Quantitative estimation of the hydrophilic–hydrophobic balance of mixed bile salt solutions

Douglas M. Heuman[1]

Divisions of Gastroenterology, Medical College of Virginia and McGuire Veterans Administration Medical Center, Richmond, VA 23298

Abstract This paper describes the derivation of a bile salt monomeric hydrophobicity index that quantitatively defines the composite hydrophilic–hydrophobic balance of a mixture of bile salts. The index is based on the logarithms of bile salt capacity factors determined using reversed phase high performance liquid chromatography (HPLC) (stationary phase octadecyl silane; mobile phase methanol–water 70:30 w/w, ionic strength 0.15). It has been standardized arbitrarily to set indices of taurocholate and taurolithocholate to 0 and 1, respectively. Indices of tauroursodeoxycholate, taurohyodeoxycholate, taurochenodeoxycholate, and taurodeoxycholate were found to be −0.47, −0.35, +0.46, and +0.59, respectively. Whereas capacity factors and hydrophobicity indices of taurine-conjugated bile salts were constant for pH 2.8–9.0, the hydrophilic–hydrophobic balance of glycine-conjugated and unconjugated bile salts was strongly influenced by pH. At alkaline pH (>8.5), hydrophobicity indices of fully ionized unconjugated (n = 4) and glycine-conjugated (n = 6) bile salts differed by only 0.14 ± 0.02 and 0.05 ± 0.01, respectively, from those of the corresponding taurine conjugates. At acid pH (<3.5) the hydrophobicity indices of four unconjugated bile acids (protonated form) exceeded those of the corresponding salts (ionized form) by 0.76 ± 0.04; indices of six glycine-conjugated bile acids exceeded those of the corresponding salts by only 0.26 ± 0.03. Capacity factors of the salt forms of cholate and its conjugates increased dramatically with increasing ionic strength of the mobile phase; retention of the protonated forms (cholic and glycocholic acids) was only minimally influenced by ionic strength. Thus the difference in hydrophilic–hydrophobic balance between a bile acid and its corresponding salt decreases with increasing ionic strength. Examples are given of calculation of hydrophobicity indices for biliary bile salts (fully ionized) from four species under conditions of intact enterohepatic circulation. Mean values, from least to most hydrophobic, were: rat (−0.31) < dog (0.11) < hamster (0.22) < human (0.32). This study provides a rational basis for calculating the hydrophilic–hydrophobic balance of mixed bile salt solutions over a broad range of pH.—**Heuman, D. M.** Quantitative estimation of the hydrophilic–hydrophobic balance of mixed bile salt solutions. *J. Lipid Res.* 1989. **30:** 719–730.

Supplementary key words reverse phase • high performance liquid chromatography • partition coefficient • bile acids and salts • cholesterol • quantitative structure–activity relationships

Bile salts are natural detergents that solubilize lipids in the intestinal tract and in bile. Their detergency derives from the fact that they are quasi-planar amphiphiles, with surfaces of differing polarity. The relative balance of hydrophobic and hydrophilic properties of the common C-24 bile salts is determined by their state of ionization, by the number, position and orientation of hydroxyl groups, and by the presence and nature of ring or side chain esters (taurine, glycine, sulfate, glucuronate) (1).

In 1982, Armstrong and Carey (2) reported that the relative hydrophobicity of individual bile salts, as determined by reversed phase HPLC, correlated directly with equilibrium micellar cholesterol solubilizing capacity. Subsequent studies suggested that many other physicochemical and biological properties of individual bile salts also may reflect their hydrophilic–hydrophobic balance as determined in this manner (see Discussion).

However, attempts to assess the importance of hydrophilic–hydrophobic balance as a determinant of biological properties of bile salts have been impeded by the fact that natural bile salt pools invariably contain multiple bile salts, with various glycine/taurine conjugation ratios and various ratios of acid:salt forms. It is possible to manipulate the hydrophilic–hydrophobic balance of the bile salt pool by altering the proportions of different bile salts in the enterohepatic circulation via feeding or infusion of exogenous bile acids. However, these manipulations do not result in a completely homogeneous bile salt pool; furthermore, their net effect on the hydrophilic–hydrophobic balance of the bile salt pool is not always straightforward. For example, feeding of chenodeoxycholic acid to humans enriches the bile salt pool with this relatively hydrophobic bile salt, but leads to the virtual disappearance of deoxycholate, a more strongly hydrophobic bile salt (3–5). In rats, the situation is rendered even more complex because

Abbreviations: HPLC, high performance liquid chromatography; HI, hydrophobicity index; C, cholate; DC, deoxycholate; CDC, chenodeoxycholate; UDC, ursodeoxycholate.

[1]Address for reprint requests: Box 711, MCV Station, Richmond, VA 23298.

chenodeoxycholate is partially converted to strongly hydrophilic α- and β-muricholates (6–8).

The bile salt hydrophobicity index derived here addresses this problem by providing a rational means by which the overall or composite hydrophilic–hydrophobic balance of a solution containing two or more bile salts may be quantified. The index is based on reversed phase HPLC capacity factors. The effects of pH and ionic strength on the bile salt hydrophobicity index are presented, along with an example of its application in comparing the overall hydrophilic–hydrophobic balance of bile salts in bile from different mammalian species.

MATERIALS AND METHODS

Materials

Unconjugated and conjugated bile salts used in these studies were obtained from Calbiochem-Behring, La Jolla, CA, with the exceptions of glycohyodeoxycholic acid and β-muricholic acid, which were obtained from Steraloids, Wilton, NH, and ursocholic acid, which was obtained from GIPharmex, Milan, Italy. All were >96% pure and >99% free of bile acid contaminants as assessed by gas–liquid chromatography (9); they were used without further purification. Radiolabeled bile salts, including [11,12-^{3}H]ursodeoxycholate (37 Ci/mmol), [24-^{14}C]cholate (52 mCi/mmol), [24-^{14}C]chenodeoxycholate (50.5 mCi/mmol), and [24-^{14}C]taurocholate (46.7 mCi/mmol), were obtained from NEN-Dupont, Boston, MA; [24-^{14}C]deoxycholate (58.2 mCi/mmol) was purchased from Amersham, Arlington Heights, IL. All were >99% free of radiolabeled bile acid contaminants as judged by reversed phase HPLC with on-line scintigraphic detection, and were used without further purification. Sodium acetate, sodium chloride, potassium phosphate monobasic, potassium hydroxide, hydrochloric acid, and Tris-HCl were obtained from Sigma, St. Louis, MO; all were of the highest grades available. HPLC-grade solvents (water and methanol) were obtained from American Burdick and Jackson, Muskegon, MI.

