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Identification of the Positional Isomers of 2-Fluorobenzoic Acid 1-*O*-Acyl Glucuronide by Directly Coupled HPLC–NMR

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Directly coupled HPLC–^{1}H NMR was used in the "stop-flow" mode to separate and rapidly identify an equilibrated mixture of ester glucuronide isomers formed spontaneously by intramolecular rearrangement reactions (internal acyl migration and mutarotation) of 2-fluorobenzoic acid β-1-glucuronide (1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid). The equilibrated mixture of isomers was obtained by incubation of the synthetic 2-fluorobenzoic acid glucuronide in buffer solution (pH 7.4) at 25 °C for 24 h. The β-anomer of the 1-*O*-acyl glucuronide, and the 2-, 3-, and 4-positional glucuronide isomers (all three as both α- and β-anomers) present in the equilibrium mixture, were all characterized after separation in an isocratic chromatographic system containing phosphate buffer at pH 7.4 and 1% acetonitrile in the mobile phase. The HPLC–NMR investigations also elucidated the mutarotation of the positional glucuronide isomers as well as showing the benefits of the HPLC–NMR technique as a primary analytical tool. This HPLC–NMR method will be of particular value in studies on the acyl migration reactions of nonsteroidal antiinflammatory drug glucuronides which may be related to their toxicological properties.

It has been shown that NMR spectroscopy of biofluids can serve as an effective approach for the detection and identification of endogenous and xenobiotic metabolites.[1,2] However, for such complex mixtures the direct hyphenation of NMR with liquid chromatography can offer clear benefits as metabolites can be separated and characterized directly.[3–8] Recent advances in NMR probe technology, improved dynamic range of receiver systems, and solvent suppression methods have resulted in major practical improvements in the HPLC–NMR technique,[3,4] resulting in increased sensitivity and decreasing the need for deuterated organic modifiers in the eluent. In the present study, we show that HPLC–NMR in the "stop-flow" mode can be used to study a complex mixture of isomers that interconvert spontaneously in aqueous solution, i.e., acyl glucuronides that undergo reversible acyl migration and mutarotation reactions.

Many carboxylate-containing drugs form β-1-*O*-acyl glucuronic acid conjugates in vivo. The acyl glucuronides formed are potentially reactive metabolites due to the susceptibility of the acyl group to nucleophilic reactions. They have been shown to undergo hydrolysis (regeneration of parent compound),[9,10] intramolecular rearrangement (isomerization by acyl migration),[11,12] and covalent adduct formation with both low molecular weight nucleophiles (such as methanol) and proteins.[11,13–15] In the rearrangement reactions of acyl glucuronides, the susceptibility of the ester linkage to nucleophilic reactions allows the drug moiety to move from one hydroxyl group to an adjacent hydroxyl group on the glucuronic acid ring (Scheme 1). The mechanism of transacylation via a tetrahedral cyclic ortho ester intermediate is well established.[10,16–21] The acyl groups migrate from C-1

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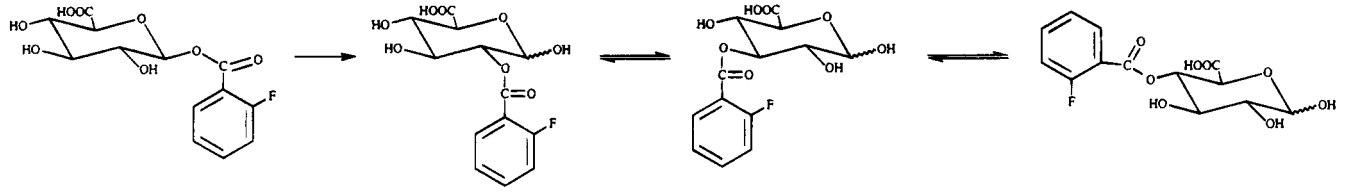
Scheme 1. General Scheme for the Acyl Migration of 1-*O*-(2-Fluorobenzoyl)-D-glucopyranuronic Acid (1)



