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BIOMEDICAL APPLICATIONS

Development of a simple liquid chromatographic method for the separation of mixtures of positional isomers and anomers of synthetic 2-, 3- and 4-fluorobenzoic acid glucuronides formed via acyl migration reactions

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Abstract

Many drugs containing carboxylate groups form β -1-*O*-acyl glucuronides as their major phase II metabolites in vivo. These ester glucuronides are potentially reactive due to the susceptibility of the acyl group to nucleophilic reactions resulting in hydrolysis, acyl migration or covalent adduct formation. In the present study, a number of synthetic fluorobenzoic acid glucuronide conjugates were chosen as models for chromatographic studies. A high-performance liquid chromatography method is presented for the simultaneous determination of the 1-, 2-, 3- and 4-positional isomers of the acyl glucuronides, and their α - and β -anomers for the 2-, 3- and 4-fluorobenzoic acids as well as each aglycone formed as a result of hydrolysis. The same elution order was found for the acyl migrated glucuronide isomers of the three fluorobenzoic acids in their equilibrium mixtures. The α -4-*O*-acyl isomer eluted first followed by the β -4-*O*-acyl isomer, then the β -1-*O*-acyl, the β -3-*O*-acyl, the α -3-*O*-acyl, the α -2-*O*-acyl and finally the β -2-*O*-acyl isomer eluted. The method was used to determine the overall degradation rates, the acyl migration rates and the hydrolysis rates of 1-*O*-(2-fluorobenzoyl)- β -D-glucopyranuronic acid, 1-*O*-(3-fluorobenzoyl)- β -D-glucopyranuronic acid and 1-*O*-(4-fluorobenzoyl)- β -D-glucopyranuronic acid in a buffer system pH 7.4 at 25°C. It was found that the order of β -1-glucuronide acyl migration rates was 2-fluorobenzoyl > 3-fluorobenzoyl > 4-fluorobenzoyl. Both the acyl migration rates and the elution order were interpreted in terms of electronic effect of the fluorine substituent on the carbonyl carbon.

Keywords: Acyl migration reactions; Fluorobenzoic acid glucuronides

1. Introduction

Xenobiotics which contain functional carboxylate

groups such as substituted benzoic acids, arylacetic acids and aryloxyacetic acids are often used as drugs. Many non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen, naproxen, diflunisal and aspirin contain carboxylate groups. Some

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NSAIDs have shown a variety of toxicological effects and a number of NSAIDs (e.g. zomiperac, indoprofen, alclofenac) have been withdrawn from clinical use because of a high incidence of allergic reactions [1,2]. It has been proposed that these allergic reactions may be caused by the formation of drug–protein adducts and that the ability of a carboxylate group-containing drug to form reactive acyl glucuronides plays an important role in the covalent protein binding properties of these compounds [3–6].

Drugs containing carboxylate groups are metabolised by conjugation to form the β -anomers of 1-*O*-acyl glucuronides in vivo by the enzyme uridine diphosphate-glucuronosyl transferase. These ester glucuronides are potentially reactive because of the susceptibility of the acyl group to nucleophilic attack and such glucuronides readily undergo hydrolysis [1,7], and isomerisation via acyl migration [8–10]. These glucuronides may also undergo covalent adduct formation with both small nucleophiles (such as methanol) and proteins [8,10–12]. The ester linkage on an acyl glucuronide is susceptible to nucleophilic reactions and this allows the acyl moiety to move from one –OH group to an adjacent –OH group on the glucuronic acid ring (see Fig. 1). The mechanism of transacylation involves a tetrahedral cyclic *ortho* ester intermediate [12,13] and the acyl group moves sequentially from C₁ to C₄ on the glucuronic acid ring forming both the α - and β -anomers of the 2-, 3- and 4-*O*-acyl positional isomers of the acyl glucuronide. The reactions are generally reversible, but acyl migration from C₂ to C₁ to reform the 1-*O*-acyl glucuronide is not usually observed. The acyl migration reactions occur spontaneously in aqueous solution, the isomerisation rates being highly dependent

upon the structure of the aglycone, pH and temperature [8]. The relationship between chemical structure of the drug and acyl migration rates has been explained by steric factors, as bulky groups on the acyl moiety can obstruct the nucleophilic attack of the adjacent hydroxyl on the carbonyl group inhibiting formation of the *ortho* ester intermediate [14]. The acyl migration reactions are inhibited under acidic conditions but, once slightly alkaline, the migration rates increase with increasing pH [8].

