HPLC/^{1}H NMR Spectroscopic Studies of the Reactive α-1-*O*-acyl Isomer Formed during Acyl Migration of S-Naproxen *β*-1-*O*-acyl Glucuronide

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A widely held view in drug metabolism and pharmacokinetic studies is that the initial 1-isomer to 2-isomer step in the intramolecular acyl migration of drug ester glucuronides is irreversible, and that α-1-*O*-acyl isomers do not occur under physiological conditions. We investigated this hypothesis using high-performance liquid chromatography directly coupled to proton nuclear magnetic resonance spectroscopy (HPLC/^{1}H NMR) and mass spectrometry (LC/MS) to probe the migration reactions of S-naproxen *β*-1-*O*-acyl glucuronide, in phosphate buffer at pH 7.4, 37 °C. We report the first direct observation of the α-1-*O*-acyl isomer of a drug ester glucuronide (S-naproxen) formed in a biosystem via the facile acyl migration of the corresponding pure *β*-1-*O*-acyl glucuronide. The unequivocal identification of the reactive product was achieved using stopped-flow one-dimensional HPLC/^{1}H NMR and two-dimensional ^{1}H-^{1}H total correlation spectroscopy (^{1}H-^{1}H TOCSY). Parallel LC/ion-trap mass spectrometry yielded the confirmatory glucuronide masses. Moreover, "dynamic" stopped-flow HPLC/^{1}H NMR experiments revealed transacylation of the isolated α-1-*O*-acyl isomer to a mixture of α/*β*-2-*O*-acyl isomers; the reverse reaction from the isolated α/*β*-2-*O*-acyl isomers to the α-1-*O*-acyl isomer was also clearly demonstrated. This application of "dynamic" stopped-flow HPLC/^{1}H NMR allows key kinetic data to be obtained on a reactive metabolite that would otherwise be difficult to follow by conventional HPLC and NMR methods where sample preparation and off-line separations are necessary. These data challenge the widely held view that the α-1-*O*-acyl isomers of drug ester glucuronides do not occur under physiological conditions. Furthermore, the similar formation of α-1-*O*-acyl isomers from zomepirac and diflunisal *β*-1-*O*-acyl glucuronides has recently been confirmed (Corcoran et al., unpublished results). Such reactions are also likely to be widespread for other drugs that form ester glucuronides in biological systems. Ultimately, the presence of significant quantities of the kinetically labile α-1-*O*-acyl glucuronide isomer may also have toxicological implications in terms of reactivity toward cellular proteins.

Introduction

Ester glucuronides of many acidic drugs are unstable in aqueous solutions at pH 7.4 due to the susceptibility of the acyl groups to internal and external nucleophilic attack. This chemical reactivity is well established and comprehensively described elsewhere (*1*−*4*). Acyl glucuronides undergo spontaneous hydrolysis (in aqueous buffer solution) and enzymatic hydrolysis (in animal biofluids and cells) along with internal intramolecular acyl migration reactions of the glucuronide ring to form the 2-, 3-, and 4-positional isomers of the *β*-1-*O*-acyl glucuronide. These positional isomers also readily ring-open and undergo mutarotation, giving α- and *β*-anomeric pairs at C1 on the sugar ring which themselves can undergo further acyl migration reactions. All these acyl migration reactions have been considered to be reversible except the initial *β*-1-*O*-acyl to *β*-2-*O*-acyl reaction (*5*), and this has been widely accepted in drug metabolism and pharmacokinetic studies (*1*−*4*). In previous kinetic studies of acyl migration using synthetic *β*-1-*O*-acyl material, the occurrence of the α-1-*O*-acyl glucuronide isomer has been reported as a synthetic impurity and has not been considered in the general kinetic models of glucuronide reactivity (*1*−*4*).

While revisiting NMR data from our earlier studies (*6*−*8*) on the reactivity of acyl glucuronides including those of isoxepac glucuronide, we observed minor ^{1}H NMR doublet signals between *δ* 5 and 6.5, with *J*-couplings of 3 Hz. These are consistent with the presence of α-1-*O*-acyl glucuronide isomers in equilibrium mixtures obtained from synthetic and biosynthetic *β*-1-*O*-acyl glucuronides. This observation appeared to be in conflict with the accepted scheme used by us (*6*−*8*) and others

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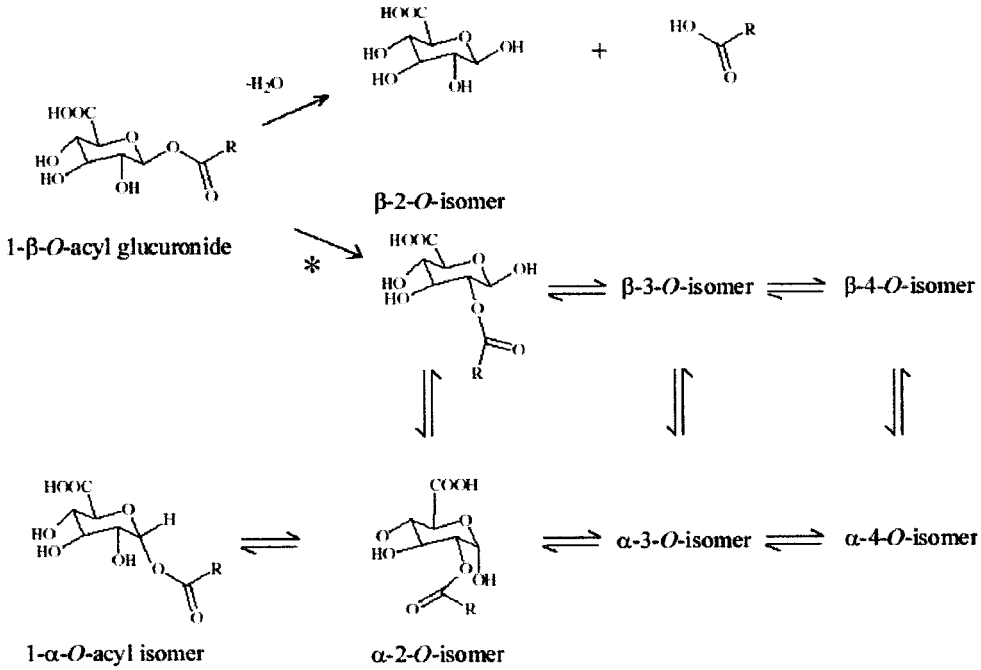
Scheme 1[a]