(1) 1-*O*-Acyl (β) isomer (2) 2-*O*-Acyl (α,β) isomer (3) 3-*O*-Acyl (α,β) isomer (4) 4-*O*-Acyl (α,β) isomer

toward C-4 on the glucuronic acid ring forming both the α- and β-anomers of the 2-, 3-, and 4-*O*-acyl positional isomers as shown in Scheme 1. All the reactions are reversible except for the reformation of the 1-*O*-acyl glucuronide, presumably because of the higher energy barrier to formation of the anomeric C–O bond. Mutarotation (α ↔ β) of the isomers occurs via the open-chain aldehyde structure of the sugar ring. The acyl migration reactions occur spontaneously in aqueous solution, the isomerization rates being highly dependent upon the structure of the aglycon, pH, and temperature.[12] Acyl migration is inhibited under acidic conditions, but the migration rates increase dramatically with increasing pH; however, mutarotation occurs at all pH values studied (pH 3–8.5).[12]

Previously in studies on drug glucuronide reactivity, it would have been necessary to separate compounds and later analyze them off-line, during which time complete mutarotation and possibly further acyl migration rearrangements would have occurred. The analytical challenge presented here is to develop a system that would allow direct detection and, hence, signal assignment of each glucuronide isomer in an isocratic HPLC–NMR system at pH 7.4. We present here a novel HPLC–NMR approach for the separation and identification of the positional isomers **(2–4)** of the acyl glucuronide of 2-fluorobenzoic acid (1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid, **1**) and their corresponding α- and β-anomers as an example of a glucuronide isomer mixture.

EXPERIMENTAL SECTION

Chemicals. All chemicals were of analytical chemical grade and purchased from Aldrich Chemical Co. Ltd. (U.K.).

Synthesis of 2-Fluorobenzoic Acid β-1-Glucuronide (1). Benzyl 2,3,4-tri-*O*-benzyl glucopyranuronate **(5)** was synthesized according to the literature.[22–24] **5** (200 mg, 0.37 mmol) and trichloroacetonitrile (0.37 mL, 3.7 mmol) were dissolved in dry dichloromethane (5 mL) under N_2 at room temperature. Sodium hydride (8 mg, 0.259 mmol, 80% in oil) was added under reflux, and the reaction was stirred for 15 min. Solvent was removed to yield a brown oil residue (0.376 g), which was purified by silica gel chromatography [petrol–ether (1:1)] to yield benzyl [1-*O*-trichloroethanimodoyl]-2,3,4-tri-*O*-benzyl-D-glucopyranuronate **(6)**, a semicrystalline yellow residue (0.118 g, 46%). **6** (0.2 mmol) and 0.4 mmol of 2-fluorobenzoic acid were dissolved in dry dichloromethane (4 mL) under Ar(g) at −20 °C. Boron trifluoride etherate (2 drops) was added, and the reaction was left at −20 °C for 48 h. The reaction mixture was washed with $NaHCO_3$ (20 mL, 10% w/v) and distilled water (20 mL). The organic extract was dried ($MgSO_4$) and filtered, and the solvent was removed to yield a residue that after purification by silica gel chromatography [petrol–ether (1:1)] gave the 2,3,4-tri-*O*-benzyl-1-*O*-(2-fluorobenzoyl)-D-glucopyranuronate **(7)**. **7** was deprotected by hydrogenation to give 1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid (**1**; yield 34%). The structure of the compound synthesized was confirmed to be **1** by ^{1}H NMR: ^{1}H NMR (D_2O) δ 7.29 (t, H5, aromatic), 7.24 (m, H3, aromatic), 8.00 (m, H6, aromatic), 7.66 (m, H4, aromatic), 5.77 (d, H1′, β-glucuronide), 3.63 (t, H2′, β-glucuronide), 3.52 (m, H3′, β–glucuronide), 3.60 (m, H4′, β-glucuronide), 3.86 (d, H5′, β-glucuronide).

Establishment of the Equilibrium Mixture of the 2-Fluorobenzoic Acid Glucuronide Isomers. A 1.5 mg sample of **1** was incubated in 1 mL of potassium phosphate buffer (100 mM) at pH 7.4, 25 °C, for up to 24 h. The equilibration was followed by HPLC; when the relative amounts of the positional isomers had stabilized, the sample was stored at −20 °C until further analysis.

