Apparent Intramolecular Acyl Migration and Hydrolysis of Furosemide Glucuronide in Aqueous Solution

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The stability of furosemide glucuronide (FG) was investigated in buffer solutions ranging from pH 1 through 10. This glucuronic acid conjugate was the major metabolite of furosemide (F) excreted in human urine. FG, obtained by extraction from human urine, was purified by ion-exchange chromatography. The concentration of FG, acyl migration isomers of FG (FG-iso), and F were determined simultaneously with an HPLC method that included fluorescence detection and gradient elution. FG was found to be unstable in highly acidic and in neutral to alkaline solutions. Hydrogen ion and hydroxy ion catalyzed the hydrolysis of FG below pH 2.8 and above pH 5.6, respectively. Above pH 3.7, FG instability led to the formation of eight FG-iso compounds. Though β-glucuronidase cleaved FG, the FG-iso compounds were resistant to the enzyme. The half-life of FG in a buffer solution at pH 7.4 and 37 °C was 4.4 h. The maximum stability of FG (half-life about 62 d) occurred at approximately pH 3.2. Below pH 3.7, acyl migration products of FG were not detected. Instead, the hydrolysis of FG to F and glucuronic acid was followed by the formation of 4-chloro-5-sulfamoylanthranilic acid (CSA), a secondary product in acidic media.

Keywords furosemide; furosemide glucuronide; acyl migration; hydrolysis; HPLC assay; pH-rate profile

Furosemide (F) is widely used as a potent diuretic agent in patients with renal failure,[1,2] hypertension,[3,4] hepatic cirrhosis,[5,6] and congestive heart failure.[7] Its major metabolite in human is the glucuronic acid conjugate, furosemide glucuronide (FG).[8-10]

The concentrations of FG in human urine specimens collected following the administration of F decreased within several hours at room temperature, even when the specimens were protected from light.[11] Such FG instability was also reported by Lovett *et al.*[12] Furthermore, the decrease in FG concentration did not correspond to the increase in F, which suggests that isomers of furosemide glucuronide (FG-iso) may be formed in biological fluids following the administration of F. (Note: FG-iso will be used to designate all 8 or more compounds represented by those peaks identified as resulting from FG and degrading by base catalyzed hydrolysis to F).

Acyl glucuronides of xenobiotics, formed by intramolecular rearrangement of the 1-*O*-acyl-β-D-glucopyranosiduronic acid, have been reported by several investigators for non-steroidal anti-inflammatory agents,[13-17] probenecid,[18] clofibric acid,[19,20] and other drugs and compounds.[21-23] The formation of isomeric acyl glucuronide often causes complications in the *in vitro* or *in vivo* pharmacokinetic description or metabolic analysis of such drugs. The evidence for glucuronidase resistant forms of FG was first reported by Rachmel *et al.*[24] However, the degradation rates of FG at various pH levels were not reported. To be able to assess the stability of FG to minimize its degradation in body fluids or in biological specimens, it is necessary to obtain such information. Basic studies of the degradation of FG in aqueous solution are also necessary in order to understand the hydrolysis characteristics of FG. We studied the pH dependent degradation of FG and the rearrangements of FG and FG-iso.

MATERIALS AND METHODS

Materials Furosemide (lot No. 122F-018) and limpet (*Patella vulgata*) β-glucuronidase (Type L-II) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Furosemide tablets (40 mg, lot No. 606193) were obtained from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ, U.S.A. Bumetanide (lot No. 8193311811) was obtained from Hoffmann-La Roche, Inc., Nutley, NJ, U.S.A., dissolved into acetonitrile, and used as an internal standard in the HPLC assay for F, FG and FG-iso. 4-Chloro-5-sulfamoylanthranilic acid (CSA) (lot No. F-1) was obtained from U.S. Pharmacopeia, Inc., Rockville, MD, U.S.A. Acetonitrile and methanol were HPLC grade from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Dowex 50WX8-100 ion exchange resin (lot No. 0319 MK) was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, U.S.A. All other chemicals were of reagent grade. Purified water was used throughout the study.

Due to the rapid photodegradation of F,[11] all processes which required handling FG, FG-iso and F were done in a darkened room or at night under fluorescent lamps covered with translucent plastic frames. For the automatic HPLC assays, glass inserts were covered with aluminum foil to protect the specimens from light.

