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NMR Spectroscopic Studies on the in Vitro Acyl Glucuronide Migration Kinetics of Ibuprofen ((±)-(*R*,*S*)-2-(4-Isobutylphenyl) Propanoic Acid), Its Metabolites, and Analogues

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Carboxylic acid-containing drugs are often metabolized to 1-β-*O*-acyl glucuronides (AGs). These can undergo an internal chemical rearrangement, and the resulting reactive positional isomers can bind to endogenous proteins, with clear potential for adverse effects. Additionally any 1-β-*O*-acyl-glucuronidated phase I metabolite of the drug can also show this propensity, and investigation of the adverse effect potential of a drug also needs to consider such metabolites. Here the transacylation of the common drug ibuprofen and two of its metabolites is investigated in vitro. 1-β-*O*-Acyl (*S*)-ibuprofen glucuronide was isolated from human urine and also synthesized by selective acylation. Urine was also used as a source of the (*R*)-ibuprofen, (*S*)-2-hydroxyibuprofen, and (*S*,*S*)-carboxyibuprofen AGs. The degradation rates (a combination of transacylation and hydrolysis) were measured using ^{1}H NMR spectroscopy, and the measured decrease in the 1-β anomer over time was used to derive half-lives for the glucuronides. The biosynthetic and chemically synthesized (*S*)-ibuprofen AGs had half-lives of 3.68 and 3.76 h, respectively. (*R*)-Ibuprofen AG had a half-life of 1.79 h, a value approximately half that of the (*S*)-diastereoisomer, consistent with results from other 2-aryl propionic acid drug AGs. The 2-hydroxyibuprofen and carboxyibuprofen AGs gave half-lives of 5.03 and 4.80 h, considerably longer than that of either of the parent drug glucuronides. In addition, two (*S*)-ibuprofen glucuronides were synthesized with the glucuronide carboxyl function esterified with either ethyl or allyl groups. The (*S*)-ibuprofen AG ethyl ester and (*S*)-ibuprofen AG allyl esters were determined to have half-lives of 7.24 and 9.35 h, respectively. In order to construct useful structure−reactivity relationships, it is necessary to evaluate transacylation and hydrolysis separately, and here it is shown that the (*R*)- and (*S*)-ibuprofen AGs have different transacylation properties. The implications of these findings are discussed in terms of structure−activity relationships.

Glucuronides of carboxylic acid-containing nonsteroidal anti-inflammatory drugs (NSAIDs) have been studied extensively in the past due to their potential reactivity in the body.[1−4] Conjugation of these drugs with UDP-α-D-glucuronic acid to form 1-β-*O*-acyl glucuronide (AG) esters is part of the generally accepted detoxification process, but it has also been shown that AGs can undergo spontaneous chemical reactions, resulting in acyl migration and hydrolysis, and can also irreversibly bind to endogenous proteins. The formation of protein adducts by acyl migration clearly has the potential to result in toxicological consequences.[2,3]

The initial biosynthetic 1-β-*O*-AG esters that are formed via metabolism can degrade in the body via hydrolysis and transacylation as depicted in Scheme 1. The positional isomers thus formed have been shown to be reactive toward proteins.[5−7] The carbonyl carbon of the 1-β-*O*-AG is susceptible to nucleophilic attack by neighboring hydroxyl groups on the glucuronic acid ring and, thus, will undergo internal transesterification. The aglycone migrates from the glucuronic acid C1-position in turn to the 2-, 3-, and 4-positions through ortho-ester intermediate structures. Each of the β-acyl isomers can undergo mutarotation via ring-opened aldehyde forms of the sugar to form α-anomers. The 1-α-*O* anomer is an exception and is formed through a back-reaction from the 2-α-*O* anomer via a cyclic ortho-acid ester intermediate with 1,2-*cis*-(ax, eq) fused rings.[8] In principle, the 4-*O*-acyl isomer can react further via formation of an internal

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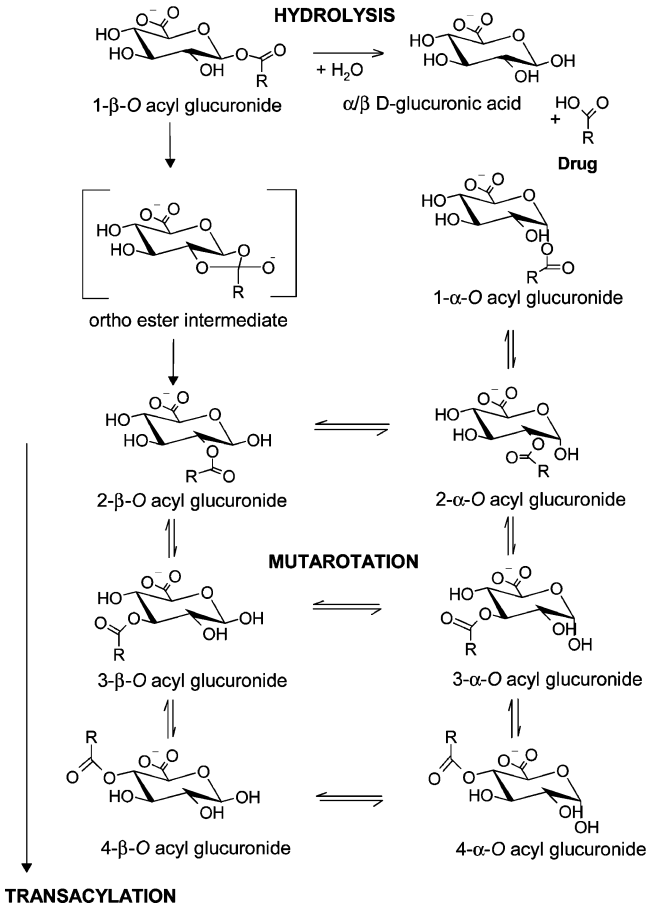
Scheme 1. Rearrangement Reactions of Acyl Glucuronides