[a] The accepted scheme, in drug metabolism and pharmacokinetics studies, for the intramolecular transesterification of reactive *β*-1-*O*-acyl glucuronide molecules. The formation of the α-1-*O*-acyl glucuronide isomer is postulated here, based for the first time on structural evidence. **Important: the reaction of the β-1-O-acyl glucuronide to the 2- positional isomer is usually denoted as irreversible.*

(*1*−*4*) in previous drug metabolism and pharmacokinetic studies (Scheme 1). The principle of microscopic reversibility used in mechanistic organic chemistry states that all organic reactions must be to a certain extent reversible (*9*). However, the formation of α-1-*O*-acyl glucuronides under physiological conditions has not been reported in the modern literature, and some reports have specifically stated that they do not exist, or only in insignificant amounts (*2*). To test the hypothesis that the 2-isomer to 1-isomer reaction is reversible to a significant extent, we have used HPLC/^{1}H NMR[1] and LC/MS to investigate the possible production of the α-1-*O*-acyl isomer for the ester glucuronide of the widely prescribed drug S-naproxen. The acyl migration reactions have been described by nine first-order reactions with double and triple parallel reactions postulated to involve acyl migration and mutarotation (anomerization) (*2*). Obtaining detailed kinetic data on glucuronide rearrangement remains a significant challenge.

Understanding and modeling such complex equilibria requires an accurate chemical description of the interacting components and an efficient means of monitoring the reaction coordinates over time. We have previously developed directly coupled HPLC/^{1}H NMR techniques for investigating the kinetics of complex intramolecular rearrangements (*6*−*8*). The advantages of using on-line NMR following HPLC separation are many: preparative HPLC prior to off-line NMR (with the attendant risk of degrading the sample) is not necessary (*10*); analytes are not destroyed which allows further experiments, e.g., tandem mass spectrometry and enzyme hydrolysis. Finally, coelution in HPLC/UV methods is overcome. Coelution, which would otherwise result in erroneous determination of kinetics, is readily highlighted by the diagnostic HPLC/^{1}H NMR spectra of the individual isomeric components. Directly coupled HPLC/^{1}H NMR thus provides a very powerful tool for investigating the kinetics of acyl migration in complex equilibrium mixtures.

In light of recent evidence linking the propensity for extensive or rapid acyl glucuronide migration to toxicity (*11*), it has become increasingly important to investigate the equilibrium concentrations and the relative reactivities of the positional isomers toward proteins. We have decided to investigate the propensity for reversibility of acyl glucuronides to the α-1-*O*-acyl glucuronide isomer, as the significant occurrence of this molecule would necessitate the use of modified kinetic models. It adds another reactive isomer to the already complex kinetic model of acyl migration for ester glucuronides of many acidic drugs, thus necessitating the modification of previously used kinetic models for drug glucuronide acyl migration (*1*−*8*, *22*−*25*). Furthermore, the presence of significant amounts of α-1-*O*-acyl glucuronide isomers presents new potential drug−protein reactivity problems in vivo.

Materials and Methods

Chemicals. GOSS Scientific Instruments, Ltd. (U.K.), supplied deuterium oxide for LC/NMR. NMR grade acetonitrile was obtained from Riedel de Haën (Sigma-Aldrich, U.K., Dorset, U.K.). All other chemicals were analytical grade, from BDH Laboratory Supplies (Dorset, UK).

Purification of Biosynthetic S-naproxen *β*-1-*O*-acyl Glucuronide (1) from Human Urine. 1 was isolated from human urine using solid-phase extraction (SPE) (*12*) followed by preparative HPLC (*13*). Structures and numbering are shown in Figure 1. Approximately 200 mg of **1** was recovered from 4 L

[1] Abbreviations: S-naproxen, (S)-6-methoxy-α-methyl-naphthalene acetic acid; HPLC/^{1}H NMR, high-performance liquid chromatography directly coupled to proton nuclear magnetic resonance spectroscopy; TOCSY, ^{1}H-^{1}H total correlation NMR spectroscopy; HPLC/UV, ultraviolet wavelength detected-high-performance liquid chromatography; SPE. solid-phase extraction; FID, free induction decay; TPPI, time proportional phase increments; SSB, sine shifted bell function; A_{230}, maximal UV absorbance at 230 nm; ESI, electrospray ionisation.

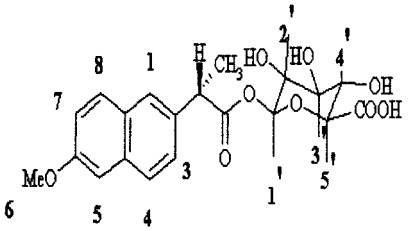


Figure 1. Chemical structure and numbering scheme for the *β*-1-*O*-acyl glucuronide of S-naproxen.

of urine 0−30 h after oral dose of 440 mg S-naproxen (Alleve) to a healthy male volunteer (27 years, 71 kg).

The identity of **1** was confirmed by the 600 MHz ^{1}H NMR spectrum of the biosynthetic product (in 25 mM potassium dihydrogen orthophosphate-D_2O buffer, pH* 7.4, 37 °C). By peak integration the purity was measured to be 97%, the remaining 3% was attributable to the α- and *β*-2 positional isomers. Shifts were referenced internally to residual HDO at *δ* 4.70. The assignments of the spectral data were (*δ* H, multiplicity, coupling constant, integration, assignment): *δ* 7.95, d, 8.8 Hz, 1H, H4; *δ* 7.93, d, 9.0 Hz, 1H, H8; *δ* 7.91, s, 1H, H1; *δ* 7.59, dd, 8.8 and 1.5 Hz, 1H, H3; *δ* 7.45, d, 2.6 Hz, 1H, H5; *δ* 7.32, dd, 9.0 and 2.6 Hz, 1H, H7; *δ* 5.67, d, 8.2 Hz, 1H, H1′; *δ* 4.21, q, 7 Hz, 1H, -CH-; *δ* 4.03, s, 3H, -OCH_3; *δ* 3.88, d, 9.5 Hz, 1H, H5′; *δ* 3.66, t, 9.5 Hz, 1H, H3′; *δ* 3.57, 2 × t, 9.5 and 8.8 Hz, 2H, H4′ + H2′; *δ* 1.70, d, 7 Hz, 3H, -CH_3.

