Self-Micellization of Gemfibrozil 1-*O*-β Acyl Glucuronide in Aqueous Solution

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Purpose. Phase II metabolism involves the conjugation of a polar moiety, such as sulfate or glucuronic acid, to a (relatively) nonpolar xenobiotic. Although it might be expected that such conjugates may exhibit amphiphilic character (e.g., surface activity and potential to form micelles), no detailed study of the micellization characteristics of any drug–glucuronide conjugates has yet been reported. Therefore, the aim of this study was to investigate the solution behavior and amphiphilic characteristics of gemfibrozil 1-*O*-β glucuronide (GG), a model drug–glucuronide conjugate.

Methods. Crude GG was extracted from the urine of volunteers dosed with 600 mg of gemfibrozil, and this material was then purified by reversed-phase high-performance liquid chromatography to yield a white solid. The amphiphilic properties of GG within the bulk aqueous phase were studied by isothermal titration microcalorimetry and ^{1}H-NMR spectrometry, whereas those at the aqueous/air interface were studied by surface tensiometry.

Results. The results of each independent analytical technique were consistent with GG in aqueous solution exhibiting amphiphilic properties typical of a hydrophilic surfactant. The titration microcalorimetry and ^{1}H-NMR spectrometry data were in excellent agreement with each other, yielding critical micellization concentrations (cmc) for GG in 0.1 M acetate buffer of 18.1 ± 0.4 mM and 18.3 ± 0.3 mM, respectively. The profile and results of the surface tension measurements were consistent with GG localizing at the aqueous/air interface.

Conclusion. These results confirm the hypothesis that a glucuronide conjugate of a relatively nonpolar xenobiotic, such as gemfibrozil, behaves as an amphiphile in aqueous solution. The implications of this observation include a likely basis for the previously observed concentration–dependence in the degradation rate of the acyl glucuronides of 2-phenylpropionic acid, as well as identifying a possible broader contributory effect to the structural dependencies in biliary choleresis of different glucuronide conjugates of xenobiotics.

KEY WORDS: gemfibrozil; glucuronides; cmc; amphiphiles; microcalorimetry; NMR.

INTRODUCTION

Glucuronidation involves the enzyme-mediated attachment of a polar sugar moiety (i.e., D-glucuronic acid) to a relatively nonpolar xenobiotic to increase its aqueous solubility and enhance its potential for excretion in the bile and/or urine. When a drug molecule is conjugated with glucuronic acid via an ester linkage, the resulting 1-*O*-β acyl glucuronide metabolite is generally considered a chemically reactive species, and much recent research has been conducted with this class of metabolites as a result of their potential immunogenic properties (1). Of particular interest has been the chemical degradation and re-arrangement of glucuronide conjugates mediated via intramolecular acyl migration, where the site of esterification can migrate around the glucuronic acid ring potentially leading to various positional isomers (1).

Recently, Shackleford et al. (2) studied the degradation of (*R*)- and (*S*)-2-phenylpropionic acid 1-O-β acyl glucuronide (37°C and pH 7.4) and reported degradation half-lives for the two diastereomers of 55 and 110 min, respectively, which were substantially shorter than in a previous report of 108 and 198 min, respectively (3). It was suggested that the observed difference in degradation rates of the acyl glucuronides could involve a specific buffer effect (1), but it may arise from the different concentrations of acyl glucuronide studied (700 μM vs. 2.3 μM) with the higher concentration of acyl glucuronide affording the longer degradation half-life. As the initial acyl migration from the 1-*O*- to the 2-*O*- position of the glucuronic acid ring is considered an intramolecular (i.e., concerted) process (1,4), the reaction rate would be expected to be concentration independent. However, a possibly analogous concentration-dependent process where the rate of degradation (mediated via hydrolysis and acyl migration) is reduced at higher drug concentration has been reported for some 21-ester prodrugs of methylprednisolone (5,6). The ester prodrugs of methylprednisolone have been shown to self-associate in aqueous solution to form micelles, and the consequent increase in prodrug stability was ascribed to stabilization of the ester group within the micellar pseudophase, that is, at concentrations above the critical micellization concentration (5,6). Given that drug glucuronides typically possess both polar and nonpolar functionalities, it is possible that some of those conjugates in aqueous solution may exhibit properties that are characteristic of amphiphiles (i.e., surface activity and bulk phase micellization). The possibility that glucuronides might form micelles has been offered as an explanation for the negligible impact of iopanoate glucuronide concentration on bile osmolarity (7); however, no detailed study of the micellization of any glucuronide conjugate has yet been reported.