lactone with the carboxyl group to form an anhydride. The acyl migration and mutarotation reaction are reversible except for the re-formation of the 1-*β*-*O*-AG, which has not been observed. All the isomers also irreversibly undergo spontaneous hydrolysis to the aglycone. Each transacylation reaction has an individual reaction rate constant which is dependent on the structure of the aglycone,[9] the solvent,[10] pH, and temperature.[11] These acyl glucuronides are most stable at low temperature and under acidic conditions. The rates of acyl migration from the 1β to the 2β position and of hydrolysis can be combined to calculate the overall rate of the disappearance of the 1-*β*-*O*-AG, termed here as the degradation rate (k_d). The rate of acyl migration is a first-order kinetic process, while in principle the rate of hydrolysis is a second-order reaction, but the large excess of water present makes this pseudo-first-order. The observed k_d is the sum of the rates of these two reactions and is essentially a first-order process.[12] AGs have been shown to have a range of degradation rates,[2,7,11,13−21] but individual hydrolysis and transacylation rates have only been calculated for naproxen[8] and for model compounds.[22]

Each of the positional isomers is capable of reacting with endogenous proteins resulting in the formation of covalent adducts, via transacylation or glycation mechanisms.[7,14,15,19,23] The transacylation mechanism occurs through nucleophilic attack of a protein functional group primarily at the acyl carbon of the 1β glucuronide and results in the drug being bound to the protein with the subsequent loss of glucuronic acid. The glycation mechanism, which is thought to be the major route for protein binding to the 2-, 3-, and 4-acyl isomers, results in the protein being irreversibly bound to the drug to form an imine (Schiff base). This imine can undergo an Amadori rearrangement to form a keto amine.[22]

The protein adducts that are formed by these two mechanisms have been implicated in various cytotoxic, carcinogenic, and idiosyncratic immunological manifestations.[24,25] Furthermore in vivo studies using mice[26] and rats[27] have shown that the presence of glucuronide−protein conjugates results in the formation of antibodies, indicating an immunological response. Several NSAIDs (tolmetin, zomepirac, benoxaprofen) have been withdrawn from the market, due to different types of adverse reactions. For example, anaphylactic reactions were seen in patients taking zomepirac[28] and hepatotoxicity has been observed with ibufenac.[29] These withdrawn drugs have fast degradation rates for the 1-*β*-*O*-AG in aqueous solutions at pH 7.4 and also exhibit protein binding.[22,23,30] Therefore, tentative links have been made between AG toxicity and the AG degradation rate, but no clear correlation has yet been made between degradation rates and the incidence of reported toxicity,[31] and it is likely that a complex interplay between AG transacylation rates and other pharmacokinetic processes is involved. It has been postulated that a $t_{1/2}$ of 1.5 h should be a cutoff value for removing a compound from a drug development process when it degrades essentially through acyl migration and also taking into account other factors,[32] but given the above arguments this is clearly oversimplistic, and a short

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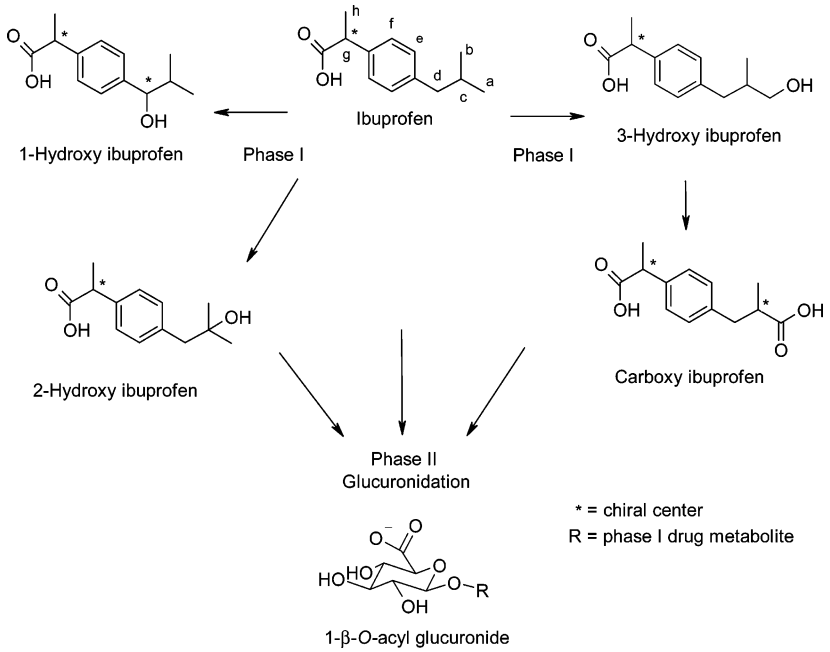

Figure 1. Phase I and phase II metabolites of ibuprofen.

half-life is not necessarily critical if the pharmacokinetics is otherwise favorable. Indeed there are marketed drugs such as naproxen, diclofenac, diflunisal with $t_{1/2} \sim 1.5$ h. Thus, in order to capture this complexity, investigations are required in order to elucidate the relationships between AG kinetics, toxicity, and the physicochemical properties of AGs and their aglycones in order to build quantitative structure−activity relationship (QSAR) models. Some preliminary studies have been carried out on model compounds,[33] but further investigations are needed in order to provide predictive models for understanding the role, if any, of these metabolites in drug toxicity for optimizing compound structure in drug design.

