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Kinetic Analysis by HPLC−Electrospray Mass Spectrometry of the pH-Dependent Acyl Migration and Solvolysis as the Decomposition Pathways of Ifetroban 1-*O*-Acyl Glucuronide

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The decomposition of both α- and *β*-anomers of ifetroban 1-*O*-acyl glucuronide in an aqueous medium was studied at ambient temperature (23 °C). An HPLC/MS technique was used to investigate the decomposition of these anomers via hydrolysis and acyl migration over a pH range of 1.0−13.0. It was found that while no acyl migration occurred at pH ≤4.0, hydrolysis occurred at both acidic and basic pH. The hydrolysis rate was the slowest within the pH range of 3.0−4.0. Outside this range, the rate of hydrolysis increased as the pH was increased or decreased. The acyl migration at pH 5.0 was very slow, but the rate increased as the pH was increased. First-order rate constants (in h^{-1}), as a function of pH, were estimated according to a simplified scheme for both hydrolysis and acyl migration processes. Methanolysis of the *β*-anomer was studied in 80% or 100% methanol in water and also in 20% methanol in aqueous buffer solutions with apparent pH values of 3.0, 6.0 and 9.0. A systematic study of varying the pH and ionic strength of the HPLC mobile phase showed that both parameters significantly affected the resolution and retention times of the positional isomers and anomers. The HPLC method developed was capable of separating all the positional isomeric glucuronides and their anomers.

Ifetroban (**I**, Figure 1), a carboxylic acid compound, is a potent antagonist of thromboxane A_2 receptors in smooth muscle and in platelets.[1,2] Ifetroban 1-*O*-acyl *β*-D-glucuronide (1*β*-gluc, **II**, Figure 1) was found to be a major metabolite in human plasma. Glucuronidation is one of the major phase II metabolic pathways of carboxylic acid compounds and serves to improve the solubility and hence the elimination of the parent compound.[3] The biosynthetic product of the acyl glucuronidation reaction has a 1-*O*-acyl *β*- conformation at the sugar moiety of the resulting conjugate.[4]

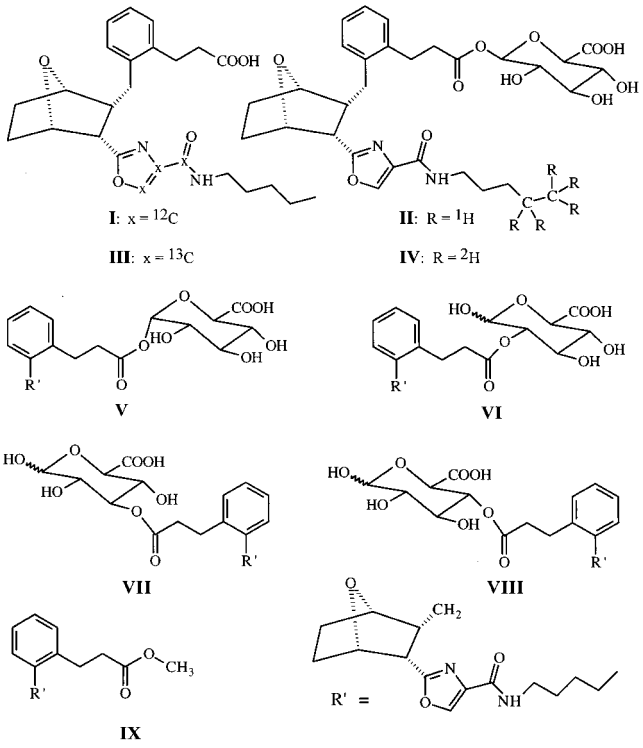

Figure 1. Structures of the various compounds used as analytes and internal standards.

Acylglucuronides are known to undergo a variety of spontaneous chemical reactions.[5−7] Hydrolysis of the ester bond and intramolecular migration of the acyl group are among the most common reactions of acyl glucuronides.[8] The in vivo biological implications of the formation of acyl glucuronides have been thoroughly reviewed.[8−11] The in vitro lability of the acyl glucuronides, due

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to hydrolysis, solvolysis, and acyl migration, is a major concern in quantitative bioanalysis of biofluids for the parent carboxylic acid compound and/or its acyl glucuronide. Enzymatic hydrolysis of glucuronides with β-glucuronidase is a routine method of sample preparation. Unlike the biosynthetic 1-*O*-acyl β-glucuronide, the positional isomers are not hydrolyzed by β-glucuronidase,[12] and therefore, the analytical results can be potentially misleading. The susceptibility of acyl glucuronides toward in vitro hydrolysis will produce results which may not represent the true concentration profile of the unconjugated drug in a biological matrix such as plasma and urine. Hence, it is essential to determine the optimum conditions required to minimize the degradation of the acyl glucuronide during the various steps of a bioanalytical assay, including the collection and storage of the biological samples.