The aim of this study was to investigate the potential for micellization of a model drug glucuronide. Gemfibrozil 1-*O*-β glucuronide (GG) was specifically chosen as a model glucuronide because of its higher resistance to *in vitro* acyl migration and hydrolysis relative to other acyl glucuronides (8). The putative amphiphilic character of GG was studied by assessing its demicellization profile by isothermal titration microcalorimetry, and the effect of GG concentration on the resulting ^{1}H-NMR spectrometry profile and aqueous surface tension.

MATERIALS AND METHODS

Preparation of GG

Urine from healthy male volunteers who had been dosed with 600 mg of gemfibrozil (Jezil, Alphapharm Pty. Ltd.,

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Glebe, N.S.W., Australia) was acidified with 11 N HCl (pH < 2) and then immediately extracted with ethyl acetate. The solvent was removed from the extract by rotary evaporation (25°C) and the residue reconstituted with mobile phase. Semipreparative reversed-phase liquid chromatography (50: 45:5 acetonitrile/H_2O/methanol + 0.00004% v/v trifluoroacetic acid; 1.8 mL/min; λ_{abs} at 235 nm; Waters Symmetry C18 5 μm 7.8 × 100 mm) of the crude material yielded a buff colored solid after lyophilization of the appropriate pooled eluent fractions. Reconstitution of the buff colored solid in mobile phase and more extensive purification using a longer column (SymmetryPrep C18 7 μm 7.8 × 300 mm, eluent fraction between 12–16 min) yielded a white fluffy solid after lyophilization.

Characterization of GG

The white fluffy solid product of the preparative procedure was confirmed to be GG by electrospray mass spectrometry (Micromass, Platform II) and $^1H/^{13}C$-nuclear magnetic resonance spectrometry (Bruker Avance, 300 MHz), and the assessment of purity via analytical high-performance liquid chromatography peak area was >98%. The major peaks present in the electrospray mass spectrum (m/z 425.4, 249.2, 175.0, and 121.1) corresponded to those reported for the fast atom bombardment-mass spectrum (FAB-MS) of GG in a glycerol matrix (9).The details and peak assignments of the $^1H/^{13}C$-NMR spectra were as follows (s = singlet, d = doublet, m = multiplet, br = broad):

^1H-NMR (δ, ppm; conducted in D_2O): 7.35 (d, 1H, J = 7.8 Hz, C_{10}), 7.12 (s, 1H, C_7), 7.04 (d, 1H, J = 7.2 Hz, C_9), 5.75 (d, 1H, J = 7.8 Hz, $C_{1'}$), 4.28 (s, 2H, C_5), 4.07 (d, 1H, J = 9 Hz, $C_{5'}$), 3.81 - 3.75 (m, 3H, $C_{4'}/C_{3'}/C_{2'}$), 2.52 (br, 3H, C13), 2.40 (br, 3H, C12), 2.00 (s, 4H, C_3/C_4), 1.48 (s, 6H, C_{14}/C_{15})

^{13}C-NMR (δ, ppm; conducted in D_2O): 173.4 (C_1), 167.8 ($C_{6'}$), 152.3 (C_6), 131.8 (C_8), 125.9 (C_{10}), 118.8 (C_9), 116.7 (C_{11}), 108.1 (C_7), 89.6 ($C_{1'}$), 71.2 ($C_{5'}/C_{3'}$), 67.6 ($C_{2'}$), 66.9 ($C_{4'}$), 63.6 (C_5), 37.6 (C_2), 32.0 (C_3), 20.2 (C_4) 20.0 (C_{14}/C_{15}), 16.6 (C_{13}), 11.2 (C_{12})

These spectral details of GG differ slightly from those reported previously in deuterochloroform (9). Although the discrepancies may partly be the result of the different solvents used (i.e., D_2O vs. deuterochloroform), they may arise as the earlier interpretation was based on spectra that were complicated by the presence of residual solvent (9). In the present case, the high purity of GG allowed for the acquisition of full ^1H, ^{13}C and heteronuclear single quantum coherence NMR spectra without such interference.

