Immobilized artificial membrane chromatography: a rapid and accurate HPLC method for predicting bile salt–membrane interactions

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Abstract To predict bile salt–membrane interactions physiologically, we used an immobilized artificial membrane HPLC column that contains dimyristoyl-phosphatidylcholine molecules covalently linked to silica microspheres. Using a 90% aqueous (10% acetonitrile) mobile phase, 22 species of bile salts and 4 species of fusidates were eluted. Glycine conjugates displayed higher affinity for the column at pH 5.5, eluting later than their taurine-conjugated congeners, but this order was reversed at pH 6.5 and 7.4 as glycine conjugates became fully ionized. Capacity factors decreased logarithmically as functions of increasing temperature, permitting determinations of interaction enthalpies, which ranged from -2.86 to -7.67 kcal/mol. A standard curve was developed from which the enthalpy for an uncommon bile salt could be inferred from its capacity factor at room temperature. Bile salt interaction enthalpies were substantially better correlated than hydrophobic indices by octadecylsilane-HPLC (D. M. Heuman, *J. Lipid Res.* 1989. **30:** 719–730) with equilibrium binding to small unilamellar vesicles and literature values reflecting bile salt–membrane interactions (e.g., biliary phosphatidylcholine secretion), but not with bile salt functions that do not require phospholipid (e.g., micellar cholesterol solubility). This new application should prove valuable for evaluating membrane-active physical–chemical properties as well as therapeutic potential of novel bile salts, particularly when they are available in quantities too small for study by conventional techniques.—**Cohen, D. E., and M. R. Leonard.** Immobilized artificial membrane chromatography: a rapid and accurate HPLC method for predicting bile salt–membrane interactions. *J. Lipid Res.* 1995. **36:** 2251–2260.

Supplementary key words phospholipid • phosphatidylcholine • partition coefficient • interaction enthalpy • reverse phase

Bile salts, a closely related family of steroid detergents formed primarily by catabolism of cholesterol in the liver and secondarily by bacterial modification in the colon, are essential for bile formation as well as for fat digestion and absorption. It has long been appreciated that physical–chemical properties of bile salts are integral to their biological activities as endogenous compounds (1, 2) and to their potential as therapeutic agents (3, 4). The detergency of a common bile salt molecule, referred to as its hydrophobic–hydrophilic balance or hydrophobicity, is governed by both the hydroxylation pattern of its steroid nucleus and the identity, position, and ionization state of a conjugated amino acid (glycine or taurine) (5). Heretofore, bile salt hydrophobicity has been quantified principally by either retention times on octadecylsilane (ODS)–HPLC columns (6–8) or octanol–water partition coefficients (9). These techniques, which measure the extent of bile salt distribution into a hydrophobic hydrocarbon versus an aqueous environment, have led to important mechanistic insights into solubilization of cholesterol in simple micelles (6), intestinal absorption and hepatic uptake of bile salts (9), and regulation of cholesterol metabolism in the liver (10).

Interactions of bile salts with membranes are central to the promotion of biliary lipid secretion, aggregation, and fat digestion (2), cytotoxicity and cytoprotection (11), as well as absorption of drugs across mucous membranes (12). Membrane binding necessitates interaction of anionic bile salt molecules with both ionized head groups of membrane phospholipids as well as a hydrophobic bilayered core. Therefore, bile salt hydrophobicity as quantified by reverse phase–HPLC or octanol–water partitioning would not be expected a priori to provide an optimal measure for predicting physiological processes that depend principally upon bile salt–membrane interactions. For example, whereas positive correlations between bile salt hydrophobicity by reverse phase ODS-HPLC and biliary lipid secretion in animal models have been reported (13, 14), others using

Abbreviations: HPLC, high performance liquid chromatography; ODS, octadecylsilane; IAM, immobilized artificial membrane chromatography.