Methods to isolate and measure the positional AG isomers of NSAIDs have been developed in the past utilizing both separation and spectroscopic methods. SPEC (solid-phase extraction chromatography) has been shown to effectively isolate drug metabolites from urine,[34,35] and the development of HPLC (high-performance liquid chromatography) methods coupled with NMR spectroscopy has been demonstrated for the separation and identification of individual AG isomers formed by acyl migration and mutarotation.[36−41] We and others have measured the degradation rates of a range of NSAID AGs using these methods in order to develop a suitable database for QSAR studies.[31]

Here we have studied the AGs of ibuprofen [(±)-(*R*,*S*)-2-(4-isobutylphenyl) propanoic acid], which is a readily available over-the-counter NSAID. It is metabolized to form two major phase 1 metabolites, 2-(4-(2-hydroxy-2-methylpropyl)phenyl) propanoic acid (2-hydroxyibuprofen) and 2-(4-(2-carboxypropyl)phenyl) propanoic acid (carboxyibuprofen), formed via 3-hydroxyibuprofen, and one minor metabolite, 1-hydroxyibuprofen, as shown in Figure 1. These metabolites, together with the parent drug, are conjugated with β-D-glucuronic acid to form AG esters.[1,4] We have also studied two synthetically modified glucuronic acid esters of (*S*)-ibuprofen, with the aim to identify any changes in reactivity that may occur with these minor modifications to the structure.

While adding to the database of experimental reactivity values, this study also probes the reactivity of the AGs of phase I metabolites of the drug, relative to the parent AG, since these can also be implicated in toxicity. The effect on AG reactivity of blocking the free glucuronic acid carboxyl group is also investigated as this could lead to insight into the effect of structure on reactivity.

EXPERIMENTAL SECTION

Materials. 3-[2,2,3,3-$^2H_4$] trimethylsilyl propionate sodium salt (TSP), sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Sigma-Aldrich Company, Ltd. (Gillingham, Dorset, U.K.). HPLC−NMR grade deuterium oxide (^2H_2O) was obtained from Goss Scientific Instruments (Essex, U.K.). Analytical grade methyl alcohol, HPLC grade water, and HPLC grade acetonitrile were purchased from BDH Laboratory supplies (Poole, U.K). C18 Bond-Elut SPEC columns (12 mL reservoir volume/2 g sorbent mass) were obtained from Varian

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Associates (Walton-on-Thames, U.K.). For high-performance liquid chromatography−mass spectrometry (HPLC−MS), deuterated acetonitrile (99.8 atom % deuterium) was purchased from Eurisotop (Gif-sur-Yvette, France) and formic acid from Fisons Scientific (Loughborough, U.K.).

Chemical Synthesis of 1-β-*O*-Acyl (*S*)-Ibuprofen Glucuronide. 1-β-*O*-Acyl (*S*)-ibuprofen glucuronide was chemically synthesized using a variation of the selective acylation method as recently documented.[31,42,43] However, here the much more acid-labile 4-methoxybenzyl (compared to benzyl) ester was used. The method involves alkylation of glucuronic acid with *p*-methoxybenzylbromide using a resin-bound fluoride (full details will be published elsewhere). Activation of the carboxylic acid was achieved by the use of Carpino's uronium reagent *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*-methylmorpholine (NMM) which helped produce the acylation product. The acyl glucuronide ester was deprotected using dilute TFA in dichloromethane.

Preparation of the Esters of 1-β-*O*-Acyl (*S*)-Ibuprofen Glucuronides. The (*S*)-ibuprofen ethyl ester AG was formed as a synthetic byproduct in the synthesized (*S*)-ibuprofen AG sample and identified by ^{1}H NMR spectroscopy. It was separated from the main product by HPLC-SPE and detected and identified using on-line MS. An Agilent 1100 series chromatographic system (Agilent Technologies U.K. Ltd., West Lothian, U.K.) consisting of a quaternary pump, an autosampler, and a single-wavelength UV detector operated at 254 nm was connected to a Bruker NMR/MS interface (BNMI) (Bruker Daltonics, Coventry, U.K). The eluent was split postcolumn in the BNMI, and 5% of the eluent flowed into an Esquire 6000 ion-trap mass spectrometer (Bruker Daltonics, Coventry, U.K), and 95% was directed to the UV detector. A Prospekt 2 automated solid-phase extraction unit was connected after the UV detector for peak collection comprised of Hysphere resin GP (10−12 μm) cartridges from Spark Holland (Emmen, Holland). A Knauer K120 pump was used for the makeup flow.

For the chromatography an Agilent eclipse αDB-C18 column (4.6 mm × 150 mm i.d., 5 μm particle size) was used. The mobile phase solvents for the separation were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A linear gradient was applied at a flow rate of 1 mL/min: 50% to 80% B for 6 min, then 80% to 50% B for the next 3 min, and finally 50% B held for 14 min. The peaks of interest were directed toward the Prospekt 2 system to be trapped onto the SPE cartridges. At this stage, the Knauer K120 pump was operated at 3 mL/min to pump solvent A into the eluent. The chromatography and peak trapping was performed four times using the same cartridges. The cartridges were dried, and deuterated acetonitrile was used to elute the samples.