To investigate the reactivity of ester glucuronides, a series of synthetic fluorobenzoic acid glucuronides were chosen as model compounds. Acyl migration can then be followed in either buffer or biological fluids. We here present a high-performance liquid chromatography (HPLC) method developed for the simultaneous determination of the 2-, 3- and 4-positional isomers of the β -1-*O*-acyl glucuronides of fluorobenzoic acids, as both their α - and β -anomers. The separations obtained for the anomers of the positional isomers are improved using the present separation method relative to the separations obtained in earlier presented HPLC methods for the separation of the acyl migrated positional isomers of ester glucuronides [15–21]. Additionally, all the positional isomers as well as their α - and β -anomers have been identified in the chromatograms obtained using directly-coupled HPLC–nuclear magnetic resonance (NMR). The HPLC method was applied to determine the overall degradation rates, the acyl migration rates and the hydrolysis rates of the 2-fluorobenzoic acid glucuronide [1-*O*-(2-fluorobenzoyl)- β -D-glucopyranuronic acid], the 3-fluorobenzoic acid glucuronide [1-*O*-(3-fluorobenzoyl)- β -D-glucopyranuronic acid] and the 4-fluorobenzoic acid

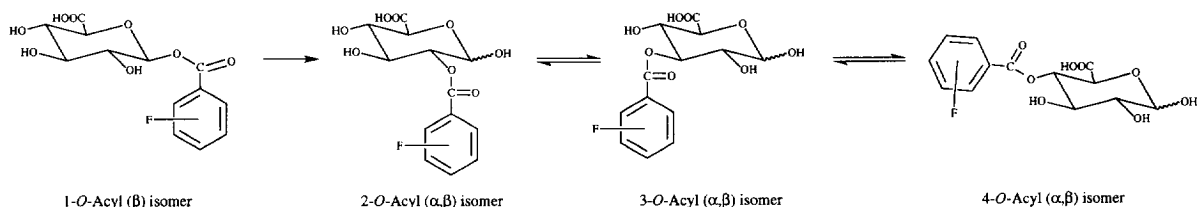


Fig. 1. General acyl migration scheme for 1-*O*-acyl mono-fluorobenzoic acid glucuronides.

glucuronide [1-*O*-(4-fluorobenzoyl)- β -D-glucopyranuronic acid] in an aqueous buffer system.

2. Experimental

2.1. Chemicals

All chemicals were of analytical chemical grade and purchased from Aldrich (Gillingham, UK).

2.2. Synthesis of 2-, 3- and 4-fluorobenzoic acid β -1-D-glucuronides (**1**, **2** and **3**)

Benzyl 2,3,4-tri-*O*-benzyl glucopyranuronate (**4**) was synthesised according to the literature [22]. Compound **4** (200 mg, 0.37 mmol) and trichloroacetonitrile (0.37 ml, 3.7 mmol) were dissolved in dry dichloromethane (5 ml) under nitrogen 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 chromatography on silica gel, with petroleum ether–diethyl ether (1:1, v/v) as the eluent, to yield benzyl 1-*O*-trichloroethanimodoyl-2,3,4-tri-*O*-benzyl-D-glucopyranuronate (**5**), a semi-crystalline yellow residue (0.118 g, 46%). A 0.2 mmol amount of compound **5** and 0.4 mmol of either 2-fluorobenzoic acid, 3-fluorobenzoic acid or 4-fluorobenzoic acid were dissolved in dry dichloromethane (4 ml) under argon gas at -20°C . Boron trifluoride etherate (two 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), filtered and the solvent removed to yield a residue which after purification by chromatography on silica gel, with petroleum ether–diethyl ether (1:1, v/v) as the eluent, gave the 2,3,4-tri-*O*-benzyl-1-*O*-(2-fluorobenzoyl)- β -D-glucopyranuronate, 2,3,4-tri-*O*-benzyl-1-*O*-(3-fluorobenzoyl)- β -D-glucopyranuronate or 2,3,4-tri-*O*-benzyl-1-*O*-(4-fluorobenzoyl)- β -D-glucopyranuronate (**6**, **7** or **8**). Compounds **6**, **7** or **8** were deprotected by hydrogenation to give 1-*O*-(2-fluorobenzoyl)- β -D-glucopyranuronic acid (**1**), 1-*O*-(3-fluorobenzoyl)- β -D-glucopyranuronic acid (**2**) and 1-

O-(4-fluorobenzoyl)- β -D-glucopyranuronic acid (**3**). Overall yields were 34%, 22% and 24%, respectively.

2.3. Structures of the synthesised compounds were confirmed by ^1H NMR

^1H NMR (D_2O) for 2-fluorobenzoic acid glucuronide (**1**): δ 7.21 (m, H5, aromatic); δ 7.27 (m, H3, aromatic); δ 7.65 (m, H4, aromatic); δ 8.05 (m, H6, aromatic); δ 5.78 (d, H1', β -glucuronide); δ 3.63 (t, H2', β -glucuronide); δ 3.52 (t, H3', β -glucuronide); δ 3.62 (t, H4', β -glucuronide) and δ 4.19 (d, H5', β -glucuronide). ^1H NMR (D_2O) for 3-fluorobenzoic acid glucuronide (**2**): δ 7.41 (m, H4, aromatic); δ 7.53 (m, H5, aromatic); δ 7.80 (m, H2, aromatic); δ 7.90 (m, H6, aromatic); δ 5.75 (d, H1', β -glucuronide); δ 3.68 (t, H2', β -glucuronide); δ 3.56 (t, H3', β -glucuronide); δ 3.62 (t, H4', β -glucuronide) and δ 3.9 (d, H5', β -glucuronide). ^1H NMR (D_2O) for 4-fluorobenzoic acid glucuronide (**3**): δ 7.21 (m, H3, H5, aromatic); δ 8.06 (m, H2, H6, aromatic); δ 5.72 (d, H1', β -glucuronide); δ 3.65 (t, H2', β -glucuronide); δ 3.53 (t, H3', β -glucuronide); δ 3.60 (t, H4', β -glucuronide) and δ 3.88 (d, H5', β -glucuronide).