Isothermal Titration Microcalorimetry (ITC)

Enthalpimetric titration was performed using an isothermal titration microcalorimeter (CSC, Spanish Forks, UT, USA) by titrating a 254 mM solution of GG in 0.1 M acetate buffer into 1.00 mL of blank buffer (pH 5.0) at 25°C. The enthalpy associated with each addition of titrant (5 μL) was measured and the data used to generate an enthalpimetric titration curve (ΔH_{dil} vs. GG concentration), and all experiments were conducted in triplicate. The critical micelle concentration (cmc) of GG was assessed by intersection of the linear sections on each side of the first discontinuity on the plot.

^1H-NMR Spectrometry

^1H-NMR spectra were recorded for solutions of GG in D_2O having concentrations well above and below the cmc determined by ITC (i.e., ranging between 4.9 and 245 mM). Solutions were buffered with 0.1 M perdeuteroacetate buffer, where the pD of the blank buffer was equivalent to a pH of 5. Spectra were recorded with a Bruker Avance 300 MHz spectrometer at 25°C. Plots of chemical shift vs the reciprocal of total GG concentration for selected proton signals were used to determine the cmc of GG by intersection of the linear sections on each side of the discontinuity on the plots. This experimental procedure was repeated using 1 M perdeuteroacetate-buffered D_2O.

Surface Tension

GG was dissolved in 6.0 mL of 0.1 M acetate buffer (pH 5.0) at a concentration well above the cmc values determined by ITC and ^1H-NMR. Triplicate measurements of the surface tension were made at room temperature (22.5°C) by the Wilhelmy plate method, using a glass plate (40 mm length) suspended from the arm of a torsion balance. The plate was cleaned in 15% nitric acid, rinsed with double-distilled H_2O, and dried under a stream of N_2 gas. An aliquot of the GG solution was then drawn from the sample reservoir and replaced with an equal volume of blank 0.1 M acetate buffer, before re-measurement of the surface tension. This procedure was repeated until surface tension data had been obtained for a range of GG concentrations above and below the cmc determined by ITC and ^1H-NMR.

RESULTS AND DISCUSSION

Selection of Buffer pH for Conducting Studies

Glucuronide conjugates are weak acids that are essentially 100% ionized at physiologic pH as the pK_a of the glucuronic acid moiety can be considered to range between 2.85 and 3.2 (10). Assessment of the amphiphilic character of GG could not be readily conducted at pH 7.4 because acyl glucuronides such as GG are subject to significant degradation (through acyl migration and hydrolysis) at near neutral pH. A pH value of 5.5 was chosen for these studies as it is considered to afford maximal chemical stability for acyl glucuronides (11) while maintaining the compound in a predominantly ionized state

Isothermal Titration Microcalorimetry

The calorimetric titration experiments involved measurement of the enthalpy change associated with addition of successive aliquots of a 254 mM solution of GG to 0.1 M acetate buffer that had initially contained no GG. The form of the enthalpimetric titration plot for GG (Fig. 1) is comparable with the sigmoidal plots typically observed for hydrophilic surfactants (12–17). The measured enthalpy changes evident in the first portion of the curve are considered to be the sum of the enthalpies of demicellization, micelle and monomer dilution, and monomer surface relocalization. At GG concentrations close to the cmc, the air-liquid interface becomes saturated with GG monomer and the monomers are no longer able to localize to the interface. Similarly, the demi-

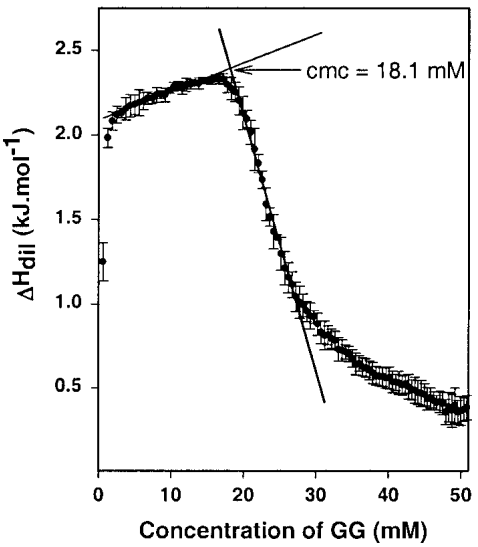

Fig. 1. Molar enthalpy changes resulting from consecutive addition (5-μL aliquots) of a concentrated gemfibrozil 1-*O*-β glucuronide solution (254 mM) into 0.1 M acetate buffer at 25°C. Data are presented as mean ± SE of n = 3 replicate titrations.

cellization of micelles added to the solution progressively becomes less as the total concentration of GG in solution is increased. At GG concentrations far above the cmc, the measured enthalpy change corresponds to that for dilution of intact micelles.