The MS was operated in the positive ion mode, and spectra were obtained by electrospray ionization using the following experimental parameters: scan range 50−850 *m*/*z*, nebulizer pressure 18.0 psi, dry gas flow 9.0 L/min, dry temperature 300 °C, skimmer −40 V, ion charge control (ICC) target 50 000, maximum accumulation time 50 ms, 5 spectra averages, high-voltage capillary 4000 V, and capillary exit −106 V.

The (*S*)-ibuprofen allyl ester AG was synthesized using selective acylation as described for the (*S*)-ibuprofen AG above, using allyl glucuronate as the carbohydrate component.

^{1}H NMR Spectroscopic Analysis of AGs in Human Urine. A single 400 mg dose of ibuprofen was administered to a healthy human female volunteer. Initially, a predose urine sample was collected for a control, and then urine collections were made at 0−2, 2−4, and 4−6 h postdose. Samples were acidified to pH 2 with 1 M HCl and stored at −40 °C until further analysis. An amount of 800 μL of each urine sample and 80 μL of TSP (0.5 mg/mL in ^2H_2O) was added into a 5 mm NMR tube. TSP provides a chemical shift reference ($\delta = 0$), and the ^2H_2O was used as a magnetic field−frequency lock signal. ^{1}H NMR spectra were acquired for each sample at 300 K using a Bruker AVANCE600 NMR spectrometer at 600.13 MHz, with a broad-band inverse (BBI) 5 mm probe. A standard water peak presaturation pulse sequence was used.[44] A total of 128 free induction decays (FIDs) were collected into 32 K data points with a spectral width of 20.017 ppm and a relaxation delay of 2 s. An exponential apodization function corresponding to a line-broadening factor of 0.3 Hz was applied to the FIDs. The data were manually phase-corrected. Inspection of the NMR spectra showed characteristic β-anomeric proton peaks for the AGs, and these were present in the urine sample taken 2−4 h postdose.

Isolation and Characterization of Ibuprofen Metabolites from Urine. Solid-phase extraction/chromatography (SPEC) of urines, on a 12 mL Bond-Elut column (Varian Associates, Walton-on-Thames, U.K.), was used to isolate the ibuprofen-related material from the sample. The column was conditioned before sample application with methanol (24 mL) and equilibrated with HPLC grade water (pH 2 water, acidified with 1 M HCl) (24 mL). A volume of 12 mL of the 2−4 h postdose urine sample was loaded onto the column, and the column was washed with pH 2 HPLC grade water (24 mL). The retained sample was eluted using 12 mL fractions of an increasing methanol/pH 2 water stepwise gradient: 20%, 30%, 40%, 50%, 60%, 80%, and 100% methanol as described previously.[45] Each fraction collected was placed under a stream of nitrogen gas to remove any methanol prior to freeze-drying. The samples were stored at −40 °C until further use.

When required for ^{1}H NMR spectroscopy, the samples were reconstituted in 1 mL of ^2H_2O. A volume of 550 μL was transferred to a 5 mm NMR tube along with 50 μL of TSP (0.5 mg/mL in ^2H_2O) and 50 μL of pH 2 HPLC grade water. The spectral data showed ibuprofen glucuronide metabolites present in the 40%, 50%, 60%, and 80% methanol SPEC fractions. The samples were freeze-dried for the subsequent degradation rate studies.

Degradation Rate Measurements. ^{1}H NMR spectroscopy was carried out at 310 K using the above pulse sequence on a Bruker AVANCE600 spectrometer equipped with a BBI probe at 600.13 MHz. Once the acquisition parameters were optimized using a standard sample, 550 μL of 100 mM sodium phosphate buffer (pH 7.4) was added quickly to one of the freeze-dried samples containing an ibuprofen AG (e.g., 1.5 mg of synthesized sample). The sample was transferred to a 5 mm NMR tube

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Table 1. First-Order Degradation Rate Constants and Half-Lives for the 1-β-*O*-acyl Glucuronides of Ibuprofen

1-β-*O*-acyl glucuronide	k_d (h^{-1})	SE[a]	$t_{1/2}$ (h)
(*R*)-ibuprofen (urine-extracted)	0.387	0.0004	1.79
(*S*)-ibuprofen (urine-extracted)	0.188	0.0011	3.68
(*S*)-ibuprofen (synthesized)	0.184	0.0002	3.76
(*S*)-2-hydroxyibuprofen	0.138	0.0003	5.03
(*S*,*S*)-carboxyibuprofen	0.144	0.0020	4.80
(*S*)-ibuprofen ethyl ester	0.095	0.0032	7.27
(*S*)-ibuprofen allyl ester	0.074	0.0032	9.35

[a] SE = standard error for the standard least-squares linear fit to the slope.

containing 50 μL of TSP (0.5 mg/mL in ^2H_2O), and 16 scans were acquired with a spectral width of 20.017 ppm. The spectrum was phased manually, and an automated program was run by which 159 spectra were acquired sequentially. The first 61 experiments were acquired with a time delay of 59.2 s between experiments; the subsequent 95 experiments were acquired with a time delay of 636.4 s between each experiment. The total reaction times for the two segments are 1.48 and 17.58 h, respectively. An exponential apodization function corresponding to a line-broadening factor of 0.3 Hz was applied to the FIDs. Spectra were chosen at different time points and manually phase- and baseline-corrected. The β-anomeric proton doublet (at $\delta \sim 5.6$ ppm) of the ibuprofen AGs was chosen to monitor this degradation. Integration was used to measure the intensity of the doublet which was referenced to that of the TSP peak set at unity. The logarithms of the intensities were plotted against time, and the slope of the graph gave the degradation rate constant assuming first-order kinetics.