Methods

Bile salt hydrophilic–hydrophobic balance was determined using reversed phase high performance liquid chromatography (HPLC); the same system was also used for bile acid analysis. Hardware consisted of a Beckman 320 chromatographic system with a Beckman 117 solid state radioactivity detector (Beckman Instruments, Columbia, MD), an Isco V4 variable wavelength detector, and an Isco Foxy fraction collector (Isco Scientific, Lincoln, NE). Bile salts were separated on an Altex Ultrasphere ODS (octadecyl silane) column (5 μm particle size, 4.5 mm × 250 mm), equipped with a 4.5 mm × 50 mm precolumn of the same material (Beckman Instruments, Columbia, MD). Because extremes of pH can damage bonded silica columns by causing detachment of stationary phase molecules from the silica support, an additional precolumn containing Ultrasphere ODS was used immediately proximal to the sample injector, as described by Atwood, Schmidt, and Slavin (10). This arrangement saturates the mobile phase with minute amounts of octadecanol and thereby protects the analytical column. No deterioration of column performance was observed over the course of these studies.

Mobile phase consisted of 70% aqueous methanol (70% methanol and 30% water, w/w). Solvents were HPLC grade and were filtered (0.2 μm Millipore) immediately prior to use. Mobile phase pH was determined using a glass pH electrode with aqueous reference solution; we refer to this measurement as "apparent pH" because true hydrogen ion activity differs slightly from the measured pH unless the solvent compositions of the reference and test solutions are identical (11). Buffering agents used included monobasic potassium phosphate (apparent pH 2.5–4.5), sodium acetate (apparent pH 4.5–7.0), and Tris-HCl (apparent pH 7.0–9.0). In order to maintain a constant ionic strength of 0.15, we adjusted the apparent pH of buffers containing 0.01 M sodium acetate/0.14 M sodium chloride or 0.01 M potassium dihydrogen phosphate/0.14 M sodium chloride by adding 0.04 M HCl/0.15 M NaCl. The apparent pH of 0.01 M Tris-HCl/0.14 M NaCl buffer was adjusted using 0.04 M KOH/0.15 M NaCl. This approach represents a modification of the constant ionic strength buffers described by Bates (11). For experiments determining the effect of ionic strength on HPLC capacity factors, buffers were prepared as above but concentrations of sodium chloride in the buffer solution and the titrating solution were increased or decreased by fixed amounts.

For determination of HPLC capacity factors, 20-μl samples containing 4 nmol of each bile salt in pure methanol were analyzed isocratically at ambient temperature (25°C) with a solvent flow rate of 1.0 ml/min. Conjugated bile salts were detected by absorbance at 200 nm. An initial deflection corresponding to the elution of the sample solvent (methanol) was observed in each chromatograph at approximately 2.76 min. Radiolabeled unconjugated bile salts were detected by collection of column effluent in 30-sec intervals; to each 0.5-ml sample was added 10 ml of ACS (Amersham, Arlington Heights, IL) with subsequent liquid scintillation counting using external standard quench correction. In later experiments, chromatographic peaks of labeled bile acids also were detected with on-line solid state scintigraphy; the two approaches gave identical measures of chromatographic retention.

For studies of mammalian bile, male Sprague-Dawley rats (250–350 g) and male Syrian hamsters (80–120 g) were obtained from Charles River, Boston, MA. Samples of gallbladder bile (hamster, n = 4) or common bile duct bile (rat, n = 5) were obtained under general anesthesia. Male mongrel dogs (n = 3) weighing 5–10 kg were obtained from local suppliers; they were fasted overnight prior to surgery. Bile was aspirated from the gallbladder at laparotomy under methoxyflurane inhalant anesthesia. Human bile samples were obtained from gallbladders of patients undergoing elective cholecystectomy for gallstones (n = 5). Conjugated bile salts were quantified by HPLC following chloroform–methanol extraction, as described by us previously (12).

Theoretical considerations

Derivation of the hydrophobicity index. Equilibrium partition of compounds between polar and nonpolar solvents is a generally accepted method of quantifying hydrophilic–hydrophobic balance (13, 14). In recent years, alternative methods using reversed phase chromatography have been utilized extensively (2, 15, 16). The two approaches in theory provide similar information. As summarized by Melander and Horvath (17) and Armstrong and Carey (2), the thermodynamic equilibrium partition coefficient K of a bile salt x from the polar mobile phase to the nonpolar stationary phase of a reversed-phase liquid chromatographic system is defined as

$$K_x = [BS_x]st/[BS_x]mo. \qquad Eq.\ 1$$

$[BS_x]st$ and $[BS_x]mo$ refer to equilibrium concentrations of bile salt x in the stationary and mobile phases, respectively. The basic measure of retention in isocratic HPLC is the capacity factor; the chromatographic capacity factor k_x of a bile salt is defined as

$$k_x = (V_x - V_0)/V_0 \qquad Eq.\ 2$$

where V_x is the volume of mobile phase required to elute the bile salt peak and V_0 is the volume required to elute a non-retained solute (typically water or methanol). The capacity factor is directly proportional to the partition coefficient K_x:

$$k_x = \phi K_x \qquad Eq.\ 3$$

where ϕ is the phase ratio, defined as the ratio of the volumes of the stationary to mobile phases within the column (17). The magnitude of ϕ depends upon the characteristics of the individual column.

The standard free energy ΔG_x (kcal/mole) for transfer of a bile salt in the partitioning process is given by

$$\Delta G_x = -RT \ln K_x = -RT \ln k_x - RT \ln \phi \qquad Eq.\ 4$$

where R and T are the gas constant and absolute temperature, respectively (17).

