs of several NSAIDs. These half-lives were previously determined by HPLC methods, and these values were obtained by several investigators over the course of many years (*5*, *23*). One of the objectives of this article is to demonstrate how the half-lives of these AGs were obtained in a relatively short period of time using NMR.

Materials and Methods

Eleven glucuronides were obtained either synthetically or through isolation of metabolites from the excreta of rats or through *in vitro* incubations with the compounds shown in Figure 3. These compounds were selected on the basis of their chemical diversity, ranges in degradation kinetics, known toxicities, or the availability of half-life data in the literature.

Materials. Compounds A and D were obtained from the Chemistry Department, Pfizer (Ann Arbor, MI). Acyl glucuronide

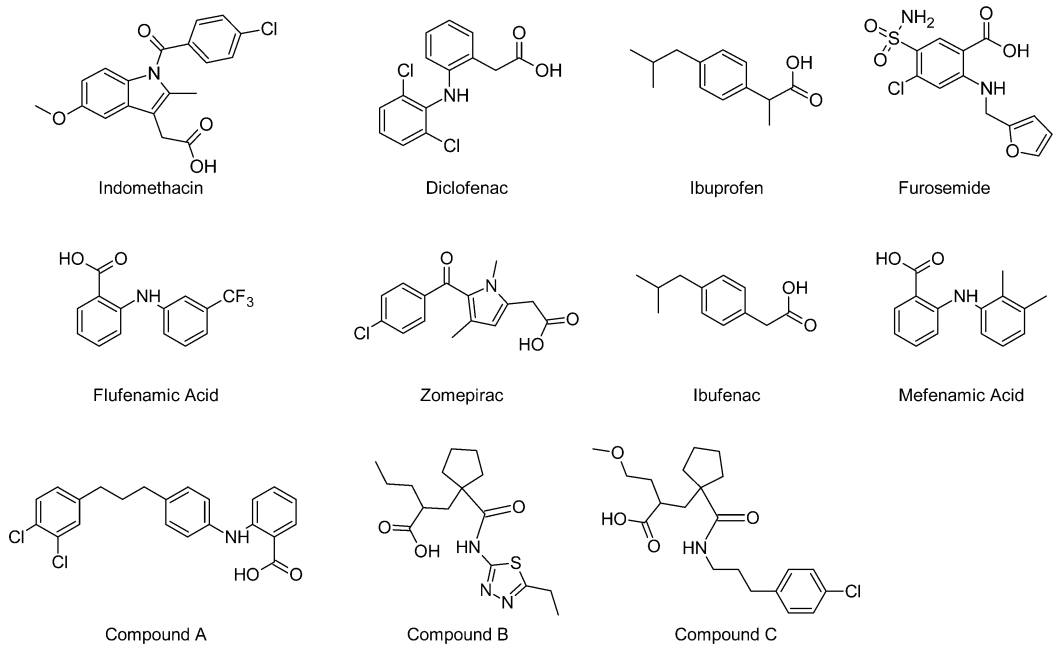

Figure 3. Structures of the 11 compounds used to generate glucuronides for this study. Glucuronides of Compounds A and D, diclofenac, flufenamic acid, furosemide acid, indomethacin, mefenamic acid, and zomepirac were isolated from rat bile. Glucuronides of Compounds B and C were synthetically prepared. Ibuprofen and ibufenac glucuonides were isolated from *in vitro* incubations.

Table 2. Compounds Administered to Rats for the Purpose of Isolating Acyl Glucuronide Conjugates

compound	dose (mg/kg)/route	dosing solution/vehicle	dose interval
ibuprofen racemate	250/PO	37.5 mg/mL; 0.5% MC[a]	QD[b]
zomiperac	100/PO	20 mg/mL; 0.5% MC	BID[c]
flufenamic acid	100/PO	20 mg/mL; 0.5% MC	BID
diclofenac	30/IP	30 mg/mL; saline	QD
furosemide	500/PO	75 mg/mL; 0.5% MC	QD
indomethacin	30/PO	6 mg/mL; 0.5% MC	BID
mefenamic acid	150/PO	45 mg/mL; saline	QD

[a] MC, methylcellulose. [b] QD, dosing once daily. [c] BID, dosing twice daily.

conjugates of compounds B and C were obtained from Pfizer, Pharmacokinetics, Dynamics and Metabolism, (Sandwich, Kent, U.K.). Diclofenac, flufenamic acid, furosemide, ibuprofen (racemic), indomethacin, mefenamic acid, zomepirac, and UDPGA were obtained from Sigma (St. Louis, MO). Ibufenac was synthesized by the Department of Chemistry, Pfizer Global Research and Development (Ann Arbor, MI).

All organic solvents and water were of HPLC grade. Formic acid was obtained from Mallinckrodt Baker Inc. (Paris, Kentucky). Pooled male dog liver microsomes were purchased from BD Gentest (Woburn, MA).

Dosing and Sample Collection. Male bile duct-cannulated Sprague−Dawley rats (weighing between 200 and 350 g) were dosed orally with 30−500 mg/kg of each compound once or twice daily for 2 days. The doses and the dosing solutions used for each compound are listed in Table 2. All animal husbandry procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication, Vol. 25, 1996, http://grants1.nih.gov/grants/guide/notice-files/not96−208.html) and all experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of the facilities in which the studies were conducted. All animals were housed in suspended stainless steel and wire-mesh cages equipped with an automatic watering system. The study room was environmentally controlled for temperature (72 ± 4 °F), relative humidity (40−70%), and light (a 12 h light/dark cycle). Rats had free access to water and were given a specific amount of certified Purina rodent chow each day. Bile and urine samples were collected in metabolism cages during the course of the study (0−8, 8−24, 24−32, and 32−48 h). Biological samples were collected in vessels containing 2 mL of 1 M acetic acid to stabilize the AGs.

Incubations *in Vitro*. Ibufenac (2 mM) and ibuprofen (2 mM) were incubated at 37 °C with 50 mM Tris-HCl (pH 7.1), 10 mM UDPGA, 10 mM $MgCl_2$, 50 μg alamethicin/mg of protein, and pooled dog liver microsomes (0.5 mg/mL) at a total volume of 100 mL. Following a 15-min preincubation on ice, the reaction was initiated with the addition of UDPGA and terminated 1 h later with 15 mL of 1 M acetic acid. The quenched samples were centrifuged and the supernatants extracted by solid-phase extraction as described below.

Isolation of Acyl Glucuronides. An equal volume of bile from three rats was pooled and centrifuged. Aliquots of pooled bile (20 mL) were loaded onto a solid-phase extraction (SPE) column (MegaBond Elute-C18, 10 g/60 mL (Varain, Walnut Creek, CA) conditioned with acetonitrile and 0.1% formic acid in water. Fractions were eluted under light negative pressure using increasing percentages of 0.1% formic acid in water/acetonitrile (%v/v). Aliquots from the SPE fractions were profiled by LC/MS using the conditions described above. The identification of glucuronide peaks was confirmed by performing MS/MS of the pseudomolecular ion, corresponding to the protonated AG. Once the fractions that contained the glucuronide were identified by LC/MS, they were evaporated to dryness in a heated water bath (30 °C) under a stream of nitrogen.

The samples were subsequently reconstituted in a mixture of acetonitrile and 0.1% formic acid in water (10:90 v/v). Isocratic conditions were used to isolate and collect the glucuronide conjugates on an Agilent 1100 HPLC system equipped with a fraction collector (Agilent Technologies Inc.). A semipreparative column (Luna C18, 150 × 21.0 mm, 5 μm, Phenomenex) with the solvent delivered at a flow rate of 20 mL/min was used to isolate sufficient quantities of AGs for NMR studies. The composition of mobile phase was varied for each glucuronide conjugate by altering the percentages of acetonitrile and 0.1% formic acid used to isocratically elute the metabolites (ACN/0.1% formic acid, 65−60:35−40, v/v). The HPLC eluent was monitored at 254 nm using a variable wavelength detector. Fractions were pooled, and the purity of the glucuronide peaks were confirmed by performing LC/MS/MS analysis. Final extracts containing the glucuronides were dried under vacuum before submitting for NMR analysis.

