Stereoselective Internal Acyl Migration of 1 β -O-Acyl Glucuronides of Enantiomeric 2-Phenylpropionic Acids

Kazuki AKIRA,*^a Hiroshi HASEGAWA,^a Yoshihiko SHINOHARA,^a Misako IMACHI,^b and Takao HASHIMOTO^a

School of Pharmacy, Tokyo University of Pharmacy and Life Science,^a 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan and Bruker Japan Co., Ltd.,^b 3-21-5 Ninomiya, Tsukuba, Ibaraki 305-0051, Japan.

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Internal acyl migration reactions of 1 β -O-acyl glucuronides of 2-arylpropionic acids (profens) are of interest because of their possible role in covalent binding to serum proteins and consequent allergic reactions. The stereoselective degradation of 1 β -O-acyl glucuronides of enantiomeric 2-phenylpropionic acids (PAs), the basic structures of profens, in phosphate buffer (pH 7.4) at 37°C, has been investigated using HPLC. Apparent first-order degradation of 1 β -O-acyl glucuronide and the sequential appearance of 2-, 3- and 4-O-acyl isomers were observed for each enantiomer. Acyl migration was observed to predominate over hydrolysis as in the other profen glucuronides. All the positional isomers and anomers were characterized using NMR and HPLC-NMR. The overall degradation half-life of (*R*)- and (*S*)-PA glucuronides was 1.8 and 3.3 h, respectively. These results suggest that (*R*)-PA glucuronide could be more susceptible to covalent binding to proteins *via* acyl migration than the corresponding antipode. The lability of the (*R*)-diastereomer over the antipode is consistent with previous reports on other profen glucuronides. Thus, the diastereomeric PA glucuronides are considered to be the best model compounds for the computation of structural physicochemical parameters to control the stereoselective internal acyl migration of profen glucuronides because PA has the simplest chemical structure of all the profens.

Key words 2-phenylpropionic acid glucuronide; acyl migration; immunotoxicity; covalent binding; structure-acyl migration relationship study

Conjugation with glucuronic acid to yield 1-O-acyl- β -D-glucopyranuronates (1 β -O-acyl glucuronides) is a major metabolic route for many carboxylate agents including non-steroidal anti-inflammatory drugs (NSAIDs).¹⁾ Acyl glucuronides are potentially reactive because of the susceptibility of the acyl group to nucleophilic attack, and readily undergo hydrolysis and isomerization *via* internal acyl migration.²⁾ The internal acyl migration involves transfer of the acyl group to the C-2, C-3 or C-4 position of the glucuronic acid ring. Acyl groups migrate away from C-1 toward C-4 *via* ortho-acid ester intermediates³⁾ which are formed by nucleophilic attack of neighboring glucuronic acid hydroxyl groups on acyl carbonyl groups. Isomeric glucuronides readily undergo ring-opening and mutarotate giving α - and β -anomers which can then undergo further acyl migration reactions. All these reactions appear to be reversible except the initial 1 β -O-acyl to 2 β -O-acyl migration. The rates of degradation by acyl migration and hydrolysis have been reported for various drug 1 β -O-acyl glucuronides.²⁾ Drug 1 β -O-acyl glucuronides differ greatly in their degradation rates, and acyl migration is generally observed to predominate over chemical hydrolysis under physiological conditions (pH 7.4, 37°C).^{4–10)}

The 1 β -O-acyl glucuronides of many carboxylate drugs have been shown to react with proteins to form covalent adducts.^{11–13)} Covalent binding appears to occur mainly *via* the formation of a Schiff's base linkage between the free aldehyde of the open-chain acyl migrated glucuronide and a nucleophilic amine group on the protein.^{11,13,14)} There is speculation that protein adducts are at least partially responsible for the immunological side-effects of carboxylate drugs.^{2,15,16)} Recently, some NSAIDs (*e.g.* benoxaprofen, indoprofen) have been withdrawn from clinical use because of a high incidence of allergic reactions,²⁾ and this may have

some relation to the above-mentioned immunotoxicity of acyl glucuronides.