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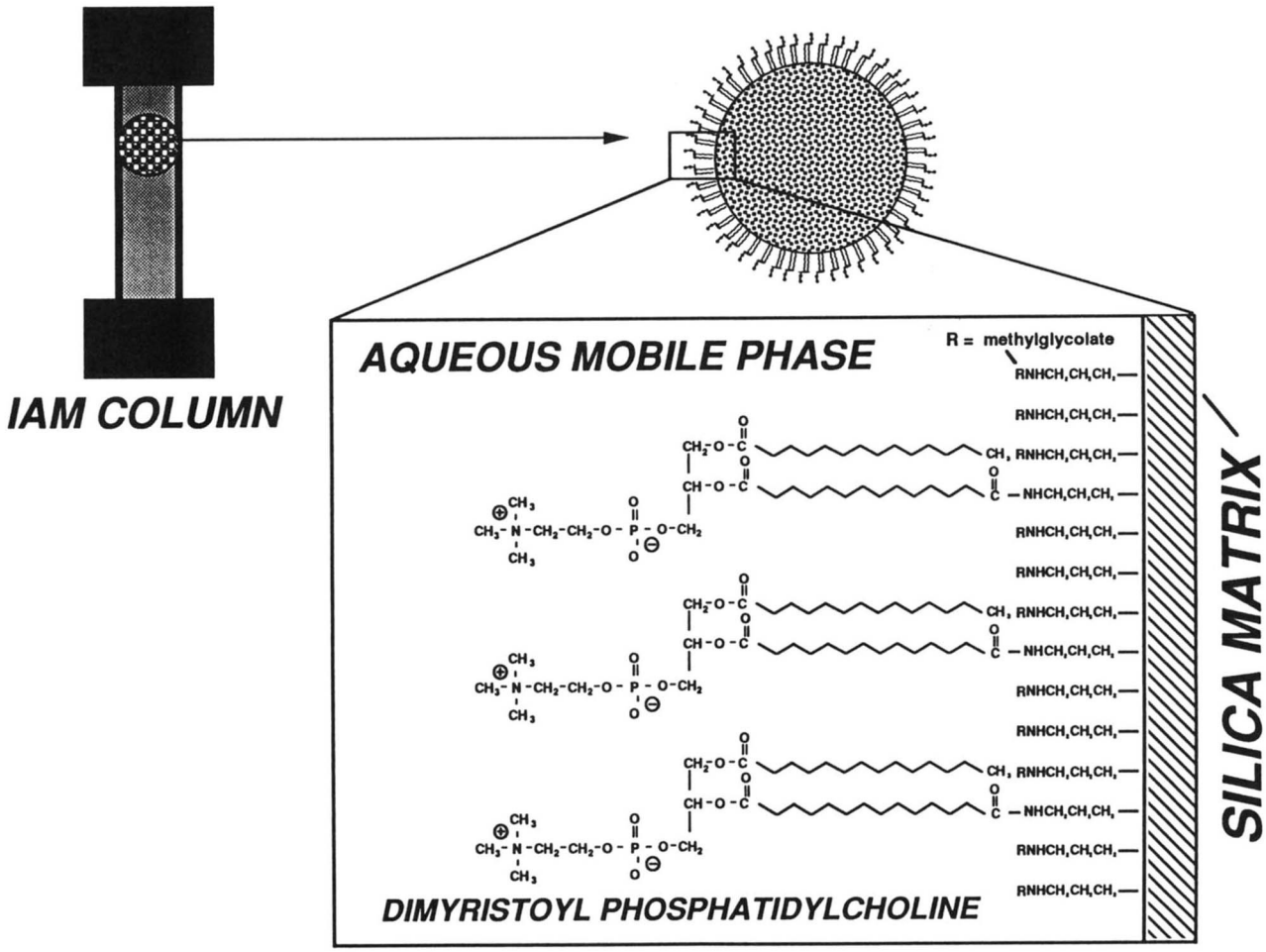

Fig. 1. Schematic diagram of the immobilized artificial membrane (IAM) stationary phase. Dimyristoyl phosphatidylcholine molecules are covalently bound to silica by the terminal carbon of *sn*-2 fatty acyl chains. Free silica amine groups are capped with methylglycolate in order to minimize their potential charge contribution (16). In the presence of an aqueous mobile phase, zwitterionic phosphorylcholine head groups face outward and simulate the outer hemileaflet of a membrane bilayer (17).

similar systems have observed this correlation to be weak (15).