The rate of acyl migration depends on the structure of the aglycon, pH, and temperature.[13] In this paper, we report the results of an extensive investigation of the hydrolysis and acyl migration as the decomposition pathways of **II** in various buffer solutions over a pH range of 1.0−13.0. The combination of serial migration of the acyl group over multiple positions across the glucuronic acid molecule and the associated equilibria makes the kinetic analysis of such a system a formidable task. This paper presents an attempt to simplify the kinetic analysis of the acyl migration in a typical acyl glucuronide.

It is known that acyl glucuronides can undergo reactions with nucleophilic functions (SH, OH, and NH_2) of peptides and proteins to covalently bond the aglycon moiety of the conjugates by substituting the glucuronic acid moiety.[14,15] Since aqueous methanol, a potential nucleophile, is one of the most popular solvent mixtures for solid-phase extraction, reconstitution, and HPLC, we studied the solvolysis of **II** in aqueous methanol under different conditions.

In this paper, we also report the results of a systematic study of the effects of pH and ionic strength of the HPLC mobile phase on the chromatographic resolution and the retention times of the positional isomeric glucuronides and their anomers derived from the decomposition of **II**.

EXPERIMENTAL SECTION

Reagents. The structures of all analytes and internal standards are shown in Figure 1. Both labeled and unlabeled ifetroban and the corresponding glucuronide conjugates were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (BMS-PRI). The labeled analogues consisted of [$^{13}C_3$]ifetroban (**III**) and [$^2H_5$]1β-gluc (**IV**), which were used as internal standards. The anomer ifetroban 1-*O*-acyl α-D-glucuronide (1α-gluc, **V**) was also obtained from BMS-PRI. Chemicals of highest available purity were used. Ammonium acetate (99.999%), acetic acid (double distilled), trifluoroacetic acid (TFA), ammonium trifluoroacetate, formic acid, ammonium formate, potassium hydrogen phosphate, ammonium hydroxide, and potassium hydroxide were from Aldrich (Milwaukee, WI). Highly pure (18.2 MΩ) water was obtained by passing house-distilled deionized water through a Barnstead Nanopure II system (Syborn/Barnstead, Boston, MA). HPLC grade acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ).

Test Mixture. At ambient temperature (23 °C), unless otherwise described, a solution of **II** (4 mg/100 mL) was subjected to a mild hydrolysis condition overnight in 20% methanol in phosphate buffer, pH 8.0 (10 mM). This allowed the production of measurable amounts of the positional isomers (**VI**−**VIII**), the aglycon **I**, and the methyl ester (**IX**) of the aglycon.

Sample Incubation. The buffer systems (10 mM) used to cover the pH range of the incubation medium were trifluoroacetic acid/ammonium trifluoroacetate (pH 1.0), formic acid /ammonium formate (pH 3.0), acetic acid/ammonium acetate (pH 4.0 and pH 5.0), and phosphate buffers (pH ≥6.0). The pH was adjusted with the corresponding acid and ammonium or potassium hydroxide. Solutions of **II** (25 μM, 15.4 μg/mL) spiked with **III** (10.0 μM, 4.43 μg/mL) were incubated at 23 °C in the pH range of 1.0−13.0. The labeled compound **III** served as the internal standard for the quantitation of **I** produced during incubation. No adjustment of pH over the incubation period was made.

The susceptibility of **II** toward methanolysis was studied by incubating **II** at 23 °C in 20% methanol in aqueous buffers with apparent pH values of 3.0, 6.0, and 9.0. Methanolysis was also studied in 100% and 80% methanol in water. Formation of the methyl ester of **I** (**IX,** Figure 1) was monitored to check the progress of methanolysis. Because no reference standard for **IX** was available, the quantitation of the methanolysis product was done with **III** as the standard.

HPLC/MS Analysis. A Hewlett-Packard (Palo Alto, CA) HP 1090 Series II HPLC system equipped with an autoinjector was used for solvent delivery and programmed sample injection. An HP 5989B MS Engine quadrupole mass spectrometer equipped with a pneumatically assisted electrospray interface, rf-only hexapole ion guide (Analytica of Branford, CT) and a Chem-Station data system, was used for detection. Positive ion mode of electrospray was used for ionization. The electrospray voltage was 4.5 kV (measured as ΔV between the needle and the cylinder), and the quadrupole temperature was set at 110 °C. The capillary exit (CapEx) voltage was ramped to find the optimum value. The operating CapEx voltage was set at 130 V. Similarly, the quadrupole entrance voltage was set at 190 V. High-purity (99.999%) nitrogen gas was used as the nebulizing gas at an operating pressure of 80 psi. The drying gas was also high-purity nitrogen flowing at 1.2 L/min and was set at 360 °C. The quadrupole was tuned and calibrated with the electrospray tuning mixture, supplied by Hewlett-Packard (Part No. 59987-60135), at unit resolution. HPLC separations were performed on a (150 × 2 mm i.d.) column packed with 3-μm Hypersil BDS-C-8 packing material (Keystone, Bellefonte, PA). A flow rate of 0.250 mL/min was used.