The extent of formation of the potentially immunotoxic adducts has been postulated to be related to the rate of the initial 1 β -O-acyl to 2 β -O-acyl migration, which is highly dependent on the structure of the aglycone as well as pH and temperature. Benet *et al.*¹⁷⁾ have shown a strong relationship between the substitution pattern on the carbon α to the ester carbonyl carbon and the rate of acyl migration for several drug acyl glucuronides. Nicholls *et al.*⁸⁾ have also suggested the influence of some steric factors on the acyl migration of 1 β -O-acyl glucuronides of 2-, 3- and 4-(trifluoromethyl)benzoic acids as models of drug acyl glucuronides. However, the relationship between the structural physicochemical properties of acyl glucuronides and acyl migration remains ambiguous. Derivation of such relationships by computational chemistry would potentially be useful in drug design studies and help avoid potential toxicological problems related to glucuronide reactivity.

The 2-arylpropionic acids (profens), an important group of NSAIDs, possess a chiral center and exist as racemates. The profens exhibit stereoselective pharmacokinetics and undergo a unidirectional bioinversion of the less active *R*-enantiomer to the active *S*-enantiomer.¹⁸⁾ The profens are generally conjugated with glucuronic acid to form diastereomeric 1 β -O-acyl glucuronides. The lability of diastereomeric acyl glucuronides under physiological conditions (in buffer at pH 7.4, 37°C) has been investigated using several kinds of profens. The half-lives of *R*- and *S*-conjugates have been reported as follows: benoxaprofen,^{5,19)} *R* 2.0 h, *S* 4.1 h; carprofen,⁴⁾ *R* 1.7 h, *S* 2.9 h; fenoprofen,¹²⁾ *R* 1.0 h, *S* 2.0 h; flunoxaprofen,²⁰⁾ *R* 4.5 h, *S* 8.0 h; naproxen,⁶⁾ *R* 0.9 h, *S* 1.7 h; ketoprofen,¹⁰⁾ *R* 0.7 h, *S* 1.3 h. It should be noted that the *R*-form degrades almost twice as rapidly as the corresponding

* To whom correspondence should be addressed.

S-form, although the degradation rates differ considerably depending upon the chemical structure of the drugs. This is of interest in connection with structure-acyl migration relationship studies. Enantiomeric 2-phenylpropionic acids (PAs), the basic structures of profens, are largely biotransformed to 1 β -O-acyl glucuronides in rats, rabbits and man.²¹⁾ Although the stereoselective pharmacokinetics of PA in rats and rabbits is known,^{22,23)} the degradation of diastereomeric PA glucuronides remains unknown. In this paper, we have synthesized diastereomeric PA glucuronides as models to compute the structural physicochemical parameters which influence the acyl migration of diastereomeric profen glucuronides and investigated their stereoselective reactivity under physiological conditions.

MATERIALS AND METHODS

Instrumentation Mass spectra were recorded on a ThermoQuest (San Jose, CA, U.S.A.) TSQ-700 spectrometer. ¹H-NMR and ¹H-decoupled ¹³C-NMR spectra were recorded on a Varian (Tokyo, Japan) Gemini-300, a Bruker (Rheinstetten, Germany) AM400 or a Bruker DRX500 spectrometer. Chemical shifts (δ , ppm) are given with reference to tetramethylsilane or sodium 3-trimethylsilyl[2,2,3,3-²H₄]propionate, unless otherwise stated. The HPLC system consisted of a Waters (Milford, MA, U.S.A.) M600E multisolvent delivery system, a Waters U6K injector and a Waters Lambda Max 481 UV absorbance detector operated at 220 nm. Analytical HPLC was carried out on a Wakosil II3C18RS column (150 $\times$ 4.6 mm i.d., 3 μ m, Wako, Osaka, Japan) using a mixture of 10 mM sodium phosphate buffer (pH 6.0) and acetonitrile (85:15, v/v) as mobile phase. The flow-rate was 1 ml/min. Preparative HPLC was carried out on an Inertsil ODS-Prep column (250 $\times$ 30 mm, i.d., 10 μ m, GL Sciences, Tokyo, Japan) using a mixture of 10 mM ammonium acetate buffer (pH 6.0) and acetonitrile (85:15, v/v) as mobile phase, where the flow rate was 15 ml/min.