It is convenient to express the free energy of partition relative to that of a bile salt standard. A taurine-conjugated bile salt is ideal for this purpose because its retention is unaffected by changes in pH (vide infra). We have chosen arbitrarily to use taurocholate (tc) as the standard. A capacity factor relative to taurocholate k' may be defined as:

$$k'x = k_x/k_{tc}. \qquad Eq.\ 5$$

Substituting in equation 4,

$$\Delta G_x - \Delta G_{tc} = -RT \ln (k'_x). \qquad Eq.\ 6$$

The use of a relative capacity factor thus has the effect of eliminating the term ϕ, and $\Delta G_x - \Delta G_{tc}$ for a given mobile and stationary phase should theoretically be independent of individual column characteristics.

The free energies of independent chemical processes are additive. We and others (16) have found that retention factors of bile salts are similar whether chromatographed singly or in various mixtures, and are independent of sample mass over a wide range. Therefore, bile salt monomers at low concentrations partition independently, and the net free energy of partition of all bile salts in a mixture (again in kcal/mole) is given by

$$\Delta G = \sum_{x=1}^{n} \Delta G_x \times F_x \qquad Eq.\ 7$$

where F_x is the mole fraction of bile salt x in a mixture of n different bile salts. Substituting equation 6 into equation 7 yields

$$\Delta G = \Delta G_{tc} - RT \sum_{x=1}^{n} F_x \ln (k'_x) \qquad Eq.\ 8$$

A bile salt monomeric hydrophobicity index may be defined arbitrarily as

$$HI = \sum_{x=1}^{n} F_x \times \ln(k'_x)/\ln(k'_{tlc}) \qquad Eq.\ 9$$

where k'_{tlc} is the relative capacity factor of taurolithocholate. Equation 8 then may be simplified to

$$\Delta G = HI \times [-RT \ln(k'_{tlc})] + \Delta G_{tc}. \qquad Eq.\ 10$$

Capacity factors (and hence also k'_x and ΔG_x) of taurine-conjugated bile salts are constant over the entire physiologic range of pH assuming ionic strength and temperature remain constant (see Fig. 2). Equation 10 therefore demonstrates that the hydrophobicity index, as defined, is a linear function of the standard free energy change for partition of a mixture of bile salts between the stationary and mobile phases.

For a homogeneous solution of a single bile salt, n = 1, and the hydrophobicity index HI_x for that bile salt is identical to $\ln(k'_x)/\ln(k'_{tlc})$. Equation 9 may therefore be simplified finally to

$$HI = \sum_{x=1}^{n} HI_x F_x. \qquad \textit{Eq. 11}$$

The factors k'_{tc} and k'_{tlc} in the above equations are arbitrary but rational. Taurine conjugates were chosen because their capacity factors are not pH-dependent, and cholate and lithocholate because they are, respectively, the most hydrophilic and most hydrophobic of the major bile salts in human bile. Because k'_x is defined relative to taurocholate, k'_{tc} is 1 by definition, and HI for taurocholate (proportional to log k'_{tc}) is therefore 0. The constant $1/\ln(k'_{tlc})$ adjusts the scale by which HI is measured to set HI for taurolithocholate to 1. Bile salts or bile salt mixtures which are less hydrophobic than pure taurocholate (for example, tauroursodeoxycholate) have negative HI values by this definition.

Effect of pH and ionic strength. Because bile acids are ionizable compounds, their relative affinity for mobile versus stationary phase may be expected to change with changes in their state of ionization. Two features of the aqueous medium strongly influence the state of bile acid ionization—pH and ionic strength (18). For most organic acids, hydrophobicity of the protonated, uncharged form is greater than that of the ionized form. The relative abundance of these two forms is a function of the ionization constant and the pH. The effect of pH on the HPLC capacity factor is described by the following formula (19):

$$k_x = \frac{k_{HA} + (k_{A^-} \times k_I/[H+])}{(1 + k_I/[H+])} \qquad \textit{Eq. 12}$$

where k_{HA} refers to the HPLC capacity factor of the protonated form (HA), k_{A^-} is the HPLC capacity factor of the ionized form (A^-), and k_I is the bile acid ionization constant:

$$k_I = [H+] \times [A^-]/[HA]. \qquad \textit{Eq. 13}$$

According to this formula, the relationship between pH and the capacity factor of an organic acid is expected to be sigmoidal. At extremes of low or high pH, the measured capacity factors approximate k_{HA} and k_{A^-} (17). At the pK_a (that is, the pH at which $[H+] = K_I$, $[A^-] = [HA]$ and equation 12 simplifies to

$$k_x = (k_{HA} + k_{A^-})/2. \qquad \textit{Eq. 14}$$

Thus from a plot of k_x versus pH, one can estimate the ionization constant and pK_a of a bile acid in a given mobile phase (17). This is illustrated in **Fig. 1.**

Ionic strength is another factor that may strongly influence partition of organic acids between polar and nonpolar media (18). Ionic strength is defined (19) as

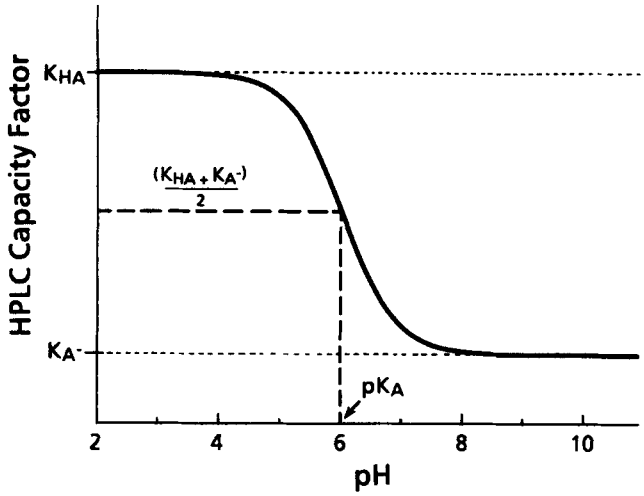

Fig. 1. Idealized plot of HPLC capacity factor (ordinate) vs. pH (abscissa) for an organic acid, from equation 12 (see Theoretical Considerations). K_{A^-} and K_{HA} represent the capacity factors of the ionized salt and protonated acid forms, respectively; the difference between these values reflects the change in hydrophilic-hydrophobic balance attributable to ionization. At extremes of pH, measured capacity factors will converge on these values asymptotically. A horizontal line midway between K_{A^-} and K_{HA} intersects the plot at an inflection point which corresponds to the pK_a ($-\log K_I$). In this example, $pK_a = 6$.