Initially the test mixture (5 μL) was isocratically chromatographed with a 10 mM ammonium formate/formic acid buffer in 50% acetonitrile (apparent pH 3.5). From the retention time, as confirmed by the deuterated internal standard (**IV**, Figure 1), and m/z value of the $[M + H]^+$ ion (617), it was concluded that all the positional isomers coeluted in one peak at 2.3 min (Figure

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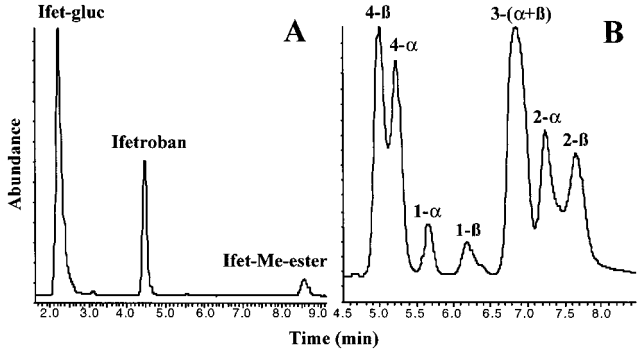

Figure 2. Separation of the decomposition products of **II**: (A) LC/MS chromatogram of ifetroban 1-*O*-acyl β-D-glucuronide, incubated overnight at 4 °C in a pH 8.0 phosphate buffer with 10 mM ammonium acetate, pH 3.5, 50% acetonitrile as the mobile phase. (B) LC/MS chromatogram of the test mixture with 10 mM ammonium acetate, pH 5.0, 30% acetonitrile as the mobile phase.

2A). Chromatographic conditions were optimized to further resolve this peak and separate all the coeluting positional isomers and most of the anomers (Figure 2B). This was achieved with an isocratic elution with a 10 mM ammonium acetate/acetic acid buffer in 30% acetonitrile (apparent pH 5.0) at 0.250 mL/min flow rate. For the kinetic studies, **II**, its positional isomers **VI**−**VIII**, the aglycon **I**, and the ester **IX** (if produced) were separated by a gradient elution. The chromatographic conditions were set such as to achieve maximum resolution of the positional isomers at the expense of no resolution of some of the anomers. Consequently, the α- and β-anomers of 4-*O*-acyl glucuronide (**VIII**) coeluted. The gradient elution was carried out with 30% aqueous acetonitrile as solvent A, buffered with ammonium acetate/acetic acid (5.0 mM NH_4OAc, apparent pH 5.5), and solvent B was 50% aqueous acetonitrile (5.0 mM NH_4OAc). Separation of the glucuronide isomers was achieved with 100% A during the first 7 min. This was followed by a gradient of 100% A to 100% B in 1.5 min. The aglycon **I** eluted with 100% B during the next 3.5 min.[16] The elution was then brought back to 100% A in 1 min. Finally, the column was reequilibrated for at least 5 more min. An isocratic elution with 35% acetonitrile and a flow rate of 0.250 mL/min was used to study the effect of pH and ionic strength of the mobile phase on the resolution of the glucuronide isomers.

To minimize the hydrolysis of **IV**, used as the internal standard for **II**, a separate solution of **IV** (20.0 μM, 12.42 μg/mL) in the pH 3.0 buffer was co-injected with the incubated sample. The autoinjector was programmed such that 5 μL of the incubated solution, prespiked with **III**, and 5 μL of the standard solution of **IV** were withdrawn sequentially into the needle and then injected (10 μL) onto the HPLC column at specified intervals. The solution of **IV** was replaced twice a day with a refrigerated stock solution.

The mass spectrometer was operated in selected ion monitoring (SIM) mode. The unlabeled **II** was monitored as its $[M + H]^+$ ion at m/z 617 and its $^2H_5$-labeled analogue (**IV**) was monitored at m/z 622. Ifetroban was monitored at m/z 441 as unlabeled $[M + H]^+$ ion and at m/z 444 as its $^{13}C_3$-labeled analogue. Occurrence of any exchange reaction between the unlabeled **II** and the $^{13}C_3$-labeled **III** was also monitored at m/z 620. The release of $^2H_5$-aglycon at m/z 446 was also monitored to correct for any hydrolysis of the labeled **IV**, which was used as an internal standard. For the data acquisition, the m/z values of the conjugates and the m/z values of aglycons were grouped together separately as timed events to correspond to the regions of their respective elution in the chromatogram. The dwell time corresponded to a scan rate of ~4.5 scans/s. Methanolysis of **II** was monitored at m/z 455.