2.4. Establishment of the equilibrium mixture of the glucuronide positional isomers.

Each fluorobenzoic acid 1-*O*-acyl glucuronide (**1**, **2**, **3**) (150 $\mu\text{g}/\text{ml}$) was incubated in potassium phosphate buffer (20 mM) at pH 7.4, 25°C , for up to 24 h. The equilibration was followed by HPLC and when the relative amounts of the trans-acylated isomers had stabilised, the sample was stored at -20°C until further analysis was carried out. Determination of the acyl migration rates of each 1-*O*-acyl glucuronide isomer was performed by incubating 40 $\mu\text{g}/\text{ml}$ of the fluorobenzoic acid 1-*O*-acyl glucuronides in potassium phosphate buffer (20 mM) at pH 7.4, 25°C and the acyl migration and hydrolysis reactions were followed by HPLC over a time period of 24 h, by injection of 20 μl into the chromatographic system every 10 min for the first hour and then every hour. The degradation rate was defined as the initial loss of the β -1-*O*-acyl com-

ponent, hydrolysis was defined as the initial formation of the aglycone and acyl migration was defined as the formation of positional isomers. The hydrolysis rate was calculated as the degradation rate corrected for formation of positional isomers and the acyl migration rate was calculated as the degradation rate corrected for hydrolysis.

2.5. Analytical chromatography

The HPLC system consisted of a LDC Milton Roy ContraMetric III pump (Staffordshire, UK) and a LDC Milton Roy variable-wavelength UV detector (operated at 200 nm). The analytical columns were (A) a Hypersil ODS 5 μm Universal cartridge column (150 \times 4.6 mm I.D.) (Capital HPLC, Broxburn, UK) and (B) a Knauer column (120 \times 4.6 mm I.D.) packed with Spherisorb ODS-2 (Phase Separations, Deeside, UK), 5 μm . The final mobile phase developed for the separation of 2-, 3- and 4-fluorobenzoic acid glucuronides and their glucuronide positional isomers was acetonitrile–0.2 M potassium phosphate (pH 7.4)–water (1:10:89, v/v). The flow-rates were 2 ml/min for column A and 1 ml/min for column B.

2.6. Identification of isomers by HPLC–NMR

Identification of 2-, 3- and 4-*O*-acyl positional isomers and their α - and β -anomers observed in the HPLC chromatograms of the equilibrium mixtures of 2-, 3- and 4-fluorobenzoic acid were determined by HPLC– ^1H NMR as earlier described [23,24]. The HPLC system consisted of a Bruker LC22C pump (Rheinstetten, Germany) and a Bruker LC33 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 polyether ether capillary (0.25 mm I.D.). A Bruker column oven was used to thermostat the column at 25°C. Chromatographic data were collected using the Bruker Chromstar HPLC data system. The NMR data were acquired using Bruker AMX-600 MHz and DRX 750 MHz NMR spectrometers equipped with a ^1H flow probe (cell of 3 mm I.D. with a volume of 120 μl).

2.7. Quantification of isomers

The glucuronic acid ring was assumed not to contribute as a chromophore to the absorption of UV light in the glucuronic acid conjugates. The glucuronic acid isomers were, therefore, assumed to have the same molar extinction coefficients as the parent compound. Quantification of the isomers was performed using the areas of the peaks and relating them to the standard curves of the parent compounds (2-, 3- and 4-fluorobenzoic acid).

2.8. Computational chemistry

Calculations were carried out on a Silicon Graphics Power Indigo² R8000 work station. The 1-*O*-(2-fluorobenzoyl)- β -D-glucopyranuronic acid glucuronides were constructed and the minimum energy forms were obtained by geometry optimisation using Spartan version 3.1 with the semi-empirical MO AM1 Hamiltonian. Three rotatable bonds were each varied over 360° in 36° intervals to sample 1000 conformers for each of which the energy was calculated. Finally, the overall minimum energy form determined from the sequential search was again subjected to optimisation.

3. Results and discussion

3.1. Chromatography

An HPLC method was developed in order to separate the equilibrium mixture of the fluorobenzoic acid 1-*O*-acyl glucuronides and their 2-, 3- and 4-positional glucuronide isomers, as well as the α - and β -anomer of each isomer, and the hydrolysis product. Acetonitrile was chosen as organic modifier, as methanol can cause the methanolysis of some glucuronides and thereby interfere with the acyl migration [21]. The effect of changing the amount of acetonitrile in the mobile phase on separation of the 1-, 2-, 3- and 4-positional isomers as their α - and β -anomers of the 4-fluorobenzoic acid glucuronide and its hydrolysis product in the equilibrium mixture can be seen in Fig. 2. The Hypersil ODS chromatographic column was used. The mobile phase that