LC/MS and LC-MS/MS Analyses. LC-MS analysis was performed on a ThermoFinnigan (Thermo Electron Corporation, Waltham, MA) LCQ Deca XP+ system equipped with an electrospray ionization interface. The capillary temperature used for analysis was 275 °C. The nitrogen gas flow rate, spray current, and voltages were adjusted to give maximum sensitivity for the parent compounds or their glucuronide conjugates. The mass spectrometer was operated in positive ion mode with a scan range from 200 to 900 amu. MS/MS was carried out using helium as the collision gas. The collision energy was set at an arbitrary value of 20 to 30%. Data were collected and analyzed by Xcaliber software (version 1.3, Thermo Electron). HPLC was conducted using a ThermoFinnigan Surveyor (Thermo Electron Corporation, Waltham, MA) system consisting of an autosampler, solvent pumps equipped with a gradient controller, and a diode array detector coupled to the mass spectrometer.

Chromatographic separation was achieved using a Luna C18 (150 × 2 mm, 5 μm, Phenomenex Inc., Torrance, CA) column used in conjunction with a gradient solvent system. Elution was performed using a mobile phase comprising 0.1% formic acid in water and acetonitrile. The initial conditions consisted of 10% acetonitrile in 0.1% formic acid. After maintaining the initial HPLC conditions for 5 min, the proportion of acetonitrile was increased to 80% from 5 to 30 min and held for an additional 5 min. The solvent was delivered at a flow rate of 0.25 mL/min. All samples were centrifuged and injected directly onto the HPLC column.

NMR Analysis. The kinetic evaluations of the AGs were conducted on a Varian Inova 800 MHz spectrometer. Samples were dissolved in 100 mM sodium phosphate buffer (pH 7.4, 90/10 v/v of H_2O/D_2O) and acquisition of spectra conducted at regular intervals for 2 to 60 h. All spectra were recorded at 37 °C. All experiments used the manufacturers' standard presaturation pulse programs. The number of scans used per acquisition for kinetic studies was adjusted for each sample and ranged between 8 and 128 scans per time point. The typical recycle time was 5 s. The spectra were phased, referenced, and integrated using an in-house program, Metabonomi (Patent pending WO2004038602A1). The initial structural characterizations of isolated glucuronides were performed by dissolving the samples in d_3-acetonitrile and conducting 1D- and 2D-NMR experiments on a Bruker Avance 600 MHz instrument.

Ibufenac Glucuronide. MH^+ at *m/z* 369; ^{1}H NMR (800 MHz, D_2O): δ 0.99 (d, J = 6.8 Hz, 6 H), 1.97 (m, 1 H), 2.61 (d, J = 7.9 Hz, 2 H), 3.66 (m, 2 H), 3.71 (t, J = 9.3 Hz, 1 H), 3.95 (d, J = 9.9 Hz, 1 H), 3.97 (s, 2 H), 5.70 (d, J = 8.2 Hz, 1 H, anomeric), 7.37 (d, J = 7.8 Hz, 2 H), 7.40 (d, J = 7.8 Hz, 2 H).

Ibuprofen Glucuronide. MH^+ at *m/z* 383; ^{1}H NMR (800 MHz, D_2O): δ 0.99 (d, J = 6.8 Hz, 6 H), 1.65 (d, J = 7.2 Hz, 3 H), 1.96 (m, 1 H), 2.61 (d, J = 7.0 Hz, 2 H), 3.61 (m, 2 H), 3.70 (d, J = 9.2 Hz, 1 H), 3.92 (d, J = 9.8 Hz, 1 H), 4.07 (q, J = 7.2 Hz, 1 H), 5.68 (d, J = 8.2 Hz, 1 H, anomeric), 7.37 (d, J = 7.7 Hz, 2 H), 7.43 (d, J = 7.7 Hz, 2 H).

Flufenamic Acid Glucuronide. MH^+ at *m/z* 458; ^{1}H NMR (800 MHz, D_2O): δ 3.62 (t, J = 9.5 Hz, 1 H), 3.69 (t, J = 9.3 Hz, 1 H), 3.73 (t, J = 8.3 Hz, 1 H), 3.93 (d, J = 9.8 Hz, 1 H), 5.82 (d, J = 8.1 Hz, 1 H, anomeric), 6.97(m, 1 H), 7.30 (m, 1 H), 7.46 (d, J = 7.6 Hz, 1 H), 7.49−7.53 (m, 2 H), 7.57 (m, 1 H), 7.61 (s, 1 H), 8.13 (d, J = 8.1 Hz, 1H).

Furosemide Glucuronide. MH^+ at *m/z* 507; ^{1}H NMR (800 MHz, D_2O): δ 3.61 (t, J = 9.3 Hz, 1 H), 3.67 (t, J = 9.5 Hz, 1 H), 3.74 (t, J = 8.5 Hz, 1 H), 3.92 (d, J = 10.0 Hz, 1 H), 5.78 (d, J = 8.1 Hz, 1 H, anomeric), 6.40(s, 1 H), 6.45 (s, 1 H), 7.17 (s, 1 H), 7.5 (s, 1 H), 8.58 (s, 1 H).

Diclofenac Glucuronide. MH^+ at *m/z* 472; ^{1}H NMR (800 MHz, D_2O): δ 3.67 (m, 2 H), 3.72 (m, 1 H), 3.96 (d, J = 9.9 Hz, 1 H), 4.14 (d, J = 17.3 Hz, 1 H), 4.17 (d, J = 17.3 Hz, 1 H), 5.74 (d, J = 8.1 Hz, 1 H, anomeric), 6.61 (m, 1 H), 7.13 (m, 1 H), 7.33 (m, 2 H), 7.46 (d, J = 7.8 Hz, 1 H), 7.63(d, J = 8.1 Hz, 2 H).

Indomethacin Glucuronide. MH^+ at *m/z* 534 and $[M + NH_4]^+$ at *m/z* 551; ^{1}H NMR (800 MHz, D_2O): δ 2.26 (s, 3 H), 3.56 (m, 2 H), 3.61 (m, 1 H), 3.83 (d, J = 9.8 Hz, 1 H), 3.86 (s, 3 H), 3.97 (s, 2 H), 5.61 (d, J = 8.0 Hz, 1 H, anomeric), 6.77 (d, J = 8.4 Hz, 1 H), 7.05 (d, J = 8.4 Hz, 1 H), 7.10(s, 1 H), 7.61 (d, J = 8.2 Hz, 2 H), 7.68(d, J = 8.2 Hz, 2 H).

Zomiperac Glucuronide. MH^+ at *m/z* 468; ^{1}H NMR (800 MHz, D_2O) for glucuronic acid moiety and the aromatic signals: δ 3.97 (d, J = 9.8 Hz, 1 H), 5.75 (d, J = 8.1 Hz, 1 H, anomeric), 6.26 (s, 1 H), 7.69 (d, J = 8.3 Hz, 2 H), 7.78 (d, J = 8.3 Hz, 2 H). The rest of the proton signals in the upfield region were obscured by other contaminants in the sample.

