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Rapid Internal Acyl Migration and Protein Binding of Synthetic Probenecid Glucuronides

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Internal acyl migration reactions of drug 1-*O*-acyl-*β*-D-glucopyranuronates (1*β*-acyl glucuronides) are of interest because of their possible role in covalent binding to proteins and consequent adverse effects. The reactivity of the synthetic probenecid 1*β*-acyl glucuronide (PRG), the principal metabolite of probenecid (PR) in humans, has been investigated in terms of acyl migration, hydrolysis, and covalent binding to proteins in phosphate buffer (pH 7.4) and human plasma at 37 °C. PRG primarily degraded by acyl migration according to apparent first-order kinetics and the 2-, 3-, and 4-acyl isomers sequentially appeared as both α- and *β*-anomeric forms. In addition, small amounts of PRG and extremely labile 1α-acyl isomer existed in the equilibrated mixture favoring the 2α/*β*-acyl isomer, that provided significant information regarding the mechanism of acyl migration. All of the positional isomers and anomers were characterized using preparative HPLC and NMR spectroscopy. Acyl migration was observed to predominate over hydrolysis in both media although the extent of hydrolysis in plasma was larger than that in the buffer. The overall degradation half-lives (h) in the buffer and plasma were 0.27 ± 0.003 and 0.17 ± 0.007, respectively. The covalent binding rapidly proceeded mainly via the Schiff's base mechanism and reached a plateau after 2 h of incubation. The maximal binding was 146 ± 4.8 pmol/mg of protein, and ca. 10% of the initial concentration of PRG. These results indicated that PRG is most labile and susceptible to acyl migration of all the drug acyl glucuronides reported to date in the physiological conditions, and highly reactive to plasma proteins, that could provide a possible explanation for the immunologically based adverse effects of PR.

Introduction

Conjugation with glucuronic acid to yield 1-*O*-acyl-*β*-D-glucopyranuronates (1*β*-acyl glucuronides) is a major metabolic route for many carboxylate drugs (*1*). It is well-known that 1*β*-acyl glucuronides are labile and reactive. These compounds undergo both hydrolysis and internal acyl migration. In the acyl migration, the aglycon is transferred to the C-2, C-3, or C-4 position of the glucuronic acid ring (*1*) (Figure 1). In general, acyl migration has been observed to predominate over hydrolysis under physiological conditions (*2*−*11*). Furthermore, the 1*β*-acyl glucuronides of many carboxylate drugs have been shown to form covalent adducts with proteins (*12*−*16*). Currently, there is speculation that the protein

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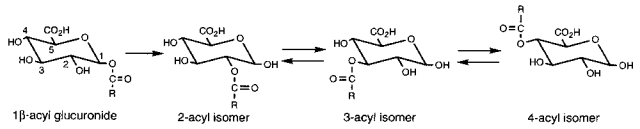

Figure 1. General scheme for the acyl migration of 1β-acyl glucuronides. R = drug moiety. Mutarotation is possible in the isomeric glucuronides.

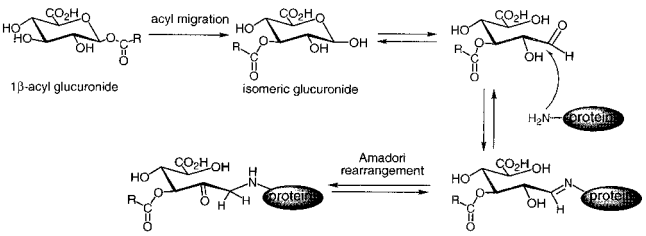

Figure 2. Schiff's base mechanism proposed for covalent binding of 1β-acyl glucuronides to proteins.

adducts are at least partially responsible for immunological side effects of carboxylate drugs (*1*, *17*, *18*). Recently, a number of NSAIDs (e.g., benoxaprofen, indoprofen, tolmetin, zomepirac) have been withdrawn from clinical use because of a high incidence of allergic reactions, and this may have some relation to the immunotoxicity induced by the protein adducts. Toxicity mechanisms involving modification of active sites of enzymes (*19*) and interaction with structural proteins (*20*) have been also suggested. It is now accepted that the covalent binding can occur by either transacylation or Schiff's base mechanisms shown in Figure 2 (*1*). The covalent binding appears to occur mainly by the Schiff's base mechanism, in the drug acyl glucuronides which readily undergo acyl migration (*12*, *15*, *21*, *22*).

p-(Dipropylsulfamoyl)benzoic acid (probenecid, PR) has been used for many years as a therapeutic agent for chronic gout. PR inhibits glucuronidation of some drugs and the active transport of organic acids in the renal tubules (*23*−*25*). Recently, these effects have therapeutic advantages in the medication of patients with acquired immunodeficiency syndrome; PR prolongs the duration of serum levels of zidovudin (*26*, *27*) and zalcitabine (*28*) by decreasing the glucuronidation and the renal tubular excretion, respectively, and reduces the cidofovir-induced nephrotoxicity by decreasing its renal tubular concentration (*29*, *30*). However, PR causes a high frequency (11−25%) of hypersensitivity reactions among patients with human immunodeficiency virus whereas in other patients the incidence is low (2−4%) (*30*). It has been suggested that changes in drug metabolism and responsiveness to some reactive metabolites of PR could explain the high rate of adverse effects (*31*). In addition, PR-induced immune hemolytic anemia has been reported, that is speculated to be mediated by an immune complex with some PR metabolites (*32*, *33*).