Purification of Furosemide Glucuronide Crude FG was obtained as follows: FG was obtained from the urine of human volunteers who were given an 80 mg oral dose of furosemide tablets. Combined urine samples, adjusted to pH 4.8 with 0.1 M acetate buffer, were washed twice with ethyl acetate, acidified to pH 2 with dilute hydrochloric acid and extracted twice with ethyl acetate. The ethyl acetate fractions were combined, dried with magnesium sulfate, filtered, and then concentrated using a rotary evaporator at about 40 °C. Toluene was added and the solution was again concentrated. The resultant precipitate was filtered, washed with water and dried in a vacuum

desiccator for 24 h at room temperature.

Purification of FG was made by ion-exchange chromatography (10 g of Dowex 50WX8-100) in a glass column (10 mm × 15 cm) set in a dark and cold room (4 °C). About 20 mg of crude FG was dissolved in 20 ml of water. The solution was passed through the ion-exchange column and the FG was eluted with water. The elute was occasionally checked for the presence of FG by HPLC. The HPLC chromatograms showed a clear single peak for FG and the absence of peaks for F and FG-iso. Samples used for the studies were diluted to 20 μg/ml as F equivalent in water at pH 4.0 and stored in the refrigerator (4 °C).

Assay Procedure for Furosemide Glucuronide HPLC was performed with an Altex Ultrasphere-ODS, 5 μm reversed phase column (4.6 × 250 mm) from Beckman Instruments, Inc., Berkeley, CA, U.S.A. A fluorescence spectrophotometer, Model 650-10s, with a 150B Xenon power supply (Perkin-Elmer Co., Norwalk, CT, U.S.A.) was used as a detector. Excitation and emission wavelengths were 345 and 425 nm, respectively. In order to obtain the separation of FG-iso from FG, and for the simultaneous determination of F, a gradient elution program was used. Mobile phases used in the gradient method were: solvent A— 17.6% acetonitrile, 3.6% methanol, 0.05% phosphoric acid and water at pH 3.5; and solvent B— 100% acetonitrile. The flow rate was 1.3 ml/min. The gradient program was set as follows: from 0 to 34.9 min, the mobile phase consisted of 100% solvent A; from 35.0 to 49.9 min, the mobile phase consisted of 70% solvent A and 30% solvent B; and from 50.0 to 60.0 min, 100% solvent A was used. The internal standard was eluted at 48.0 min. Sample solutions were injected into the HPLC system using a WISP 710B automatic sampler (Waters Associates, Inc., Milford, MA, U.S.A.) with a run time of 60 min. A Varian 5000 pump (Varian Associates, Palo Alto, CA, U.S.A.) was used for the gradient elution. An SP 4100 computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.) was used to obtain peak height ratios from the chromatograms.

Stability Studies of Furosemide Glucuronide The stabilities of FG and FG-iso were evaluated in samples held at 37 ± 0.1 °C in a constant temperature water bath and shaker. One milliliter of FG solution (20 μg/ml as F equivalents) was diluted to 20 ml with pre-warmed (37 °C) buffer solution. Buffer solutions between pH 1.2 and 10.0 were prepared according to U.S. Pharmacopeia XXII standards.[25] To prepare the solutions below pH 1.2, a proper amount of 0.2 M hydrochloric acid was added to the acidic buffer. The pH of each buffer solution was found to be constant throughout the study. During the study, the pH of the solutions was not adjusted. Samples from the reaction mixtures, obtained prior to analysis and at appropriate time intervals, were adjusted to pHs between 4 and 5 by the addition of 0.05 M phosphoric acid or 0.01 M sodium hydroxide to prevent further degradation of FG and FG-iso. Portions of the solution (250 μl) were mixed with 25 μl of internal standard solution (2 μg/ml bumetanide in acetonitrile), and injected into the HPLC system.

Enzymatic Hydrolysis of Furosemide Glucuronide Enzymatic hydrolysis of FG in solution was done as follows: β-Glucuronidase was dissolved in 0.1 M acetate buffer (pH 4.5, 650 units/ml). The resulting solutions (250 μl) were incubated with 250 μl of enzyme solution for 6 h at 37 °C. After the addition of 250 μl of the internal standard solution, the contents of the test tubes were mixed, centrifuged, and transferred into glass inserts for HPLC assay.