Kinetic Analysis. Kinetic analysis of the key steps of the decay of **II** was undertaken with the assumption that the initial decay followed first-order kinetics and that initially only **II** was the source of **I**. Area ratios (analyte response/internal standard response) of chromatographic peaks were used for quantitation. For the kinetic analysis, area ratios calculated for the peaks of **I**, **II**, and **VI** were used. Because no conversion of 1β- to 1α-anomer, or vice versa, was noticed, only the peak corresponding to the 1β-anomer was integrated for the kinetic analysis of the decomposition of **II**. For **VI**, the partially resolved anomers were summed in the integration process.

RESULTS AND DISCUSSION

Chromatography. The test mixture, obtained via overnight incubation, was anticipated to contain **I** and the positional isomers of **II**. In Figure 2A, the peak eluting at 4.5 min is that of **I**, which is produced by the hydrolysis of **II**. The identity of this peak was confirmed by comparing with the authentic compound. The late eluting peak at 8.6 min was identified as **IX**, which was produced by methanolysis of the glucuronide. Because acyl glucuronides are known to undergo positional isomerization,[8,9,17] the early-eluting peak (2.3 min) was suspected to be a composite peak of the coeluting glucuronide isomers (**II** and **V**−**VIII**). This prompted the necessity to establish chromatographic conditions to further resolve this glucuronide peak.

Ionic strength and pH of the mobile phase are major factors in the separation of carboxylic acids. Solvent strength and flow rate may also be utilized to improve the resolution. Thus the variables that were manipulated to achieve the intended resolution were (i) pH of the mobile phase (3.5−6.5); (ii) the buffer concentration (1.0−10.0 mM ammonium acetate); (iii) the organic content of the mobile phase (20−50% acetonitrile); and (iv) flow rate of the mobile phase (0.2−0.4 mL/min). Consequently not only all the four positional isomers but also most of the anomers were completely or partially resolved (Figure 2B).

Effect of pH of the Mobile Phases. At a constant ionic strength of 1.0−10.0 mM NH_4OAc, a pH range of 3.5−6.5 of the mobile phase was investigated to accomplish an optimum resolution of the glucuronide isomers. The chromatographic peaks corresponding to the aglycon and the different glucuronide isomers progressively shifted to longer retention times with decreasing pH. This was expected due to the protonation of the carboxylic acid groups of the aglycon and the glucuronic acid moiety of the different positional isomers.

It was anticipated that the longer retention times achieved by the lower pH would result in enhanced resolution of the isomeric mixture. However, it was found that the resolution was progressively lost with decreasing pH. Consequently, the longer the retention time, the worse the resolution. The effect of pH on the

(16) In the methanolysis experiments, 6 min more was added to the elution time with 100% B to elute the methyl ester **IX**.

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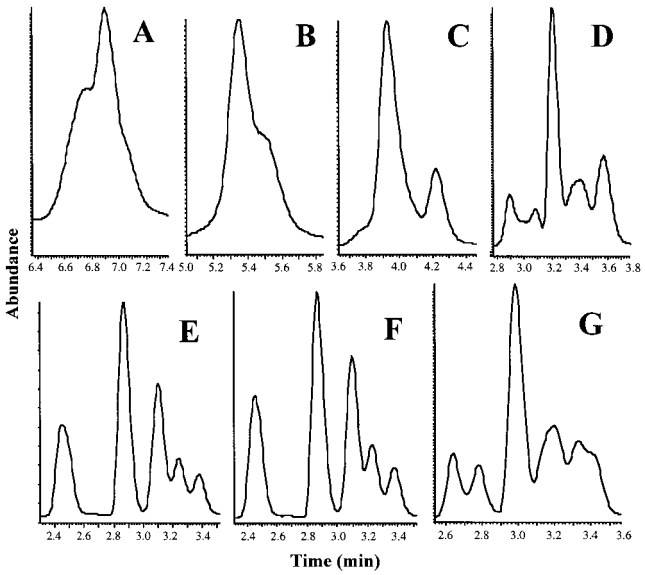

Figure 3. Resolution vs pH at 7.5 mM [NH_4OAc] with 35% aqueous acetonitrile and a flow rate of 0.250 mL min: (A) pH 3.5, bandwidth at the baseline 0.8 min; (B) pH 4.0, bandwidth at the baseline 0.5 min; (C) pH 4.5, bandwidth at the baseline 0.6 min; (D) pH 5.0, bandwidth at the baseline 1.0 min; (E) pH 5.5, bandwidth at the baseline 1.1 min; (F) pH 6.0, bandwidth at the baseline 1.1 min; (G) pH 6.5, bandwidth at the baseline 1.0 min.