RESULTS

Studies on Ibuprofen AGs Synthesized by Selective Acylation. The degradation of synthetic 1-β-*O*-acyl (*S*)-ibuprofen glucuronide, produced via the selective acylation method, was first investigated as described in the Experimental Section. The anomeric proton peaks used for degradation rate calculation decreased in intensity over the course of the experiment, while other peaks, corresponding to the other isomers appeared. On the basis of the assumption of first-order kinetics, the degradation rate constant and corresponding half-life were calculated by measuring the peak integrals. (*S*)-Ibuprofen AG had a k_d of 0.184 h^{-1} as shown in Table 1, and this is in good agreement with previous studies.[21,30] The chemical shifts and coupling constants for this sample are shown in Table 2 and were used to confirm the assignment of (*S*)-ibuprofen AG extracted from human urine. The hydrolysis rate of the AG was also obtained by integration of the aglycone methyl doublet at 1.39 ppm. This is a larger signal than that of the α-glucuronic acid doublet and provides better sensitivity. The hydrolysis rate was determined to be 0.036 mol dm^{-3} h^{-1}, as displayed in Table 3, and follows a zero-order reaction. From these results we can predict that the first-order transacylation rate is approximately 0.148 h^{-1}, and the ratio of hydrolysis to acyl migration is 1:4. The hydrolysis rate was also calculated using product ratios at the 2 h time point as described by Gavaghan,[20] (the 2β isomer was hidden under the water peak and assumed to be present as a 1:1 ratio of α/β isomers), as seen in Table 3.

(*S*)-Ibuprofen AG ethyl ester was purified from the synthetic (*S*)-ibuprofen AG sample using HPLC−MS, as described in the Experimental Section. ^1H NMR spectroscopy was used to confirm and identify the AG as an ethyl ester at the glucuronic acid carbonyl group of 1-β-*O*-acyl (*S*)-ibuprofen glucuronide. (*S*)-Ibuprofen AG allyl ester was synthesized by selective acylation, and the structures of both the allyl and ethyl esters are shown in Figure 2; the chemical shifts and *J*-couplings for these molecules are also given in Table 2. The k_d's of these esters were found to be considerably slower than that of the (*S*)-ibuprofen AG, at 0.095 and 0.074 h^{-1} for the ethyl and allyl esters, respectively. The hydrolysis rates of these ester AGs could not be calculated, as the concentration of the hydrolysis products was too low.

Degradation Half-Lives of Ibuprofen Glucuronides Extracted from Human Urine. The ibuprofen glucuronide metabolites were extracted from urine as described in the Experimental Section and identified using ^1H NMR spectroscopy. On the basis of the 1D ^1H NMR spectrum, (*S*)- and (*R*)-1-β-*O*-acyl ibuprofen AGs were found to be present in the 80% methanol fraction. The 2-hydroxyibuprofen and carboxyibuprofen 1-β-*O*-acyl ibuprofen AGs were present in the 40% methanol fraction obtained from the SPE columns (the chemical shifts and coupling constants

Table 2. 600 MHz ^1H NMR Chemical Shifts δ, Multiplicities, and Coupling Constants *J* (Hz) of Synthesized 1-β-*O*-acyl Glucuronides of Ibuprofen in ^2H_2O[a,b]

1-β-*O*-acyl glucuronide	−CH_3 a + b δ	−CH− c δ	−CH_2− d δ	−CH− e δ	−CH− f δ	−CH− g δ	−CH_3 h δ	H1′ δ
(*S*)-ibuprofen	0.87 (d) *J* = 6.8	1.85 (m) *J* = 6.8	2.49 (d) *J* = 7.0	7.26 (d) *J* = 7.9	7.31 (d) *J* = 7.9	3.95 (q) *J* = 7.3	1.51 (d) *J* = 7.3	5.56 (d) *J* = 8.2
ethyl ester[c] (*S*)-ibuprofen	0.86 (d) *J* = 6.8	1.84 (m) *J* = 6.8	2.48 (d) *J* = 7.3	7.25 (d) *J* = 8.2	7.29 (d) *J* = 8.2	3.95 (q) *J* = 7.0	1.49 (d) *J* = 7.0	5.61 (d) *J* = 7.9
allyl ester[d] (*S*)-ibuprofen	0.88 (d) *J* = 6.8	1.85 (m) *J* = 6.8	2.50 (d) *J* = 7.2	7.28 (d) *J* = 7.9	7.22 (d) *J* = 7.9	3.96 (q) *J* = 7.0	1.50 (d) *J* = 7.0	5.62 (d) *J* = 8.2

[a] δ referenced to internal TSP at δ 0.0. [b] Multiplicities: d, doublet; s, singlet; q, quartet; m, multiplet. [c] Ethyl ester at carbonyl carbon on glucuronide ring CH_2CH_3: CH_2 (q) δ 4.23, $J = 7.0$ Hz; CH_3 (t) δ 1.24, $J = 7.0$ Hz. [d] Allyl ester at carbonyl carbon on glucuronide ring $CH_2CHCHCH$: CH_2 (dd) δ 4.7, $J = 3.4$; CH (ddt) δ 5.89 cis $^3J_{HH} = 10.5$ Hz and δ 5.88 trans $^3J_{HH} = 17.4$ Hz; CH (dd) δ 5.29 and δ 5.27 due to $^2J_{HH}$ gem coupling of 1.5 Hz and trans $^3J_{HH}$ coupling of 17.4 Hz; CH (dd) seen at δ 5.19 and δ 5.18 due to $^2J_{HH}$ gem coupling of 1.2 Hz and cis $^3J_{HH}$ of 10.5 Hz.