Isolation of Acyl Migration Isomers of Furosemide Glucuronide In order to isolate each FG-iso compound, a solution of FG was incubated for 48 h in pH 7.4 phosphate buffer. The solution was fractionated with an analytical column and solvent system described above at a flow rate of 1.0 ml/min, but fluorescence monitoring was omitted in order to avoid the photodegradation of FG or FG-iso. Each isolate was reinjected into the HPLC systems to confirm purity.

RESULTS AND DISCUSSION

Degradation of Furosemide Glucuronide in Neutral Solution Figure 1 shows the chromatogram of FG solution 48 h after incubation in a pH 7.4 phosphate buffer at 37 °C. Eight peaks other than those of FG (peak 8, retention time 33.5 min) and F (peak 10, retention time 41.0 min) were observed. Retention times for the peaks were: 1) 15.8; 2) 16.4; 3) 17.3; 4) 21.6; 5) 22.4; 6) 23.1; 7) 31.1; and 9) 34.0 min. Hydrolysis of the starting FG solution and the samples from the incubation solutions by β-glucuronidase resulted in the disappearance of peak 8. All other peaks remained after enzyme hydrolysis. Hydrolysis of the incubated solution with 0.5 M sodium hydroxide resulted in an increase in peak 10 (F) and the disappearance of all peaks 1 through 9.

In earlier studies concerning the intramolecular rearrangement of acyl glucuronide, 2, 3 and 4 position

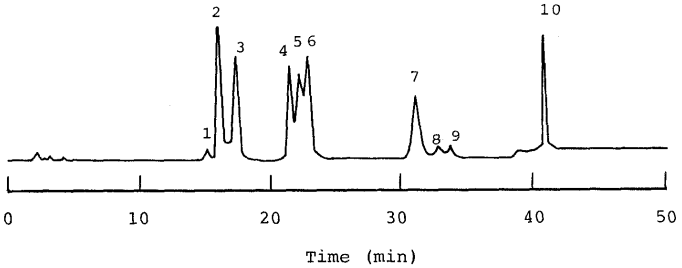

Fig. 1. Chromatogram Developed for a Solution of Furosemide Glucuronide, Its Isomers and Furosemide Following the Incubation of Furosemide Glucuronide for 48 h at pH 7.4 Phosphate Buffer and 37 °C

Furosemide glucuronide, 8; isomers of furosemide glucuronide, 1—7 and 9; furosemide, 10.

isomers were reported for bilirubin-IX,[23] probenecid,[18] clofibric acid[19,20] and Wy-18,251.[21] FG is considered to be a C-1 as 1-*O*-acyl-*β*-D-glucopyranosiduronic acid, since it is cleaved specifically by *β*-glucuronidase. Several authors have reported the presence of more than three isomers of the 1-*β*-D-glucuronides of drugs. Four isomers have been found for the glucuronide of zomepirac[13,14] and isoxepac.[17] Seven structural isomers of the glucuronide of valproic acid were found.[22] Smith and Benet[26] specifically identified 3 of the 4 fractions of acyl migration products of zomepirac glucuronide using high field NMR (one fraction was not stable enough to be isolated). They were 2, 3, and 4-*O*-acyl-*α* and *β*-isomers of zomepirac glucuronide. Dickinson *et al.* reported the intramolecular rearrangement of valproic acid glucuronide and speculated on the presence of isomers containing 2, 3 and 4-*O*-acyl-*α* and *β*-glucopyranosyluronates and on the presence of furanose type and lactone type derivatives formed from the open-chain intermediates.[22]

The maximum number of *α*- and *β*-forms of 2, 3, and 4-*O*-acyl-positional isomers of FG would account for only six acyl migration products. Because we found eight FG-iso compounds (those reverted to F upon base hydrolysis), and although they were not identified, the rearranged compounds could not be limited to products of simple acyl migration from position 1 to positions 2, 3, and 4 on the glucuronide ring. In addition, the possibility remains that unstable isomers or isomers with similar retention times were not detected. Thus, our finding of eight peaks lends support to some of the pathways hypothesized by Dickinson and coworkers.[22]

Rachmel *et al.* reported five glucuronidase resistant peaks due to an acyl migration rearrangement of FG.[24] Column and mobile phase selection probably explains the larger number of peaks found here.