Establishment of an Equilibrium Mixture Containing the Isomeric Rearrangement Products of S-Naproxen *β*-1-*O*-Acyl Glucuronide (1). A sample of the purified product **1** (2.8 mg/mL) was incubated in potassium dihydrogen orthophosphate buffer (25 mM) at pH 7.4, 37 °C, for up to 24 h. The acyl migration reaction was monitored by HPLC (UV detection at 230 nm) and the concentration of the putative α-1-*O*-acyl isomer was found to be maximal at 6 h. A sample of this mixture was injected directly onto the analytical column for HPLC/^{1}H NMR in stopped-flow mode.

Analytical Chromatography. The HPLC system consisted of a Bruker LC22C pump (Rheinstetten, Germany), a Bruker photodiode array detector (J & M Analytische Messund Regeltechnik GmbH, Aalen, Germany) and a Bruker BPSU-36/BSFU-0 flow control unit. The outlet of the detector was connected to the NMR flow probe via an inert polyether-ether ketone (PEEK) capillary (3.1 m × 0.25 mm i.d.). LC/NMR software (Hystar v1.1, Bruker, Germany) controlled the flow dynamics of the system and stored the chromatographic data obtained. Analytical chromatography was performed on a Hibar Lichrospher 100 RP-C18 column of particle size 5 *μ*m (250 mm × 4 mm i.d. purchased from Merck, Darmstadt, Germany). Isocratic reversed-phase chromatographic separation of the isomers was achieved using acetonitrile/ammonium formate in D_2O (50 mM, pH* 5.75), 25/75 v/v at a flow rate of 1.0 mL/min. The separation was achieved at a controlled temperature of 25 °C using a Bruker column oven compartment. The HPLC/^{1}H NMR spectrum of **1** was obtained on injection of 50 *μ*L equilibrium mixture, while that for the α-1-*O*-acyl isomer of **1** required 100 *μ*L injection. Assignment of components seen in the HPLC/UV profile was based on the stopped-flow HPLC/^{1}H NMR results.

To investigate the reactivities of the α-1-*O*-acyl isomer and the α/*β*-2-*O*-acyl isomers, the dynamic stopped-flow experiments were carried out in acetonitrile/potassium phosphate buffer in D_2O (25 mM, pH* 7.4), 20/80 v/v. The α-1-*O*-acyl and α/*β*-2-*O*-acyl isomers eluted at 12 and 15 min respectively (data not shown).

NMR Spectroscopy. The HPLC/^{1}H NMR spectra were acquired using a Bruker Avance 600 MHz spectrometer equipped with a ^{1}H-^{13}C inverse-detection *z*-gradient LC flow probe containing a 4 mm i.d., 120 *μ*L cell. NMR spectra of the α-1-*O*-acyl isomer and the *β*-1-*O*-acyl glucuronide of S-naproxen were obtained in stopped-flow mode (*13*) at 600.13 MHz and probe temperature 25 °C. To achieve effective solvent suppression, the spectra were acquired using a presaturation pulse sequence (*14*) with double presaturation for the acetonitrile and residual water signals using relaxation and mixing delays of 2.0 and 0.1 s, respectively. Between 128 and 512 free induction decays (FIDs) were collected into 64 K computer data points with a spectral width of 20 ppm, corresponding to an acquisition time of 2.73 s. Prior to Fourier transformation, an exponential apodization function was applied to the FID, corresponding to a line broadening of 2 Hz. Chemical shifts for the glucuronide (**1**) in the ammonium formate mobile phase (pH* 5.75) at 25 °C were referenced internally to the acetonitrile signal at *δ* 2.00 and thus differ slightly from those obtained in deutero-phosphate buffer (pH* 7.4) at 37 °C, referenced internally to residual HDO at *δ* 4.70.

The 2D ^{1}H-^{1}H TOCSY spectrum of the α-1-*O*-acyl isomer was acquired in stopped-flow HPLC/^{1}H NMR mode (pH* 5.75) after injecting 100 *μ*L of the equilibrium mixture onto the analytical column. Probe temperature was maintained at 25 °C during the 3 h experiment. This minor peak, representing 3% of the total glucuronic-acid-related material, was estimated to contain 8.5 *μ*g. Twenty-four scans per increment were collected for 160 increments, with spectral widths of 15 ppm in both dimensions and spin-lock and relaxation delays of 65 ms and 2.4 s, respectively. The spectrum was processed in phase-sensitive (TPPI) mode using squared sine functions (SSB = 3) in both dimensions and forward linear prediction to supplement these data in the F1 dimension.

Dynamic stopped-flow HPLC/^{1}H NMR experiments were conducted by separating the α-1-*O*-acyl isomer from the other glucuronide isomers in potassium phosphate buffer at pH 7.4 as described above and transferring to the flow probe where rearrangement kinetics were followed as a function of time. The probe temperature was set to 37 °C and a time zero NMR spectrum was acquired using the spectral parameters as above but accumulating only 32 scans. Thereafter, spectra obtained by accumulation of 256 scans were sequentially acquired over a 16 h period. To compensate for the low signal-to-noise ratio resulting from the ongoing reactions, the 16 h spectrum was acquired on accumulation of 1024 scans. Similarly the kinetic reactions of the α/*β*-2-*O*-acyl isomers were also monitored.

LC/MS Studies. LC/MS was performed using a Bruker Esquire ion-trap multipole mass spectrometer coupled to the HPLC system described above. Ionization was by positive-ion electrospray (ESI) and centroid mass spectra were acquired over the range 100−1000 *m*/*z* using capillary exit and skimmer voltages of 100 and 40 V, respectively.