$$IS = \sum_{i=1}^{n} C_i \times z_i^2/2 \qquad \textit{Eq. 15}$$

where z_i is the charge and C_i is the concentration of ionic species i in a solution containing n different ions. Apparent hydrophobicity of bile salts increases with increasing ionic strength, a phenomenon that Small (18) has attributed to partial neutralization of the negative charge on the bile salt by association with cations. An ionic strength of approximately 0.15 M approximates that of blood and bile under normal circumstances, and this "physiological" value therefore has been employed in these studies.

RESULTS

Capacity factors (k_x) of four common bile salts in their unconjugated, glycine-conjugated, and taurine-conjugated forms are plotted as a function of pH in **Fig. 2.** For taurine-conjugated bile salts, k_x was unaffected by varying pH from 2.8 to 9.0, provided that ionic strength was held constant. This is consistent with the previous observation that pK_as of taurine conjugates are extremely low, probably on the order of −1.5 to 1.5 (20); thus taurine conjugates are expected to remain fully ionized even at acid pH. In contrast, retention of glycine-conjugated and unconjugated bile acids varied with pH. At pH ≥ 8.5 (unconjugated) or ≥7.5 (glycine-conjugated), the plot of k_x versus pH is essentially flat; thus k_x at alkaline pH approximates the capacity factor of the pure ionized bile

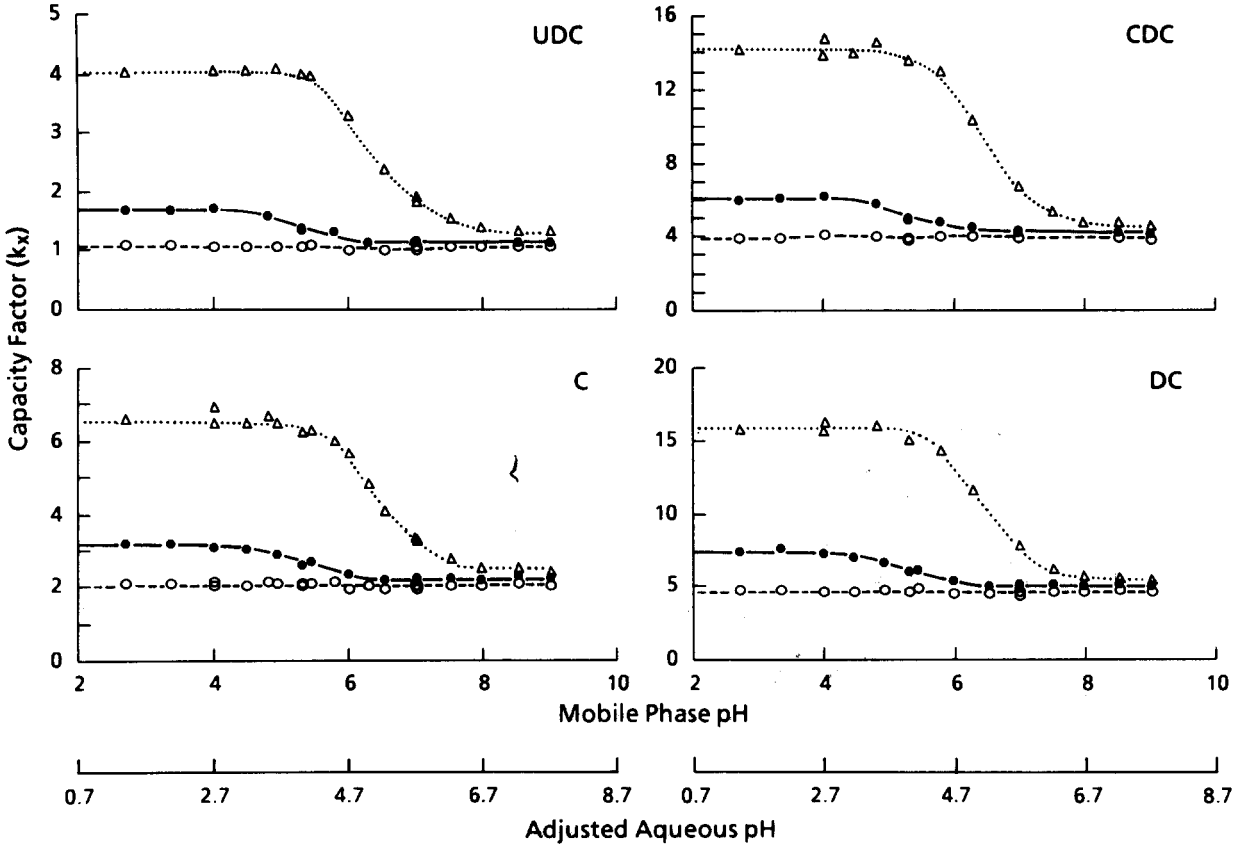

Fig. 2. HPLC capacity factors (k_x) as a function of pH for four bile salts and their glycine and taurine conjugates. Shown on the abscissa in this and the subsequent figure are both the measured apparent pH and the adjusted aqueous pH; the latter has been corrected to compensate for the increase in bile acid pK_a caused by the presence of methanol in the mobile phase (see Results). Analytical conditions were as described under Methods. Ionic strength was fixed at 0.15 by use of constant ionic strength buffers. Abbreviations: UDC, ursodeoxycholate; C, cholate; CDC, chenodeoxycholate; DC, deoxycholate. Legend: (△) unconjugated bile salt; (●) glycine conjugate; (○) taurine conjugate.