Synthesis of 1 β -O-Acyl Glucuronide of (R)-PA ((R)-1PG) (2) Benzyl 2,3,4-tri-O-benzyl-D-glucopyranuronate (**1**)²⁴⁾ (444 mg, 0.80 mmol) was coupled with (R)-PA (100 μ l, 0.75 mmol) as described in our previous paper⁹⁾ to give an anomeric mixture (579 mg) of benzyl (2'*R*)-1 α -O-(2'-phenyl)propionyl-2,3,4-tri-O-benzyl-D-glucopyranuronate and benzyl (2'*R*)-1 β -O-(2'-phenyl)propionyl-2,3,4-tri-O-benzyl-D-glucopyranuronate. The product was purified by flash column chromatography over 190 g silica-gel with hexane-diethyl ether (13/1, v/v) as the eluent to give the β -anomer (153 mg, 28% based on **1**) as a white solid; *Anal.* Calcd for C₄₃H₄₂O₈: C, 75.20; H, 6.16. Found: C, 74.93; H, 6.23. ¹H-NMR (500 MHz; C²HCl₃) δ : 1.51 (3H, d, *J*=7.1 Hz, 3'-H), 3.78 (1H, q, *J*=7.1 Hz, 2'-H), 3.51, 3.64, 3.79 (each 1H, each t, 2-H, 3-H, 4-H), 4.09 (1H, d, *J*=9.6 Hz, 5-H), 4.0—5.2 (8H, m, CH₂), 5.65 (1H, d, *J*=7.9 Hz, 1-H), 6.9—7.3 (25H, m, Ph). MS (electron ionization) *m/z*: 595 ([M-C₆H₅CH₂]⁺), 445 ([M-C₆H₅CH₂-C₆H₅CH(CH₃)CO₂H]⁺). The benzylated β -anomer (143 mg) was hydrogenated with 10% Pd/C as described in our previous paper,⁹⁾ to give **2** (60 mg, 88%) as a white solid; *Anal.* Calcd for C₁₅H₁₈O₈ 2/3H₂O: C, 53.25; H, 5.76. Found: C, 53.33; H, 6.00. ¹H-NMR (500 MHz; ²H₂O) δ : 1.52 (3H, d, *J*=7.2 Hz, 3'-H), 3.4—3.6 (3H, m, 2-H, 3-H, 4-H), 3.98 (1H, q, *J*=7.2 Hz, 2'-

Chart 1

H), 4.10 (1H, d, *J*=9.6 Hz, 5-H), 5.59 (1H, d, *J*=8.0 Hz, 1-H), 7.3—7.5 (5H, m, Ph). MS (electrospray ionization) *m/z*: 327 ([M+H]⁺), 344 ([M+H₂O]⁺), 349 ([M+Na]⁺), 365 ([M+K]⁺).