Recently, a novel immobilized artificial membrane (IAM)-HPLC column was developed principally for purposes of purification of integral membrane proteins by affinity chromatography (16). As depicted in **Fig. 1,** the IAM stationary phase consists of phosphatidylcholine molecules covalently bound to silica particles by the terminal carbons of their *sn*-2 fatty acyl chains. In the presence of an aqueous mobile phase, immobilized phosphatidylcholines orient outward simulating an outer membrane hemileaflet (17). In contrast, ODS-HPLC consists of hydrophobic straight-chain hydrocarbons bonded to silica that project into an organic (methanolic) mobile phase (18). Due to the more physiological membrane-like structure of the IAM stationary phase, we have explored the hypothesis that IAM-HPLC would resolve individual molecular species of conjugated bile salts and fusidates, anionic steroid detergent-like molecules (19), and more accurately predict their interactions with model and native membranes.

EXPERIMENTAL PROCEDURES

Materials

Taurine- and glycine-conjugated bile salts were from Sigma Chemical Company (St. Louis, MO) and/or Calbiochem (La Jolla, CA) with the exception of the following generous gifts: tauroursocholate from Dr. Ashok Batta (Newark, NJ), glycoursocholate from GIPharmex (Milan, Italy), taurohyodeoxycholate from Dr. Mario Angelico (Rome, Italy), glycodehydrocholate, glycohyocholate, and glycohyodeoxycholate from Dr. Alan Hofmann (San Diego, CA), and conjugates of muricholates from Tokyo Tanabe Co. (Tokyo, Japan). Conjugates of sodium fusidate and dihydrofusidate were gifts from Drs. W. O. Godtfredsen and W. von Daehne (Leo

Pharmaceutical Products, Ballerup, Denmark). Taurocholate was purified by the method of Pope (20), whereas other bile salts and fusidates were used as received or purified by charcoal treatment, ether washing, or recrystallization. [24-^{14}C]taurocholate (50 mCi/mmol) was from New England Nuclear (Boston, MA). [^{14}C]taurodeoxycholate and [^{14}C]taurochenodeoxycholate were synthesized by conjugation of the free bile salt (21) to [U-^{14}C]taurine (115 mCi/mmol) (Amersham, Arlington Heights, IL). Similarly prepared [^{14}C]tauroursodeoxycholate was a generous gift from Dr. Joanne Donovan (West Roxbury, MA). Purities (and radiopurities) exceeded 98% as judged by HPLC (6) and/or thin-layer chromatography (22). Egg yolk phosphatidylcholine was purchased from Lipid Products (South Nutfield, Surrey, UK) and was >99% pure as assessed by HPLC (23). All other chemicals and solvents were of ACS or HPLC grade and were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Medford, MA). Water was filtered, ion exchanged, and glass distilled (Corning Glass Works, Corning, NY). Pyrex glassware was washed overnight in a solution of 2 M KOH in 50% ethanol, followed by overnight washing in 3 M HNO_3 and thorough rinsing in glass-distilled, deionized H_2O.

Instrumentation

An isocratic HPLC apparatus (Beckman Instruments, Inc., Wakefield, MA) consisted of a model 110B pump, a model 166 UV-Visible detector, and an AI406 Analog Interface to an IBM computer utilizing System Gold software. The HPLC apparatus was fitted with an analytically sized (15 cm × 4.6 mm) IAM column (Regis Chemical, Morton Grove, IL) together with an IAM guard column (1 cm × 3.0 mm) placed between the injector and the analytical column. Elution temperatures (15–37°C) were controlled to within ±1°C by enclosing both precolumn and column in a water jacket (Alltech Associates, Deerfield, IL) attached to a circulating water bath.