salt. At more acid pH the capacity factors of unconjugated or glyco-bile salts increased sigmoidally, as predicted (Fig. 1), again flattening at low pH; at pH <4.5 (unconjugated) or <3.5 (glycine-conjugated), retention is determined entirely by the nonionized form. Values for kA^- and kHA, determined as the average of two or three determinations at pH 8.5–9.0 or pH 2.8–3.5, respectively, are shown in **Table 1.**

Using the methods outlined in Fig. 1 and the data shown in Fig. 2, the pK_as of unconjugated ursodeoxycholate, cholate, chenodeoxycholate, and deoxycholate in 70% aqueous methanol were determined to be 6.3, 6.4, 6.5, and 6.5, respectively. The pK_a values of glycine conjugates of these four bile salts were 4.9, 5.1, 4.9, and 5.1; glycine conjugates of hyodeoxycholate and lithocholate exhibited pK_as of 5.1 and 5.2 (data not shown). By comparison, Fini and Roda (21) found the true pK_as of unconjugated and glycine-conjugated bile acids in water to be approximately 5.0 and 3.9, respectively. It is well established that addition of methanol to an aqueous medium increases the pK_as of acids (11, 19). Based on the mean differences between our data cited above and the findings of Fini and Roda, one may conclude that the pK_as of bile salts in the chromatographic mobile phase used in these studies averaged 1.3 ± 0.1 pH units greater than the corresponding pK_as in true aqueous solution. Thus, on each figure we have amended the abscissa to show both the measured apparent pH of the mobile phase, as well as a calculated scale adjusted by 1.3 pH units to compensate for the decrease in ionization caused by the presence of methanol (adjusted aqueous pH). The latter scale should be employed in calculating the effect of pH on hydrophilic-hydrophobic balance of bile salts in biological systems.

Like k', HI varied sigmoidally with pH. The difference between HI values determined at extremes of pH, where the sigmoidal curve is flat, is a quantitative measure of the change in hydrophilic-hydrophobic balance which accompanies ionization. **Fig. 3** illustrates the effect of pH on HI values of ursodeoxycholate (UDC), cholate (C), chenodeoxycholate (CDC), and deoxycholate (DC) in their unconjugated and conjugated forms. HI of taurocholate is 0 by definition; HIs of taurine conjugates of UDC, CDC, and DC averaged −0.47 ± 0.01, +0.46 ± 0.01, and +0.59 ± 0.01, respectively, and did not change with pH.

TABLE 1. Hydrophobicity indices of individual bile salts

Bile Acid/Salt	Taurine Conjugate (A^-)	Glycine Conjugate (A^-)	Glycine Conjugate (HA)	Unconjugated (A^-)	Unconjugated (HA)
Ursocholic	− 0.94				
α-Muricholic	− 0.84				
β-Muricholic	− 0.78				
Ursodeoxycholic	− 0.47	− 0.43	− 0.15	− 0.31	+ 0.49
Hyocholic	− 0.45				
Hyodeoxycholic	− 0.35	− 0.30	+ 0.01		
Cholic	0.00	+ 0.07	+ 0.30	+ 0.13	+ 0.83
Chenodeoxycholic	+ 0.46	+ 0.51	+ 0.77	+ 0.59	+ 1.37
Deoxycholic	+ 0.59	+ 0.65	+ 0.93	+ 0.72	+ 1.46
Lithocholic	+ 1.00	+ 1.05	+ 1.34		

Hydrophobicity indices of the ionized forms (A^-) of glyco- and free bile salts were determined at apparent pH 8.5–9.0; indices of the protonated forms (HA) were determined at apparent pH 2.8–3.5. Hydrophobicity indices of taurine conjugates (A^- at all physiologic pHs) were constant between pH 2.8 and 9.0. Analytical conditions and calculations were as described in the text. As chromatographic standards for taurine conjugates of ursocholic, hyocholic and α- and β-muricholic acids, we used samples of bile from rats fed hyocholic or ursocholic acids or from controls (58). In these animals, taurine conjugates of the bile acids in question were the predominant components of the bile, as confirmed by GLC analysis of bile (9); identities of all major components except for α-muricholic acid also were confirmed by mass spectrometric comparison with commercial standards (9). Missing data: conjugated or radiolabeled standards not obtainable.