Table 3. Hydrolysis Rate Constants for the 1-β-*O*-acyl Glucuronides of Ibuprofen

1-β-*O*-acyl glucuronide	hydrolysis rate constant (mol dm^{-3} h^{-1}) integration method	SE[a]	hydrolysis rate constant (mol dm^{-3} h^{-1}) product ratio method	SE[a]
(*R*)- and (*S*)-ibuprofen (urine-extracted)[b]	0.042	0.0001	0.045	0.0015
(*S*)-ibuprofen (synthesized)	0.036	0.0001	0.032	0.0002
(*S*)-2-hydroxyibuprofen and (*S*,*S*)-carboxyibuprofen[b]	0.099	0.0001	N/A	

[a] SE = standard error for the standard least-squares linear fit to the slope. [b] Examined an approximately equal two-component mixture.

Table 4. 600 MHz ^{1}H NMR Chemical Shifts δ, Multiplicities, and Coupling Constants *J* (Hz) of the 1-β-*O*-acyl Glucuronides of Ibuprofen in ^2H_2O[a,b]

1-β-*O*-acyl glucuronide	−CH_3 a + b δ	−CH− c δ	−CH_2− d δ	−CH− e δ	−CH− f δ	−CH− g δ	CH_3 h δ	H1′ δ
(*S*)-ibuprofen	0.88 (d) *J* = 6.4	1.85 (m) *J* = 6.8	2.50 (d) *J* = 6.9	7.27 (d) *J* = 7.9	7.32 (d) *J* = 7.9	3.96 (q) *J* = 7.0	1.52 (d) *J* = 7.0	5.57 (d) *J* = 8.3
(*R*)-ibuprofen	0.88 (d)	ND[c]	2.49 (d)	7.22 (d) *J* = 8.3	7.30 (d)	ND	1.51 (d)	5.55 (d) *J* = 8.3
(*S*)-2-hydroxyibuprofen	1.19 (s)		2.79 (s)	7.26 (d) *J* = 8.3	7.31 (d) *J* = 8.3	3.97 (q) *J* = 7.2	1.51 (d) *J* = 7.2	5.60 (d) *J* = 8.1
(*S*,*S*)-carboxyibuprofen	1.15 (d) *J* = 6.6	2.91 (m)	2.80 (m)	7.24 (d) *J* = 8.1	7.29−7.31 (d)	3.95 (q) *J* = 7.2	1.45 (d) *J* = 7.2	5.56 (d) *J* = 8.1

[a] δ referenced to internal TSP at δ 0.0. [b] Multiplicities: d, doublet; s, singlet; q, quartet; m, multiplet. [c] ND = not detected.

measured for each metabolite are shown in Table 4). The (*R*)- and (*S*)-ibuprofen AG assignments were based on the known (*R*) to (*S*) conversion in the body, and the assignments for the biosynthetic (*S*)-isomer were confirmed by agreement with the NMR parameters found for the synthesized (*S*)-ibuprofen AG. The 2-hydroxyibuprofen and carboxyibuprofen AGs are thus assumed to be the (*S*)- and (*S*,*S*)-isomers, respectively. The ratio of the (*S*)- to (*R*)-1-β-*O*-acyl ibuprofen glucuronides present in the 2−4 h postdose urine sample was about 8:1 and would support the assumption that the carboxy and 2-hydroxyibuprofen glucuronides observed would be metabolites of the (*S*)-enantiomer. The (*S*)-2-hydroxyibuprofen glucuronides have also been shown to have a urine recovery 13.3 times that of the (*R*)-form, and the (*S*,*R*)-, (*R*,*R*)-, (*R*,*S*)-, (*S*,*S*)-carboxyibuprofen glucuronides have urine recoveries of ratios 1:1.8:5.7:7.2, respectively,[46] confirmed by a second study,[47] which supports the assumptions that the phase I metabolites are the (*S*)-isomer.

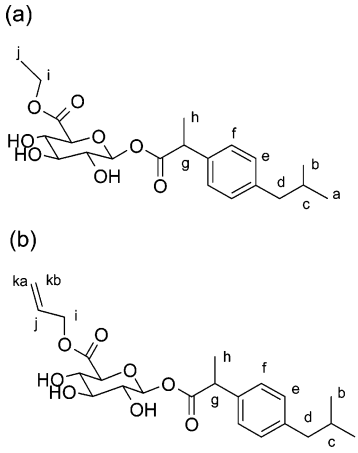

Figure 2. Structures of (a) ethyl ester and (b) allyl ester 1-β-*O*-acyl (*S*)-ibuprofen glucuronides.