Mefenamic Acid Glucuronide. MH^+ at *m/z* 418; ^{1}H NMR (800 MHz, D_2O): δ 2.14 (s, 3 H), 2.35 (s, 3 H), 3.64(t, J = 9.4 Hz, 1 H), 3.70(t, J = 9.6 Hz, 1 H), 3.76(t, J = 8.0 Hz, 1 H), 3.95 (d, J = 9.8 Hz, 1 H), 5.61 (d, J = 8.1 Hz, 1 H, anomeric), 6.70 (d, J = 8.6 Hz, 1 H), 6.82 (t, J = 7.6 Hz, 1 H), 7.19(d, J = 8.1 Hz, 1 H), 7.23 (m, 2 H), 7.41(t, J = 8.2 Hz, 1 H), 8.11(t, J = 8.1 Hz, 1 H).

Compound A Glucuronide. MH^+ at *m/z* 577; ^{1}H NMR (800 MHz, D_2O): δ 1.5 (bs, 2 H), 2.21 (bm, 4 H), 3.58(t, J = 8.8 Hz, 1 H), 3.61(t, J = 8.9 Hz, 1 H), 3.67(t, J = 8.6 Hz, 1 H), 3.95 (d, J = 9.0 Hz, 1 H), 5.62 (d, J = 7.5 Hz, 1 H, anomeric), 6.46 (t, J = 6.7 Hz, 1 H), 6.64 (d, J = 7.9 Hz, 1 H), 6.71 (m, 3 H), 6.73 (m, 3 H), 6.91 (s, 1 H), 7.00 (d, J = 8.2 Hz, 1 H), 7.91 (d, J = 8.0 Hz, 1 H).

Compound B Glucuronide. MH^+ at *m/z* 516; ^{1}H NMR (800 MHz, D_2O for the glucuronic acid moiety only: δ 3.35−3.5 (cm, 3 H), 3.61 (d, J = 9.9 Hz, 1 H), 5.27 (d, J = 7.5 Hz, 1 H, anomeric).

Compound C Glucuronide. MH^+ at *m/z* 573; ^{1}H NMR (800 MHz, D_2O): δ 1.48 (bm, 1 H), 1.57 (bm, 3 H), 1.69 (bm, 2 H), 1.77 (bm, 1 H), 1.89 (bm, 5 H), 1.95 (s, 3 H), 2.11 (m, 1 H), 2.53 (bm, 1 H), 2.69 (t, J = 7.3 Hz, 2 H), 3.24 (t, J = 6.2 Hz, 2 H), 3.28 (s, 3 H), 3.46 (t, 2 H), 3.58 (m, 3 H), 3.81 (d, J = 9.5 Hz, 2 H), 5.52 (d, J = 8.1 Hz, 1 H, anomeric), 7.30 (d, J = 8.7 Hz, 2 H), 7.40 (d, J = 8.7 Hz, 2 H).

Compound D Glucuronide. ^{1}H NMR (800 MHz, D_2O) for the glucuronic acid moiety only: δ 3.6−3.75 (cm, 3 H), 3.95 (d, J = 9.8 Hz, 1 H), 5.65 (d, J = 7.8 Hz, 1 H, anomeric).

Results

NMR was used to determine the structures and relative stabilities of 11 acyl glucuronides, which were either obtained synthetically or isolated from biological sources. The degradation pathways and kinetics of each AG were obtained and compared with each other. Furthermore, the calculated half-lives (by NMR) of AGs produced from well-known NSAIDs were compared with those reported in literature. Because of the similarity in the approach taken to isolate, characterize and subsequently use the isolated AGs to determine the half-lives, we will limit our discussion to only a few examples, including Compound B, ibufenac, ibuprofen, indomethacin, and mefenamic acid.

In all cases, except for the zomiperac glucuronide conjugate, the isolated conjugates were found to be pure by LC/MS and NMR. Each of the AG displayed a characteristic anomeric proton resonance in the NMR spectra. These anomeric resonances were distinguished from other proton signals by characteristic chemical shifts (between 4.9 and 6.0 ppm), multiplicity (must be a doublet), and coupling constant (between 7 and 9 Hz). These observed ^{1}H NMR signals for the β-anomeric proton are consistent with those previously reported as being indicative of a β-glycosidic linkage (*24*). The structures of the acyl glucuronides were confirmed by either 1D- and/or by 2D-NMR experiments prior to any kinetic studies.

The zomiperac glucuronide isolate contained significant impurities that masked the high field resonances (0−4 ppm); however, the anomeric resonance was free from any interferences, allowing us to monitor its degradation over a time course. Molecular weights, determined by LC-MS, for all 11 glucuronides were consistent with published structures.

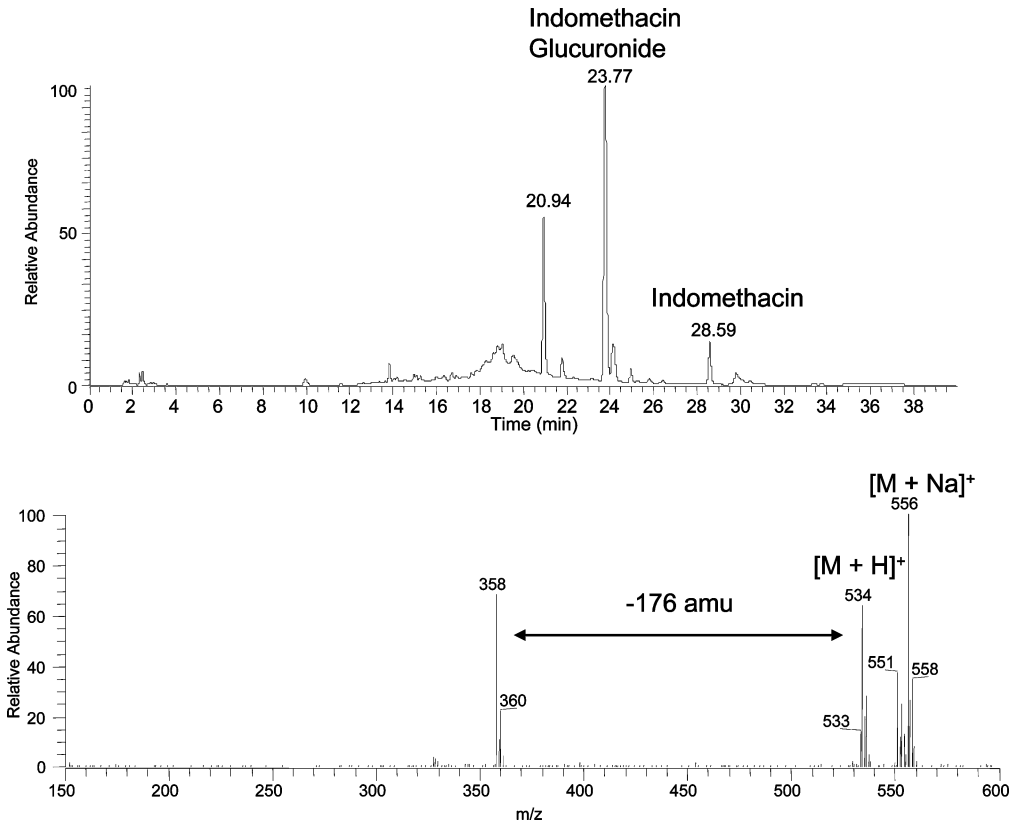

Figure 4. LC/MS analysis of the bile from a rat dosed with 30 mg/kg of indomethacin. The UV chromatogram ($\lambda = 254$ nm) and positive ion MS spectrum of the indomethacin acyl glucuronide ($t_R = 23.7$ min) are shown in the top and bottom panels, respectively.