Synthesis of 1 β -O-Acyl Glucuronide of (S)-PA ((S)-1PG) (3) Compound **1** and (S)-PA were coupled as described above to give an anomeric mixture (549 mg) of benzyl (2'*S*)-1 α -O-(2'-phenyl)propionyl-2,3,4-tri-O-benzyl-D-glucopyranuronate and benzyl (2'*S*)-1 β -O-(2'-phenyl)propionyl-2,3,4-tri-O-benzyl-D-glucopyranuronate. The mixture was purified by flash chromatography over 180 g silica-gel with hexane-acetone (10:1, v/v) as the eluent to give the β -anomer (196 mg, 36% based on **1**) as a white powder; *Anal.* Calcd for C₄₃H₄₂O₈: C, 75.20; H, 6.16. Found: C, 74.74; H, 6.12. ¹H-NMR (500 MHz; C²HCl₃) δ : 1.52 (3H, d, *J*=7.1 Hz, 3'-H), 3.70 (1H, q, *J*=7.1 Hz, 2'-H), 3.58, 3.70, 3.82 (each 1H, each t, 2-H, 3-H, 4-H), 4.06 (1H, d, *J*=9.6 Hz, 5-H), 4.4—5.1 (8H, m, CH₂), 5.67 (1H, d, *J*=7.7 Hz, 1-H), 7.1—7.3 (25H, m, Ph); MS (electron ionization) *m/z*: 595 ([M-C₆H₅CH₂]⁺), 445 ([M-C₆H₅CH₂-C₆H₅CH(CH₃)CO₂H]⁺). Hydrogenation of the benzylated β -anomer (190 mg) gave **3** (97 mg, 97%) as a white powder; *Anal.* Calcd for C₁₅H₁₈O₈ 3/4H₂O: C, 53.02; H, 5.78. Found: C, 53.11; H, 5.92. ¹H-NMR (500 MHz; ²H₂O) δ : 1.52 (3H, d, *J*=7.1 Hz, 3'-H), 3.47—3.62 (3H, m, 2-H, 3-H, 4-H), 3.99 (1H, q, *J*=7.1 Hz, 2'-H), 4.03 (1H, d, *J*=9.5 Hz, 5-H), 5.60 (1H, d, *J*=8.1 Hz, 1-H), 7.3—7.5 (5H, m, Ph). MS (electrospray ionization) *m/z*: 327 ([M+H]⁺), 349 ([M+Na]⁺), 365 ([M+K]⁺).

Isolation of the Degradation Products (R)-1PG (20 mg) was dissolved in water (1 ml) and the pH of the solution was adjusted to 7.5 with 0.5% aqueous ammonia. The solution was incubated at 37°C for 24 h to obtain a degradation mixture. The pH of the solution was then adjusted to 4 with 10% acetic acid, and the solution was lyophilized to give a powder. The powder was redissolved in water (1 ml) and aliquots (10—100 μ l) were subjected to preparative HPLC. The eluates corresponding to the five major peaks at *t_R* 10.0, 18.4, 20.6, 23.1 and 25.8 min were separately collected (fraction 1, 2, 3, 4, and 5, respectively). Each fraction was cooled in an ice-bath immediately after collection and the pH was adjusted to 4 with 10% acetic acid. Each fraction was concentrated to remove acetonitrile under reduced pressure using a rotary evaporator and the remaining solvent was removed by lyophilization to give a powder (yield, 2—4 mg). Fractions 1, 2, 3, 4 and 5 were confirmed to correspond to the peaks at *t_R* 3.3, 5.5, 7.8, 8.5 and 9.8 min, respectively, in the analytical HPLC. Five degradation products from (S)-1PG (20 mg) corresponding to the peaks at *t_R* 3.2, 5.5, 8.0, 9.0 and 10.2 min in the analytical HPLC were similarly isolated.

HPLC-NMR Analysis of the Degradation Mixture of (S)-1PG A degradation mixture prepared from (S)-1PG (3 mg) as described above was redissolved in deuterium

oxide (200 μ l) and aliquots (10–50 μ l) were subjected to HPLC-NMR. The HPLC-NMR data were acquired in the stopped-flow mode (accumulation time, 28–50 min) according to the literature⁷⁾ except that a Bruker DPX300 NMR spectrometer was used. The HPLC conditions were the same as in the analytical HPLC except that a mixture of 50 mM sodium phosphate buffer in deuterium oxide (pH 6.0) and acetonitrile (85 : 15, v/v) was used as mobile phase.