The degradation rate constants and half-lives were measured using the same method as that for the synthesized glucuronides and are displayed in Table 1. A comparison of the rates shows that 1-β-*O*-acyl (*R*)-ibuprofen AG degraded approximately twice as fast as the (*S*)-enantiomer. This result is consistent with previously published work where similar conditions have been used to measure the degradation rates of (*R*)- and (*S*)-1-β-*O*-AGs.[2,13,14,16,17,20,48] The k_d of 0.188 h^{-1} calculated for the (*S*)-enantiomer is also consistent with previous studies[21,30] and with that for the synthesized (*S*)-ibuprofen AG. When comparing the hydrolysis rate of the urine-extracted (*R*)- and (*S*)-ibuprofen AG with that of the synthesized (*S*)-ibuprofen AG in Table 3, it can be seen that they are similar. This indicates that the rate of hydrolysis of the (*R*)-isomer must also be close to that of the (*S*)-isomer. This has also been shown in previous work with (*R*)- and (*S*)-ketoprofen[49] and naproxen[13] AGs where the hydrolysis rates of the (*R*)- and (*S*)-isomers are the same, within the experimental

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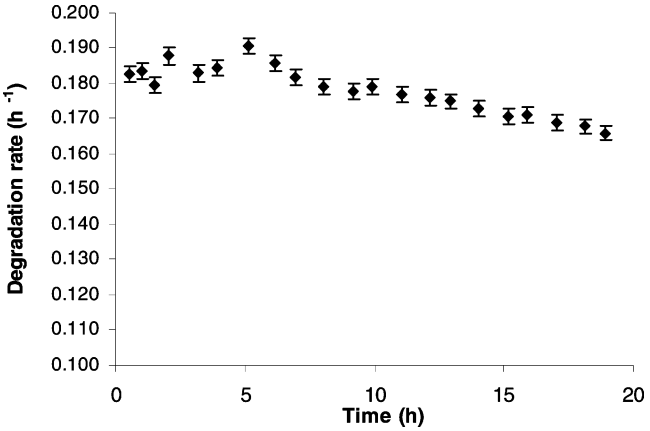

Figure 3. Degradation rate of 1-β-*O*-acyl (*S*)-ibuprofen glucuronide as a function of time.

error. Therefore, the acyl migration rate for the (*R*)-isomer is predicted to be 0.345 h^{-1}, which is approximately twice as fast as the (*S*)-isomer.

The k_d values of the 2-hydroxyibuprofen and carboxyibuprofen AGs at 0.138 and 0.144 h^{-1}, respectively, were considerably slower than either the (*R*)- or (*S*)-ibuprofen AGs. This result is somewhat surprising as these sites of metabolism are distant from the site of acyl glucuronidation, and as a result, a major difference in the degradation rates compared to (*S*)-ibuprofen glucuronide was not anticipated. However, it is perhaps not unreasonable to conjecture that the molecular conformation is changed by metabolism in such a way that the introduced carboxyl and hydroxyl groups come into proximity with the glucuronide ring moiety and in some way interact with it, thereby affecting the reactivity. To investigate whether this difference is due to changes in the transacylation or hydrolysis rates, the relative rates of production of free glucuronic acid were also measured from the α-glucuronic acid anomeric doublet NMR signal intensity, as signals from the free aglycones could not be fully resolved from the NMR spectra. The carboxyibuprofen and 2-hydroxyibuprofen glucuronides, measured as a mixture, have a weighted average hydrolysis rate constant of 0.099 mol dm^{-3} h^{-1} as displayed in Table 3, showing that hydrolysis accounts for approximately 70% of the degradation, and transacylation occurred at a much slower rate compared to the (*S*)-ibuprofen AG. The product ratio method was not used here to determine the hydrolysis rate as the 2-*O*-acyl isomer was not visible at the 2 h time point.

The degradation experiments were run for a 20 h time period, and the integrals of the β-anomeric proton peaks were recorded for as long as they were detectable. Previous papers have documented the degradation kinetics of 1-β-*O*-acyl ibuprofen glucuronide to be first-order in experiments recorded for up to 4[18] and 6 h.[21] However, when data are recorded for 20 h, it can be seen that the degradation rate starts to slow down after 6 h (Figure 3). The kinetics are thus more complex than a simple first-order reaction. This is not unexpected given the overall reaction scheme and the fact that the degradation rate reflects both transacylation and hydrolysis. As transacylation proceeds over the course of the degradation, the isomers produced degrade at different rates slowing down the overall reaction.

DISCUSSION

Differences between the degradation rates of the (*R*)- and (*S*)-NSAID glucuronides have been observed before and clearly show that the degradation is stereoselective with the (*R*)-AG degrading approximately twice as fast as the corresponding (*S*)-AG.[2,7,13,14,16,17,20,48] In this study the results show that the (*S*)- and (*R*)-isomers also have very similar hydrolysis rates; therefore, the difference between the degradation rates must be due to faster transacylation for the (*R*)-isomer. Previous suggestions for the differences observed in degradation rates have been largely related to steric reasons. One suggestion is that the distance between the aglycone at position C1 on the glucuronide ring and the attacking hydroxide nucleophile at C2 is greater for the (*S*)-isomer than that for the (*R*)-isomer and this would cause a slowing down of the reaction.[48] A previous study comparing the free energy of activation ΔG for the two diastereoisomers showed that the ΔG for the transacylation of the (*R*)-isomer was smaller than that of (*S*)-isomer and it would therefore be more reactive.[50] Incidentally Bischer et al.[51] have also observed a stereochemical effect on protein binding, with stronger binding for (*R*)-naproxen AG than for the (*S*)-isomer. Naproxen is in fact marketed as the (*S*)-form only since the (*R*)-enantiomer has been reported to cause liver toxicity.[52,53] but this property has not yet been directly correlated to protein binding to the AG. Presle et al.[54] have also shown evidence that the (*S*)-form of ketoprofen forms more protein adducts with human serum albumin (HSA) in vitro by reacting at site I via the Schiff mechanism, whereas the (*R*)-form binds to site II of the HSA by nucleophilic attack. This demonstrates that the formation of the modified protein is sensitive to the stereochemistry of the attacking glucuronide[7,55,56] and that the three-dimensional structure of the AG is important for protein binding.