Figure 4 contains the LC-MS chromatogram of the rat bile extract of an animal dosed with 30 mg/kg indomethacin. The extracted ion chromatogram clearly shows a protonated molecular ion (*m*/*z* 534) with its associated ammonium (*m*/*z* 551) and sodium (*m*/*z* 556) adducts corresponding to the AG of indomethacin. Additionally, the aglycone ion (*m*/*z* 358) is produced from the loss of the glucuronic acid due to in-source fragmentation. SPE fractions of rat bile were analyzed by LC/MS for the presence of AG conjugate. It was found that the 1:1 (H_2O/ACN, v/v) fraction contained the largest proportion of the glucuronide and hence was subsequently used to isolate the conjugate for further characterization. An isocratic method was developed on a semipreparative HPLC column to isolate approximately 2 mg of the AG of indomethacin. The LC-MS chromatogram of the final isolate contained one peak, which was confirmed to be the AG of indomethacin.

Figure 5 shows the representative zero time ^{1}H NMR spectra of the indomethacin glucuronide. The assignments of the protons shown in Figure 5 were based on the 2D-NMR experiments. As evident, the spectrum showed no resonances indicative of acyl migration prior to conducting kinetic studies using the phosphate buffer (pH 7.4). Figure 6 contains a composite of the time course data acquired for the indomethacin acyl glucuronide. The zero time spectrum is at the bottom of the Figure, with other spectra displayed with time progression up the *y*-axis (15 min between spectra). It is clearly visible in Figure 6 that some of the resonances are quite dynamic in their magnitude, while others remain relatively constant. The anomeric proton (*O*-1-β) resonance (5.62 ppm) decreases with time, while all resonances between 5.4 and 5.2 ppm (believed to be due to the anomeric resonances from the migrated products) increase in magnitude over time (*15*, *25*). Integration values from the 5.60 to 5.65 ppm region of each individual spectrum were taken, normalized to the zero time, and natural log values plotted against time (Figure 7). The correlation coefficient was 0.996, and the calculated $t_{1/2}$ for the indomethacin AG was 1.5 h. The $t_{1/2}$ based on NMR data was within 10% of the value published in the literature, ($t_{1/2} = 1.4$ h) (*23*). Similar results, in terms of both linearity and agreement with literature values, were obtained for all other AGs evaluated using this technique (Table 1 and Figure 8).

In addition to the assessment of degradation rates, these experiments also allowed the determination of the route of degradation. In some cases, AGs decompose through simple hydrolysis, and in others, the degradation is through acyl migration. Additionally, there may be a combination of migration and hydrolysis that leads to the disappearance of the anomeric resonance. To demonstrate this, the ^{1}H NMR profiles of mefenamic acid (degradation largely through acyl migration) and Compound C (degradation largely through hydrolysis) were obtained and compared. In the case of Compound C, hydrolysis is the dominant pathway, resulting in the appearance of proton resonances corresponding to glucuronic acid and presumably the anomeric proton of the α-2-*O*-acyl positional isomer (Figure 9). In the spectra of mefenamic acid, which predominantly undergoes acyl migration, multiple resonances appear in the 4.7−5.6 ppm region of the ^{1}H spectra (Figure 9). These resonances correspond to the multiple forms of the AG produced as a result of the transacylation reaction depicted in Figure 2 (pathway A).

Studies conducted with dog liver microsomes were done principally to illustrate the application of this NMR technique to assess AGs produced by an *in vitro* system. Ibufenac was used as a model compound that was metabolized by liver microsomes to produce sufficient quantities of AG for isolation and subsequent characterization. As reported previously in the literature, ibufenac has a relatively short half-life ($t_{1/2} = 1.1$ h) compared to that of its close analogue, ibuprofen ($t_{1/2} = 3.3$ h)

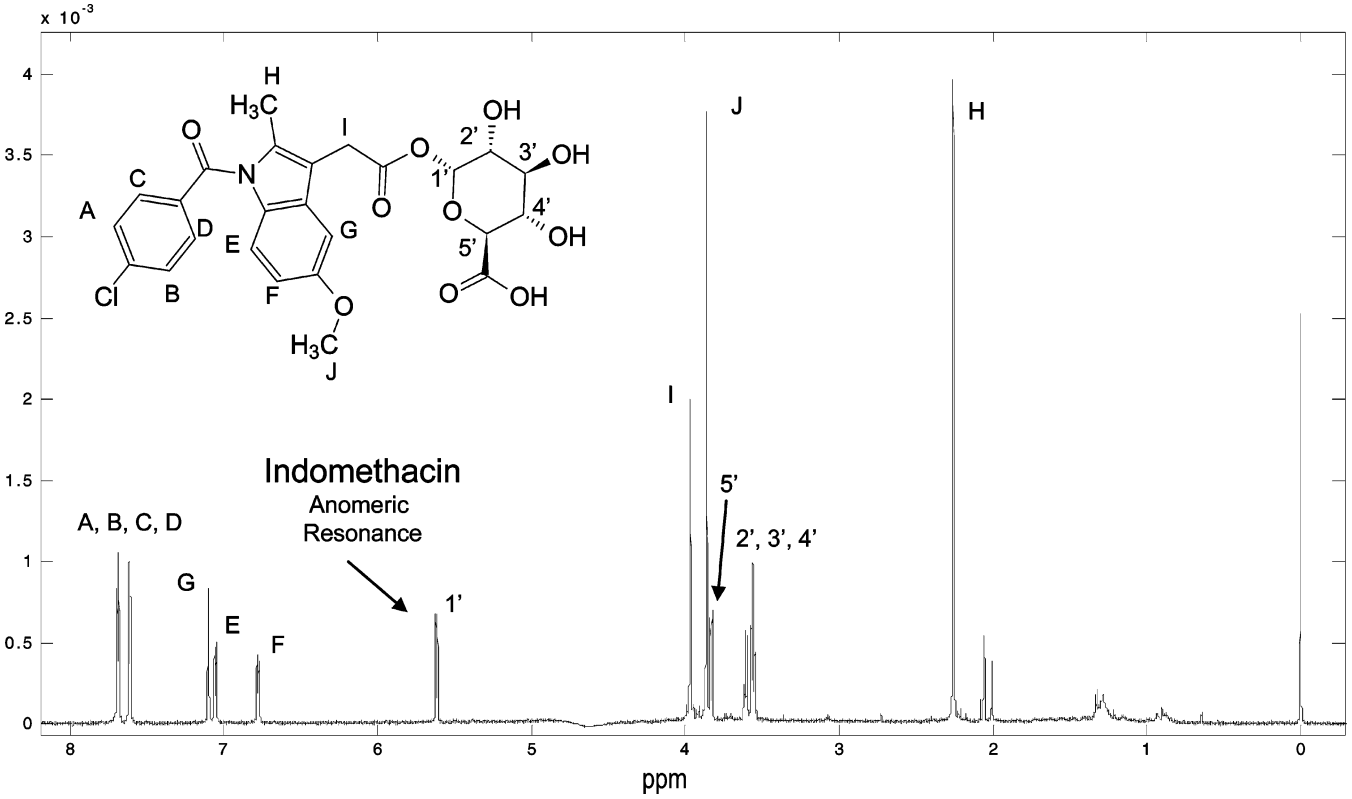

Figure 5. [1]H NMR spectra (800 MHz) of the isolated indomethacin glucuronide, at $t = 0$, in a mixture of 0.1 M PO_4 buffer (pH 7.4) made with H_2O/D_2O(90:10, v/v).