pH-Dependent Rearrangement of Furosemide Glucuronide in Buffer Solutions Qualitative time-dependent profiles of the concentrations of peak heights of the FG-iso compounds, F, FG and CSA in buffer solutions at various pHs are illustrated in Figs. 2 through 4. The numbers of the peaks in Fig. 2 correspond to those in Figs. 3 and 4. Although other compounds were formed first, the apparent final product of FG-iso degradation was F, when pH values were greater than 6.0. While FG degraded rapidly, FG-iso formed rapidly and hydrolyzed to F slowly in slightly alkaline solutions. For example, in buffer solutions of pH 9.8 and 8.9, FG degraded with half lives of 2.1 and 11.7 min, respectively, but the complete formation of F took at least one day (Fig. 2). FG was completely hydrolyzed within one minute in 0.5 M sodium hydroxide solution.

In all cases, the peaks from compounds 5 and 7 were

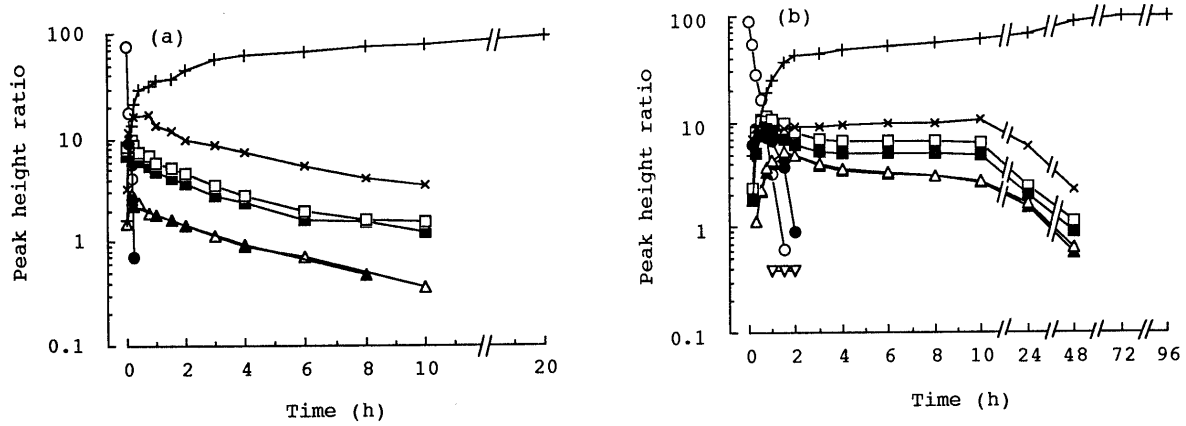

Fig. 2. Time Dependent Degradation of Furosemide Glucuronide Incubated in Buffer Solution at pH Values of 9.8 (a) and 8.9 (b) at 37 °C

○, furosemide glucuronide (fraction 8); +, furosemide (fraction 10); ▽, fraction 1; △, fraction 2; ▲, fraction 3; □, fraction 4; ●, fraction 5; ■, fraction 6; ×, fraction 7; ▼, fraction 9. Peak heights are represented relative to the initial value for furosemide glucuronide which is assigned a value of 100.