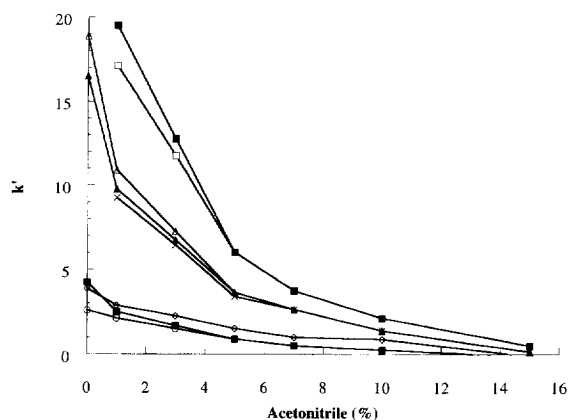


Fig. 2. Effect of the percentage of acetonitrile on the separation of glucuronide positional isomers of 1-*O*-(4-fluorobenzoyl)- β -D-glucopyranuronic acid. The analytical column was a Hypersil ODS Universal cartridge column (150 \times 4.6 mm I.D.). The hold-up volume t_M was estimated as the retention time of D₂O. The final mobile phase developed for the separation of the 2-, 3- and 4-fluorobenzoic acid glucuronides and their glucuronide isomers was acetonitrile–0.2 M potassium phosphate (pH 7.4)–water (1:10:89, v/v). The flow-rate was 2 ml/min. Symbols: (○) α -4-*O*-acyl isomer; (●) β -4-*O*-acyl isomer; (◇) aglycone; (×) β -1-*O*-acyl isomer; (△) β -3-*O*-acyl isomer; (▲) α -3-*O*-acyl isomer; (□) α -2-*O*-acyl isomer; (■) β -2-*O*-acyl isomer.

gave optimal resolution of the 4-fluorobenzoic acid 1-*O*-acyl glucuronide, its hydrolysis product and its 2-, 3- and 4-positional glucuronide isomers as their α - and β -anomers contained 1% acetonitrile and resulted in a total analysis time of 12 min. Alternatively, a column containing Spherisorb ODS-2 was used. With this column, the same selectivity was obtained, but similar retention times were obtained with a flow-rate of only 1 ml/min.

Compared to earlier published chromatographic methods for separation of acyl migrated positional isomers of acyl glucuronides, the selectivity obtained here is greatly improved and the problem of a late eluting aglycone has been omitted. The earlier published methods all used C₁₈ columns, eluents with acidic pH and even higher contents of organic modifier resulting in long retention times and with a narrow retention time window within which the positional isomers elute [17,19–21]. One example is the separation of positional isomers of the diflunisal acyl glucuronide where anomeric separations were obtained but overlap was observed between the 1-*O*-

acyl isomer and one of the anomers of the 2-*O*-acyl isomers, also overlap was observed between the last eluting anomer of the 3-*O*-acyl isomer and the first eluting anomer of the 2-*O*-acyl isomer [19,20]. Another example is the separation of the positional isomers of the 3-(*p*-chlorophenyl)thiazolo(3,2-*a*)benzimidazole 2-acetic acid ester glucuronide where no anomeric separations were obtained [21]. Similarly, an HPLC method was developed for the separation of the positional isomers of the furosemide acyl glucuronide [17]. Again no separation of the α - and β -anomers was observed.

The chromatographic method developed here was applied to the equilibrium mixtures of the 2-fluorobenzoyl, the 3-fluorobenzoyl and the 4-fluorobenzoyl glucuronides using the Spherisorb ODS-2 column. The resulting chromatograms for the equilibrium mixture of all of the fluorobenzoyl glucuronides can be seen in Fig. 3. The plateau observed between the last two peaks in each of the chromatograms indicates that the compounds are interconverting as they pass down the chromatographic column. In the present case it is a result of mutarotation. This phenomenon also explains why baseline separations of the other pairs of α - and β -anomers could not be obtained. Thus, the high pH of the eluent (pH 7.4) is a disadvantage if it is necessary to obtain sharp peaks of the single anomers (because of their interconversion). However, the chromatographic system allows fast and highly selective separations of the positional isomers and their anomers in less than 10–15 min.

3.2. Identification of glucuronide isomers and investigations of their elution order

The structures of the various glucuronide positional isomers and anomers in the 8 HPLC peaks of the equilibrium mixtures of the 2-, 3- and 4-fluorobenzoic acid glucuronides were determined by directly coupled 600 MHz and 750 MHz HPLC-¹H NMR [23,24]. The elution order of the positional isomers was the same for the 2-, 3- and 4-fluorobenzoic acid glucuronides. The α -4-*O*-acyl isomers eluted first, immediately followed by the β -4-*O*-acyl isomers and then the β -1-*O*-acyl isomers. These peaks were followed by the β -3-*O*-acyl isomers and then the α -3-*O*-acyl isomers and finally the 2-*O*-acyl