Results

Chromatographic Separation of the *β*-1- and α-1-*O*-acyl Isomers of S-Naproxen Glucuronide. Biosynthesized S-naproxen *β*-1-*O*-acyl glucuronide **(1)** was purified from human urine by solid-phase extraction chromatography (SPE) and preparative HPLC. The purified glucuronide was incubated in nondeuterated phosphate buffer at pH 7.4 and 37 °C to give a mixture of the glucuronide isomers (containing the *β*-1-*O*-acyl glucuronide, the α/*β*-2-*O*-acyl anomers, the α/*β*-3-*O*-acyl anomers, the α/*β*-4-*O*-acyl anomers, the aglycone and, as a minor component, the α-1-*O*-acyl isomer). Chromatographic conditions were then developed to allow direct HPLC/^{1}H NMR detection of the various positional isomers in a dedicated NMR flow probe. The mobile phase consisted of ammonium formate buffer in D_2O (50 mM, pH* 5.75) and acetonitrile (75/25 v/v). Both the mobile phase pH and the column oven temperature (25 °C) were selected so as to minimize intramolecular rearrangement on-column during the chromatography. Formate buffer (as opposed to phosphate) was chosen for use in both the HPLC/^{1}H NMR and LC/MS experiments. Using photodiode array detection it was found that UV absorbance

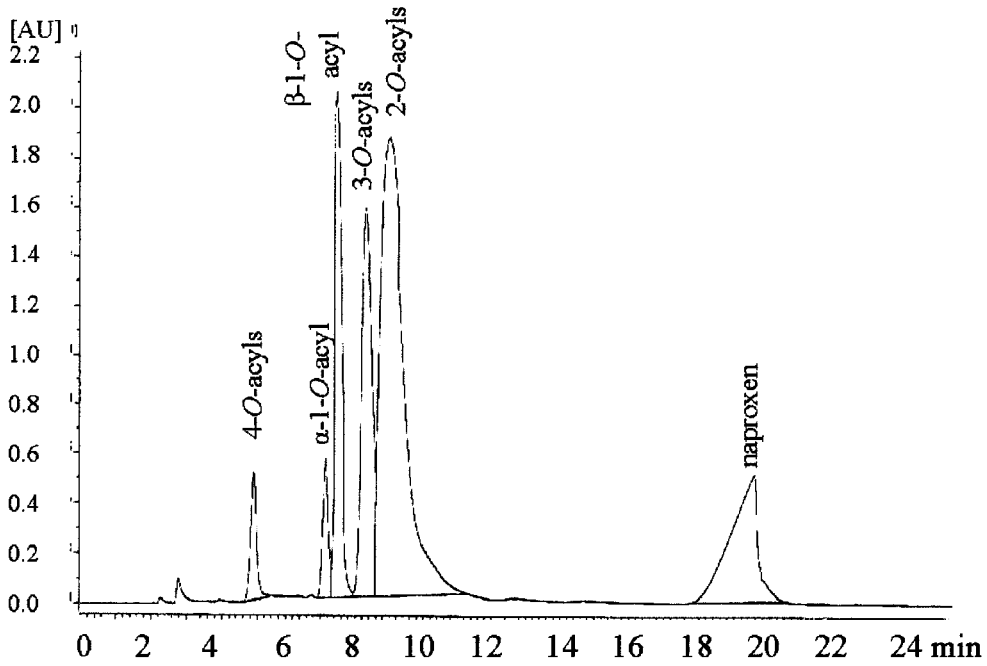


Figure 2. Resulting HPLC chromatogram of S-naproxen acyl glucuronide and isomers, on injection of an equilibrium incubation mixture (6 h). The isomers eluted with the following retention times (min): $t_{R,4-O-acyl(\alpha,\beta)isomers} = 4.83$, $t_{R,1-O-acyl(\alpha)isomer} = 6.89$, $t_{R,1-O-acyl(\beta)isomer} = 7.20$, $t_{R,3-O-acyl(\alpha,\beta)isomers} = 8.10$, $t_{R,2-O-acyl(\alpha,\beta)isomers} = 8.77$, $t_{R,S-naproxen} = 19.30$. Chromatographic system: Lichrosorb C18 analytical column, 5 μm, (250 mm × 4.6 mm i.d.), mobile phase was acetonitrile/ammonium formate (50 mM) D_2O, pH* 5.75, 25/75 v/v; flow rate 1 mL/min; UV detection (230 nm); column temperature 25 °C; injection volume 100 μL.

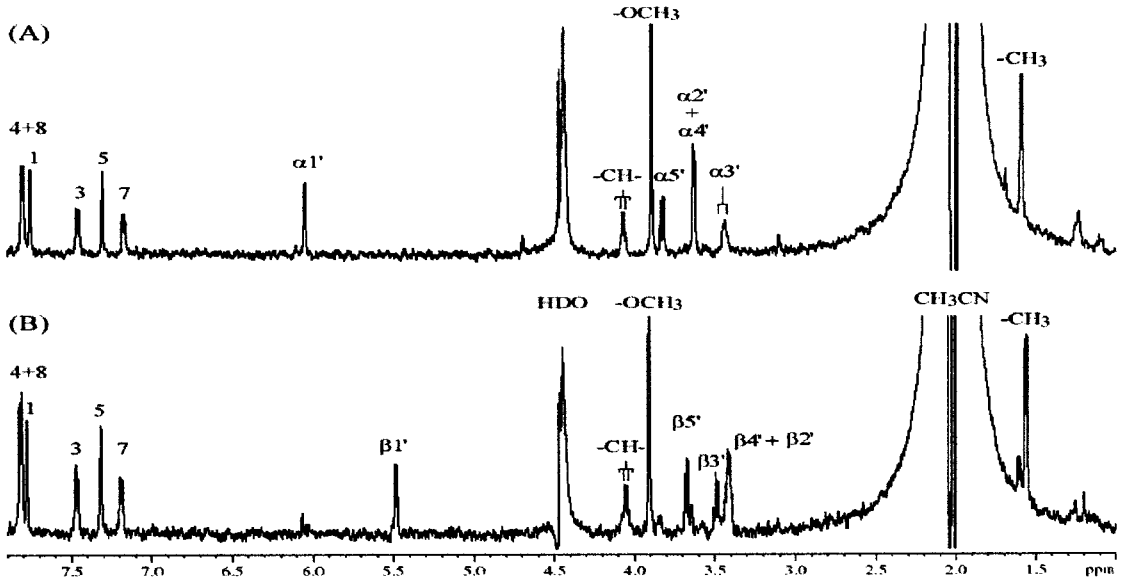


Figure 3. Stopped-flow 600 MHz HPLC/^{1}H NMR spectra, on injection of the equilibrium incubation mixture of S-naproxen acyl glucuronide and isomers in phosphate buffer, pH 7.4, 37 °C: (A) peak eluting at 6.89 min corresponding to the α-1-*O*-acyl glucuronide isomer (256 scans) and (B) peak eluting at 7.20 min corresponding to the *β*-1-*O*-acyl glucuronide of S-naproxen (128 scans).

at 230 nm gave the maximal response for S-naproxen-related analytes and hence the A_{230} signal was used to trigger the transfer of peaks to the NMR flow probe for acquisition of ^{1}H NMR spectra. The HPLC chromatogram of the equilibrium mixture of glucuronide isomers after 6 h incubation is shown in Figure 2. Integration of the areas under these HPLC peaks indicated that the minor component eluting at 6.9 min, corresponding to the putative α-1-*O*-acyl isomer, represented 3% of the total glucuronide-related material in the complex equilibrium mixture. In addition, the integration of the anomeric protons in the 1D ^{1}H NMR spectrum of the mixture confirmed the HPLC integration values. The 100 μL injection of the mixture contained a total of 280 μg of glucuronide-related material on-column. The resolution of the α-1-*O*-acyl isomer from the *β*-1-*O*-acyl glucuronide was found to be compromised on injecting more sample.