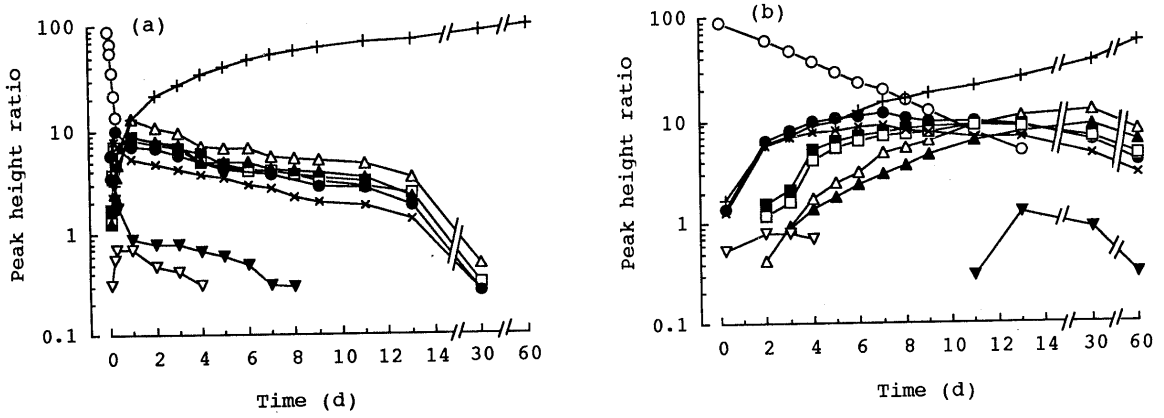

Fig. 3. Time Dependent Degradation of Furosemide Glucuronide Incubated in Buffer Solution at pH Values of 7.4 (a) and 6.0 (b) at 37 °C

Symbols are the same as Fig. 2.

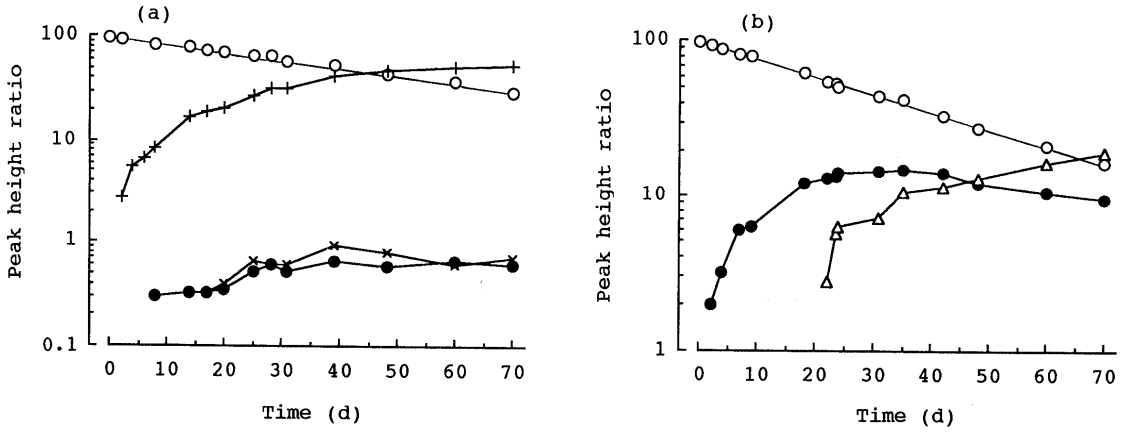

Fig. 4. Time Dependent Degradation of Furosemide Glucuronide Incubated in Buffer Solution at pH 3.7 (a) and 2.5 (b) at 37 °C
Symbols of Fig. 4 (a) are the same as Fig. 2. (b) ○, furosemide glucuronide; ●, furosemide; △, 4-chloro-5-sulfamoylanthranilic acid.

formed first, followed by the formation of the other FG-iso compounds. In weakly alkaline solutions (pHs 8.9 and 9.8), the peak 5 compound disappeared rapidly, while peak 2, 3, 4, 6 and 7 compounds disappeared slowly. The compound of peak 1 appeared under neutral and slightly alkaline conditions (pH 5 to pH 9). Both peaks 1 and 9 were small when present, and were not observed for solutions below pH 4.5 nor above pH 8.9.

The peak areas of fractions 2 and 3 were nearly identical at each pH. When the elute of peak 2 was reinjected into the HPLC, both peaks 2 and 3 appeared in the chromatogram. Likewise, when the elute of peak 3 was reinjected, both peaks 2 and 3 appeared. Thus, the compounds of peaks 2 and 3 interconverted rapidly in solution. The same results were obtained for peaks 4 and 6.

In acidic solutions, FG degraded slowly (Fig. 4). Peaks due to the acyl migration isomers of FG were not detected below pH 3.0 (Fig. 4b. at pH 2.5), but the increase in F did not correspond to the decrease in FG. Especially in a strongly acidic solution, the furosemide peak was found to be quite small. A new peak, with a retention time of 4.3 min, corresponded to that of an authentic sample of CSA. F was formed first following the hydrolysis of FG in acidic media, then F was hydrolyzed to CSA. CSA was also detected in all solutions below pH 6.0 after long incubation periods.