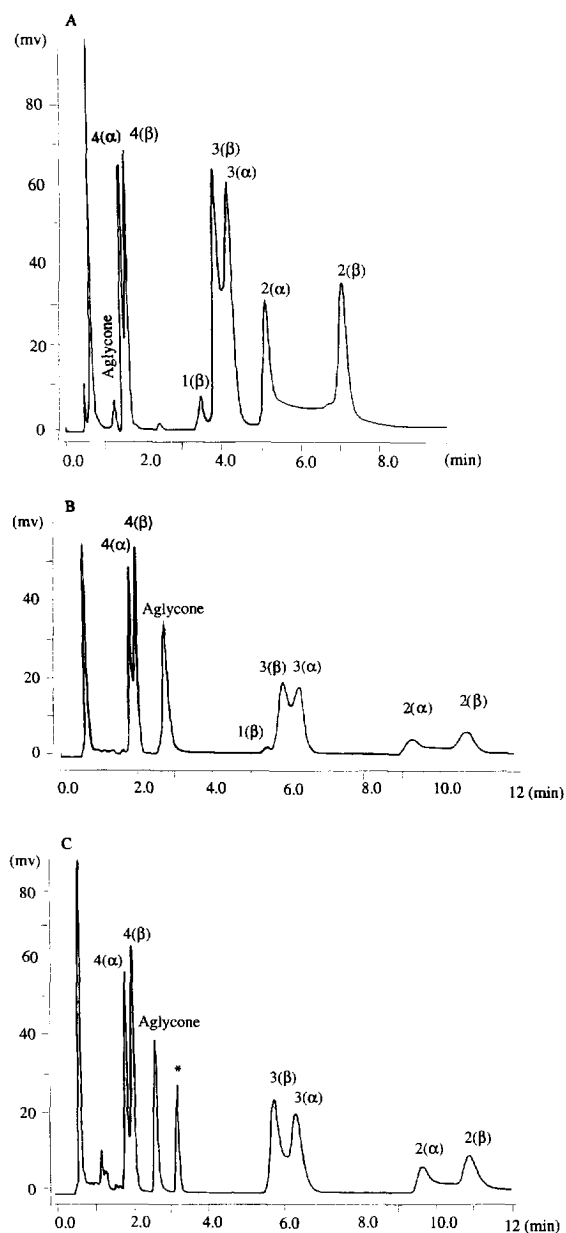


Fig. 3. Chromatograms for the equilibrium mixtures of 1-*O*-(2-fluorobenzoyl)-*D*-glucopyranuronic acid (A), 1-*O*-(3-fluorobenzoyl)-*D*-glucopyranuronic acid (B) and 1-*O*-(4-fluorobenzoyl)-*D*-glucopyranuronic acid (C). Chromatographic system: analytical column was a Knauer column (120×4.6 mm I.D.) packed with Spherisorb ODS-2, 5 μ m; mobile phase was acetonitrile–0.2 M potassium phosphate (pH 7.4)–water (1:10:89, v/v); the flow-rate was 1 ml/min. * = synthetic impurity.

isomers eluted, the α -anomer followed by the β -anomer (see Fig. 3). The free fluorobenzoic acid eluted before the glucuronide isomers in the case of the 2-fluorobenzoyl glucuronides but in the case of the 3- and 4-fluorobenzoyl glucuronides, the free fluorobenzoic acid eluted just after the 4-*O*-acyl isomers (see Fig. 3). As the glucuronides of all three fluorobenzoic acids elute in the same order, this is indicative of an underlying structure–chromatography relationship. Firstly, it would appear that the overall retention properties of the glucuronides are dependent on the non polar properties of the aglycone. However, the elution order of the positional isomers will depend upon the local conformational and polarity properties of the glucuronic acid ring leading to differential adsorption onto the C_{18} surface. There is an obvious effect of the position of the fluorine atom on the aromatic ring on the separation of the anomers of the 2-*O*-acyl isomer (see Fig. 3). When fluorine is in the 2-position on the aromatic ring the separation of the α - and the β -anomers of the 2-*O*-acyl isomers is greater. This probably results from the fact that the fluorine atom is closer to the anomeric hydroxyl group compared to when the fluorine atom is in the 3- and 4-position on the aromatic ring and therefore can interact more effectively with the anomeric centre. In the minimum energy structure calculated by molecular modelling (see Fig. 4) of the α -2-*O*-acyl glucuronide of 2-fluorobenzoic acid, the fluorine atom and the anomeric -OH group are pointing towards each other and the distance between them is 4.38 Å. For the β -2-*O*-acyl glucuronide of 2-fluorobenzoic acid, the fluorine atom and the anomeric -OH group are further apart at 6.87 Å. Thus, there is a significant difference between the calculated minimum energy structures of the two anomers of the 2-*O*-acyl-glucuronic acid conjugates (Fig. 4), which may explain the large separation observed for these two α - and β -anomers.

3.3. Assay validation

The quantification limits for the 2-, 3- and 4-fluorobenzoic acids were estimated as ten times the standard deviation (σ) of the peak-to-peak noise (N_{p-p}), $\sigma = (N_{p-p}/5)$, as this is a good estimate when the noise is assumed to be normally distributed.