Shackleford et al.[57] have provided evidence of another stereochemical effect—the ability of AGs to cross the sinusoidal membrane of hepatocytes. The difference in the ability of the diastereoisomers to gain access to intracellular proteins could possibly cause stereoselective toxicity. The differences between the (*R*)- and (*S*)-isomers may be the result of specific interactions with the components of the sinusoidal membrane domain of the hepatocytes, particularly with membrane transport proteins, in particular monocarboxylate transporter 2 (MCT2), which is capable of stereoselective substrate recognition.

It is thus evident that in general (*R*)- and (*S*)-drug AGs have different reaction rates and cellular transport abilities, which may be related to their toxic effects on the body.

An unexpected finding was the considerable increase in the half-life of the (*S*,*S*)-carboxyibuprofen and (*S*)-2-hydroxyibuprofen AGs compared to ibuprofen AGs. The carboxyl and hydroxyl

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groups are far from the reactive carbonyl carbon in chemical bond terms and so do not exert a large electronic effect. Previous studies where metabolism is also distant to the site of glucuronidation[48] have shown that 3- and 4-hydroxyketoprofen AGs had similar degradation rates, compared to ketoprofen AG itself. Further studies involving molecular modeling to elucidate potential reasons for these observation are ongoing.

The degradation rate data also shows that when the carboxyl group at the H5′ position on the glucuronide ring has an ethyl or allyl ester function the degradation rate is significantly slowed down by factors of 2 and 3, respectively. This shows that minor modifications of the chemical structure can have marked effect on the reactivity. The presence of the ethyl group increases the half-life by 3.5 h, and the addition of the rather similar allyl function increases the half-life by a further 2.1 h. The reason for the longer degradation rates for the esters remains unknown at this stage and requires further investigation, but these results give a clue to a possible mechanism. Not only do they give evidence of the operation of long-range electronic effects in these systems but also they lend weight to the idea that intermolecular general base catalysis by carboxylate anion may be involved.

Given that the postulated toxic effects of AGs are unlikely to arise as a result of hydrolysis of AGs back to aglycones,[31] it is likely that the transacylated AGs will modify proteins resulting in potential cytotoxic effects. Benet et al.[15] and Spahn-Langguth and Benet[2] have shown that a lower degree of covalent binding occurs with a longer half-life of the AG. Thus, drugs that have been withdrawn from the market due to toxicity and which display a high degree of AG covalent binding, such as tolmetin and zomepirac, have very short half-lives of 0.26[2] and 0.44 h,[23] respectively. However, the AGs of (*S*)-benoxaprofen and (+)-beclobrate (both withdrawn from the market due to idiosyncratic side effects and irreversible protein binding)[58,59] have half-lives of 4.1[16] and 25.7 h,[59] respectively, and therefore toxicity cannot be solely dependent on $t_{1/2}$. In this respect the cutoff value for $t_{1/2}$ recently suggested for allowing inclusion for drug development[32] is arbitrary. The formation of protein adducts is thus probably a balance between the transacylation propensity, protein binding kinetics, dose, and drug pharmacokinetics. If a drug has a very fast plasma/liver clearance it is possible that it can transacylate relatively fast but still remain innocuous. It has been shown here that 2-hydroxyibuprofen and carboxyibuprofen AGs have long half-lives and hydrolysis as the predominant mechanism for degradation and, hence, would be less likely to be involved in protein binding[31] and thus be eliminated from the body without any potentially toxic reactions. (*S*)-Ibuprofen AG has a half-life similar to that of (*S*)-benoxaprofen and undergoes degradation via hydrolysis and transacylation at a ratio of 1:4, but the incidence of protein binding for ibuprofen has been found to be very low,[60] and no serious complications have been reported. The allyl and ethyl ester ibuprofen AGs have long half-lives and appear to undergo both transacylation and hydrolysis. Protein binding experiments would be of interest here as differences in toxicity have been previously seen between ibuprofen and ibufenac, which is the structurally similar isobutylphenylacetic analogue of ibuprofen. The drug was withdrawn from the market due to hepatotoxicity. It degrades 3 times as fast as ibuprofen, with a half-life of 1.1 h, and shows a higher degree of covalent binding to plasma and liver proteins.[18] In addition to identifying adverse reactions in the liver, the effects of transacylating glucuronides spending considerable periods of time in the bladder might be usefully studied, as the formation of reactive intermediates may promote the formation of cancers in the bladder.

The series of compounds studied here have allowed us to try to understand the relative reactivities of a related series of compounds in vitro and to identify factors that affect reactivity. Ultimately it would be of interest to study this reactivity in vivo, possibly using hepatocytes as an in vitro system to look for in vivo versus in vitro differences. It is known that acyl migration predominates in vitro, whereas hydrolysis is the main reaction in vivo due to the action of nonspecific esterases and β-glucuronidase.[61] However, a major difference between the in vivo and in vitro acyl migration rates would not be expected, as enzymes do not affect this process.[48]

Hence, this study has provided additional transacylation and hydrolysis data on a range of structurally related aryl-propionic acid AGs. Further work, currently in progress, is required in order to provide a full explanation of the findings. Intriguing differences in reaction rates have been observed that will be of importance in deriving qualitative structure−reactivity relationships.

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