Analytical Chromatography. The HPLC system consisted of a Bruker LC22C pump (Rheinstetten, Germany) and a Bischoff 1000 Lambda variable-wavelength UV detector (operated at 200 nm). The outlet of the UV detector was connected to the HPLC–NMR flow probe via an inert poly(ether ketone) capillary (0.25 mm i.d.). A column oven was used for thermostating the column at 25 °C. Data were collected using the Bruker Chromstar HPLC data system. Analysis was performed on a Knauer column (120 × 4.6 mm, i.d.) packed with Spherisorb ODS-2 (Phase Separations Ltd.), 5 μm. The final mobile phase developed for the separation of 2-fluorobenzoic acid glucuronides and their positional glucuronide isomers was acetonitrile–0.2 M potassium phosphate (pH 7.4)–deuterium oxide (1:10:89, v/v/v), with a flow rate of 1 mL/min.

NMR Spectroscopy. The HPLC–NMR data were acquired using a Bruker AMX-600 spectrometer equipped with a ^{1}H flow probe (cell of 3 mm i.d., with a volume of 100 μL). ^{1}H NMR spectra were obtained in the stop-flow mode[5] at 600.14 MHz. In order to suppress the solvent signals, the 1D ^{1}H NMR spectra were acquired using a pulse sequence based on a one-dimensional version of the nuclear Overhauser effect spectroscopy experiment (NOESYPRESAT),[25] with double presaturation for suppression of the water and the acetonitrile signals. Free induction decays (FIDs) were collected into 32K computer data points with a spectral width of 7246 Hz, 90° pulses were used with an acquisition time of 2.26 s, and the spectra were acquired by accumulation of 128 scans. Prior to Fourier transformation, an exponential

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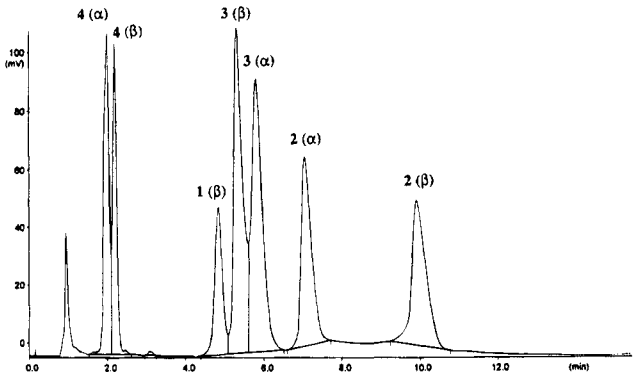

Figure 1. Resulting chromatogram of 1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid (**1**), its 2-, 3-, and 4-positional isomers (**2**–**4**), and their α- and β-anomers. The isomers eluted with the following retention times (in minutes): $t_{R4\text{-}O\text{-}acyl(\alpha)isomer}$ = 1.99, $t_{R\ 4\text{-}O\text{-}acyl\ (\beta)\ isomer}$ = 2.19, $t_{R\ 1\text{-}O\text{-}acyl\ (\beta)\ isomer}$ = 4.84, $t_{R3\text{-}O\text{-}acyl\ (\beta)\ isomer}$ = 5.31, $t_{R\ 3\text{-}O\text{-}acyl\ (\alpha)\ isomer}$ = 5.80, $t_{R\ 2\text{-}O\text{-}acyl\ (\alpha)\ isomer}$ = 7.05, $t_{R\ 2\text{-}O\text{-}acyl\ (\beta)\ isomer}$ = 9.91. Chromatographic system: Spherisorb ODS2 column, 5 μm (120 × 4.6 mm i.d.), mobile phase, CH_3CN–D_2O–KD_2PO_4 (0.2 M, pH 7.4) 1:89:10 by volume; flow rate, 1 mL/min; UV detection (200 nm); column temperature, 25 °C.

apodization function was applied to the FID corresponding to a line broadening of 1.1 Hz.