Various approaches are described in the literature for the determination of cmc ranges from calorimetric titration data, with one of the most frequently used being based on the relationship between cumulative enthalpy and the surfactant concentration with the cmc being the point of intersection of lines extrapolated from the linear regions on either side of the discontinuity (12). However, it has been noted (13) that this treatment is not straightforward when the change in enthalpy of dilution (ΔH_{dil}) is not constant at surfactant concentrations above and below the transition region, as is the case for GG. Hence, we have chosen to use an alternative method, which determines the cmc as the point of intersection of the lines extrapolated from the linear regions on either side of the first discontinuity in a plot of the ΔH_{dil} vs. concentration (14–16), and these data are presented in Fig. 1. Using this approach, the apparent cmc for GG was determined to be 18.1 ± 0.4 mM. A further calculation method assumes that the cmc corresponds to the inflexion point of the transition region observable in Fig. 1, and it is typically determined as the minimum (or maximum) of the first derivative of the ΔH_{dil} vs. concentration plot (13,17,18). However, this interpretation of the bulk surfactant property profile suggests that the cmc is the concentration range where micelle formation is maximal. This is at variance with the long held meaning of the cmc, which is regarded as the concentration range where micellization first begins. To confirm the results of the ITC study, an independent method for estimating the cmc of GG was required and we chose to study the concentration–dependence of the ^{1}H-NMR profile of GG.

NMR Spectrometry

NMR spectrometry has been used extensively to study the self-association of compounds in aqueous solution as the signals evident in NMR spectra may change their chemical shift (δ) in accordance with the molecular state of association in solution (i.e., monomeric or micellar; Ref. 19). Figure 2 presents the GG concentration-dependent changes in the chemical shift of a number of (^{1}H) signals of GG when present at concentrations below (Fig. 2A, 4.9 μM) and above (Fig. 2B, 245 μM) the cmc value determined by ITC.

Qualitatively, each of the signals assigned to protons on the gemfibrozil portion of GG experienced an upfield shift with increasing GG concentration, whereas the chemical shifts of (most) signals assigned to protons on the sugar moiety were largely independent of the GG concentration. This suggests that only those protons attached to the gemfibrozil moiety experienced a change in their magnetic environment upon micellization, and this is consistent with GG being oriented within aggregated structures where the gemfibrozil "tail" is positioned within the hydrophobic core of the micelle whereas the glucuronic acid "head" is positioned within the peripheral surface region of the micelle.

The magnitudes and direction of the changes in chemical shift as a function of GG concentration (and micellization) described in Table I suggest that the protons on the gemfibrozil tails within the interior of the micelle were shielded by ring current effects from the aromatic groups of GG. Similar behavior has been observed previously for ω-phenylalkyltrimethylammonium bromides (19), a number of penicillins (20,21), and ω-phenyldecanoate (22).