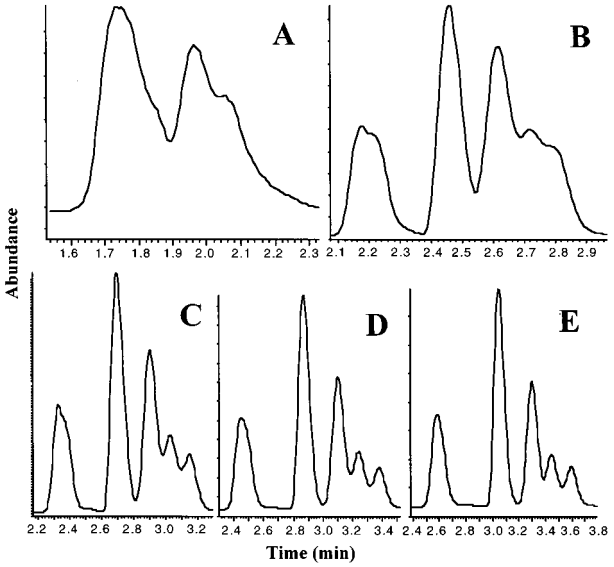

Figure 4. Resolution vs [NH_4OAc] at pH 5.5 with 35% aqueous acetonitrile and a flow rate of 0.250 mL min: (A) 1.0 mM, bandwidth at the baseline 0.6 min; (B) 2.5 mM, bandwidth at the baseline 0.8 min; (C) 5.0 mM, bandwidth at the baseline 1.0 min; (D) 7.5 mM, bandwidth at the baseline 1.1 min; (E) 10.0 mM, bandwidth at the baseline 1.2 min.

Table 1. Induction Times of the Positional Isomers **VI**–**VIII** as a Function of pH over a One-Week-Long Incubation

	pH 1.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.4	pH 9.0
VI	none[a]	none	none	72 h	5 h	0.5 h	0.2 h
VII	none	none	none	none	75 h	3 h	1.5 h
VIII	none	none	none	none	none	7.5 h	4.5 h

[a] None, represents no formation of the respective isomer.

separation of the isomers, at a constant ionic strength of 7.5 mM ammonium salt, is shown in Figure 3. At pH 3.5, all the isomers coeluted in one broad band (Figure 3A). This band started to compress at the baseline as the pH of the mobile phase was increased from 3.5 to 4.5 (Figure 3B,C). Further increase in pH resulted in the expansion of the bandwidth to a constant value of 1.1 min (Figure 3D–G), notwithstanding the shorter retention times. This led to the resolution of the isomers. The data indicate that the extent to which the retention of an isomer is affected by pH is different for different isomers. Thus, the order of elution of the isomers could be subject to change with pH. This is in contrast with the effect of pH on the retention behavior of the aglycon-based α- and *β*-anomers of an ether-linked *β*-D-glucuronide. The retention of these anomers is reported to be equally affected by a change in pH of the mobile phase causing them to move in unison.[18]

Effect of Ionic Strength of the Mobile Phase. Increasing the concentration of an ammonium salt (1.0–10.0 mM) at a given pH (in the pH range 3.5–6.5) increased the retention times of the glucuronide isomers, but the increase was not as dramatic as with the lowering of pH. Increasing ionic strength also improved the resolution of the late-eluting isomers. Figure 4 depicts the effects of changing the ammonium acetate concentration, at a fixed pH of 5.5. The retention times increased and the resolution improved with the increase in ammonium acetate concentration. The bandwidth gradually increased with increasing ammonium acetate concentration. The order of elution of the isomers did not appear to change.

A pH value of 5.0–5.5 and ammonium acetate concentration of 5.0–10.0 mM provided acceptable resolution for a mobile phase of 30–35% aqueous acetonitrile, with an isocratic elution and a flow rate of 0.250 mL/min. Under these isocratic elution conditions, the aglycon eluted at ~18 min, which was deemed to be too long. A gradient elution, described in the Experimental Section, was thus used in order to elute the aglycon in less than 12 min.

Identification of the Positional Isomers and Their Respective Anomers. In Figure 2, the chromatographic peaks obtained from the test mixture, an incubated solution of **II**, have been assigned to their respective glucuronide isomers. The 1-*O*-acyl *β*- and α-glucuronide anomers (**II** and **V**, respectively) were identified by matching their retention times with those of the authentic compounds. The 1-*O*-acyl α-anomer (**V**) was present as a synthetic impurity in **II** and was not a product of mutarotation. Interconversion between **II** and **V** is not expected because the anomeric hydroxyl in **II** or **V** is already locked by the acyl substituent. The assignment of 2-O-, 3-O-, and 4-O-acyl positional isomers (**VI**, **VII**, and **VIII**, respectively) was based on the respective induction time, which is the time elapsed between the onset of the incubation of **II** and the emergence of the corresponding peak in the chromatograms (Table 1). The partially resolved doublet which eluted at 7.5 min was identified as the 2-*O*-acyl α/*β*-glucuronide (**VI**) because it was the first new glucuronide peak to appear in the course of the incubation of **II**. The two peaks of the doublet emerged simultaneously; however,