Stopped-Flow HPLC-^{1}H NMR Assignments of the *β*-1- and α-1-*O*-Acyl Isomers of S-Naproxen Glucuronide. The α-1-*O*-acyl isomer was assigned in the UV-detected chromatogram (Figure 2) using stopped-flow HPLC/^{1}H NMR. The HPLC/^{1}H NMR spectra of the α-1-*O*-acyl isomer and *β*-1-*O*-acyl glucuronide of S-naproxen glucuronide are shown in Figure 3 and signal assignments are summarized in Table 1. These assignments were based on the characteristic chemical shift values

Table 1. ^{1}H NMR Chemical Shifts of S-Naproxen α/*β*-1-*O*-acyl Glucuronides Obtained by Stopped-Flow HPLC-^{1}H NMR in Ammonium Formate Buffer pH* 5.75/CH_3CN (75/25) V/v

		aglycone protons (δ, pattern)									glucuronosyl protons (δ, pattern)				
isomer	t_R (min)	-CH_3	-CH-	H1	H3	H4	H5	-OCH_3	H7	H8	H1′	H2′	H3′	H4′	H5′
α-1-*O*-acyl	6.9	1.60 d	4.06 q	7.76 s	7.46 d	7.80 d	7.30 s	3.89 s	7.17 d	7.80 d	6.05 d	3.62 d	3.44 m	3.62 d	3.82 d
β-1-*O*-acyl	7.2	1.56 d	4.05 q	7.78 s	7.46 d	7.82 d	7.32 s	3.91 s	7.19 d	7.82 d	5.49 d	3.41 t	3.49 t	3.41 t	3.68 d

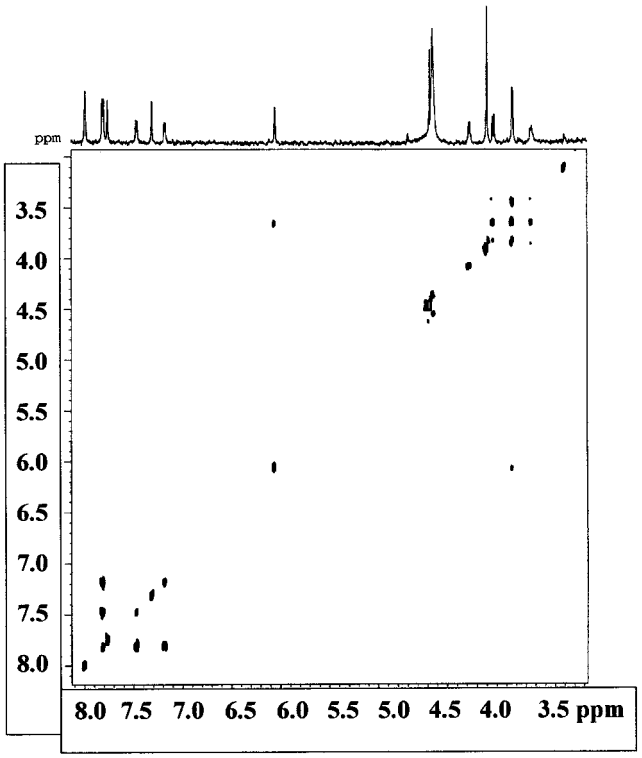


Figure 4. Stopped-flow 600 MHz two-dimensional ^{1}H-^{1}H TOCSY spectrum of the peak eluting at 6.89 min, corresponding to 8.5 μg of α-1-*O*-acyl glucuronide isomer injected onto the analytical column. Connectivities of the sugar ring are observed in the region δ 6.10−3.60 ppm.

and coupling constants of acyl glucuronides (*15*). To confirm the connectivities in the sugar ring, a 2D ^{1}H-^{1}H TOCSY spectrum was acquired for the α-1-*O*-acyl isomer (equivalent to 8.5 μg on-column). The result is shown in Figure 4. The correlation between the protons at sugar positions 1 and 2 is easily seen, confirming that the doublet (J = 3.4 Hz) signal at δ = 6.08 was from the sugar residue. Further correlations within the isolated naphthalenyl- and glucuronosyl-substructures are also visible. The weak correlations between the sugar H1 signal and those from protons beyond position 2 on the ring is due to the small coupling constants in relation to the modest spin-lock time (65 ms) used. That the experiment required less than 3 h demonstrates the ready practicability of using stopped-flow 2D experiments for identifying low-level metabolites and degradation products in mixtures.

LC/MS Analysis of the *β*-1- and α-1-*O*-Acyl Isomers of S-Naproxen Glucuronide. Naproxen glucuronide isomers in D_2O have a molecular weight of 410 by virtue of the four exchangeable protons. Under the conditions of this positive-ion LC/MS method, the major ion observed for these compounds was the sodium adduct, having a *m*/*z* value of 433. Figure 5A illustrates the extracted-ion chromatogram at *m*/*z* 433 for the glucuronide-related compounds and Figure 5, panels B and C, show the mass spectra of the peaks corresponding to the α-1-*O*-acyl isomer and *β*-1-*O*-acyl glucuronide, respectively. These experiments confirmed that the *β*-1-*O*-acyl glucuronide of S-naproxen and the prior-eluting minor peak both showed a major ion at *m*/*z* 433, confirming the latter to be a glucuronide isomer. Other fragments were common to both spectra, e.g., *m*/*z* 233 is the deuterium cation adduct of deuterated naproxen, presumably formed by thermal degradation of the glucuronides; the major cluster seen in the range *m*/*z* 185−188 may be the methoxynaphthylethyl carbonium ion with varying degrees of deuterium substitution. The fragments at *m*/*z* 343 and 454 in the spectrum of the α-1-*O*-acyl epimer have not been assigned and probably arise from coeluting impurities. The LC/MS retention times, 7.8 and 8.7 min, are marginally longer than those obtained with stopped-flow HPLC/^{1}H NMR (6.9 and 7.2 min), the discrepancy being due to the different total volume of the LC/MS system. However, although the MS data clearly identified this minor peak at 7.8 min (*m*/*z* 433) as a sodium adduct of naproxen glucuronide, and thus an isomer of the *β*-1-*O*-acyl glucuronide at 8.7 min, no further structural information was apparent. Only by stopped-flow HPLC/^{1}H NMR experiments was it possible to attribute this minor component to the α-1-*O*-acyl isomer of S-naproxen ester glucuronide.