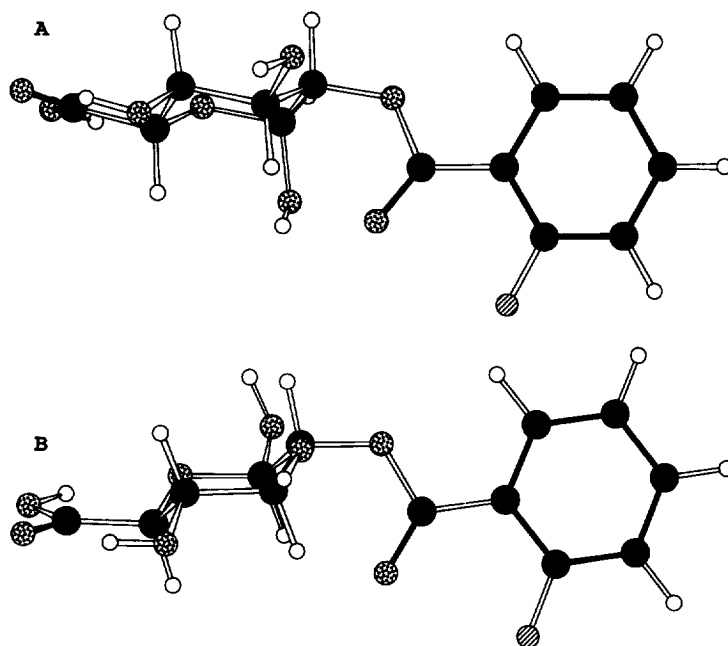


Fig. 4. Comparison of the calculated minimum energy structures of the α -2-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid (A) and the β -2-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acid (B). The atoms are colour coded as follows: Dark grey (C); striped (F); dotted (O); white (H).

Detection limits were: 3.0 ng/ml for 2-fluorobenzoic acid; 3.1 ng/ml for 3-fluorobenzoic acid and 3.5 ng/ml for 4-fluorobenzoic acid. Quantitation limits were: 10 ng/ml for 2-fluorobenzoic acid; 10 ng/ml for 3-fluorobenzoic acid and 12 ng/ml for 4-fluorobenzoic acid. The calibration curves in buffer for 2-fluorobenzoic acid, 3-fluorobenzoic acid and 4-fluorobenzoic acid were linear within the concentration range of interest with respect to the samples analysed, 0.2 μ g/ml–40 μ g/ml ($r > 0.99$).

4. Measurement of acyl migration and hydrolysis rates

The developed HPLC method was used to estimate the overall acyl migration and hydrolysis rates of the 2-, 3- and 4-fluorobenzoyl 1-*O*-acyl glucuronides in buffer solution (pH 7.4) at 25°C over 24 h. The concentrations of the positional isomers, the hydrolysis product produced and the disappearance of each of the fluorobenzoyl β -1-*O*-acyl glucuronides as a function of time is seen in Fig. 5A, B and

C. When the logarithm of the concentration of the β -1-*O*-acyl isomers corresponding to the first 5 h were plotted against time, a straight line was obtained, and the degradation rate of the β -1-*O*-acyl isomer could be estimated as the slope of this line in agreement with first-order kinetics. In order to estimate the actual acyl migration rates, the concentration of the β -1-*O*-acyl isomer at the different time points was corrected for hydrolysis by adding the amount of aglycone formed, the acyl migration rate was then estimated by plotting the logarithm of the corrected concentrations of β -1-*O*-acyl isomer as a function of time (0–5 h). The initial hydrolysis rates were similarly estimated from the straight line observed when the logarithm of the concentration of the β -1-*O*-acyl isomer, corrected for the amount of positional isomers formed as a consequence of acyl migration, was plotted as a function of time (0–5 h). The acyl migration rates, the degradation rates and the hydrolysis rates for the β -1-*O*-acyl- 2-, 3- and 4-fluorobenzoyl glucuronides are shown in Table 1. The accuracy of these could be questioned considering the fact that acyl migration may occur at pH 7.4