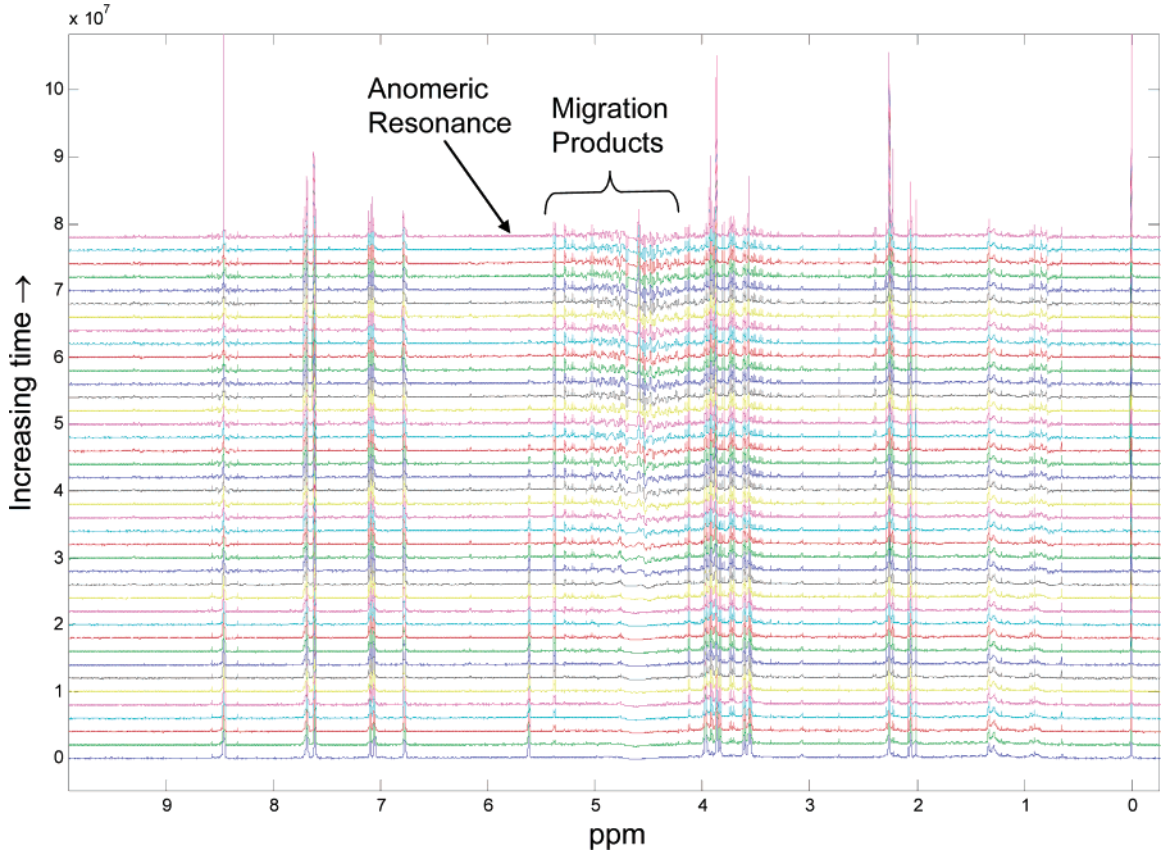

Figure 6. [1]H NMR spectra acquired for the indomethacin acyl glucuronide over 15 min intervals.

(*17*). We wanted to evaluate the feasibility of applying the NMR technique to an AG of a compound that was fairly unstable under physiological conditions. Ibufenac AG was detected as multiple peaks in the dog microsomal extract (data not shown).

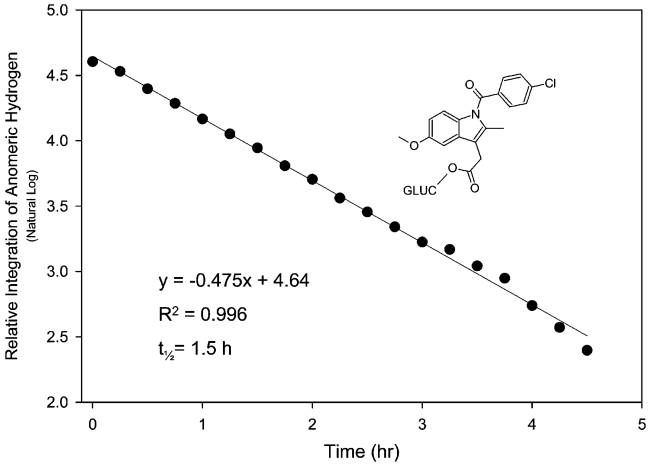

Figure 7. Natural log of the relative integration of the anomeric ^{1}H (5.60−5.65 ppm) of indomethacin plotted against time.

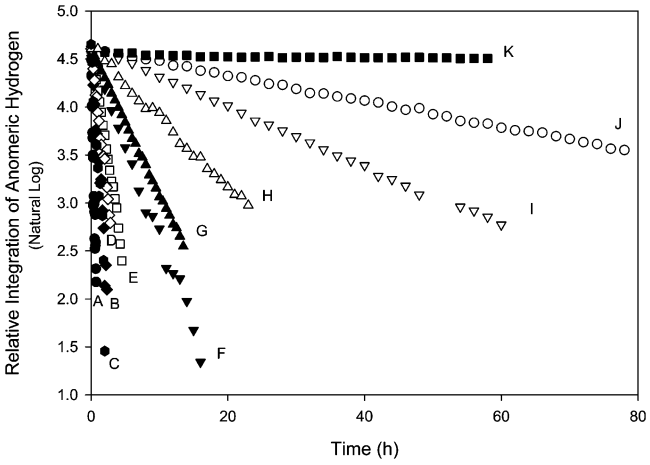

Figure 8. Natural log of the relative integration of the anomeric ^{1}H of all 11 test compounds plotted against time; Compound B (A), diclofenac (B), zomepirac (C), ibufenac (D), indomethacin (E), ibuprofen (F), furosemide (G), flufenamic acid (H), mefenamic acid (I), compound C (J), compound A (K).

Hence, some of the AG of ibufenac had transacylated during the course of incubation, making it difficult to isolate pure 1-*O*-β form. However, because the NMR technique is used specifically to determine the half-lives of the anomeric protons, it did not matter if the sample was already contaminated with the migrated products. The important point to note is that as long as the anomeric resonance is isolated from other components in the NMR spectrum, the half-lives could be easily achieved, irrespective of the degree of purity of the samples.

This NMR technique was used to evaluate the stability of the AG of a number of early discovery compounds considered for further advancement in development. As an example, Compound D was isolated from rat bile and evaluated by this technique. It was shown to be fairly stable under the conditions used with a half-life of greater than 24 h (Figure 10). Hence, on the basis of this data, the reactivity of the AG of this compound was considered to not be an issue, and the compound was progressed to the next stage of development process.

Discussion

In recent years, there has been increasing pressure within the pharmaceutical industry to reduce the attrition rate of compounds in development. Consequently, many assays designed to demonstrate compound liabilities such as metabolic instability or potential reactive metabolites are being performed earlier in the drug discovery process. This necessitates new methodologies that can provide rapid assessment of these potential liabilities. Because of the large numbers of compounds that need to be screened in early stages of drug discovery, these assays must be amenable to automation.