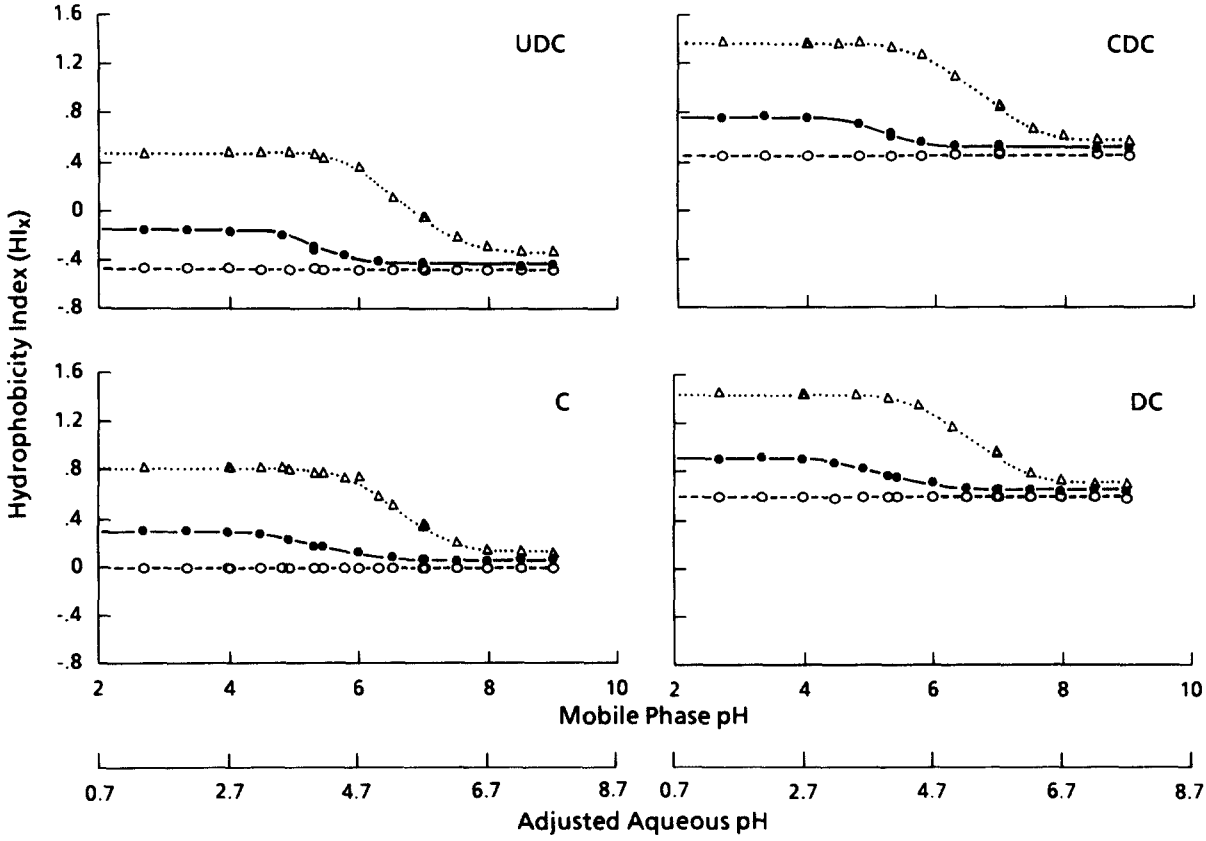

Fig. 3. Hydrophobicity indices (HI_x) as a function of pH for four unconjugated bile salts and their glycine and taurine conjugates. HI_x were calculated as described in the text, using the capacity factor data illustrated in Fig. 2. Abbreviations and abscissa labels are identical to those used in Fig. 2.

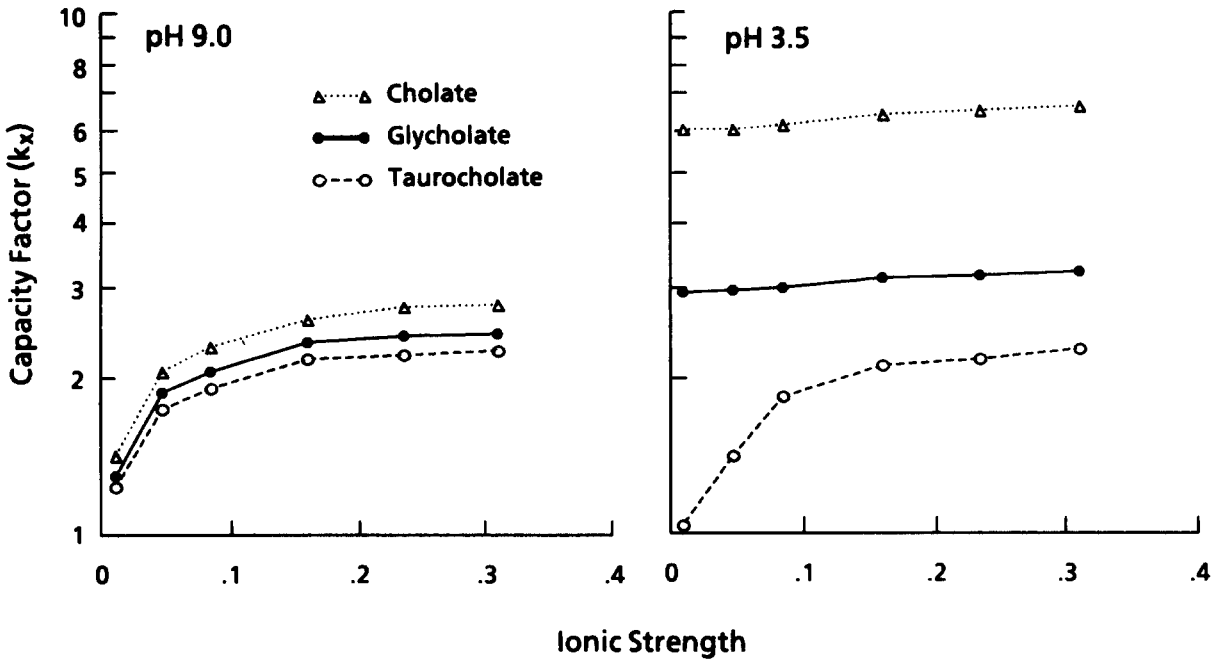

Fig. 4. Effect of changing ionic strength on capacity factors of cholic acid and its glycine and taurine conjugates. Buffers were prepared as described under Methods; ionic strength was adjusted by varying the concentrations of sodium chloride. Experiments were performed both at pH 9.0 (unconjugated and conjugated bile salts fully ionized) and at pH 3.3 (taurine conjugates fully ionized; unconjugated and glyco-bile acids fully protonated).

At pH ≥ 8.5, HIs of six glycine-conjugated bile salts (ursodeoxycholic, hyodeoxycholic, cholic, chenodeoxycholic, deoxycholic, and lithocholic) exceeded HIs of the corresponding taurine conjugates by only 0.05 ± 0.01; HIs of four unconjugated bile salts (ursodeoxycholic, cholic, chenodeoxycholic, deoxycholic) exceeded those of the corresponding taurine conjugates by 0.14 ± 0.02. When apparent pH was lowered to ≤3.5, HI values of these six glycine-conjugated or four unconjugated bile acids, now fully protonated, increased by 0.26 ± 0.02 or 0.76 ± 0.04, respectively, as compared to the corresponding ionized bile salts. HI values of these and several additional bile acids and salts are shown in Table 1.