PR is metabolized to form an acyl glucuronide, 1-*O*-[*p*-(dipropylsulfamoyl)benzoyl]-β-D-glucopyranuronic acid (PRG), and several phase-I metabolites, which are excreted in the urine together with a small amount of the parent compound in humans (*34*). Of the dose, 25−70% has been reported to be excreted as PRG (*34*−*37*). Protein adducts of PR have been detected in plasma from human subjects after dosing PR and diflunisal, that is formed probably through the acyl migaration of PRG (*38*), although the presence of PRG in plasma has not been demonstrated (*36*, *38*). The protein binding to serum albumin has been found to occur also in vitro (*35*). The protein adduct formation may be responsible for the above-mentioned adverse effects of PR.

The reactivity of PRG has been investigated in buffer solutions with or without serum albumin, urine, and plasma using high-performance liquid chromatography (HPLC) (*35*, *36*). However, the reactivity is still unclear, and the contribution of acyl migration to the lability is entirely unknown, because PRG seems to not be clearly discriminated from its isomeric glucuronides in the HPLC method used in the literature. In this paper, we have investigated the reactivity of the synthetic PRG in buffer solutions and human plasma using an HPLC method to simultaneously determine PR, PRG, and its isomeric glucuronides.

Materials and Methods

Materials. Ammonium-$^2H_4$ deuterioxide [26% (w/v), >99.0 atom % ^2H] and sulfaphenazole were purchased from Sigma-Aldrich (Tokyo, Japan). β-Glucuronidase (>76 × 10^4 Fishman units/g) was purchased from Funakoshi (Tokyo, Japan). Silica gel (Wakogel C-300) and PR were purchased from Wako Pure Chemical Industries (Osaka, Japan). Deuterated solvents and thin-layer chromatography plates (silica gel $60F_{254}$) were purchased from Merck (Darmstadt, Germany). Other reagents were purchased from Kanto Chemical (Tokyo, Japan). Plasma was obtained by centrifugation of heparinized blood from healthy male volunteers not on any medication. The plasma was immediately used for incubation following pH measurements. Protein was measured with Lowry method using bovine serum albumin as the standard.

Instrumentation. ^1H NMR, ^1H-decoupled ^{13}C ($^{13}C\{^1H\}$) NMR, and two-dimensional NMR spectra [correlated spectroscopy (COSY)] of 1-*O*-[*p*-(dipropylsulfamoyl)benzoyl]-α-D-glucopyranuronic acid (1α-acyl isomer), PRG, and the degradation products were recorded in ^2H_2O on a Bruker DPX400 spectrometer (9.4 T) or a Bruker DRX500 spectrometer (11.75 T), and chemical shifts were referenced to that of sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-propionate (TSP, δ^1H 0), unless otherwise stated. ^1H NMR spectra of other compounds were recorded in C^2HCl_3 on a Varian GEMINI300 spectrometer, and chemical shifts were referenced to that of tetramethylsilane (δ^1H 0). All the NMR spectra were obtained at 300 K using 5-mm NMR tubes. Mass spectra (MS) were recorded on a ThermoQuest (San Jose, CA) TSQ700 spectrometer in the electron impact ionization mode (EI) or a Hitachi (Tokyo, Japan) M-1000 spectrometer in the electron spray ionization mode (ESI). Melting points were determined on a Yanako (Kyoto, Japan) MP-S3 melting point apparatus and were uncorrected. HPLC was performed using a Waters (Milford, MA) M600E multisolvent delivery system, a Rheodyne (Cotati, CA) 7125 injector, a Shimadzu (Kyoto, Japan) SPD-6A UV Spectrophotometric Detector set at 254 nm, and a LiChrospher 100 RP-18 (e) column (250 × 4 mm i.d., 5 μm, Kanto Chemical, Tokyo, Japan) or an Inertsil PREP-ODS column (250 × 30 mm i.d., 10 μm, GL Sciences, Tokyo, Japan). The mobile phase for the LiChrospher column was 10 mM sodium phosphate buffer (pH 5.0)−acetonitrile (72/28, v/v) at a flow rate of 1 mL/min. The mobile phase for the Inertsil column was 10 mM ammonium acetate buffer (pH 5.0)−acetonitrile (72/28, v/v) at a flow rate of 15 mL/min. Unless otherwise noted, the former analytical column (LiChrospher) was used.

Synthesis of 1-*O*-[*p*-(Dipropylsulfamoyl)benzoyl]-α-D-glucopyranuronic Acid (1α-Acyl Isomer) (4) and 1-*O*-[*p*-(Dipropylsulfamoyl)benzoyl]-β-D-glucopyranuronic Acid (PRG) (5) (See Figure 3). PR (202 mg) and benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate **1** (*39*) (444 mg) were condensed using the trichloroacetimidate method (*40*) according to the previously reported manner (*9*). The product, which showed a single spot (R_f 0.5) on thin-layer chromatography [hexane/ethyl acetate = 3/1 (v/v)], was isolated from the reaction mixture by