Methods utilizing NMR and mass spectrometry to study the degradation of acyl glucuronides were described (*26*, *27*). The decomposition of α- and β-anomers of ifetroban 1-*O*-acyl glucuronide in an aqueous medium was studied by the HPLC-ESI/MS technique (*26*). This technique was used to investigate the degradation of these AG via hydrolysis and acyl migration over a pH range of 1.0 to 13.0. It was found that no acyl migration occurred at pH < 4.0. Kinetic studies on the intramolecular acyl migration of β-1-*O*-acyl glucuronides of ketoprofen and tolmetin, two NSAIDs still on the market, were conducted using ^{1}H NMR as the analytical tool (*27*). Tolmetin was shown to have the faster degradation rate compared to that of ketoprofen, exhibiting first-order kinetics by NMR. The $t_{1/2}$ of 0.4 h obtained by the NMR method was close to what was previously obtained by a HPLC method ($t_{1/2}$ = 0.44 h) (*28*). Directly coupled HPLC/^{1}H NMR techniques have also been used in recent years to investigate the kinetics of complex intramolecular rearrangements exhibited by acyl glucuronides (*15*, *29*−*31*). The advantage of using HPLC/NMR is that the HPLC isolation step is avoided. However, in our experience, we have found that we can stabilize the acyl glucuronides by keeping the pH of the mobile phases well below 4, consistent with what has been reported in the literature. The quality of spectra and the reliability of data interpretation increase with cleaner samples submitted for NMR analysis. Often, we have found that direct injection of bile or urine onto LC/NMR does not produce quality spectra, as there is considerable interference from other endogenous components. Further limitations of kinetic analysis are centered around chromatography. In many cases, the loading capacity of an analytical column is not adequate enough to separate material in sufficient quantity for the reliable acquisition of kinetic data. Having said this, changes in NMR and LC-NMR technology (cryoprobes, inline SPE, VHPLC etc.) may in the near future decrease the required amount of material sufficiently to allow easy acquisition of NMR kinetic data routinely. However, organic mobile phase modifiers may still interfere with the acquisition of kinetic data reflective of physiological conditions. Many separations require an organic modifier, typically acetonitrile or methanol, which would dramatically interfere with the solution pH resulting in data not relevant to biological systems. An off-line semi-purification step using SPE and/or HPLC separation of the acyl glucuronides provided us with reasonable amounts of material in sufficiently pure form to conduct structural characterization as well as study the degradation kinetics. The excessive presence of impurities in the sample can make interpretation of the data difficult at times. It must be emphasized that the NMR method used to study the degradation of the AG measures a combined contribution from the hydrolysis and transacylation reactions. However, by studying the NMR spectra of the AG undergoing degradation in the NMR tube allows one to distinguish whether one or the other competing pathways (Figure 1) were operative during the study. This was demonstrated by following the degradation of mefenamic acid and Compound C (Figure 9), where it was clearly shown that Compound C degraded largely by hydrolysis under the conditions used in the study. Mefenamic acid, however, degraded almost exclusively by acyl migration. Similarly, for the acyl glucuronides showing very short half-

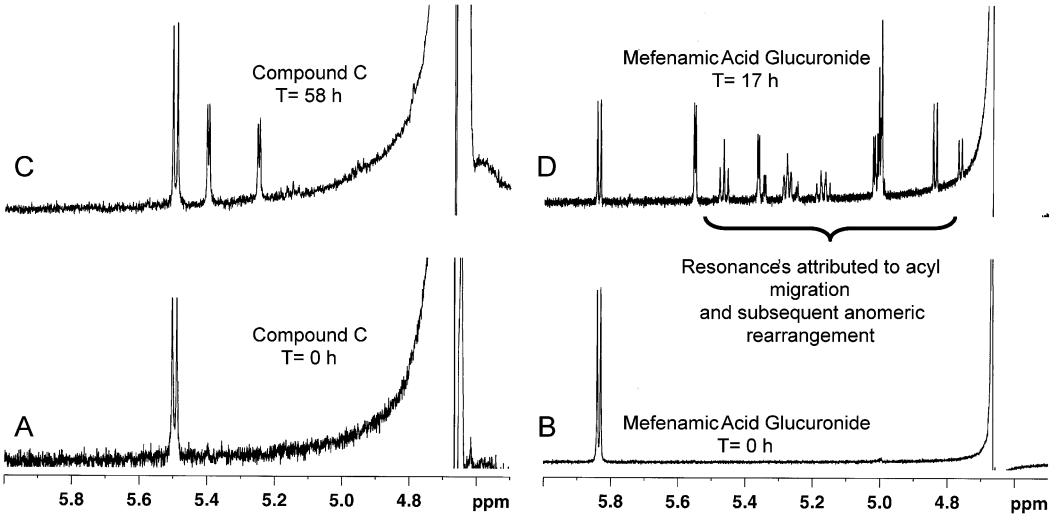

Figure 9. (A) Anomeric region of the ^{1}H spectrum of the Compound C acyl glucuronide, $t = 0$ h. (B) Anomeric region of the ^{1}H spectrum of the mefenamic acyl glucuronide, $t = 0$ h. (C) Anomeric region of the ^{1}H spectrum of Compound C, $t = 58$ h. (D) Anomeric region of the ^{1}H spectrum of the mefenamic acyl glucuronide, $t = 17$ h.

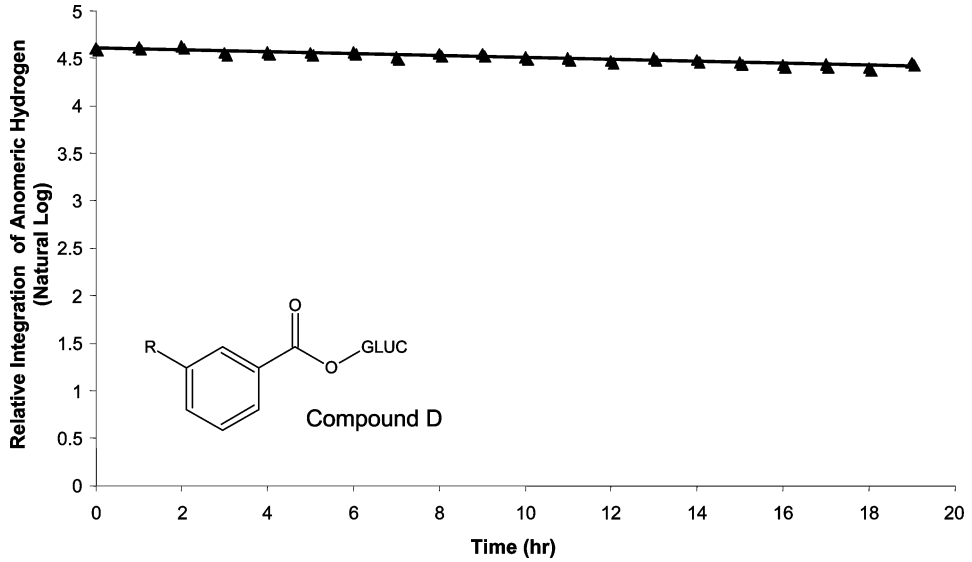

Figure 10. Natural log of the relative integration of the anomeric ^{1}H of Compound D (Pfizer discovery compound) plotted against time, showing a very long half-life ($t_{1/2} > 24$ h).

lives *in vitro*, diclofenac ($t_{1/2} = 0.6$ h), ibufenac ($t_{1/2} = 1.1$ h), and indomethacin ($t_{1/2} = 1.5$ h) all showed degradation through acyl migration. There was very little or no evidence of hydrolysis to parent aglycones, as was observed with Compound C. In general, acyl migration has been found as the predominant reaction occurring *in vitro* (*31*, *32*). Hydrolysis of the AG to the parent aglycone has been considered to be more significant *in vivo* on the basis of previous observations and studies conducted with nonspecific esterases and β-glucuronidase (*33*), and albumin (*34*). Although the hydrolysis reaction appears to be affected by these factors, the transacylation reactions are relatively unaffected; hence, it is expected that the $t_{1/2}$ calculated for the transacylation reaction from *in vitro* studies should be close to what is expected *in vivo*. In our study with the AG of various compounds, we found that the transacylation reaction was the dominant reaction at pH 7.4 in phosphate buffer with no evidence of hydrolysis, hence confirming previous observations. However, one exception to this rule was Compound B, which displayed high instability under these conditions, decomposing principally by hydrolysis. Irrespective of the degree of hydrolysis that may take place *in vivo*, the $t_{1/2}$ value obtained from the *in vitro* studies (principally due to transacylation reaction) can give an idea of the relative stability and potential reactivity of AG produced from a series of compounds. The transacylation reaction is important as it leads to intermediates capable of interacting covalently with proteins. Recently, an LC/MS/MS-based technique was used to understand the reactivity of the AG of several compounds (*35*). That study confirmed earlier findings by Benet et al. (*21*) that the covalent binding to proteins could be related to the rate of AG rearrangement. Wang et al. demonstrated that the rearrangement of AG was a prerequisite for the formation of Schiff base−peptide links. It was proposed that the extent of formation of Schiff base−peptide adducts from AG was proportional to the rearrangement rate of the primary AG. The ranking of the reactivity using the peptide technique for seven compounds was found to correlate well with rankings reported previously in the literature (*23*, *36*). Furthermore, a good correlation was found between the formation of the peptide adduct and the rearrangement rate of the AG of the seven tested compounds. However, the LC/MS/MS method, while apparently reliable, consists of the derivatization of the Schiff base−adduct formed by reacting the biologically produced AG with a peptide. A more direct and easier approach in assessing the relative reactivities of AG is needed. The NMR