Dynamic Stopped-Flow HPLC/^{1}H NMR Studies on the Reactivity of the α-1-*O*-Acyl Isomer. Mechanistically, the most likely route of α-1-*O*-acyl formation is the reverse rearrangement from the α-2-*O*-acyl glucuronide isomer. To investigate this hypothesis, dynamic stopped-flow HPLC/^{1}H NMR experiments were used to assess the reactivity of these compounds. However, whereas an acidic mobile phase of pH* 5.75 and probe temperature of 25 °C stabilized the α-1-*O*-acyl isomer to allow spectroscopic identification, physiological temperature and pH (using phosphate buffer) were needed for the reactivity studies. Although the elution order of the isomers remained the same in the phosphate buffer, the elution times were extended to 12 and 15 min for the α-1-*O*-acyl isomer and α/*β*-2-*O*-acyl isomers respectively (data not shown). The resulting dynamic stopped-flow spectra are illustrated in Figure 6. The α-1-*O*-acyl isomer rapidly transacylates first to α-2-*O*-acyl isomer, with mutarotation to the *β*-2-*O*-acyl isomer (Figure 6, panels A and B). Clearly, the α-1-*O*-acyl isomer is highly reactive toward acyl migration. It does not appear to hydrolyze to give free S-naproxen, as evidenced by the lack of methyl doublets diagnostic for S-naproxen (δ 1.40−1.80, spectral region not shown).

Dynamic Stopped-Flow HPLC/^{1}H NMR Studies on the Reactivity of the α-2-*O*-Acyl Isomer. The time zero stopped-flow spectrum for the α-2-*O*-acyl isomer shows a mixture of α/*β*-2-*O*-acyl isomers, indicating rapid mutarotation in the probe at 37 °C, Figure 6C and the α-1-*O*-acyl isomer is clearly absent. The 16 h spectrum Figure 6D shows both the presence of the α-1-*O*-acyl isomer via the reverse migration and the α/*β*-3-*O*-acyl

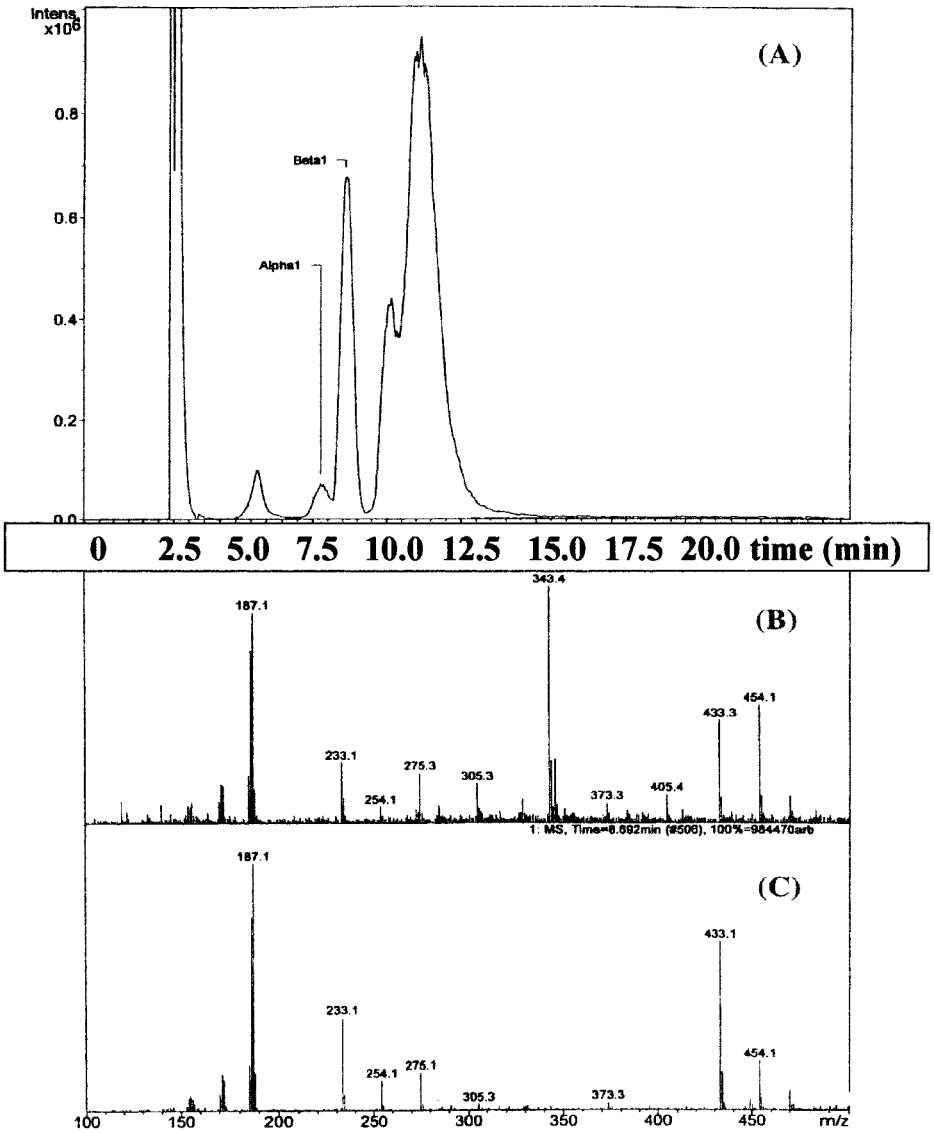


Figure 5. LC/MS data obtained by ESI in positive ion mode, demonstrating identical molecular weights for all the chromatographic peaks observed in the UV trace (Figure 3), further confirming the assignment of the α-1-*O*-acyl glucuronide isomer. (A) Selected ion mass chromatogram at *m*/*z* 433 (sodium adduct of the acyl glucuronide and isomers). (B) mass spectrum of the peak at 7.8 min, corresponding to the α-1-*O*-acyl glucuronide isomer and (C) mass spectrum of the peak at 8.7 min corresponding to the *β*-1-*O*-acyl glucuronide.