Although the majority of those signals that change

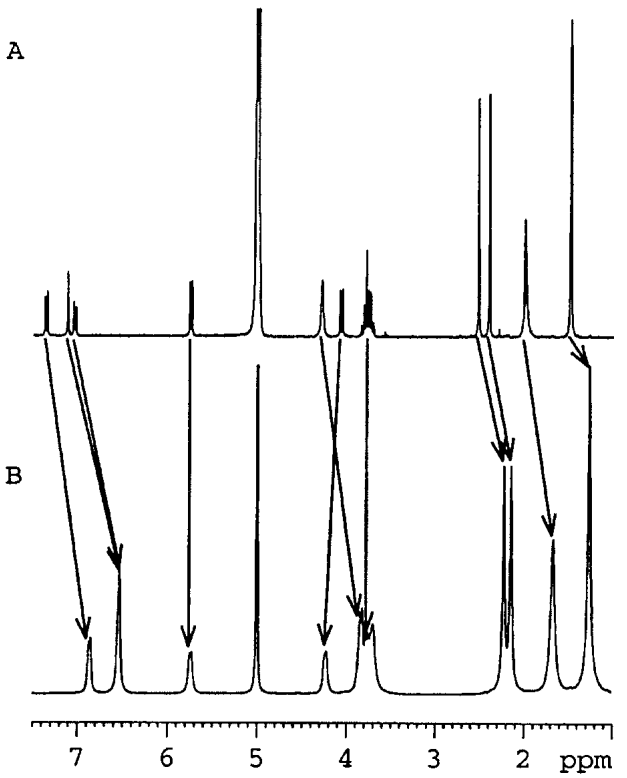

Fig. 2. ^{1}H NMR spectra of gemfibrozil 1-*O*-β glucuronide in 0.1 M perdeuteroacetate-buffered D_2O at concentrations above and below the critical micelle concentration determined by titration microcalorimetry. Arrows indicate the change in chemical shift of a number of signals on increasing the gemfibrozil 1-*O*-β glucuronide concentration from 4.9 mM (A) to 245 mM (B)

Table I. Magnitude of the Total Change in Chemical Shift of Each ^{1}H-NMR Signal When GG Concentration Increases from 4.9 to 245 mM

Proton	Δδ (ppm)
H_7	–0.58
H_5	–0.579
H_{10}	–0.487
H_9	–0.49
H_3 and H_4	–0.323
H_{13}	–0.291
H_{12}	–0.253
H_{14} and H_{15}	–0.218
$H_{5'}$	0.172

Note: Negative values indicate an upfield shift. Numbering of protons corresponds to that presented in Fig. 1.

chemical shift do so in an upfield direction, the $H_{5'}$ signal was curious in that it moved downfield as GG concentration increased above the cmc. In a similar manner, the ω-methyl proton signal of *n*-hexyl sulfate was found to move in the opposite direction to the other proton signals (i.e., downfield rather than upfield) as concentration of that solute increased above the cmc (23). No explanation was offered for that observation, presumably because despite its trend being opposite, the plot of δ vs. 1/C for that signal was comparable with those of the other protons within the molecule. However, this is not the case for the $H_{5'}$ signal of GG because the postmicellar region of its δ vs. 1/C plot is clearly nonlinear as evident in Fig. 3. Given that $H_{5'}$ is positioned α to the carboxyl group of the glucuronic acid ring, we investigated the possibility that the nonlinearity was caused by a change in the extent of ionization of the acid as GG concentration approached that of the deuteroacetate present in the buffer (0.1 M). When the ^{1}H-NMR experiment was repeated at the same pH but higher buffer capacity (1.0 M), the $H_{5'}$ chemical shift was independent of GG concentration, suggesting that the shift observed when 0.1 M buffer was used was probably an artefact arising from a small change in extent of ionization of the carboxylic acid group.

The plots presented in Fig. 3 show the relationship between δ and GG concentration for a number of the ^{1}H-NMR signals and they are entirely consistent with that expected for a compound undergoing micellization in solution (21–25), i.e., δ is essentially independent of GG concentration below the cmc; however, it changes linearly with the reciprocal of GG concentration above the cmc. As the δ vs. 1/C plot for each signal can be separated into distinct pre- and postmicellar regions, the intersection of the linear regions on each side of the discontinuity of each plot provides an estimate of the cmc. The mean (± SD) value calculated from all the signals for GG was 18.3 ± 0.3 mM when the experiment was conducted using 0.1 M buffer, and this value is in excellent agreement with the estimate of 18.1 mM, determined from the position of the first discontinuity on the enthalpimetric titration plot (Fig. 1).

Interestingly, when the experiment was repeated using 1 M buffer (data not shown), the cmc was reduced to the extent that the plateau of the plot was not clearly evident at the lowest concentration of GG measured (6.7 mM). One possibility is that the reduced cmc was the result of the increased