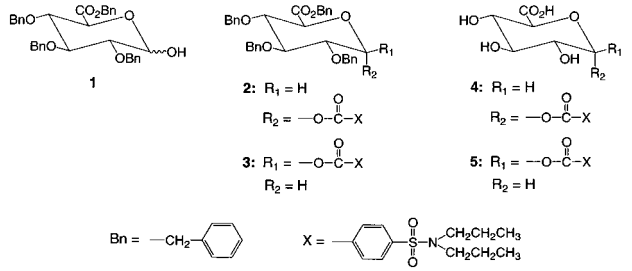

Figure 3. Probenecid glucuronides and the synthetic precursors. **1** = benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate; **2** = benzyl 2,3,4-tri-*O*-benzyl-1-*O*-[*p*-(dipropylsulfamoyl)benzoyl]-α-D-glucopyranuronate; **3** = benzyl 2,3,4-tri-*O*-benzyl-1-*O*-[*p*-(dipropylsulfamoyl)benzoyl]-β-D-glucopyranuronate; **4** = 1-*O*-[*p*-(dipropylsulfamoyl)benzoyl]-α-D-glucopyranuronic acid (1α-acyl isomer); **5** = 1-*O*-[*p*-(dipropylsulfamoyl)benzoyl]-β-D-glucopyranuronic acid (PRG).

flash column chromatography over 200 g of silica gel with hexanes/ethyl acetate (5/1, v/v) as the eluent to give an oil (267 mg). The oily product was found to be a mixture of one minor (R_f 0.2) and one major (R_f 0.15) component by further thin-layer chromatographic analyses [benzene/diethyl ether = 50/1 (v/v)]. The minor and major compounds were suggested to be the benzylated derivatives of the title compounds **2** and **3**, respectively, by ^{1}H NMR spectroscopy (data are not shown). Thus, the oily product was subjected to flash column chromatography over 40 g of silica gel with benzene/diethyl ether (100/1) as the eluent to give a minor benzylated compound (43 mg) and then a major benzylated compound (160 mg) as colorless oils. The benzylated compounds were deprotected by catalytic dehydrogenation as described previously (*9*) to give **4** (8.5 mg) and **5** (69 mg) as pale yellow amorphous powders. **4**: ^{1}H NMR δ 0.82 (6H, t, J = 7.4 Hz, CH_3), 1.52 (4H, m, CH_2), 3.18 (4H, t, J = 7.5 Hz, CH_2), 3.67 (1H, dd, J = 10.1 and 9.0 Hz, H4), 3.91 (1H, dd, J = 9.8 and 3.6 Hz, H2), 3.98 (1H, t, J = ~9 Hz, H3), 4.19 (1H, d, J = 10.1 Hz, H5), 6.45 (1H, d, J = 3.6 Hz, H1), 7.99 (2H, d, J = 8.6 Hz, aromatic protons), 8.28 (2H, d, J = 8.6 Hz, aromatic protons). MS (ESI): m/z 462 $[M + H]^+$. Anal. Calcd for $C_{19}H_{27}O_{10}NS$: C, 49.45; H, 5.90; N, 3.04. Found: C, 49.34; H, 5.99; N, 3.01. **5**: ^{1}H NMR δ 0.81 (6H, t, J = 7.4 Hz, CH_3), 1.52 (4H, m, CH_2), 3.18 (4H, t, J = 7.5 Hz, CH_2), 3.6−3.8 (3H, m, H2, H3, H4), 4.11 (1H, d, J = 9.3 Hz, H5), 5.88 (1H, d, J = 7.7 Hz, H1), 7.99 (2H, d, J = 8.6 Hz, aromatic protons), 8.29 (2H, d, J = 8.6 Hz, aromatic protons). ^{13}C NMR [$(CH_3)_2SO$]: δ 11.1 (CH_3), 21.7 (CH_2), 49.7 (CH_2), 71.5, 72.4, 75.7, 76.3 (C2, C3, C4, C5), 95.4 (C1), 127.4, 130.8 (protonated aromatic carbons), 132.4, 144.3 (quaternary aromatic carbons), 163.7 (CO), 170.0 (CO). The chemical shifts of ^{13}C resonances were referenced to that of dimethyl sulfoxide. These ^{13}C chemical shifts of PRG were identical to those reported for PRG isolated from human urine (*41*). MS (ESI): m/z 462 $[M + H]^+$. Anal. Calcd for $C_{19}H_{27}O_{10}NS\cdot 2/3H_2O$: C, 48.21; H, 6.03; N, 2.95. Found: C, 48.21; H, 6.31; N, 2.76.

The protons of the glucuronide ring of β-anomer **5** resonated at a higher field than those of α-anomer **4** in the ^{1}H NMR spectra as described in the literature (*42*). Compounds **4** and **5** showed a single peak in HPLC. The β-anomer **5** contained no appreciable amount of α-anomer **4** in the ^{1}H NMR spectra and vice versa. When compound **5** was treated with β-glucuronidase, the peak of **5** disappeared and that of PR emerged, which showed that compound **5** was the natural, biosynthetic 1β-acyl glucuronide.

***β*-Glucuronidase Treatment.** A stock solution (300 μL) of PRG in acetonitrile (200 μM) was placed in a test tube and evaporated to dryness by a stream of dry nitrogen. To the residue was added 1 mL of 10 mM ammonium acetate buffer (pH 5.0) followed by mixing. The solution was incubated at 37 °C for 45 min with or without β-glucuronidase (1 mg, 760 Fishman units). Ten milliliter aliquots of the solution were analyzed by HPLC.