Mobile phase

Because our objective was to use IAM-HPLC to predict physiological bile salt–membrane interactions, we empirically designed an aqueous-based mobile phase. In preliminary experiments using 0.01 M PO_4 as the mobile phase, bile salts were applied to the IAM column but failed to elute even at long (>3 h) intervals. Elution of bile salts was achieved by addition of acetonitrile (CH_3CN) to the mobile phase in small (2.5 vol%) increments. For purposes of this study, we selected a mobile phase composed of 90% 0.01 M PO_4:10% CH_3CN (v/v) which provided sufficient resolution of bile salt retention times while eluting the most hydrophobic bile salts within a ~1-h time span. The mobile phase was prepared daily and filtered through 0.4 μm polycarbonate membranes (Nuclepore, Pleasanton, CA). Effects of partial ionization of bile salts were explored by varying apparent pH (pH_{app}) of the mobile phase over the range 4.5 to 7.4 within which phospholipids and silica comprising the IAM stationary phase were stable to hydrolysis. Preliminary experiments demonstrated that addition of acetonitrile (10 vol%) resulted in values of pH_{app} that exceeded the pH of the corresponding pure 0.01 M PO_4 by ≤0.3 log-units. Although the mobile phase was not a true buffer at all experimental values of pH_{app} between 4.5 and 7.4, careful monitoring demonstrated that pH_{app} remained constant during elution of bile salts.

HPLC conditions

Bile salts were prepared for IAM-HPLC by dissolution in H_2O. To ensure solubility, the pH of each glycine conjugate was adjusted to greater than two pH units above its respective pK_a (24). In preliminary experiments, retention times of bile salts decreased up to 7.5% as functions of large increases (100-fold) in quantities of bile salts applied to the IAM column. Therefore, all experiments were performed using 50 nmol of bile salts (20 microliters of 2.5 mM solutions). Bile salts were eluted using a mobile phase flow rate of 1.0 ml/min, and retention times were detected by absorbance at 200 nm (6). Capacity factors k_x for retention of bile salts or fusidates on the IAM column were calculated as $k_x = (t_x - t_o)/t_o$, where t_x represents the elution time of the bile salt and t_o the elution time of an unretained solute (water). To correct for slight decreases in retention times that occurred over periods of several months due to gradual loss of the stationary phase by hydrolysis of phosphatidylcholines, capacity factors of individual bile salts were normalized to the capacity factor of taurocholate eluted the same day under identical experimental conditions.

Bile salt binding to model membranes

To assess whether IAM-HPLC predicts bile salt–membrane interactions in vitro, equilibrium binding of ^{14}C-labeled bile salts to model membranes was measured by centrifugal ultrafiltration (25). Briefly, we prepared small unilamellar vesicles of egg yolk phosphatidylcholine in 0.15 M NaCl, 5 mM Tris-HCl, 3 mM NaN_3, pH 7.4, by bath sonication as previously described (26). Bile salt solutions were prepared in the same buffer and then mixed with small unilamellar vesicles to achieve final concentrations of 0.17 mM phosphatidylcholine and 0.3 mM bile salts. Samples were incubated overnight at room temperature and then subjected to centrifugal ultrafiltration. Concentrations of free bile salts in samples of ultrafiltrate were determined by scintillation counting, and bound bile salt concentrations were cal-