method employed in the current study provides the means to directly obtain the half-lives of AG as a measure of the rates of acyl migration. (Except for Compound B, the rest of the compounds showed only acyl migration and no hydrolysis under the conditions used.)

The NMR method described here provides a rapid and specific alternative to HPLC and HPLC/MS methods for the determination of acyl glucuronide degradation kinetics. The $t_{1/2}$ values obtained for the AGs of a number of NSAIDs by this method were in good agreement with what has been reported in the literature (Table 1) (*23*). Furthermore, the NMR technique was able to provide comparable data in a relatively short period of time as compared to the HPLC methods that were used in the past. It was also demonstrated that pure samples of AGs are not necessary to obtain the half-lives using this technique. Because the calculation of half-life is based on the disappearance of the characteristic anomeric resonance for an AG, the presence of other endogenous components in the sample is not a concern, provided that they do not generate any ^{1}H resonance that will overlap with the anomeric proton signal. The robustness of this assay was demonstrated by performing the kinetic studies with partially purified AG of a number of compounds. Furthermore, one does not have to obtain the 1-*O*-β form of the AG as an absolutely pure standard to conduct the kinetic evaluation by NMR. NMR can be used to analyze a sample that is a mixture of various isomeric forms of the AG, but containing a certain measurable proportion of the 1-*O*-β form, to obtain the half-life. This was illustrated by the studies conducted with ibufenac, which produced an AG that was fairly unstable. The study with ibufenac also showed that *in vitro* systems such as liver microsomes or recombinant UGTs can be used to generate sufficient quantities of AG of a compound that can be studied by NMR. This is particularly important if one needs to evaluate carboxylic acid-containing compounds early in discovery, where there is a limited supply of compounds. Additionally, this method allows the distinction between hydrolysis and acyl migration for compounds with apparent short $t_{1/2}$. This technique is amenable to automation and could easily be used to screen and rank order discovery drug candidates capable of forming AGs.

It is interesting to note that the half-lives we obtained with Compounds A, B, and C were consistent with previous observations and predictions for molecules with similar structural moieties (*23*, *37*). Compound A is a benzoic acid derivative, which produced an AG with a relatively long half-life (Table 1). Neither Compound B nor C were acetic acid derivatives, hence their AG did not exhibit the relative high instabilities due to acyl migration as observed with other acetic acid derivatives, such as diclofenac and ibufenac (Table 1 and Figure 3). Compounds B and C were both substituted at the α-position (to carboxylic acid) in contrast to diclofenac, ibufenac, indomethacin, and zomepirac, all of which produce highly unstable AG with $t_{1/2}$ of less than 1.5 h.

On the basis of the relative ease with which we can obtain the degradation half-lives of the AG, we are proposing that compounds capable of forming relatively unstable glucuronide conjugates with $t_{1/2} \leq 1.5$ h should not be pursued further in the drug discovery process. As shown in Table 1, AG of compounds with $t_{1/2}$ of less than 1.5 h have either been withdrawn from the market or have been issued black box warnings. Using the NMR technique, one should be able to ensure that the degradation is taking place primarily through acyl migration and not through hydrolysis. This is a critical piece of information that needs to be taken into consideration before a decision is made to terminate a compound or a related series from development. We have demonstrated, with an example, where we could have a compound that degrades principally through hydrolysis leading to a short half-life. Such a compound can be considered relatively unreactive, as the hydrolysis process will dominate and not permit the acyl migration to take place. Compounds with half-lives greater than 1.5 h should be progressed through the discovery process with an understanding that the AG with shorter half-lives can potentially be more problematic than the more stable AG producing compounds. However, one must fully consider other factors such as the dose size, patient population, duration of therapy, and the nature of the disease being treated before decisions are made to either terminate or progress an AG-producing compound in development (*38*). Minimizing the formation of reactive metabolites and subsequent formation of drug−protein adducts has become important as we attempt to reduce the attrition rate of compounds in early discovery (*38*). The study described in this article constitutes one of the approaches to minimize drug−protein adduction potentially caused by reactive intermediates from NCE possessing the carboxylic acid functional group. An assessment of the relative reactivities of AG, considered as potential precursors to reactive intermediates capable of reacting with proteins, can be easily achieved using *in vitro* metabolism and NMR techniques.

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References

(1) Maguire, J. H., Butler, T. C., and Dudley, K. H. (1982) 5-Ethyl-5-phenylhydantoin N-glucuronide, the major urinary metabolite of 5-Ethyl-5-phenylhydantoin (Nirvanol) in the dog. *Drug Metab. Dispos. 10*, 595−598.

(2) Mohri, K., Uesugi, T., and Kamisaka, K. (1985) Bucolome N-glucuronide: Purification and identification of a major metabolite of bucolome in rat bile. *Xenobiotica 15*, 615−621.

(3) Lehman, J. P., Fenselau, C., and Depaulo, J. R. (1983) Quarternary ammonium linked glucuronides of amitriptyline, imipramine and chlorpromazine. *Drug Metab. Dispos. 11*, 221−225.

(4) Spahn-Langguth, H., and Benet, L. Z. (1992) Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab. Rev. 24*, 5−48.

(5) Fenselau, C. (1994) Acyl Glucuronides as Chemically Reactive Intermediates. Conjugation-Deconjugation Reactions in Drug Metabolism and Toxicity. In *Handbook of Experimental Pharmacology* (Kauffman, F.C., Ed) Vol. 112, pp 367−389, Springer-Verlag, Berlin, Germany.

(6) Boelsterli, U. A, Zimmerman, H. J, and Kretz-Rommel, A. (1995) Idiosyncratic liver toxicity of nonsteroidal anti-inflammatory drugs: molecular mechanisms and pharmacology *Crit. Rev. Toxicol. 25*, 207−235.

(7) Faed, E. M. (1984) Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev. 15*, 1213−1249.

(8) Tremaine, L. M., Stroh, J. G., and Ronfeld, R. A. (1989) Characterization of a carbamic acid ester glucuronide of the secondary amine setraline. *Drug Metab. Dispos. 17*, 58−63.

(9) Delbressine, L. P. C., Funke, C. W., van Tilborg, M., and Kaspersen (1990) On the formation of carbamate glucuronides. *Xenobiotica 20*, 133−134.

(10) Mutlib, A. E., Shockcor, J., Chen, S. Y., Espina, R. J., Pinto, D. J., Orwat, M. J., Prakash, S. R., and Gan, L. S. (2002) Disposition of 1-[3-(aminomethyl)phenyl]-N-[3-fluoro-2′-(methylsulfonyl)-[1,1′-biphenyl]-4-yl]-3-(trifluoromethyl)-1H-pyrazole-5-carboxamide (DPC 423) by novel metabolic pathways. Characterization of unusual metabolites by liquid chromatography/mass spectrometry and NMR. *Chem. Res. Toxicol. 15*, 48−62.