Incubation of Glucuronides in Phosphate Buffer. A stock solution (100 μL) of PRG, 1α-acyl isomer or 3-acyl isomer in acetonitrile (each 200 μM), was placed in a test tube and evaporated to dryness by a stream of dry nitrogen. To the residue was added 1 mL of 100 mM sodium phosphate buffer (pH 7.4) followed by mixing. Triplicate incubations were performed at 37 °C. The reaction was monitored by analyzing 10 μL portions of the solution using HPLC. The ratio of each peak area to the total area of peaks due to PR, PRG, and its isomeric glucuronides was calculated. The reactivity of PRG was also examined in 10 mM sodium phosphate buffer (pH 3.0 and 5.0) at 37 °C.

Identification of Reaction Products from PRG. To a solution of PRG (20 mg) dissolved in H_2O (1 mL) was added 1% NH_3 in H_2O so that the pH value of the solution becomes approximately 7.4. The solution was incubated at 37 °C for 45 min, and then acidified to pH 3−4 with 10% acetic acid in H_2O in order to stabilize the products, followed by freeze-drying to give a white curdy solid. The solid was redissolved in methanol (1 mL), and 50−100 μL portions of the solution were injected onto the HPLC column (Inertsil PREP-ODS). Four major peaks at t_R 12.1, 19.9, 22.9, and 26.6 min (PR) and two minor peaks at t_R 16.0 and 17.5 min (PRG) were observed after injection. The eluates corresponding to the peaks at t_R 12.1, 16.0, 19.9, and 22.9 min were collected (fractions 1, 2, 3, and 4, respectively) followed by immediate acidification (pH ~3) with 10% acetic acid in H_2O to stabilize the products. The eluates were evaporated by a rotary evaporator to remove acetonitrile and then freeze-dried. The residue was redissolved in ^2H_2O, and ^{1}H NMR and COSY spectra were obtained. Fractions 1, 3, and 4 were confirmed to correspond to the peaks at t_R 3.9, 6.9, and 7.9 min, respectively, in the analytical HPLC.

Direct ^{1}H NMR Spectroscopic Detection of Reaction Mixture. PRG (3 mg) dissolved in ^2H_2O (ca. 5 mL) was lyophilized to replace the protons of hydroxyl and carboxyl groups by deuterons. The residue was redissolved in 500 μL of ^2H_2O and transferred to an NMR tube following filtration. To the solution was added 2.6 M N^2H_3 in ^2H_2O (~2 μL) to decompose PRG, monitoring the reaction with ^{1}H NMR spectroscopy. The resulting mixture was measured by ^{1}H NMR spectroscopy for 2.5 h.

Incubation of PRG in Plasma. A stock solution (0.5 mL) of PRG in acetonitrile (218 μM) was placed in a test tube and evaporated to dryness by a stream of dry nitrogen. To the residue was added 1 mL of freshly collected human plasma (pH 7.8, 7.5 g of protein/dL) prewarmed at 37 °C for 5 min. The solution (PRG, 109 μM) was incubated triplicately at 37 °C. Fifty microliter portions were taken with time, and immediately mixed with 50 μL of acetonitrile/85% phosphoric acid in H_2O (5/0.02, v/v) (*14*) containing 12.8 μM sulfaphenazole as an internal standard in order to stop the reaction and deproteinize. The mixture was centrifuged (3000g × 10 min), and the supernatant and the precipitate were separated. The recovery of protein was 95 ± 5.1%. The supernatant was subjected to HPLC, and the precipitates was stored at −20 °C until analyzed. Linear calibration curves with correlation coefficients of 0.999 were obtained using blank plasma (50 μL) containing known amounts of PR (10−2083 ng) and PRG (10−2518 ng). The isomers of PRG were assumed to have the same molar extinction coefficients as that of PRG and were quantitated using the calibration curve for PRG.

Analysis of Covalently Bound PR. The precipitates from the plasma incubated with PRG were washed 7 times with 300 μL of methanol/diethyl ether (3/1, v/v) in order to remove PR, PRG, and isomeric glucuronides irreversibly unbound to protein, and then dried by a stream of dry nitrogen. At the last washing step, the supernatant was evaporated under nitrogen, and the residue was analyzed by HPLC to determine the effectiveness of the washing procedures. No PR, PRG, and isomeric glucuronides were detected in the last wash solution. The loss of protein by washing was negligible. The washed precipitates were suspended to 200 μL of 1 M sodium hydroxide by vortexing

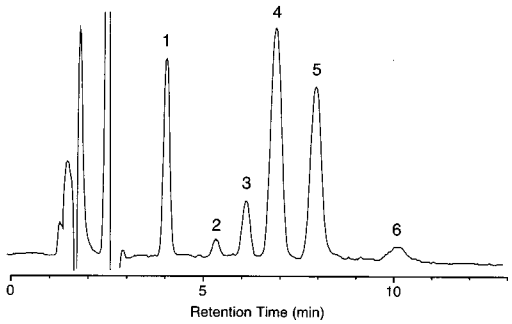

Figure 4. HPLC chromatogram of PRG after incubation in 100 mM phosphate buffer (pH 7.4) at 37 °C for 1 h. 1, 4-acyl; 2, 1α-acyl; 3, PRG; 4, 3-acyl; 5, 2-acyl; 6, PR.