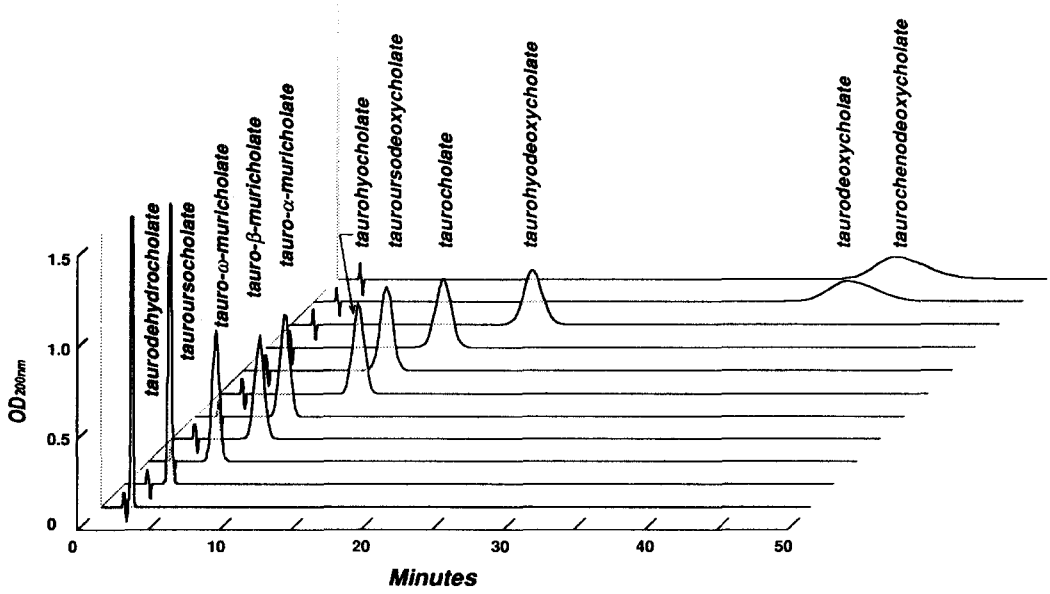

Fig. 2. IAM elution profiles of taurine-conjugated bile salts. Fifty nmol of each bile salt was eluted individually using an aqueous mobile phase (90% 0.01 M PO_4:10% CH_3CN (v/v), 23°C, pH_{app} 7.4) at a flow rate of 1.0 ml/min.

culated as the total minus the free bile salt concentration. Equilibrium partition coefficients of bile salts into membranes were determined as ratios of bound-to-free bile salt concentrations.

Thermodynamics of bile salt-IAM binding

During elution, bile salts partition into the IAM stationary phase with partition coefficients $K_x = k_x/\phi$ (18). The phase ratio, ϕ, is defined as the ratio of the volume of the stationary phase to the volume of the mobile phase. Partitioning of a bile salt from mobile to stationary phase is characterized by a free energy change, ΔG, which equals the sum of the changes in enthalpy (ΔH), the heat absorbed during bile salt–membrane binding, and entropy (ΔS), the change in molecular disorder of the system: $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature. The ΔG due to bile salt partitioning is given by $\Delta G = -RT\ln(K_x)$, where R is the gas constant (18). This relation may be expressed:

$$\ln(k_x) = -\Delta H/RT + \Delta S/R + \ln(\phi)$$

Theoretically, a linear van't Hoff plot (i.e., a plot of $\ln(k_x)$ as a function of 1/T) permits direct calculations of both ΔH and ΔS from its slope and intercept values, respectively. However, in the commercially prepared IAM column, a value for ϕ was unavailable and, consequently, values of ΔS could not be determined. Although the inability to measure ΔS directly prohibited calculations of ΔG, others have shown that binding of amphiphiles to membrane surfaces is generally dominated by a large negative ΔH term (27). Therefore, values of ΔH, calculated from the slope of an experimentally determined van't Hoff plot as $\Delta H = -RT\{d(\ln(k_x))/d(1/T)\}$, were used as a thermodynamic measure for binding affinity to the IAM stationary phase.

RESULTS

Figure 2 displays IAM elution profiles of taurine-conjugated bile salts (pH_{app} 7.4, 23°C). Because preliminary experiments demonstrated that early elution times of the most hydrophilic bile salts were similar in magnitude, bile salts were eluted individually for purposes of precisely defining their HPLC capacity factors. As shown in **Fig. 3,** capacity factors of taurocholate increased with decreases in both temperature and pH_{app}. **Figure 4** demonstrates the influence of ionization states of glycine versus taurine conjugation on IAM capacity factors by normalizing bile salt capacity factors relative to capacity factors of taurocholate obtained at identical temperatures and pH_{app} (Fig. 3). Whereas normalized capacity factors for tauroursodeoxycholate displayed only slight increases as functions of decreasing pH_{app}, values for glycoursodeoxycholate increased sharply as pH_{app} decreased from 7.4 to 5.5. Between pH_{app} values of 5.5 and 6.5, the elution order of tauroursodeoxycholate and glycoursodeoxycholate was reversed, with glycoursodeoxycholate eluting earlier at higher pH_{app} values. The same effects were observed for glycine and taurine conjugates of dehydrocholate, ursocholate, cholate, and chenodeoxycholate which were also examined systematically as functions of pH_{app} (data not shown).