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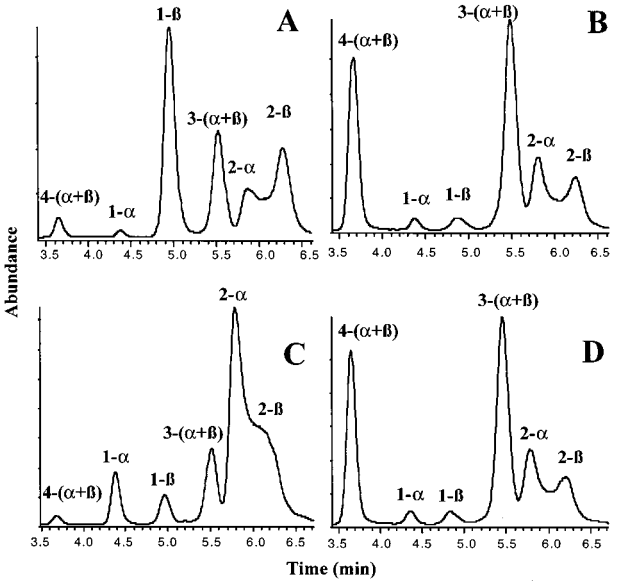
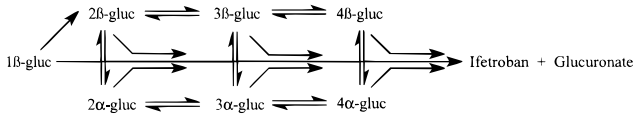


Figure 5. Isomeric transformation of the 1-*O*-acyl β- and α-D-glucuronide at pH 11.0, chromatographed with 30% acetonitrile (5.0 mM NH_4OAc, pH 5.5): 1-*O*-acyl β-D-glucuronide incubated for (A) 0.5 and (B) 5.0 min; 1-*O*-acyl α-D-glucuronide incubated for (C) 0.5 and (D) 5.0 min.

Scheme 1



in the beginning the late-eluting one was bigger than the earlier one. The emergence of the peak eluting at 6.8 min followed that of the 7.5-min doublet. Thus, the peak at 6.8 min was identified as 3-*O*-acyl glucuronide (**VII**). Attempts to further resolve this peak entailed the coelution and broadening of other peaks. The doublet at 5.1 min was the last one to emerge. Thus it was identified as the 4-*O*-acyl glucuronide (**VIII**).

The distinction between the 2-*O*-acyl α- and β-anomers was based on the assumption that the 1-*O*-acyl β-glucuronide (**II**) first transforms to 2-*O*-acyl β positional isomer which then converts reversibly to the corresponding 2-*O*-acyl α- anomer. The sequence of events related to the 1-*O*-acyl β- to 2-*O*-acyl β- and 1-*O*-acyl α- to 2-*O*-acyl α-conversion is depicted in Scheme 1. This is supported by the isomeric transformation profile obtained from the incubation of **II** and **V** in a pH 11.0 buffer (Figure 5). In Figure 5A, where the starting glucuronide is **II**, the last peak (6.3 min) of the partially resolved doublet decayed immediately to yield the early peak (5.8 min) of the doublet, as shown in Figure 5B. Thus, the peak at 6.3 min represents the initial 1-*O*-acyl β- → 2-*O*-acyl β- ↔ 2-*O*-acyl α-conversion. However,- when **V** was the starting glucuronide, the sequence of the anomeric conversions reversed (Figure 5C and D), as now the 5.8-min peak of the doublet represents the initial 1-*O*-acyl α- → 2-*O*-acyl α- ↔ 2-*O*-acyl β-conversion. The assignment of α- and β-anomers of **VII** and **VIII** could be achieved based on the same reasoning.

The sequential migration of an acyl substituent on a glucuronic acid can be rationalized by considering the structural characteristics of the closed ring pyranosyl structure of glucuronic acid. A six-membered ring structure of β-D-glucuronic acid, having a chair conformation with all the hydroxyl groups in the equatorial position, can be easily anticipated as the most stable conformation. Thus, vicinal equatorial hydroxyl groups of glucuronic acid will have staggered conformation. In an undistorted chair conformation, the torsion angle between vicinal equatorial trans groups is the same as that between the axial and equatorial cis groups. Consequently, the migration of an acyl substituent on the sugar moiety to an adjacent trans hydroxyl, via the formation of the cyclic orthoester intermediate,[14] is likely, although the energy required for the intermediate cyclization process involving the acyl group and an adjacent hydroxyl group is lower for *cis*-(*eq,ax*) than the *trans*-(*eq, eq*).[19] Therefore, a sequential migration of the acyl group from position 1 toward position 4 can be anticipated. Unlike **II**, where all the ring hydroxyl groups and the acyl substituent are trans, the acyl substituent in **V** at C-1 is axial and has a cis conformation relative to the C-2 hydroxyl function. Therefore, a relatively fast 1-*O*-acyl α → 2-*O*-acyl α-conversion is expected.[20] This is in agreement with our experimental observation (data not reported).