Table 1. ^{1}H NMR Chemical Shifts of the Glucuronide Ring of Isomeric Glucuronides in ^2H_2O[a]

isomer	H1	H2	H3	H4	H5
2α-acyl	5.54 d	5.03 dd	4.12 t	3.70 t	4.20 d
2β-acyl	∼5.0[b]	∼5.0[b]	3.91 m	3.70 t	3.85 d
3α-acyl	5.35 d	3.96 dd	5.46 t	3.89 t	4.26 d
3β-acyl	4.84 d	3.66 dd	5.28 m	∼3.9[b]	∼3.9[b]
4α-acyl	5.34 d	3.77 dd	4.11 t	5.15 t	4.38 d
4β-acyl	*c*	3.47 dd	3.93 t	5.17 t	4.03 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 (*42*). The splitting patterns are indicated as follows: d, doublet; dd, double of doublets; t, triplet; m, multiplet. Coupling constants fall into characteristic ranges of 3.6−3.8 Hz for *J* (H1−H2) in α isomers, 7.7−8.0 Hz for *J* (H1−H2) in β isomers, and 9.2−10.2 Hz for couplings between H2−H3, H3−H4, and H4−H5. For all isomers, the protons of the probenecid moiety have similar chemical shifts: δ 8.25−8.30 (d, aromatic protons), δ 7.99−8.00 (d, aromatic protons), δ 3.18−3.20 (t, CH_2 of side chain), δ 1.52−1.55 (m, CH_2 of side chain), δ 0.81−0.83 (t, CH_3 of side chain). [b] The specific assignment of the H1 and H2 resonances of 2β-acyl and the H4 and H5 resonances of 3β-acyl cannot be made because of their closeness and overlap. [c] The signal is presumed to be concealed behind the H^2HO signal.

and sonication, and incubated at 65 °C for 12 h to hydrolyze and release covalently bound PR. To the incubation mixture were added 100 μL of 10% hydrochloric acid to acidify (pH ca. 1) and 50 μL of a solution of ketoprofen in acetonitrile (4 μM) as an internal standard. The mixture was extracted with 500 μL of cyclohexane/diethyl ether (1/1, v/v) followed by centrifugation (3000*g* × 10 min). The organic layer was separated and evaporated to dryness by a stream of dry nitrogen. The residue was redissolved in 50 μL of the mobile phase, and 10 μL portions of the solution were subjected to HPLC. A linear calibration curve with a coefficient of variation of 0.999 was obtained using blank plasma containing known amounts of PR (36−730 pmol) and ketoprofen (197 pmol).

Results

Reactivity of PRG in Buffer Solutions. The reactivity of PRG in phosphate buffer (pH 7.4) at 37 °C was examined using HPLC. Four major peaks due to degradation products including the aglycon emerged after 1 h of incubation as shown in Figure 4. The eluates corresponding to individual peaks were collected by preparative HPLC and analyzed by ^{1}H 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 were anomeric pairs of 2-acyl (t_R 7.9 min), 3-acyl (t_R 6.9 min), and 4-acyl isomers (t_R 3.9 min). The anomeric ratios (α/β) were aproximately 1/1 in all cases. When ammonium-

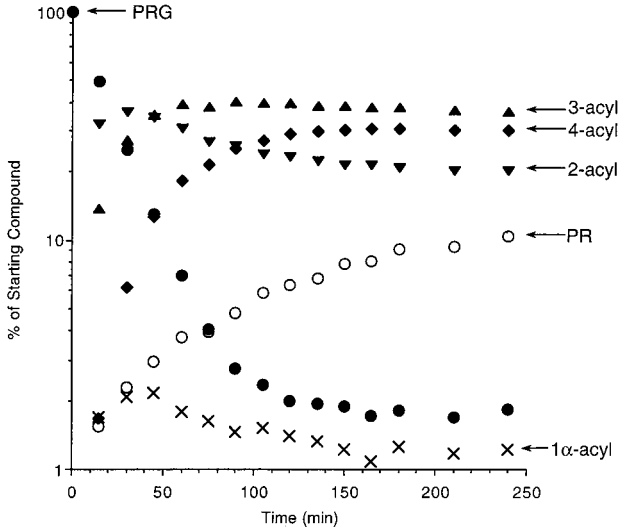

Figure 5. Profiles for acyl migration and hydrolysis of PRG in 100 mM phosphate buffer (pH 7.4) at 37 °C. Each point represents the mean of values obtained from triplicate incubations.

$^2H_4$ deuterioxide was added to the deuterium oxide solution of PRG contained in an NMR tube followed by ^{1}H NMR analysis, the compound degraded to form an equilibrated mixture of the α- and β-anomers (α/β = ca. 1) of 2-acyl, 3-acyl, and 4-acyl isomers. This experiment directly demonstrated the occurrence of acyl migration followed by rapid mutarotation.

A minor peak observed at t_R 5.2 min in Figure 4 was identified to be the 1α-acyl isomer by co-chromatography with the authentic compound synthesized here. When the peak was analyzed by ^{1}H NMR following the collection by preparative HPLC, the 2-acyl, 3-acyl, and 4-acyl isomers were observed, and the spectral pattern was similar to that of the equilibrated mixture formed from PRG in the phosphate buffer. On the other hand, the direct ^{1}H NMR spectroscopic analysis of the equilibrated mixture from PRG showed a doublet at δ 6.45 corresponding to the H1 proton chemical shift of the PR 1α-acyl glucuronide, which demonstrated the formation of the 1α-acyl isomer from PRG. From these experimental results, it was found that the compound was extremely unstable and decomposed during the collection procedures. The degradation of the 1α-acyl isomer in phosphate buffer (pH 7.4) at 37 °C was followed by HPLC. With time, the peak due to the 1α-acyl isomer very rapidly decreased with concurrent and sequential appearance of the 2-acyl, 3-acyl, and then 4-acyl isomers. The acyl migration was predominant over hydrolysis. It was ambiguous whether the degradation follows first-order kinetics because the degradation was too rapid to follow the initial decrease. The compound decreased to half its initial concentration after 1 min of incubation.