Fig. 1. HPLC Separation of Regioisomers and Aglycone of (R)-1PG (A) and (S)-1PG (B)

Degradation mixtures were prepared by incubation of (R)-1PG or (S)-1PG in phosphate buffer (pH 7.4) at 37 °C for 24 h, and then subjected to analytical HPLC following addition of each substrate.

Time-Dependent Study of the Degradation A solution of (R)- or (S)-1PG (350 nmol) and 3-phenylpropionic acid (100 nmol) as an internal standard (I.S.) in 0.5 ml 10 mM sodium phosphate buffer (pH 7.4) was incubated at 37 °C and aliquots (10 μ l) were subjected to analytical HPLC at 1 min and then at 40 min intervals after dissolution. Calibration curves for (R)-1PG, (S)-1PG and (R)-PA were prepared using solutions containing known amounts of these compounds and I.S. (100 nmol) in mobile phase. The regioisomers of 1PG were assumed to have the same molar extinction coefficients as that of 1PG and were quantitated using the calibration curve for 1PG.

RESULTS AND DISCUSSION

Compounds, 2 and 3, were synthesized in a similar manner as described in our previous paper.⁹⁾ Benzyl-protected glucopyranuronate 1, which was prepared from D-glucose in seven steps,²⁴⁾ was condensed with (R)-PA and (S)-PA by the trichloroacetimidate method.²⁵⁾ The condensation proceeded stereoselectively to give anomeric mixtures of benzylated glucuronides, whose anomeric ratios (α : β) were found to be 1 : 3 for (R)-PA and 1 : 5.5 for (S)-PA by ¹H-NMR spectroscopic analysis. The β -anomers were separated from their α -anomers by flash chromatography which required a large amount of silica-gel because the retention times of the anomers were very close to each other. ¹H-NMR spectroscopic analysis of the purified β -anomers showed that contamination by the α -anomers was negligible (<1%). The benzylated β -anomers were quantitatively deprotected by hydrogenation to give 2 and 3.

The reactivity of (R)- and (S)-1PGs in phosphate buffer (pH 7.4) at 37 °C was examined using HPLC. For both glucuronides, five peaks due to degradation products including the aglycone emerged after a 24 h incubation as shown in Fig. 1. The eluates corresponding to individual peaks were collected by preparative HPLC and analyzed by ¹H-NMR and COSY in order to identify the degradation products. The detected NMR resonances and assignments are summarized in Table 1. The NMR spectra showed that the products formed from (R)-1PG were anomeric pairs of 4PG (t_R 3.3 min), 3PG (t_R 7.8 min), 2PG (t_R 8.5 min), and 2PG (t_R 9.8 min), and the products formed from (S)-1PG were anomeric pairs of 4PG (t_R 3.2 min), 3PG (t_R 8.0 min), 2PG (t_R 9.0 min) and 2PG (t_R 10.2 min), respectively. The anomeric ratios (α : β) were approximately 1 : 1 in all cases. The finding that both the last two peaks in the chromatograms were

Table 1. ¹H-NMR Chemical Shifts of the Glucuronide Ring of the Positional Isomers of (R)- and (S)-1PG in Deuterium Oxide^{a)}