isomers via the forward acyl migration reaction. The reverse reaction, though slow, is clearly demonstrated here. The possibility of the reformation of the *β*-1-*O*-acyl glucuronide from the *β*-2-*O*-acyl isomer exists, as suggested by Hansen-Moller et al (*10*). However, our NMR experimental data on this parallel reverse migration from the *β*-2-*O*-acyl to the *β*-1-*O*-acyl isomer provides no evidence of this occurrence.

Discussion

Formation of the α-1-*O*-Acyl Glucuronide of S-Naproxen. This can be rationalized on the basis that formation of the α-1-*O*-acyl isomer from the α-2-*O*-acyl glucuronide isomer proceeds via a stable *ortho*-acid ester intermediate with 1,2-*cis*-(*ax*,*eq*) fused rings, whereas formation of the *β*-1-*O*-acyl glucuronide from the *β*-2-*O*-acyl glucuronide isomer would require a higher energy intermediate with 1,2-*trans*-(*eq*,*eq*) fused rings (Scheme 2) (*16*−*19*). The former reaction would be the more energetically favorable of the two, because of stabilization due to the anomeric effect (*20*). The results of these dynamic stopped-flow experiments support this mechanistic theory, with the α-2- to α-1-*O*-acyl isomer a more significant reaction than the *β*-2- to *β*-1-*O*-acyl isomer.

It is widely accepted in drug metabolism and pharmacokinetic studies that all the acyl migration reactions are reversible except for the reformation of the 1-*O*-acyl isomer (*2*), supposedly because of the higher energy barrier to formation of the anomeric C−O bond (*2*, *6*, *20*). To this end, most papers showing the rearrangement reaction scheme have depicted the 1-*O*-acyl isomer to 2-*O*-acyl isomer reaction as unidirectional or essentially irreversible. In fact, there are two reasons why the α-1-*O*-acyl isomer is not usually considered as part of the rearrangement scheme for *β*-1-*O*-acyl ester glucuronides. One depends on whether the glucuronide has been obtained by synthesis (*2*, *6*−*8*, *21*, *22*), by isolation from enzymatic sources (*2*) or by isolation from biofluids such as urine (*2*, *10*, *12*, *13*) (biosynthetic sources). The other depends on the analytical methodology used to assign the purity of the starting material and to measure the rearrangement kinetics.

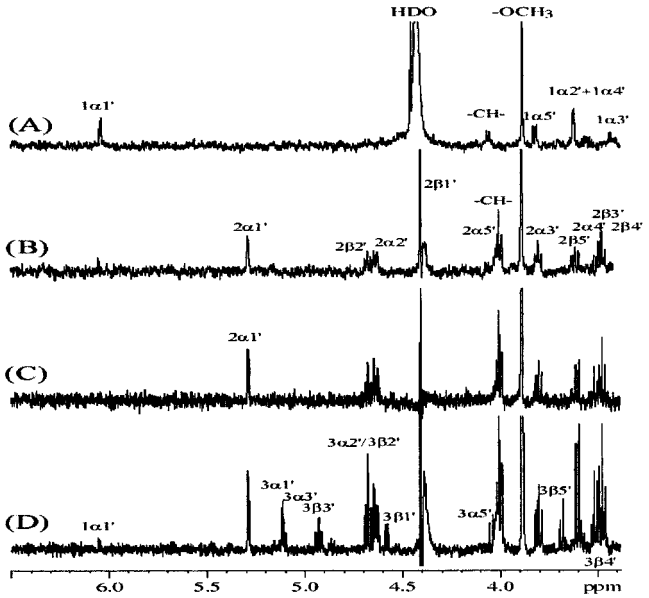
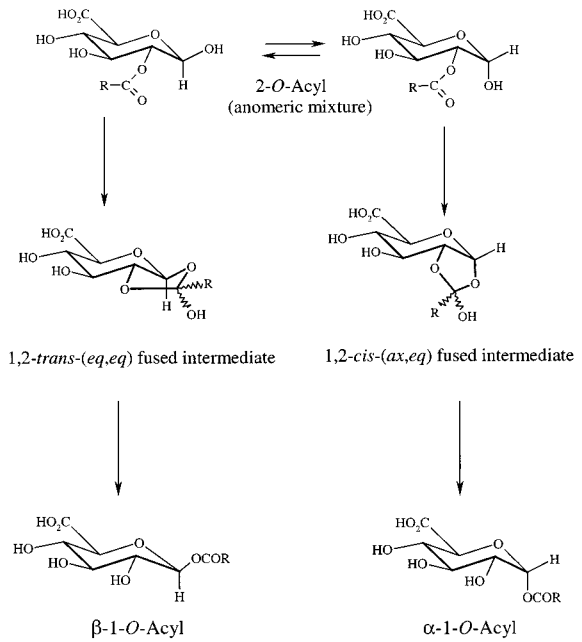


Figure 6. Dynamic stopped-flow 600 MHz HPLC/^{1}H NMR spectra to illustrate the reactivities of the α-1-*O*-acyl and 2-positional glucuronide isomers in potassium phosphate buffer, pH* 7.4 at 37 °C. (A) time zero spectrum (32 scans) for the α-1-*O*-acyl glucuronide isomer of S-naproxen, eluting at 12 min, (B) the resulting spectrum after 25 min of acquisition (256 scans) showing a mixture of the α/*β*-2-*O*-acyl glucuronide isomers, (C) time zero spectrum (128 scans) for the α/*β*-2-*O*-acyl glucuronide isomers of S-naproxen, eluting at 15 min and (D) the resulting spectrum after 16 h of acquisition (1024 scans), illustrating the slow formation of the α-1-*O*-acyl glucuronide isomer over time.