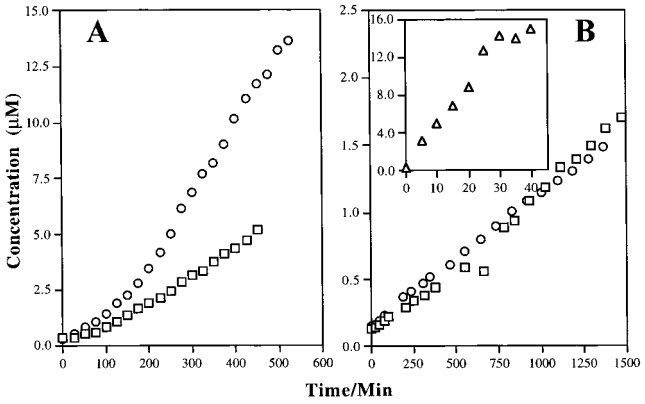


Figure 6. Methanolysis of a 25 μM (15.4 μg/mL) solution of 1-*O*-acyl β-D-glucuronide incubated at 23 °C in (A) 100% methanol (○) and 80% aqueous methanol (□); (B) 20% methanol in aqueous buffer solutions at pH 3.0 (□), 6.0 (○), and 9.0 (△, inset).

Methanolysis of II. Figure 6A represents methanolysis of a 25 μM (15.4 μg/mL) solution of **II** in 100% methanol and 80% methanol in water. As shown in Figure 6A, the rate of methanolysis increases with increasing concentration of methanol. Analysis of the data (Figure 6B) for the methanolysis of **II** in 20% methanol in aqueous buffer solutions with apparent pH values of 3.0, 6.0, and 9.0 revealed that the rate of methanolysis increased with increasing pH. While at pH 3.0 and pH 6.0 the methanolysis was not very extensive, at pH 9.0 the original glucuronide predominantly converted to **IX** in 1 h. No direct esterification of free ifetroban (**I**) to the ester **IX** was detected under the conditions of methanolysis of **II**.

Decomposition Kinetics. In Figure 7, the concentrations of different positional isomers are plotted as a function of incubation time of **II** in a pH 9.0 buffer. Scheme 1 depicts the steps involved in the hydrolysis and transacylation of the acyl glucuronides. The kinetic model for such a scheme will comprise a complex

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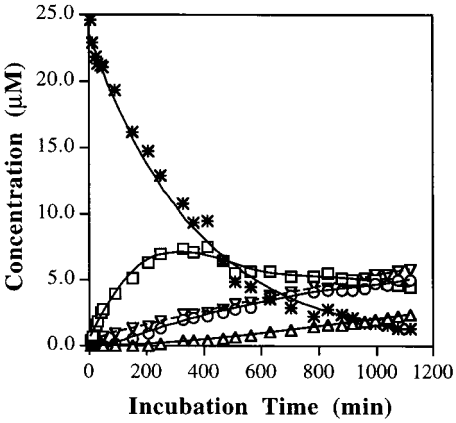
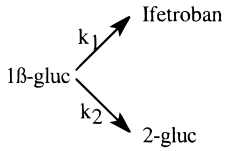


Figure 7. Typical decay curves of 25 μM (15.4 μg/mL) solution of 1-*O*-acyl β-D-glucuronide incubated in pH 9.0 phosphate buffer at 23 °C: 1β-gluc (*); 2-gluc (□); 3-gluc (○); 4-gluc (△); ifetroban (▽).

Scheme 2



combination of consecutive, opposing, and parallel reactions.[21] Because the acyl migration proceeds in a sequential fashion, Scheme 1 can be simplified to Scheme 2 to represent the initial decomposition of **II**. Even with this simplified scheme, the kinetic analysis is still complicated by the concurrent hydrolysis of **VI**.

At pH ≤4.0, only ifetroban was produced. Thus, at pH ≤4.0, there was only hydrolysis and no acyl migration occurred. At pH 5.0, the transformation of 1-*O*-acyl to the 2-*O*-acyl positional isomer was noticeable only after 72 h (Table 1). Therefore, only the deconjugation kinetics due to hydrolysis is reported here in the pH range 1.0−5.0. Above pH 5.0, **II** was consumed in two parallel processes: (i) hydrolysis, and (ii) 1-*O*-acyl → 2-*O*-acyl migration. Therefore, at pH >5.0, both hydrolysis and transacylation are considered to account for the loss of **II**.