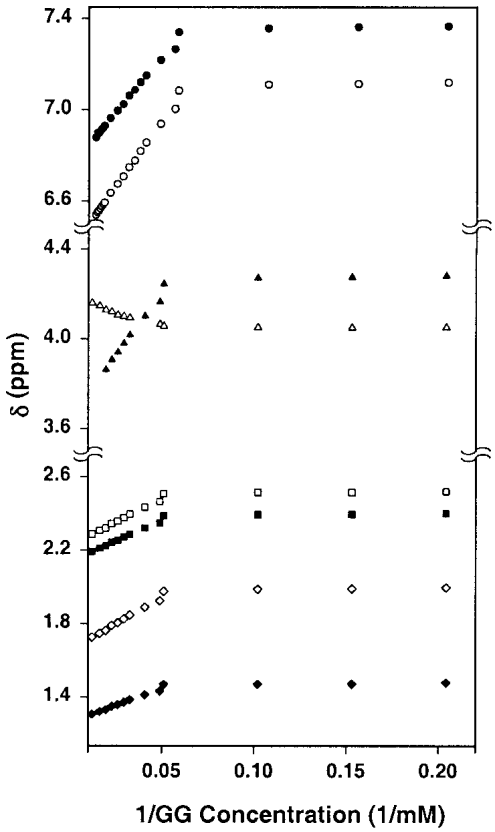

Fig. 3. Variation of the ^{1}H chemical shift (δ) with the reciprocal of the solute concentration for a number of the protons attached to gemfibrozil 1-*O*-β glucuronide dissolved in 0.1 M perdeuteroacetate-buffered D_2O. (●) H_{10}, (○) H_7, (△) $H_{5'}$, (▲) H_5, (□) H_{13}, (■) H_{12}, (◇) H_3 and H_4, and (◆) H_{14} and H_{15}.

ionic strength, as such behavior has been observed previously with numerous other surfactants (20,21,23). Alternatively, the cmc determined in 0.1 M buffer may have been artefactually elevated by changes in the extent of ionization of the glucuronic acid carboxyl as the concentration of GG exceeded the buffer capacity. The pH of a 0.1 M buffer solution containing 103 mM GG was 3.79, whereas at a GG concentration of 14.8 mM, the pH was 4.65. Assuming the pK_a of GG to be 3.0, the measured pH values correspond to fractional ionizations of GG of 86.0% to 97.8%, respectively. Although the influence of pH changes on the cmc determination could potentially have been minimized by conducting the experiments in buffer at a pH far greater than the pK_a of GG, it was impossible to do so because of the significant chemical instability of acyl glucuronides outside the pH range of 3–5 (1,11). The use of buffers at higher pH would only have caused GG to degrade to a number of possible impurities, and each of these could potentially affect any cmc estimate obtained.

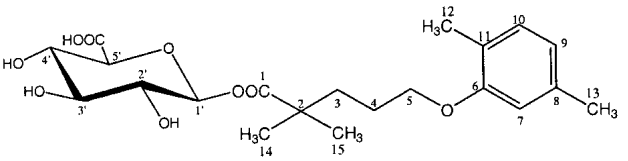

Fig. 4. Structure of gemfibrozil 1-*O*-β glucuronide.

Surface Tension

Given that the structure of GG is similar to sugar surfactants possessing a hydrophobic tail with a polar carbohydrate head group, it was expected that the relationship between surface tension and GG concentration would be comparable to that found for those compounds (26–28). In light of the results of the ^{1}H-NMR study, separate surface tension experiments were performed using solutions containing 0.1 M and 1 M acetate, and the data are presented in Fig. 5.

The qualitative relationship between surface tension and GG concentration was similar at each buffer concentration, although there was an obvious difference in the position of the plots relative to GG concentration. As the concentration of GG increased, surface tension initially fell to a minimum value before increasing slightly to an apparent plateau value of approximately 40 mN/m. The fall in surface tension with increasing solute concentration is attributed to the effect of increasing amounts of surfactant localized to the aqueous/air interface (29), thus one expects the measured surface tension to plateau at the point where the interface becomes saturated with solute. It was therefore unexpected that upon reaching a minimum value, surface tension began to rise before reaching a plateau. However, plots showing similar trends have recently been published for some sucrose monoether surfactants (27), and it was suggested that the observed behavior was caused by the presence of an impurity, possibly the surfactant's α-anomer arising from mutarotation of the sugar moiety. Although the trend observed for GG may have resulted from the presence of an unidentified impurity, it definitely could not have been the result of an α-anomer because mutarotation of the sugar moiety is prevented by the fact that the glucuronic acid is cyclized through an acetal (rather than a hemiacetal) carbon. It could, however, arise through a 2-*O* rearrangement isomer of GG (via acyl migration from the 1-*O* position), although there was no evidence of the formation of that product in the previous NMR experiments.