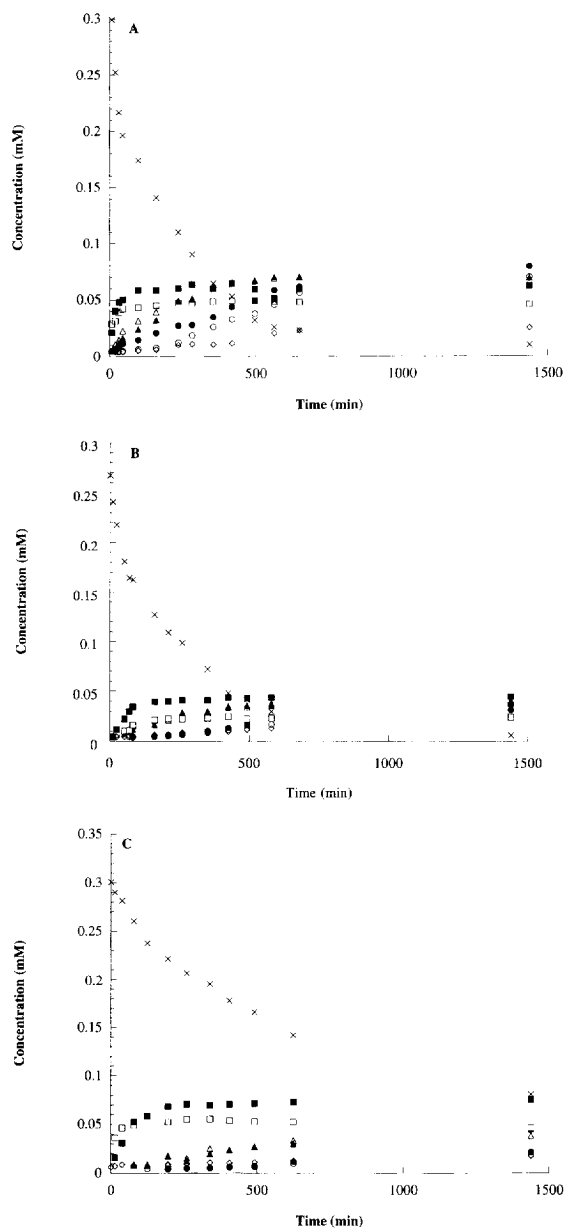


Fig. 5. Acyl migration and hydrolysis of the 1-*O*-(2-fluorobenzoyl)-*D*-glucopyranuronic acid (A), 1-*O*-(3-fluorobenzoyl)-*D*-glucopyranuronic acid (B) and 1-*O*-(4-fluorobenzoyl)-*D*-glucopyranuronic acid (C) determined by HPLC. Symbols: (×) β -1-*O*-acyl isomer; (○) α -4-*O*-acyl isomer; (●) β -4-*O*-acyl isomer; (△) β -3-*O*-acyl isomer; (▲) α -3-*O*-acyl isomer; (□) α -2-*O*-acyl isomer; (■) β -2-*O*-acyl isomer; (◇) aglycone.

during the chromatographic separation time. However, the chromatographic retention time of the positional glucuronide isomers are short in comparison with the acyl migration half lives estimated (Table 1) and if some degree of acyl migration should occur during the run time, it would surely be reproducible with each chromatographic run. Thus, as the acyl migration rates are measured from the relative concentration of the glucuronide isomers on the time scale, the rates estimated would still be the same.

The observed differences in the rates reveal a strong dependence of the structure of the aglycone on the acyl migration rates, i.e., the position of the fluorine atom on the aromatic ring. The fastest rate is observed with the 2-fluorobenzoic acid glucuronide and the slowest with the 4-fluorobenzoic acid glucuronide. Therefore, in this case the acyl migration rates mainly are dependent on inductive electronic effects on the reactivity of the carbonyl carbon. Studies on trifluoromethylbenzoic acid glucuronides show the opposite effect of the position of the trifluoromethyl group on acyl migration rates [14]. In that case, the 4-trifluoromethylbenzoic acid glucuronide showed the fastest acyl migration rates with the 2-trifluoromethylbenzoic acid glucuronide being the slowest, indicating that for this series, the steric hindrance from the trifluoromethyl group in the 2-position was dominant in determining the acyl migration rate.

The hydrolysis rates presented in Table 1 show the same order as the acyl migration rates, the hydrolysis of 2-fluorobenzoic acid glucuronide being the fastest and hydrolysis of 4-fluorobenzoic acid glucuronide the slowest. This indicates that the same physico-chemical factors, as were observed above for the acyl migration rates, control the hydrolysis reactions. The hydrolysis rates observed are lower than the acyl migration rates as can also be seen from Fig. 6 where the initial parts of the linear curves (for the β -1-*O*-acyl 2-fluorobenzoic acid glucuronide) from which the acyl migration and hydrolysis rates were estimated are shown. As the concentration of the glucuronide positional isomers increases the hydrolysis rates decrease, this indicates that the hydrolysis rates of the glucuronide positional isomers are slower than the hydrolysis of the β -1-*O*-acyl isomer.

In this study, we have shown that with very simple

Table 1

Overall degradation, acyl migration and hydrolysis rates of β -1-*O*-acyl glucuronides of 2-, 3- and 4-fluorobenzoic acids

Compound	Overall degradation rate		Acyl migration rate ^a		Hydrolysis rate ^b	
	$k \times 10^{-3}$ (/min)	$t_{0.5}$ (h)	$k \times 10^{-3}$ (/min)	$t_{0.5}$ (h)	$k \times 10^{-3}$ (/min)	$t_{0.5}$ (h)
4-FBAG	1.3±0.2	9.2	1.2±0.1	9.8	0.2±0.2	47.4
3-FBAG	3.7±0.2	3.1	3.3±0.2	3.6	0.8±0.1	14.6
2-FBAG	4.9±0.3	2.3	3.7±0.3	3.2	2.8±0.8	4.13

Acyl migration rates were measured at 25°C, pH 7.4 in potassium phosphate buffer (0.02 M). FBAG=fluorobenzoic acid glucuronide. The standard deviations of the estimated rate constants are presented in the table.

^a Degradation corrected for hydrolysis.

^b Degradation corrected for formation of positional isomers.

chromatographic conditions, it is possible to separate a series of glucuronides and hydrolysis products formed spontaneously in aqueous solution at pH 7.4. The improved separations obtained for the anomeric pairs of the positional isomers may result from the fact that the separations were performed at pH 7.4, thereby decreasing the amount of organic modifier needed in order to elute the glucuronides from the C₁₈ column as the glucuronides are present in their dissociated form. As seen in Fig. 2 the amount of organic modifier is crucial for the anomeric separations even at pH 7.4. Secondary chromatographic separation mechanisms between the dissociated form

of the glucuronides and the ionised free silanol groups may be important in this context. The acyl migration study of the monofluorobenzoic acid glucuronides emphasises the applicability of the chromatographic method developed, as it was possible to follow the acyl migration kinetics of 1-*O*-acyl glucuronides, hydrolysis kinetics and the anomerisation of each of their positional glucuronide isomers.