RESULTS AND DISCUSSION

Chromatography. An HPLC method was developed in order to separate the equilibrium mixture of 2-fluorobenzoic acid 1-*O*-acyl glucuronide and the 2–4 positional glucuronide isomers as well as the α- and β-anomer of each isomer. As the method is intended to be used to investigate the individual acyl migration rates of the 1-*O*-acyl glucuronide and its 2–4 positional glucuronide isomers, the pH of the buffer in the mobile phase was titrated to pH 7.4. The HPLC method was designed to keep the solvent systems as simple a possible to reduce problems with solvent suppression in the HPLC–NMR experiment.

A reversed phase ODS-2 column was chosen, as the glucuronides are very polar compounds and are ionized at pH 7.4; only small amounts of organic modifier are needed to elute the analytes from the column. Acetonitrile was chosen as organic modifier, as methanol can obscure the acyl migration rates by methanolysis.[26] A flow rate of 1 mL/min is most optimal with respect to filling the NMR flow cell.

The resulting optimized mobile phase contained 1% acetonitrile, and this enabled optimal resolution of the 2-fluorobenzoic acid 1-*O*-acyl glucuronide and its 2–4 positional glucuronide isomers as well as the α- and β-anomers. The chromatogram, obtained by UV detection, resulting from injection of 75 μg of 2-fluorobenzoic acid glucuronides in total, can be seen in Figure 1.

^{1}H HPLC–NMR Identification of the Chromatographic Peaks. Identification of the different peaks in the HPLC chromatograms was achieved by one-dimensional ^{1}H proton HPLC–NMR in the stop-flow mode. Assignment of the NMR resonances for the various structures from the seven HPLC peaks of the equilibrium mixture (seen in Figure 1.) is summarized in Table 1. The numbering system of the protons used for assignment of the ^{1}H signals resulting from the glucuronide isomers and anomers of 2-fluorobenzoic acid glucuronide is indicated in Figure 2. The 4-*O*-acyl isomers eluted first from the chromatographic column, the α-anomer eluting before the β-anomer. The β-anomer of the 1-*O*-acyl isomer eluted next, followed by the 3-*O*-acyl isomers, this time the β-anomer before the α-anomer. Finally, the 2-*O*-acyl isomers were eluted, the α-anomer before the β-anomer as seen with the 4-*O*-acyl isomers. In Figure 3. the ^{1}H NMR spectra obtained from peaks corresponding to 4α, 3α, and 2α in Figure 1 are shown and compared with the ^{1}H NMR spectrum obtained from the equilibrium mixture.

The mutarotation rates were fast in comparison to the acyl migration rates. In the situation where all peaks were to be measured by NMR in a single chromatographic run, the later eluting peaks underwent mutarotation while they were stopped on the analytical column (in the stop-flow mode) and the two peaks corresponding to the α- and β-anomer of the 2-*O*-acyl isomer turn into four peaks, as can be seen in the chromatogram in Figure 4. The α- and β-anomers separate into two peaks on the first major part of the analytical column. When the flow is stopped, the α- and β-anomers are still on the column and they mutarotate into their respective equilibrium mixtures α/β, which then, as the flow is started again, separate into two new peaks. As there is only a minor part of the column left, this results in four peaks. The chromatographic peak shape corresponding to the β-anomer of the 3-*O*-acyl isomer is also obscured as it is converting into its α-anomer when the flow is stopped. Even in the second peak of the 4-*O*-acyl isomer (its β-anomer), traces of the α-anomer can

Table 1. ^{1}H NMR Chemical Shifts of the Positional Isomers of 2-Fluorobenzoic Acid 1-*O*-Acyl Glucuronide Obtained by Stop-Flow HPLC–NMR Analysis[a]