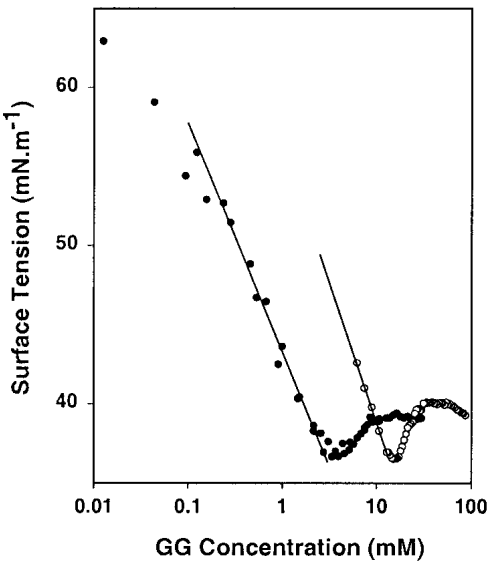

Fig. 5. Relationship between surface tension and gemfibrozil 1-*O*-β glucuronide concentration when gemfibrozil 1-*O*-β glucuronide is dissolved in 0.1 M (open symbols) and 1 M (closed symbols) acetate-buffered H_2O. Each data point is the mean of n = 3 measurements, and error bars are within the diameter of the data points. Straight lines correspond to the fits from which the surface area per molecule was calculated.

According to the Gibbs isotherm, the limiting slope of a plot of surface tension vs. ln C can be used to calculate the area occupied per molecule (nm^2) localized to the aqueous/air interface (29). That analysis yielded values of 0.53 and 0.65 $nm^2 \cdot molecule^{-1}$ from the data generated at 0.1 and 1 M buffer concentrations, respectively. The cross-sectional and in plane dimensions of GG (calculated by hypothetical gas-phase molecular modeling using CHEM-3D Ultra) predicted an area/molecule for GG of 0.28 nm^2 and 1.44 nm^2 when oriented perpendicular or parallel to the interface, respectively. The fact that the experimentally determined values for the area/molecule were between these extremes suggests that GG orients at an angle of 60–70° to the plane of the aqueous/air interface.

IMPLICATIONS AND CONCLUSIONS

In this communication, we have shown that GG forms micelles in aqueous solution, and assuming that other glucuronides behave in a similar manner, then these observations may explain the basis for the previously observed concentration-dependence for the degradation of the glucuronides of (R)- and (*S*)-2-phenylpropionic acid (2). However, in addition to being of interest with regard to studies of degradation for these compounds, these results are also of interest from a physiologic perspective. For example, as glucuronides are often avidly excreted into bile (such as valproate glucuronide), it has been suggested that the high concentrations of conjugate elicit a choleretic effect because of the increase in the osmotic pressure of the fluid within the bile canaliculi (30,31). It is noteworthy, however, that choleresis is not observed for all glucuronides excreted in bile, with GG being a glucuronide for which bile flow appears to be independent of biliary glucuronide concentration (32). Similarly, it was reported that after administration of iopanoic acid (a cholecystographic agent) to dogs, although iopanoate glucuronide was excreted into bile, there was no measurable concomitant change in biliary osmolarity despite the expectation that the measured concentrations of conjugate should have caused a significant increase in biliary osmolarity (7). It was speculated that the discrepancy was most likely the result of iopanoate glucuronide forming osmotically inactive micelles (7). In the present study, we have conclusively demonstrated that GG, as a model glucuronide conjugate, forms micelles in aqueous solution. Our current data concerning glucuronide micellization may offer an explanation for the different choleretic effects between the glucuronides of valproate and a number of its structural analogues (33), and support for the hypothesis of Mudge and Cooke (7) that iopanoate glucuronide forms micelles in bile, thereby behaving in a manner similar to bile salts. One might expect that the glucuronides of the longer chain valproate analogues (>C5) will form micelles (either by self-micellization or by co-micellization with bile salts) at lower concentrations, preventing them from eliciting a choleretic effect because of increased biliary osmolarity. Conversely, the apolar region of valproate glucuronide (and its short-chain congeners) may be too small for the molecules to form micelles at relevant concentrations in bile, thus the concentration of conjugates may result in increased bile osmolarity.

It is unclear at this point whether micellization of glucuronides in bile will alter those compounds' pharmacokinetic or toxicological properties. However, given that some glucuronides are subject to enterohepatic recirculation (34,35), differences might arise if gastrointestinal processing of micellar glucuronides was different to that for monomeric glucuronides.

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