As shown in **Fig. 4,** capacity factors (and hence apparent hydrophobicity) of cholate and its taurine and glycine conjugates in their fully ionized forms increased with increasing ionic strength; the effect was most striking at low ionic strengths. This finding is in agreement with the recent observation of Cantafora and di Biase (22). At the opposite extreme of pH (3.5), where taurine conjugates remain fully ionized but unconjugated and glyco-bile acids are fully protonated, the capacity factors of cholate and glycocholate (fully protonated) are only minimally influenced by ionic strength, whereas taurine conjugates (fully ionized) continue to show a marked increase in capacity factor with increasing ionic strength. These data suggest that positive ions in solution may partially neutralize the negative charge of bile salts in monomeric form, as has been shown to be true for bile salts in micelles (18). Because the hydrophobicity index is calculated relative to the capacity factor of taurocholate, a plot of hydrophobicity index versus ionic strength at acid pH would be misleading, showing an apparent decline in hydrophobicity of unconjugated and glycine-conjugated bile acids as ionic strength increases. Thus the hydrophobicity index values for unconjugated and glycine-conjugated bile acids presented in Fig. 2 and 3 and in Tables 1 and 2 are valid only at the specific ionic strength at which the data were obtained (0.15 M).

An example of an application of the hydrophobicity index is presented in **Table 2,** which shows the composition and calculated HI values for bile samples obtained from four mammalian species: rat, dog, hamster, and human. In bile, bile salts are >99.5% conjugated with taurine or glycine (23). At a physiologic bile pH of 7 to 8 (24) these conjugated bile salts are >99% ionized. Therefore HI_x values of fully ionized bile salts (determined chromatographically at apparent mobile phase pH ≥ 8.5) are used in calculating the HI of bile salt monomers in bile.

Different species exhibited a marked variation in the composite hydrophilic-hydrophobic balance of their biliary bile salts. The rat, which can hydroxylate bile acids in the 6β and 7α positions (6, 7), has a bile salt pool consisting primarily of very hydrophilic α- and β-muricholic acids and cholic acid; its biliary HI was consequently low (−0.31 ± 0.05). In contrast, hamster bile was composed

TABLE 2. Hydrophobicity indices of bile from various mammalian species

Bile Salt	Hydrophobicity Index of Pure Bile Salt (HIx)	Mole Fraction of Biliary Bile Salts (Fx) Rat (n = 5)	Dog (n = 3)	Hamster (n = 4)	Human (n = 5)
Taurine conjugates					
α-Muricholic	−0.84	0.015 ± 0.020			
β-Muricholic	−0.78	0.361 ± 0.056			
Ursodeoxycholic	−0.47	0.023 ± 0.005			0.013 ± 0.010
Hyocholic	−0.45				
Hyodeoxycholic	−0.35	0.064 ± 0.015			
Cholic	0.00	0.424 ± 0.064	0.705 ± 0.060	0.135 ± 0.040	0.062 ± 0.013
Chenodeoxycholic	+0.46	0.044 ± 0.006	0.068 ± 0.004	0.085 ± 0.032	0.103 ± 0.070
Deoxycholic	+0.59	0.018 ± 0.018	0.184 ± 0.036	0.006 ± 0.007	0.036 ± 0.030
Lithocholic	+1.00		0.012 ± 0.011		
Glycine conjugates					
α-Muricholic	−0.79*	0.003 ± 0.003			
β-Muricholic	−0.73*	0.017 ± 0.024			
Ursodeoxycholic	−0.43				0.034 ± 0.012
Hyocholic	−0.40*				
Hyodeoxycholic	−0.30	0.002 ± 0.003			
Cholic	+0.07	0.026 ± 0.019		0.487 ± 0.068	0.304 ± 0.070
Chenodeoxycholic	+0.51	0.001 ± 0.001		0.263 ± 0.064	0.248 ± 0.073
Deoxycholic	+0.65			0.024 ± 0.027	0.200 ± 0.101
Lithocholic	+1.05				0.001 ± 0.001
Hydrophobicity index (mean ± SD)		**−0.31 ± 0.06**	**+0.14 ± 0.04**	**+0.22 ± 0.02**	**+0.32 ± 0.05**

Conjugated bile salts were quantified by HPLC as previously described (14). Hydrophobicity index was calculated as described in the text (equation 11). Data shown are mean ± SD; *, estimated value. Where glycine-conjugated standards were unavailable, HIx values of glycine-conjugated salts (A^-) were estimated to exceed those of the corresponding taurine conjugates by 0.05, which was the empirically determined mean difference in hydrophobicity index between six glycine-conjugated bile salts (ursodeoxycholate, hyodeoxycholate, cholate, chenodeoxycholate, deoxycholate, and lithocholate) and their corresponding taurine conjugates (see Results).

primarily of glycine conjugates of cholate and chenodeoxycholate, and exhibited an HI of +0.22 ± 0.02. The samples of dog bile analyzed contained primarily taurocholate, consistent with the findings of other authors (25). This species had a biliary HI of 0.14 ± 0.04. Human bile salts as expected (26) were primarily glycine conjugates, and human bile salt pool contained a substantial fraction of chenodeoxycholates and deoxycholates; human bile, therefore, was the most hydrophobic of the four species tested (HI 0.32 ± 0.04). As determined by group *t*-testing, interspecies differences in biliary HI were all significant at $P < 0.02$ with the single exception of dog versus hamster, for which $P = 0.066$. It should be noted that HI values for biological samples are subject to the specific experimental conditions prevailing at the time bile samples were obtained. The composition of the bile salt pool, particularly with regard to hydrophobic secondary bile acids such as deoxycholate, varies with age, diet, and integrity of the enterohepatic circulation; variations in these parameters would be expected to change biliary HI.

DISCUSSION

Many biological, physical, and chemical properties are related to the ability of compounds to bind to or dissolve in hydrophobic domains such as membranes, micelles, or certain receptor sites. The free energy of partition of a compound between water and nonpolar solvents, which is proportional to the logarithm of the equilibrium partition coefficient, often exhibits a linear relationship to these properties. This fact has led to widespread use of partition coefficients to elucidate and predict quantitative structure–activity relationships. This concept of linear free energy relationships as formulated by Hansch (13) has been most widely applied in development of pharmacological agents (27), but similar principles apply equally well to groups of structurally related compounds of endogenous origin. For example, Shaw and associates (16) have applied the Hansch approach to analyze the contribution of different structural features of bile acids in determining their retention factors in reversed phase HPLC.