References

- [1] H. Spahn-Langguth and L.Z. Benet, *Drug Metab. Rev.*, 24 (1992) 5.
- [2] D.M. Clive and J.F. Stoff, *N. Engl. J. Med.*, 310 (1984) 563.
- [3] L.R. Pohl, H. Satoh, D.D. Christ and J.G. Kenna, *Annu. Rev. Pharmacol.*, 28 (1988) 367.
- [4] A.M. Williams, S. Worall, J. Jersey and R.G. Dickinson, *Biochem. Pharmacol.*, 43 (1992) 745.
- [5] P.C. Smith, A.F. McDonagh and L.Z. Benet, *J. Clin. Invest.*, 77 (1986) 934.
- [6] P.C. Smith, W.Q. Song and R.J. Rodriguez, *Drug Metab. Dispos.*, 20 (1992) 962.
- [7] G. Bradow, L.S. Kan and C. Feneslau, *Chem. Res. Toxicol.*, 2 (1989) 316.
- [8] A.R. King and R.G. Dickinson, *Biochem. Pharmacol.*, 42 (1991) 2289.
- [9] C. Feneslau, in F.C. Kauffman (Editor), *Conjugation–Deconjugation Reactions in Drug Metabolism and Toxicity*, Springer-Verlag, Berlin, 1994, p. 367.
- [10] P.C. Smith, L.Z. Benet and A.F. Modonagh, *Drug Metab. Dispos.*, 18 (1990) 639.
- [11] J.S. Weiss, A. Guatam, J.J. Lauff, M.W. Sundberg, P. Jatlow, J.L. Boyer and D. Saligson, *N. Engl. J. Med.*, 309 (1983) 147.
- [12] H.W. Ruelius, E.M. Young, S.K. Kirkman, R.T. Schillings, S.F. Sisenwine and F.W. Janssen, *Biochem. Pharmacol.*, 34 (1985) 451.
- [13] E. Fisher, *Chem. Ber.*, 53 (1920) 1621.

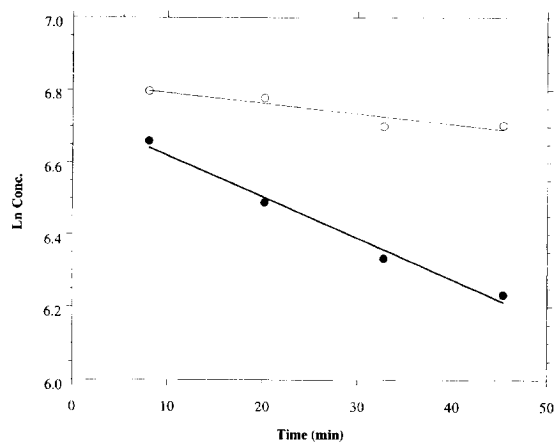


Fig. 6. The initial part of the linear curves from which the acyl migration and hydrolysis rates of the β -1-*O*-acyl 2-fluorobenzoic acid glucuronide were estimated. The natural logarithm of the concentration was plotted as a function of time (min) and the correlation coefficient of the linear fits were 0.99 and 0.92 for the acyl migration and the hydrolysis curves respectively. Key: (○) hydrolysis; (●) acyl migration.

- [14] A.W. Nicholls, K. Akira, R.D. Farrant, J.C. Lindon, I.D. Wilson and J.K. Nicholson, *Chem. Res. Toxicol.*, in press.
- [15] P.C. Smith and J.H. Liu, *Xenobiotica*, 23 (1993) 337.
- [16] H. Komura, H. Fukui, H. Sasaki and A. Morino, *Drug. Metab. Dispos.*, 4 (1992) 585.
- [17] A. Rachmel, G.A. Hazelton, A.L. Yergey and D.J. Liberato, *Drug Metab. Dispos.*, 13 (1985) 705.
- [18] R.G. Dickinson, P.V. Baker and A.R. King, *Biochem. Pharmacol.*, 47 (1993) 469.
- [19] J. Hansen-Møller, C. Cornett, A.S. Dalgaard and S.H. Hansen, *J. Pharm. Biochem. Anal.*, 6 (1988) 229.
- [20] R.G. Dickinson and A.R. King, *Ther. Drug Monit.*, 11 (1989) 712.
- [21] F.W. Jannssen, S.K. Kirkman, C. Fenselau, M. Stogniew, B.R. Hofman, E.M. Young and H.W. Ruelius, *Drug Metab. Dispos.*, 10 (1982) 599.
- [22] C.C.A. Van Boeckel, L.P.C. Delbressine and F.M. Kaspersen, *Recl. Trav. Chim. Pays-Bas*, 104 (1985) 259.
- [23] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, R.D. Farrant, J.C. Lindon, I.D. Wilson and J.K. Nicholson, *Anal. Chem.*, 67 (1995) 3401.
- [24] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, M. Spraul, M. Hofman, J.C. Lindon, I.D. Wilson and J.K. Nicholson, *Anal. Chem.*, 67 (1995) 4441.