		aromatic protons (δ, pattern)				glucuronic acid protons (δ, pattern)				
isomer	t_R	3	4	5	6	1′	2′	3′	4′	5′
4-*O*-acyl (α)	1.99	7.21 dd	7.62 m	7.27 t	7.94 m	5.25 d	3.68 dd	3.99 t	5.07 t	4.27 d
4-*O*-acyl (β)	2.19	7.21 dd	7.62 m	7.27 t	7.94 td	*b*	3.39 t	3.81 t	5.09 t	3.93 d
1-*O*-acyl (β)	4.84	7.24 m	7.66 m	7.29 t	8.00 m	5.77 d	3.63 t	3.52 m	3.60 m	3.86 d
3-*O*-acyl (β)	5.31	7.23 t	7.63 m	7.28 t	7.98 t	*b*	3.80 t	5.20 t	3.55 t	3.83 d
3-*O*-acyl (α)	5.80	7.23 t	7.63 m	7.28 t	7.98 t	5.27 d	3.85 dd	5.38 t	3.78 t	4.17 d
2-*O*-acyl (α)	7.05	7.24 dd	7.64 m	7.29 t	7.96 t	5.45 d	4.93 dd	4.00 t	3.60 t	4.10 d
2-*O*-acyl (β)	9.91	7.24 dd	7.64 m	7.29 t	7.96 m	4.89 d	4.92 t	3.80 t	3.61 t	3.75 d

[a] t_R refers to the retention time (min) in the chromatographic run. An HPLC–NMR experiment was performed in the stop-flow mode. All δ-values are in ppm. The splitting patterns of the ^{1}H NMR signals are indicated as follows: m, multiplet; d, doublet; dd, doublet of doublets; *t*, apparent triplet; td, triplet of doublets. [b] Not observed (obscured by HDO resonant, δ 4.75).

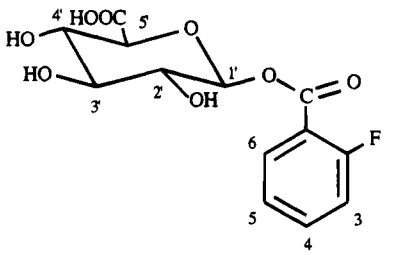

Figure 2. Numbering scheme for the protons of the 1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid (**1**).

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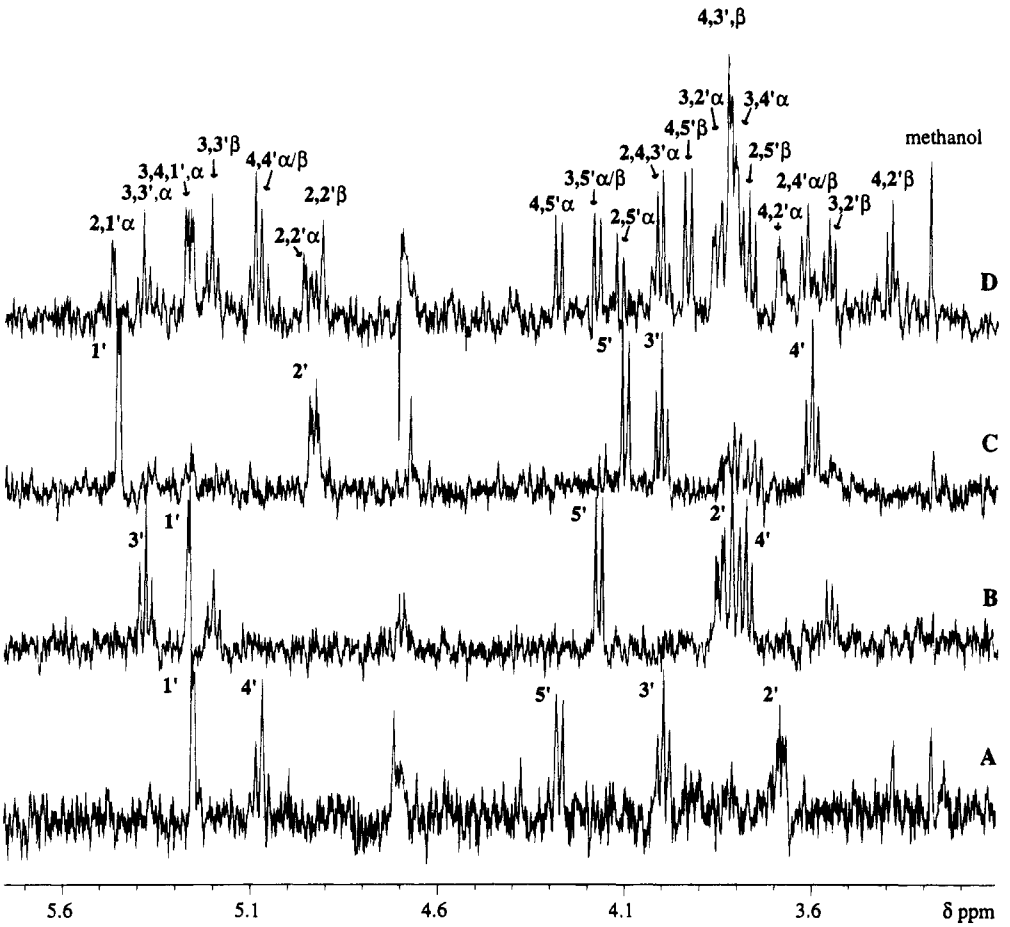