In earlier studies, CSA had been hypothesized to be the major metabolite of F. However, Smith *et al.* showed that CSA was an artifact which appeared when biological specimens were strongly acidified prior to their extraction into organic solvents in the analysis.[8] Our results confirm the Smith *et al.* conclusion that F or FG in biological specimens is hydrolyzed to CSA in strongly acidic solutions.

Apparent pH Rate of Degradation Profiles of Furosemide Glucuronide in Buffer Solution The decrease in FG concentration with time was found to follow first-order kinetics. As shown in Fig. 5, both below pH 2.8 and above pH 5.6, the pH rate profiles of FG degradation were linear with slopes −0.96 and +0.95, respectively. These results suggested that the degradation of unionized FG was catalyzed by hydrogen ion below pH 2.8. The degradation of ionized FG was catalyzed by hydroxy ion above pH

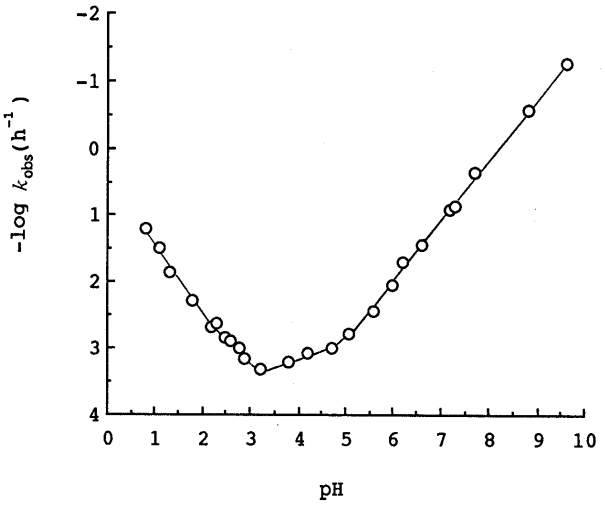

Fig. 5. pH-Rate Profile for the Hydrolysis of Furosemide Glucuronide Degradation at 37 °C

5.6. The portion between pHs 2.8 and 5.6 was probably due to the partial deprotonation of the FG molecule. From a study of FG extraction into ethyl acetate from buffer solutions at various pHs, we estimated the pK_a of FG by Eq. 1, to be 3.3.

$$P_{obs}=\frac{P}{1+K_a/[H_3O^+]} \quad (1)$$

where, P and P_{obs} are the partition coefficient and apparent partition coefficient, respectively.

The maximum stability of FG was at pH 3.2 (37 °C), where the rate constant was $4.66\times10^{-4}h^{-1}$, which corresponded to a half-life of about 62 d. Rachmel *et al.* reported that FG was quite stable at pH values less than 6.[24] In our study, 3% of the FG fraction was found to be degraded at pH 6.0 after 3 h, and FG-iso peaks were detected. The decomposition of many drugs (*e.g.* aspirin, procaine) by both base and acid catalyzed hydrolysis is well recognized.[27]

From these results, shown in Figs. 2 to 3, we concluded that the rapid degradation of ionized FG in neutral or alkaline solution is due to acyl migration into the first degradation product. Acyl migration was catalyzed

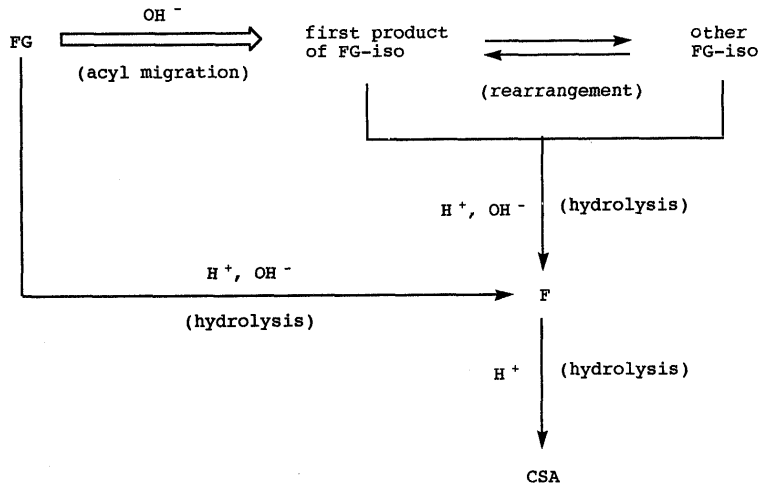

Fig. 6. Schematic Model for the Hydrolysis Pathways of Furosemide Glucuronide

TABLE I. pH-Dependent Degradation of Furosemide Glucuronide in Buffer Solutions at 37 °C