Isomer	(R)-PA glucuronide					(S)-PA glucuronide				
	1-H	2-H	3-H	4-H	5-H	1-H	2-H	3-H	4-H	5-H
2 α -O-Acyl	5.24 d	4.73 dd	3.86 t	3.57 t	4.05 d	5.41 d	^{b)}	3.91 t	3.60 t	4.14 d
2 β -O-Acyl	4.61 d	4.71 dd	3.66 t	3.57 t	3.68 d	^{b)}	^{b)}	3.66 t	3.60 t	3.76 d
3 α -O-Acyl	5.24 d	3.72 dd	5.18 t	3.56 t	4.11 d	5.26 d	3.69 dd	5.23 t	3.72 t	4.21 d
3 β -O-Acyl	4.71 d	3.43 dd	4.99 t	3.59 t	3.76 d	4.73 d	3.38 dd	5.04 t	3.74 t	3.86 d
4 α -O-Acyl	5.24 d	3.62 dd	3.78 t	^{b)}	4.19 d	5.30 d	3.70 dd	3.93 t	4.94 t	4.22 d
4 β -O-Acyl	4.63 d	3.32 dd	3.59 t	^{b)}	3.84 d	4.69 d	3.41 dd	3.73 t	4.96 t	3.86 d

a) The signals are assigned based on chemical shifts, splitting patterns, spin-spin coupling constants, and integrals and on the connectivity information from the COSY experiments. Chemical shifts are given with reference to H²HO (δ 4.8 ppm). The splitting patterns are indicated as follows: d, doublet; dd, double of doublets; t, triplet. b) not observed (obscured by H²HO resonance).

due to the anomeric mixture of 2PG implies that one of the peaks was originally due to the α -anomer and the other to the β -anomer. They then mutarotated during the fractionation procedures. The rise in the baseline between the two 2PG peaks in Fig. 1 also confirms mutarotation on the HPLC column.²⁶⁾

The degradation mixture prepared from (*S*)-1PG was further analyzed by HPLC-NMR in the stopped-flow mode. The HPLC conditions were virtually the same as in the analytical HPLC. When the eluate corresponding to each regioisomer arrived at the ¹H flow probe, the flow of mobile phase was stopped and NMR spectra were obtained. This allowed direct observation of each isomeric glucuronide. Consequently, 3PG and 4PG existed as 1:1 anomeric pairs as in the above experiments using preparative HPLC. The first and second eluting peaks due to (*S*)-2PG were found to be due to the α - and β -anomers, respectively. The regioisomers appear to be relatively stable to acyl migration in the mobile phase used for analytical HPLC, since the NMR spectrum of each regioisomer showed no appreciable resonance due to acyl migrated products.

The time-dependence of degradation of (*R*)- and (*S*)-1PG in buffer (pH 7.4) at 37 °C is shown in Fig. 2. Both (*R*)- and (*S*)-1PG decreased with time by pseudo first-order kinetics to form 2PG, 3PG and 4PG in that order. This sequential appearance is in accordance with successive acyl migration between the neighboring hydroxyl groups *via* ortho-acid ester intermediates. The acyl migration predominated over hydrolysis. The degradation half-lives of (*R*)- and (*S*)-1PG were calculated to be 1.8 and 3.3 h, respectively, and *R*-conjugate degraded twice as rapidly as the *S*-conjugate. To date, the lability of diastereomeric glucuronides of benoxaprofen,^{5,19)} carprofen,⁴⁾ fenoprofen,¹²⁾ flunoxaprofen,²⁰⁾ naproxen⁶⁾ and ketoprofen¹⁰⁾ has been investigated under physiological conditions. The half-life of the *R*-conjugate is almost half that of the corresponding *S*-conjugate. As the acyl glucuronides of benoxaprofen, carprofen, naproxen and ketoprofen degrade mainly *via* acyl migration, their overall instability roughly reflects the susceptibility to acyl migration. Thus, insight into the structure-acyl migration relationships would be gained if one investigated the structural physicochemical properties controlling the stereoselective acyl migration of diastereomeric profen glucuronides. 1PGs are considered to be the best models for computing such physicochemical parameters because PA has the simplest chemical structure of all the profens.

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Fig. 2. Representative Profiles Illustrating the Acyl Migration and Hydrolysis of (*R*)-1PG (A) and (*S*)-1PG (B) in Sodium Phosphate Buffer (pH 7.4) at 37 °C

●, 1PG; ■, 2PG; ▲, 3PG; ◆, 4PG; ○, PA.

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