(11) Elvin, A. T. (1980) Tocainide conjugation in humans. Novel biotransformation pathway for a primary amine. *J. Pharm. Sci. 69*, 47−49.

(12) Straub, K., Davis, M., and Hwang, B. (1988) Benzazepine metabolism revisited. Evidence for the formation of novel amine conjugates. *Drug Metab. Dispos. 16*, 359−366.
(13) Sallustio, B. C., Sabordo, L., Evans, A. M., and Nation, R. L. (2000) Hepatic disposition of electrophilic acyl glucuronide conjugates. *Curr. Drug Metab. 1*, 163−180.
(14) Hasegawa, J., Smith, P. C., and Benet, L. Z. (1982) Apparent intramolecular acyl migration of zomepirac glucuronide. *Drug Metab. Dispos. 10*, 469−473.
(15) Sidelmann, U. G., Gavaghan, C., Carless, H. A. J., Farrant, R. D., Lindon, J. C., Wilson, I. D., and Nicholson, J. K. (1995) Identification of the positional isomers of 2-fluorobenzoic acid 1-*O*-acyl glucuronide by directly coupled HPLC-NMR. *Anal. Chem. 67*, 3401−3404.
(16) Corcoran, O., Mortensen, R. W., Hansen, S. H., Troke, J., Nicholson, J. K. (2001) HPLC/1H NMR spectroscopic studies of the reactive α-1-O-acyl isomer formed during acyl migration of S-naproxen *β*-1-O-acyl glucuronide. *Chem. Res. Toxicol. 14*, 1363−1370.
(17) Park, B. K., and Kitteringham, N. R. (1990) Drug protein conjugation and its immunological consequences. *Drug Metab. Rev. 22*, 87−144.
(18) Castillo, M., and Smith, P. C. (1995) Disposition and reactivity of ibuprofen and ibufenac acyl glucuronides in vivo in the rhesus monkey an *in vitro* with human serum albumin. *Drug Metab. Dispos. 23*, 566−572.
(19) Smith, P. C., Benet, L. Z., and McDonagh, A. F. (1990) Covalent binding of zomiperac glucuronide to proteins: evidence for a Schiff base mechanism. *Drug Metab. Dispos. 18*, 639−644.
(20) Rabinovitz, M., and Van, Thiel, D. H. (1992) Hepatotoxicity of nonsteroidal anti-inflammatory drugs. *Am. J. Gastroenterol. 87*, 1696−1704.
(21) Vanderhoeven, S. J., Lindon, J. C., Troke, J., Nicholson, J. K., Wilson, I. D. (2006) NMR spectroscopic studies of the transacylation reactivity of ibuprofen *β*-1-O-acyl glucuronide. *J. Pharm. Biomed. Anal. 41*, 1002−1004.
(22) Bailey, M. J., and Dickinson, R. G. (2003) Acyl glucuronide reactivity in perspective: biological consequences. *Chem.-Biol. Interact. 145*, 117−137.
(23) Benet, L. Z., Spahn-Langguth, H., Iwakawa, S., Volland, C., Mizuma, T., Mayer, S., Mutschler, E., and Lin, E. T. (1993) Predictability of the covalent binding of acidic drugs in man. *Life Sci. 53*, 141−146.
(24) Fischer, B., Nudelman, A., Ruse, M., Herzig, J., and Gottlieb, H. E. (1984) A novel method for stereoselective glucuronidation. *J. Org. Chem. 49*, 4988−4993.
(25) van Breeman, R. B., and Fenselau, C. (1985) Acylation of albumin by 1-O-acyl glucuronides. *Drug Metab. Dispos. 13*, 318−320.
(26) Khan, S., Teitz, D. S., and Jemal, M. (1998) Kinetic analysis by HPLC-electrospray mass spectrometry and pH dependent acyl migration and solvolysis as the decomposition pathway of ifetroban 1-O-acyl glucuronide. *Anal. Chem. 70*, 1622−1627.
(27) Skordi, E., Wilson, I. D., Lindon, J. C., and Nicholson, J. K. (2005) Kinetic studies on the intramolecular acyl migration of *β*-1-O-acyl glucuronides: Application to the glucuronides of (*R*)- and (*S*)-ketoprofen, (*R*)- and (*S*)-hydroxy-ketoprofen metabolites, and tolmetin by ^{1}H-NMR spectroscopy. *Xenobiotica 35*, 715−725.
(28) Hyneck, M. L., Munafo, A., and Benet, L. Z. (1988) Effect of pH on acyl migration and hydrolysis of tolmetin glucuronide. *Drug Metab. Dispos. 16*, 322−324.
(29) Sidelmann, U. G., Gavaghan, C., Carless, H. A. J., Spraul, M., Hofmann, M., Lindon, J. C., Wilson, I. D., and Nicholson, J. K. (1995) 750 MHz directly coupled HPLC-NMR: Application to the sequential characterization of the positional isomers and anomers of 2-, 3- and 4-fluorobenzoic acid glucuronides in equilibrium mixtures. *Anal. Chem. 67*, 4441−4445.
(30) Sidelmann, U. G., Hansen, S. H., Gavaghan, C., Carless, H. A. J., Farrant, R. D., Lindon, J. C., Wilson, I. D., and Nicholson, J. K. (1996) Measurement of internal acyl migration reaction kinetics using directly coupled HPLC-NMR: Application for the positional isomers of synthetic (2-fluorobenzyl)-D-glucopyranuronic acid. *Anal. Chem. 68*, 2564−2572.
(31) Mortensen, R. W., Corcoran, O., Cornett, C., Sidelmann, U. G., Lindon, J. C., Nicholson, J. K., and Hansen, S. H. (2001) S-naproxen *β*-1-O-acyl glucuronide degradation kinetic studies by stopped flow high-performance liquid chromatography: ^{1}H-NMR and high-performance liquid chromatography-UV. *Drug Metab. Dispos. 29*, 375−380.
(32) Akira, K., Taira, T., Hasegawa, H., Sakuma, C., and Shinohara, Y. (1998) Studies on the stereoselective internal acyl migration of ketoprofen glucuronides using ^{13}C labeling and nuclear magnetic resonance spectroscopy. *Drug Metab. Dispos. 26*, 457−464.
(33) Shipkova, M., Armstrong, V. W., Oellerich, M., and Weiland, E. (2003) Acyl glucuronide drug metabolites: Toxicological and analytical implications. *Ther. Drug Monit. 25*, 1−16.
(34) Wells, J. D., Janssen, F. W., and Ruelius, H. W. (1987) Interactions between oxaprozin glucuronide and human serum albumin. *Xenobiotica 17*, 1437−1449.
(35) Wang, J., Davis, M., Li, F., Azam, F., Scatina, J., and Talaat, R. (2004) A novel approach for predicting acyl glucuronide reactivity via Schiff base formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. *Chem. Res. Toxicol. 17*, 1206−1216.
(36) Bolze, S., Lacombe, O., Durand, G., Chaimbault, P., Massiere, F., Gay-Feutry, C., Bromet, N., and Hulot, T. (2002) Standardization of a LC/MS/MS method for the determination of acyl glucuronides and their isomers. *Curr. Sep. 20*, 55−59.
(37) Evans, D. C., Watt, A. P., Nicoll-Griffith, D. A., and Baillie, T. A. (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol. 17*, 3−16.
(38) Rachmel, A., Hazelton, G. A., Yergey, A. L., and Liberato, D. J. (1986) Furosemide 1-O-acyl glucuronide. *In vitro* biosynthesis and pH-dependent isomerization to beta-glucuronidase-resistant forms. *Drug Metab. Dispos. 13*, 705−710.

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