pH	k_{app} (h^{-1})	$t_{1/2}$ (h)
1	0.0324	21.4
2	0.00398	174
3	0.000562	1230
4	0.000759	914
5	0.00135	514
6	0.0100	69.3
7	0.0794	8.72
7.4	0.158	4.37
8	0.562	1.23
9	3.98	0.174
10	31.6	0.0219

specifically by the hydroxy ion.[28] We did not detect FG-iso in an acidic solution below pH 3.

Most stability studies of acyl glucuronides were carried out in neutral to alkaline solutions. However, Hasegawa *et al.* found that the maximum stability of zomepirac glucuronide was at pH 2.[13] The pH-rate profile of the degradation of zomepirac glucuronide displayed typical hydrogen ion and hydroxy ion catalyzed degradation. No evidence of acyl migration in acidic solutions was found.

Dickinson *et al.* described the degradation of valproic acid glucuronide in bile over a wide pH range (from 0 to 13).[22] Valproic acid glucuronide was not stable in solution below pH 2 after incubation for 3 h at 37 °C, and the products formed were resistant to β-glucuronidase. They characterized this process as acid catalyzed acyl migration. However, we believe that it is unlikely that hydrogen ions could catalyze acyl migration in glucuronide and suggest that the observed peaks might indicate other degradation products.

The apparent degradation rates and half-lives of FG are presented in Table I. The value of degradation half-life at pH 7.4 was somewhat smaller than the value reported by Rachmel *et al.* (5.3 h).[24] This difference might be due to the improved separation of FG and FG-iso we achieved. During the finding of HPLC conditions for the separation of FG and FG-iso compounds using other columns or mobile phases, we found fewer peaks in the chromatogram. Some of the peaks of FG-iso compounds overlapped the peak of FG in those cases.

The large number of FG-iso compounds formed from FG made it difficult to obtain individual formation and degradation rates. Figure 6 presents a simplified schematic indication of the potential degradation pathways. FG is converted to F and FG-iso by hydrolysis and through acyl migration, respectively. Under neutral to alkaline conditions, we found no degradation of F to CSA. It was obvious from Figs. 2 and 3 that the degradation of FG to F and FG-iso occurred much more rapidly than the FG-iso to F reaction. Summarizing all FG-iso compounds together, we can gain a possible understanding of the relative rates of reaction in Fig. 6. Irreversible apparent FG reaction rate constants (k_{app}), the sum of rate constants of acyl migration (k_1) and hydrolysis (k_2) were determined (Fig. 5 and Table I). The rate constants of the hydrolysis of FG-iso to F (k_3) may be determined at each pH from the simultaneous fitting of FG and F data, particularly at later time points, when FG concentrations approached zero. For example, at pH 8.9 (Fig. 2b) the calculated values for k_{app} and k_3 were 3.324 and 0.0331 h^{-1}, respectively. These values suggested that the degradation rate constant of FG to F and FG-iso was about 100 times larger than that of FG-iso to F.

The degradation of FG in solution at a neutral pH is of importance, since the pH values of blood (pH 7.4) and urine (pH 5—8) are almost neutral. At pH 7.4 and 37 °C, the half-life of FG was about 4.4 h. The half-life was about 3 d at pH 6.0. An increase of 1 pH unit for a neutral pH solution resulted in a ten-fold increase in the degradation rate constant. When the pH decreased from 7.4 to 3.2, the stability of FG increased 340 times. Thus, a wide variation existed in FG degradation rates for the solution having pH values that are usually found in collected and treated biological specimens. Although the degradation characteristics of FG in actual biological fluids were not studied here, Smith *et al.* reported an increased degradation of zomepirac glucuronide in plasma *versus* that found in a pH 7.4 buffer solution.[29]

The formation rates of FG-iso in the body are not known. However, we found small amounts of FG-iso in

human urine and plasma, suggesting that FG is partially transformed to its acyl migration isomers in the body. These β-glucuronidase resistant acyl migration isomers will be further quantified in F disposition studies, in which specific methods for stabilizing F, FG and FG-iso are utilized as described here.

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