The time-depencence of degradation of PRG in buffer (pH 7.4) at 37 °C is shown in Figure 5. PRG decreased according to apparent first-order kinetics to form the 2-acyl, 3-acyl, and 4-acyl isomers in that order, and the acyl migration predominated over hydrolysis, as reported for other drug acyl glucuronides (*1*). At 75 min, 96% of PRG had disappeared, and only 4% hydrolyzed to PR. The degradation of PRG did not follow the apparent first-order kinetics after 75 min, and a small amount of PRG remained for many hours. The degradation half-life was calculated to be 0.27 ± 0.003 h using the first five points.

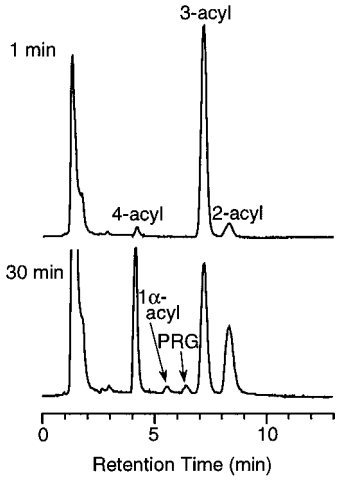

Figure 6. HPLC chromatograms of the 3-acyl isomer after incubation in 100 mM phosphate buffer (pH 7.4) at 37 °C. Incubation periods are indicated by the chromatograms.

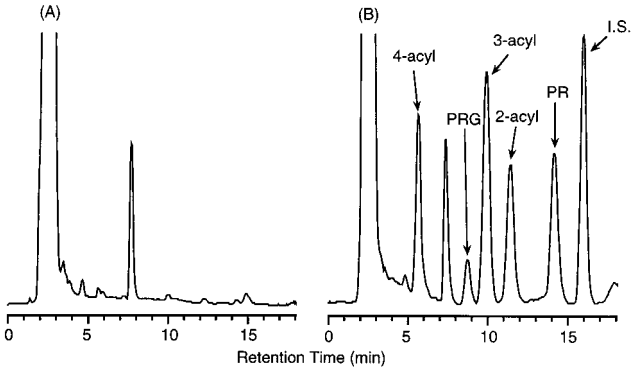

Figure 7. HPLC chromatograms of (A) blank plasma and (B) plasma spiked with PRG after incubation at 37 °C for 1 h. I.S., internal standard (sulfaphenazole).

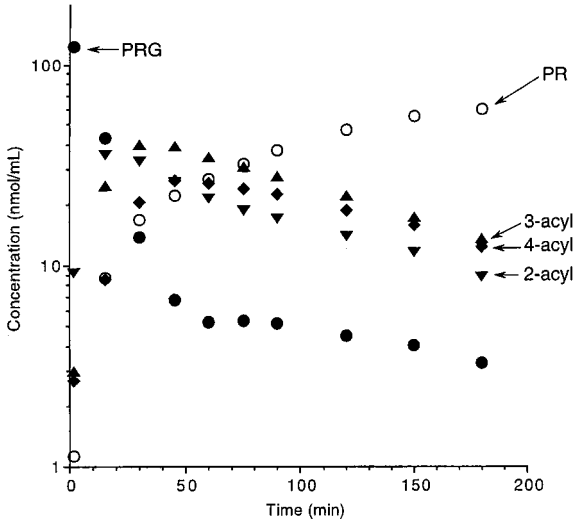

Figure 8. Profiles for acyl migration and hydrolysis of PRG in plasma at 37 °C. Each point represents mean of values obtained from triplicate incubations.

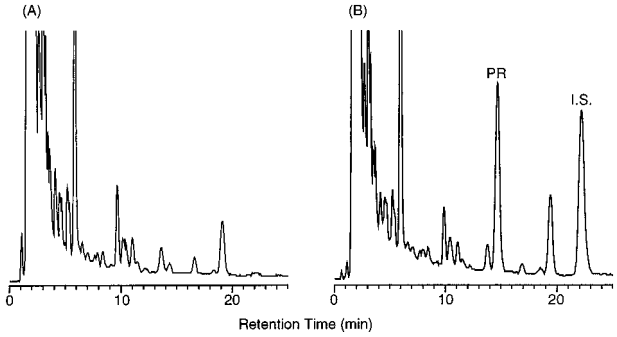

Figure 9. HPLC chromatograms of hydrolyzed pellets obtained from (A) blank plasma and (B) plasma spiked with PRG after incubation at 37 °C for 1 h. I.S., internal standard (ketoprofen).

The 1α-acyl isomer appeared as rapidly as the 2-acyl isomer, and the concentration closely followed that of the 2-acyl isomer, which means the rapid equilibrium between the 1α-acyl isomer and the 2α/β-acyl isomer. The 2-acyl, 3-acyl, and 4-acyl isomers were relatively stable to hydrolysis under the present conditions.

PRG was stable in phosphate buffer (pH 3.0) at 37 °C during 24 h incubation. However, it underwent hydrolysis to PR (2%) and rearrangement to 2-acyl isomer (13%) in phosphate buffer (pH 5.0) at 37 °C during 24 h incubation. When the 3-acyl isomer (α/β = ca. 1), that was not contaminated with the 1α- and 1β-acyl isomers at all, was incubated in phosphate buffer (pH 7.4) at 37 °C, the compound degraded with the concurrent appearance of the 2-acyl and 4-acyl isomers, and after 30 min, small amounts of PRG and 1α-acyl isomer appeared, as shown in Figure 6.