The rate law governing the decomposition processes depicted in Scheme 2, is represented by

$$-\mathrm{d}[1\beta\text{-gluc}]/\mathrm{d}t = k[1\beta\text{-gluc}] \quad (1)$$

where

$$k = (k_1 + k_2) \quad (2)$$

$$[1\beta\text{-gluc}] = [1\beta\text{-gluc}]_0 \mathrm{e}^{-kt} \quad (3)$$

$$\ln([1\beta\text{-gluc}]/[1\beta\text{-gluc}]_0) = -kt \quad (4)$$

Eq 4 represents a simple first-order reaction and can be used to determine the apparent overall decomposition rate constant, k. In the pH range 1−5, $k_2 = 0$ and thus $k = k_1$.

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Table 2. Variation of the First-Order Rate Constants (h^{-1}) of the Decay of Ifetroban 1-*O*-Acyl β-D-Glucuronide as a Function of pH

	pH 1.0	pH 3.0	pH 5.0	pH 7.4	pH 9.0
k	4.1×10^{-2}	7.1×10^{-4}	2.7×10^{-3}	8.8×10^{-2}	1.5×10^{-1}
k_1				1.2×10^{-2}	2.6×10^{-2}
k_2				7.6×10^{-2}	1.3×10^{-1}

To calculate the individual rate constants, k_1 and k_2 at a pH >5, the following expressions can be derived.[22]

$$\mathrm{d}[\text{ifetroban}]/\mathrm{d}t = k_1[1\beta\text{-gluc}] \quad (5)$$

Substituting for [1β-gluc] from eq 3, eq 5 can be rewritten as

$$\mathrm{d}[\text{ifetroban}]/\mathrm{d}t = k_1[1\beta\text{-gluc}]_0 \mathrm{e}^{-kt} \quad (6)$$

$$[\text{ifetroban}]/[1\beta\text{-gluc}]_0 = (k_1/k)(1 - \mathrm{e}^{-kt}) \quad (7)$$

Similarly the initial decay of **II** via the k_2 route can be represented by

$$[2\text{-gluc}]/[1\beta\text{-gluc}]_0 = (k_2/k)(1 - \mathrm{e}^{-kt}) \quad (8)$$

Dividing eq 7 by eq 8 gives

$$[\text{ifetroban}]/[2\text{-gluc}] = (k_1/k_2) = S \quad (9)$$

where S is the slope of the plot of [ifetroban (**I**)] vs [2-gluc (**VI**)]. Equation 2 and eq 9 can be combined to obtain

$$k_1 = kS/(S + 1) \quad (10)$$

$$k_2 = k/(S + 1) \quad (11)$$

The rate constants for the decay of **II** by hydrolysis (pH range 1.0−5.0) and by the combined mechanism (pH 7.4 and 9.0) are listed in Table 2. The plot of the concentration of **I** vs the concentration of **VI**, to calculate k_1 and k_2 from the decomposition data of pH 7.4 and pH 9.0, showed small upward curvature. This small positive deviation from the expected straight line, even in the initial part of the decay process, indicated that the hydrolysis product **I** at pH >5.0 is not exclusively produced by the k_1 route of Scheme 2. The hydrolysis of **VI**, produced via the k_2 route, also contributed to the concentration of **I**. Thus, the biased concentration of **I** would result in the overestimation of k_1. This is a clear demonstration of the complexity of kinetic analysis of the decomposition of acyl glucuronides as a consequence of the hydrolyzability of each of the positional isomers.

CONCLUSION

Depending on pH of the solution, 1-*O*-acyl β-D-glucuronides in aqueous solutions can undergo hydrolysis or hydrolysis and

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intramolecular acyl migration. Adjusting the pH to 3.0−4.0 drastically reduces the rate of both hydrolysis and acyl migration. At pH ≤4.0, only the hydrolysis occurs. At pH ≥5.0, both hydrolysis and acyl migration occur, the rate for both increasing with increase in pH. At high pH values (pH >11.0), not only the rate of acyl migration but also the rate of hydrolysis of 1-O-acyl glucuronide and its positional isomers is greatly enhanced. Hence, the production of the parent acid compound is fast since the half-lives of all isomers of the glucuronide are relatively short. The chromatographic resolution of the positional isomers is highly affected by the pH and ionic strength of the HPLC mobile phase. Thus, it is important to select the appropriate chromatographic conditions depending on whether resolving the positional isomers is desired. Finally, it should be stressed that for the accurate determination of an 1-*O* acyl-*β*-D-glucuronide and/or the parent acid compound in biological matrixes, it is essential to minimize the in vitro hydrolysis and acyl migration reactions. The choice of a solvent for preparing the stock solutions, reconstitution, and chromatographic mobile phase has to be made such as to avoid the solvolysis of the glucuronide.

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