Figure 3. ^{1}H NMR spectra obtained from the chromatographic peaks: (A) peak corresponding to the α-4-*O*-acyl isomer, (B) peak corresponding to the α-3-*O*-acyl isomer, and (C) peak corresponding to the α-2-*O*-acyl isomer shown and compared with (D) the ^{1}H NMR spectrum obtained from the equilibrium mixture.

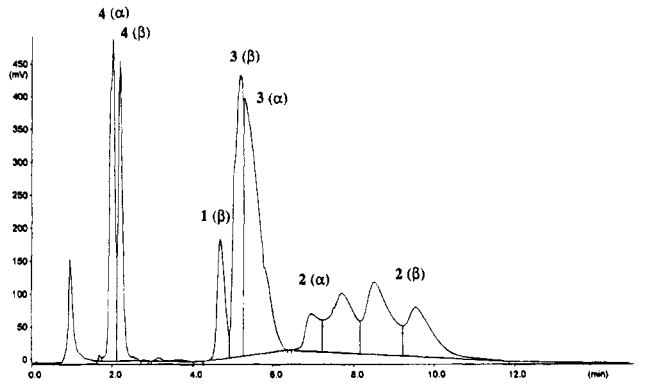

Figure 4. Resulting chromatogram of the 1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid **(1)**, its 2–4 positional isomers **(2–4)**, and their α- and β- anomers resulting from the HPLC–NMR experiment in the stop-flow mode. Time on the column for the 2-O-acyl isomers (last eluting peaks) was approximately 90 min. For identification of peaks, see Figure 1. Chromatographic system: Spherisorb ODS2 column, 5 μm (120 × 4.6 mm i.d.); mobile phase, CH_3CN–D_2O–KD_2PO_4 (0.2 M, pH 7.4) 1:89:10 by volume; flow rate, 1 mL/min; UV-detection (200 nm) and column temperature, 25 °C.

be seen in the ^{1}H NMR spectrum in amounts that cannot be accounted for by chromatographic peak overlap.

In order to identify the last three peaks eluting from the HPLC column, a further chromatographic injection was made (skipping NMR acquisition of the first four peaks). Because of their longer retention times, there was still a significant amount of on-column mutarotation of the α- and β-anomers of the 2-*O*-acyl isomers. Furthermore, as their chromatographic peaks were broader compared to the other isomers, the concentration of the isomer in the NMR flow probe was low. This had a detrimental effect on the signal-to-noise ratios of the NMR spectra, thus demanding longer NMR scanning times. In order to determine the elution order of the α- and β-anomers of the 2-*O*-acyl isomer, the amount of organic modifier in the mobile phase was increased (5% acetonitrile instead of 1%). Under these conditions, the 2-*O*-acyl isomers eluted with retention times of 3.5 and 4 min for the α- and β-anomers, respectively, and thereby allowing their structure assignment by HPLC–NMR.

We have demonstrated that directly coupled HPLC–NMR can be a highly effective analytical tool in situations that would otherwise require the extremely time-consuming purification of seven compounds that are in dynamic equilibrium and are constantly interconverting. HPLC-MS would be unrevealing in this situation as it is insensitive to the detection of positional isomerizm. The HPLC–NMR approach to studying acyl migration should therefore be of considerable value in the investigations of drug glucuronide reactivity and related protein binding and toxicological problems.

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