Reactivity of PRG in Plasma. The reactivity of PRG in intact plasma (unbuffered) at 37 °C was investigated. Aliquots of the reaction mixture were analyzed by HPLC after deproteinization. As shown in Figure 7, the 2-acyl, 3-acyl, and 4-acyl isomers and PR were well resolved although no 1α-acyl isomer was observed because of the hindrance by endogenous peaks. To quantitate PRG, the isomeric glucuronides, and PR, the suitability of various sulfa drugs as the internal standard was examined, as Selen et al. have successfully determined PR in plasma by HPLC using sulfamethazine as the internal standard (*43*). Consequently, sulfaphenazole was found to be most suitable. The peak due to PRG decreased with time according to apparent first-order kinetics with concurrent and sequential appearance of the 2-acyl, 3-acyl, and 4-acyl isomers as in the buffer, as shown in Figure 8. The half-life of PRG, 0.17 ± 0.007 h, was lower than that found in the buffer solution (pH 7.4) at 37 °C. A small amount of PRG remained for many hours after most of the PRG degraded, as in the buffer. Although the acyl migration predominated over hydrolysis, the rate of hydrolysis was much larger than that in the buffer at pH 7.4. The accelerated hydrolysis appeared to be responsible for the decrease in half-life. At 45 min, more than 90% of PRG had disapperaed, and 20% hydrolyzed to PR. PRG and the isomeric glucuronides almost completely hydrolyzed to PR after 8 h of incubation. This instability to hydrolysis in plasma is probably due not only to the higher pH value but also to enzymic hydrolysis by esterase and albumin (*44*, *45*).

The in vitro irreversible binding of PRG to plasma proteins was examined. The protein pellets obtained from the incubated plasma were exhaustively washed followed by hydrolysis of the adducts and extraction of the liberated PR. A typical chromatogram of the extract is shown in Figure 9. Ketoprofen was useful as the internal standard for the determination of PR (*46*). The covalent binding rapidly proceeded and reached a plateau after 2 h of incubation as shown in Figure 10, where the maximal binding, 146 ± 4.8 pmol/mg of protein, was achieved. The protein binding was found to be a signifi-

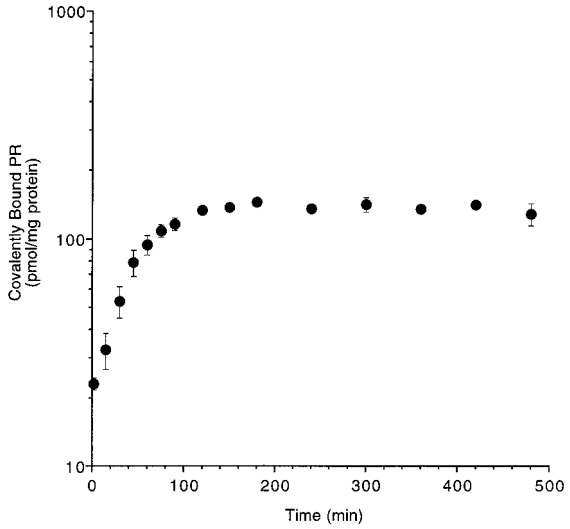

Figure 10. Time course of covalent binding of PRG to proteins in human plasma at 37 °C. initial concentration of PRG, 109 mM; bars indicate ±SD ($n = 3$).

cant pathway in the reaction of PRG in plasma since the maximum amount corresponded to approximately 10% of the initial concentration of PRG. The adduct was at least stable for more than 8 h. The protein binding was considered to proceed mainly via the Schiff's base mechanism because the adducts were largely formed after most of the PRG degraded to the isomeric glucuronides.

Discussion

The half-life of PRG (0.27 h) in phosphate buffer (pH 7.4) at 37 °C was found to be much smaller than that (ca. 0.4 h) reported in the literature (*35*). In the previous report, relatively impure PRG was used, and the HPLC separation of PRG from its isomeric glucuronides was not considered. Thus, it is considered that the measured amounts of PRG included the amounts of acyl-migrated products and the half-life was overestimated. Rates of degradation of 1β-acyl glucuronides depend on the structure of the aglycon as well as pH and temperature (*8*, *47*). The published first-order degradation half-lives in buffer at pH 7.4 and 37 °C vary from one compound to another in the range of 0.27−79 h (*48*). The half-lives (h) of relatively unstable acyl glucuronides have been reported as follows: tolmetin (*49*) 0.27; zenarestat (*50*) 0.42; zomepirac (*51*) 0.45; diclofenac (*52*) 0.51; (*R*)-ketoprofen (*10*) 0.66; diflunisal (*53*) 0.67; (*R*)-naproxen (*6*) 0.92. The lability of PRG was comparable to that of tolmetin that is the most labile acyl glucuronide reported to date. Tolmetin was withdrawn from the market because of severe adverse events that were most likely due to the formation of new antigens followed by autoimmune responses. Thus, PRG is least stable of all the drug 1β-acyl glucuronides clinically used. The extent of formation of the potentially immunotoxic protein adducts has been postulated to be ralated to the extent of acyl migration (*54*). As PRG largely degrades by acyl migration, it may be most reactive to proteins of all the drug 1β-acyl glucuronides although in vivo the extent of systemic exposure toward the acyl glucuronide and the participation of some enzymes must be also considered (*55*).