The detergent properties of bile salts are a consequence of their amphiphilicity. It would be logical to predict that differences in hydrophilic–hydrophobic balance might account for differences in the avidity with which bile salts interact with each other and with other lipids. Indeed, this appears in general to be the case for properties dependent upon hydrophobic self-aggregation of bile salts such as critical micellar concentration and mean micellar aggregation number (18, 28, 29). It also appears to be the case with regard to the ability of bile salts to solubilize less polar lipids. Armstrong and Carey (2) found a log linear

relationship between HPLC capacity factors of bile salts and their equilibrium micellar cholesterol solubilizing capacity. Similarly, the studies of Lindenbaum and Rajagopalan (30, 31) and Salvioli, Lugli, and Pradelli (32) have shown that phospholipid solubilization, alone or in combination with cholesterol, increases with increasing bile salt hydrophobicity.

Hydrophilic-hydrophobic balance of bile salts also correlates well with their toxicity in biological systems. The propensity of bile salts to induce cholestasis clearly increases with increasing hydrophobicity as quantified by HPLC (33, 34) and increasing hydrophobicity also is associated with increasing cytotoxicity toward isolated hepatocytes, gastric mucosa or colonic mucosa (35-40), at least for the most common 5β cholanoates. The common denominator in these effects may be disruption of membrane integrity secondary to bile salt solubilization of membrane lipids (32, 41-43). Increasing hydrophobicity of bile salts is associated with increasing ability to permeabilize artificial phospholipid vesicles (44). Bile salt-induced cholestasis is associated with release of integral membrane phospholipids and proteins into bile; the functional impairment may result from detergent solubilization of the canalicular membrane (41, 45). Several groups recently have shown that bile salt toxicity is associated with increased cytosolic calcium (46-48). Gordon et al. (49, 50) found that HPLC capacity factors of bile salts correlated well with their ability to promote absorption of insulin across the nasal mucosa.

It has been suggested that hydrophilic-hydrophobic balance also may determine many physiological properties of bile acids; however this remains an area of controversy. Tavoloni, Sarkozi, and Jones (51) and Tavoloni (52) found that choleretic potency of taurine-conjugated bile salts in the guinea pig was correlated with bile salt hydrophobicity as determined by HPLC. Carulli and associates (53) and Loria et al. (54) similarly noted a close relationship between HPLC capacity factors and the potency of bile salts as promoters of biliary cholesterol and phospholipid secretion following acute bile salt pool replacement in humans, and Bilhartz and Dietschy (55) have recently described a similar relationship between bile salt hydrophobicity and biliary cholesterol secretion in the rat. However, no such relationships were noted by Guarantz and Hofmann (56) in studies of biliary water and lipid secretion in the hamster, and only weak associations were found in the rat by Roda and associates (57). The reason for these discrepancies remains unclear. We (12, 58) have shown recently that the potency of bile salts as suppressors of cholesterol 7α-hydroxylase and HMG-CoA reductase, the enzymes that regulate rates of bile acid and cholesterol synthesis, increases with increasing bile salt hydrophobicity following either acute infusion or chronic feeding in the rat.

Although the largest body of data regarding hydrophilic-hydrophobic balance of organic compounds has been obtained using organic/aqueous partition in systems such as octanol/water (13), in recent years reversed phase HPLC has come into widespread use for this purpose (15). HPLC measurements are more precise and reproducible than octanol/water partition coefficients, are not dependent upon precise quantitative analysis, require only minute amounts of sample which need not be pure, and are simple to perform (59). In many cases, HPLC and solvent partition methods give equivalent information. However, as regards bile acids, octanol/water partition and reversed phase HPLC provide qualitatively different measures of hydrophilic-hydrophobic balance. Octanol/water partition coefficients appear to be influenced strongly by the number of hydroxyl groups present on the sterol nucleus and only weakly by the orientation of these groups; in contrast, reversed phase HPLC capacity factors are very sensitive to differences in orientation of ring hydroxyls (60). Thus, by reversed phase HPLC (using methanol/water or isopropanol/water mobile phases), the dihydroxy bile salts ursodeoxycholate and hyodeoxycholate are more hydrophilic than cholate (2, 16), whereas by octanol/water partition the opposite is true (14). The reason for these differences is not known.

Our choice of reversed phase HPLC as the basis for the bile salt monomeric hydrophobicity index derived from the fact that in numerous studies cited above, ursodeoxycholate and hyodeoxycholate have been found to be weaker (less hydrophobic) than cholate as regards solubilization of cholesterol and phospholipid (2, 30-32), stimulation of biliary cholesterol and phospholipid secretion (52-57), suppression of cholesterol 7α-hydroxylase and HMG-CoA reductase activities (12, 58), and cytotoxicity (35-40). Thus reverse-phase liquid chromatography capacity factors may be better predictors of many properties related to bile salt hydrophilic-hydrophobic balance than are octanol/water partition coefficients. This remains an area of controversy (1), and more extensive studies to critically evaluate different methods of quantifying bile salt hydrophilic-hydrophobic balance are currently in progress in several laboratories.

The hydrophobicity index proposed in this paper is a simple and rational quantitative measure of the composite hydrophilic-hydrophobic balance of nonhomogeneous bile salt pools. As derived, the index is linearly related to the standard free energy change for partition of bile salts between a polar mobile phase and a nonpolar stationary phase, and this linear relationship is true for solutions containing multiple bile salts as well as for individual bile salts. The index thus provides a descriptive tool for quantifying bile salt structure-activity relationships. To the extent that biological or physicochemical properties of individual bile salts are determined solely by their hydro-

philic-hydrophobic balance as measured using this reversed phase HPLC system, the hydrophobicity index would be expected to predict the properties of mixed bile salt solutions. Similar composite indices based on aqueous/organic partition coefficients have proven of value in the study of other complex systems (i.e., hydrophobic moment of proteins (61)).

Quantitative structure-activity relationships based on hydrophilic-hydrophobic balance are inherently empirical, and their utility depends upon their predictive values (27). If the hydrophobicity index proves in future studies to be an accurate predictor of biological and physicochemical properties of mixed bile salt solutions, then insights derived from its application may help to clarify the mechanisms by which changes in bile salt pool composition affect the solubilization, transport, and metabolism of lipids in living systems.

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