Scheme 2[a]



[a] The postulated mechanism for α-1-*O*-acyl glucuronide isomer formation on intramolecular rearrangement of *β*-1-*O*-acyl glucuronides. The energy barrier to the formation of the 1,2-*cis*-(*ax*,*eq*) fused intermediate is lower than that for the equivalent 1,2-*trans*-(*eq*,*eq*)fused intermediate, hence favoring the back reaction to the α-1-*O*-acyl glucuronide isomer.

With regard to the source of the starting glucuronide material, it is widely accepted that the α-1-*O*-acyl glucuronide isomer is not produced by in vivo enzymes (*2*), therefore, this isomer is assumed to be absent in the starting material obtained from biosynthetic sources. In contrast, for synthetic ester glucuronides, the α-1-*O*-acyl isomer is normally present as a synthetic impurity when measuring a time zero spectrum or chromatogram. Of course, the effective "time zero" in a kinetic experiment depends on the time required to complete the measurement. However, if exact purity data (and the solvents used to measure the purity) are not reported, it is difficult to determine whether a significant amount of α-1-*O*-acyl isomer or even of the α/*β*-2-*O*-acyl isomers is present in the original sample. Here, we have shown that the acyl group of the α-1-*O*-acyl isomer of (1) can rapidly migrate to the α-2-*O*-acyl isomer, which may explain why the synthetic α-1-*O*-acyl products of other such ester glucuronides appear to degrade within minutes when incubated at pH 7.4, 37 °C. In fact, our data concurs with the LC/MS data reported by Khan et al. (*23*) on the rapid acyl migration of α-1-*O*-acyl isomer of ifetroban ester glucuronide, present as a synthetic impurity in the starting material, although the exact ratio of isomers in the starting material was omitted. However, these authors could not measure the backward reaction from the α-2-*O*-acyl glucuronide isomer using the LC/MS method reported, neither did their overall kinetics scheme include the α-1-*O*-acyl glucuronide isomer of ifetroban. If, under similar conditions for other synthetic ester glucuronides, the α-2-*O*-acyl isomer rapidly migrates forward to the α-3-*O*-acyl isomer, or mutarotates rapidly followed by forward migration to the *β*-3-*O*-acyl isomer then, depending on the analytical method used, the α-1-*O*-acyl isomer may not be observed at pH 7.4, 37 °C. In fact, the α/*β*-2-*O*-acyl isomer thus formed from the α-1-*O*-acyl isomer (present at time zero) will be erroneously attributed to degradation of the *β*-1-*O*-acyl isomer, resulting in the false interpretation of the overall kinetics. The second reason the α-1-*O*-acyl isomer is not usually considered as part of the rearrangement scheme for *β*-1-*O*-acyl ester glucuronides concerns the analytical methodology used.

Of analytical significance is the fact that the formation of the α-1-*O*-acyl isomer has not been reported in previous HPLC/UV based methods (*2*) (except as a synthetic impurity as evidenced by ^{1}H NMR). This is explained by the difficulty in resolving these isomeric components using UV-detected chromatography, which can result in coelution of the minor α-1-*O*-acyl isomer with the *β*-1-*O*-acyl glucuronide. Therefore, the published rate and adduct formation data based on HPLC/UV methods are based on net kinetic effects which include α-1-*O*-acyl isomer formation/degradation rates but which do not account for them as integral rates. Only two notable exceptions to the generally accepted schematic have been proposed. Hansen-Moller et al. (*10*) proposed reformation of the *β*-1-*O*-acyl glucuronide of diflunisal on the basis of analytical HPLC/UV and enzyme hydrolysis experiments. However, the α-1-*O*-acyl isomer was not reported, possibly as a result of the lengthy preparative HPLC approach. Even then the resolution between isomers limited definitive assignment, as the *β*-2-*O*-acyl isomer coeluted with the *β*-1-*O*-acyl glucuronide. Clearly, if the α-1-*O*-acyl isomer were present it would have coeluted somewhere under the preceding HPLC peaks. Likewise, although Dickinson and King (*24*) postulate the putative formation of the α-1-*O*-acyl isomer in their schematic [alongside the putative parallel formation of the *β*-1-*O*-acyl glucuronide as reported by Hansen-Moller (*10*)], the HPLC/UV methods used did not provide any such evidence due to coelution of the isomeric products.

With respect to the S-naproxen case in question, our data now supersedes a recently published result whereby an unidentified minor isomer, hydrolyzable by 1 M NaOH but not β-glucuronidase, eluted at 8.8 min, just prior to the S-naproxen β-1-*O*-acyl glucuronide at 9.2 min (*25*). We now infer this minor isomer to be the S-naproxen α-1-*O*-acyl glucuronide isomer identified herein. Our results, concerning the facile formation of the α-1-*O*-acyl isomer of biosynthetic S-naproxen β-1-*O*-acyl glucuronide at pH 7.4, 37 °C reveal that 10 reversible reactions are involved in the scheme (Figure 1) contrary to previous reports. Clearly, the kinetic scheme for the acyl migration reactions is complicated to interpret and relies on appropriate analytical methodology. In this respect, dynamic stopped-flow HPLC/^{1}H NMR experiments can provide a unique perspective on a reaction which has hitherto been ignored when investigating reactivity of ester drug glucuronides.

Our results conflict with the widely held view (in drug metabolism and pharmacokinetic studies) that the acyl migration of 1-*O*-acyl glucuronic acid conjugates to the 2-positional isomers is irreversible (*1*−*10*, *13*−*15*, *21*−*25*). Given that this is a general reaction in carbohydrate chemistry, it must be assumed to apply to all drug glucuronides to a certain extent. Thus, the overall kinetics scheme for any acyl migration of ester glucuronides should include the formation of the α-1-*O*-acyl glucuronide isomer from the α-2-*O*-acyl glucuronide. What were previously considered double parallel reactions are now known to be triple parallel reactions, and there are fluxes in concentrations of the α-1-*O*-acyl glucuronide isomer that were previously unaccounted for in the current kinetic models. Although the α-1-*O*-acyl glucuronide isomer is kinetically less stable, it is thermodynamically more stable than the corresponding β-1-*O*-acyl glucuronide (*16*).

In conclusion, the toxicological significance of α-1-*O*-acyl isomer formation (even at such low concentrations) by a facile chemical reaction, may be important if the 3D conformation of this reactive product allows covalent binding to important proteins in vivo. As yet the direct reactivity of the α-1-*O*-acyl glucuronide isomer toward proteins in terms of adduct formation remains unknown.

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