The scheme of acyl migration shown in Figure 1 is generally accepted, where succesive acyl migration between the neighboring hydroxyl groups occurs via *o*-acid ester intermediates, and the initial acyl migration step from the 1β-acyl glucuronide to the 2β-acyl isomer is considered to be irreversible whereas the rearrangement between the 2α/β-, 3α/β-, and 4α/β-acyl isomers is reversible (*1*). The sequential appearance of 2-acyl, 3-acyl, and 4-acyl isomers with disappearance of PRG (Figures 5 and 8) is in accordance with this mechanism. However, the concentration of PRG did not follow the first-order degradation kinetics after most of the PRG degraded. As the concentration of PRG was higher than predicted at the late time points, it was possible that the compound exists in an equilibrium favoring the corresponding 2α/β-acyl isomer. The equilibrium was demonstrated to exist by the experiments in Figure 6, where a low level of the 1β-acyl isomer was rapidly formed from the 3β-acyl isomer via the 2β-acyl isomer. Similar deviation from first-order kinetics at the late time points was also observed in (*S*)-naproxen 1β-acyl glucuronide (*54*). Hansen-Moller et al. (*3*) presented evidence for minor regeneration of diflunisal 1β-acyl glucuronide from the corresponding 4β-acyl isomer at pH 8.0 or a mixture of the acyl-migrated isomers at pH 8.5. However, all other studies on rearrangement of acyl glucuronides have concluded that this particular migration seems not to occur.

The PR 1α-acyl isomer was found to exist in an equilibrium favoring the corresponding 2α/β-acyl isomer in buffer at pH 7.4 and 37 °C. The isomer was much less stable than PRG in terms of acyl migration as well as overall degradation. The formation and rapid degradation are of great interest in connection with the mechanism and kinetics (*10*) of acyl migration and the structure−acyl migration relationships (*8*, *47*). The isomer may have special reactivity such as the direct reaction with important proteins via the transacylation mechanism because of the characteristic conformation (1,2-cis) different from PRG (1,2-trans). Thus, the 1α-acyl isomer may be a significant species in terms of bioreactivity and toxicity, even though the concentration is low compared with other acyl-migrated isomers. There was no evidence for the formation of 1α-acyl isomer until very recently, which would be because no authentic sample was obtained, and the compound was very unstable and existed in a low concentration. Corcoran et al. (*56*) have first reported that a low level of 1α-acyl isomer exists in the incubation mixture (pH 7.4, 37 °C) of (*S*)-naproxen 1β-acyl glucuronide purified from human urine. This paper presented another example of the formation of 1α-acyl isomer.

The half-life (0.17 h) of PRG in plasma at pH 7.4 and 37 °C was much lower than that (∼0.75 h) reported in the literature (*36*), where urine from a PR-treated human subject was used as the source of PRG to incubate with human plasma. The previous half-life is thought to be overestimated because the HPLC method used did not discriminate between PRG and its isomeric glucuronides. It is needless to say that the higher pH value in our study is at least partly responsible for the difference. PRG was found to be very unstable and mainly decompose to the acyl-migrated products also in plasma. The present results mean that when the concentration of PRG in blood is measured in the pharmacokinetic studies, PRG may decompose during treatments of blood samples even though appreciable amounts of PRG originally exist. On the other hand, PRG is relatively stable in urine, the pH of which is generally acidic (*35*). The high reactivity in

plasma may be thus one convincing reason PRG was not detected in human plasma after dosing of PR despite the urinary excretion of large amounts of PRG, although the high clearance and biosynthesis of the glucuronide in the kidney tubule are also other possible reasons (*36*).

There has been no report on the irreversible protein binding of PRG in plasma whereas the binding to human serum albumin in buffer has been examined by Hansen-Moller et al. (*35*). They reported that a large amount of adducts, 3.9 nmol/mg of human serum albumin, was formed in 6 h of incubation, that was more than 20 times larger than the maximum binding in plasma obtained here. Although there have been many papers on the reactivity of drug 1β-acyl glucuronides in human plasma, it is difficult to compare the results because the pH values of plasma samples as well as the treatments (buffered or unbuffered) after blood sampling are not frequently described despite the lability of plasma pH. The increase in pH will accelerate the chemical hydrolysis and relatively decrease the acyl migration and the subsequent covalent binding. The reaction rate of binding will also be influenced by the molar ratio between the acyl glucuronide and the plasma proteins. The maximum amount (pmol/mg of protein) of protein binding, the period (h) required to reach the maximum, and the initial concentration (μM) of acyl glucuronide are reported as follows: clofibric acid (*57*) 111, 4−8, 76; diflunisal (*58*) 171, 4, 198; (*S*)-ibuprofen (*59*) 150, 12, 131; ibufenac (*59*) 185, 6, 136. Although the maximum amount of protein adducts of PRG is comparable to those of other acyl glucuronides, the period to reach the maximum is relatively short. Thus, PRG appears to be among acyl glucuronides that are most reactive to plasma proteins.

In summary, PRG was found very unstable in phosphate buffer (pH 7.4) and plasma at 37 °C when compared with other acyl glucuronides. The compound primarily degraded by acyl migration to form isomeric glucuronides in both media, and also it quickly formed significant amounts of protein adducts in plasma probably via the Schiff's base mechanism. The susceptibility to acyl migration and the high reactivity to plasma proteins could provide a possible explanation for the immunologically based adverse effects of PR.

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