Table 1 summarizes the influence of temperature on normalized capacity factors of bile salts and fusidates at

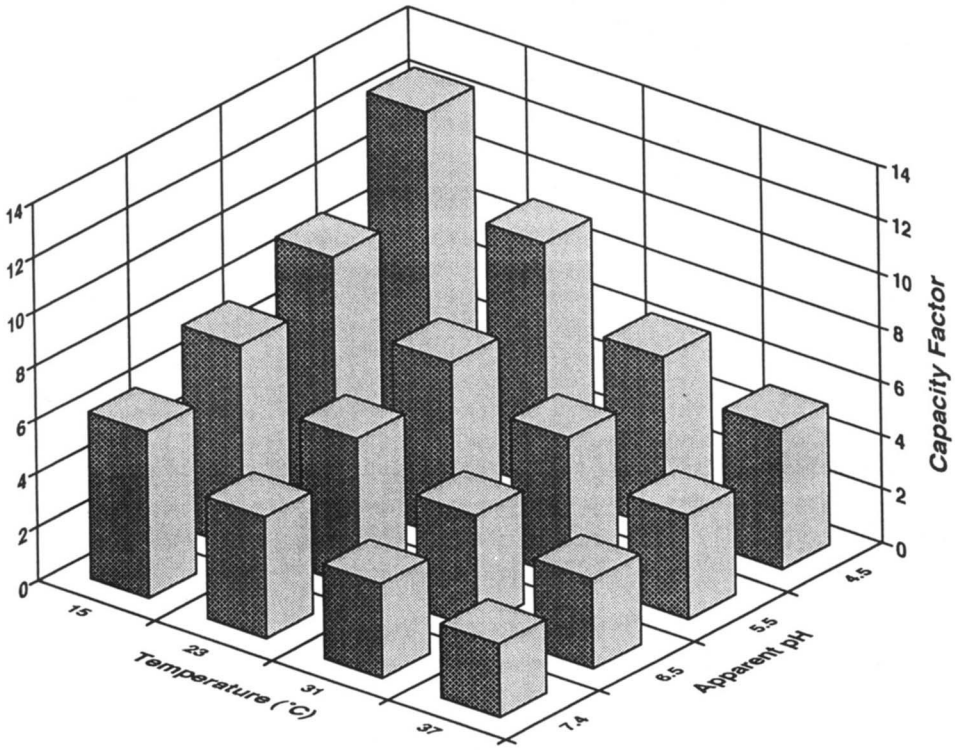

Fig. 3. IAM-HPLC capacity factors of taurocholate as functions of temperature and apparent pH.

pH_{app} 7.4. With the exception that chenodeoxycholate and deoxycholate conjugates reversed their orders of elution at 31 and 37°C compared with 15 and 23°C, bile salts maintained the same rank order of elution at each temperature. **Figure 5** illustrates in the form of van't Hoff plots that capacity factors decreased with increases in temperature. The observed linear relationships excluded a phase transition of the immobilized phosphatidylcholines in the stationary phase over the range of experimental temperatures (18) and permitted calculations of ΔH for bile salt - IAM binding. The broad range of exothermic ΔH values for bile salts (-2.86 to -7.67 kcal/mol) are listed in Table 1. Although a limited number of fusidates were available for study, a much narrower range of ΔH values (-5.78 to -5.96) was observed for this family of anionic steroid detergents. With the exception of dehydrocholate and fusidate conjugates, all taurine-conjugated bile salts demonstrated uni-

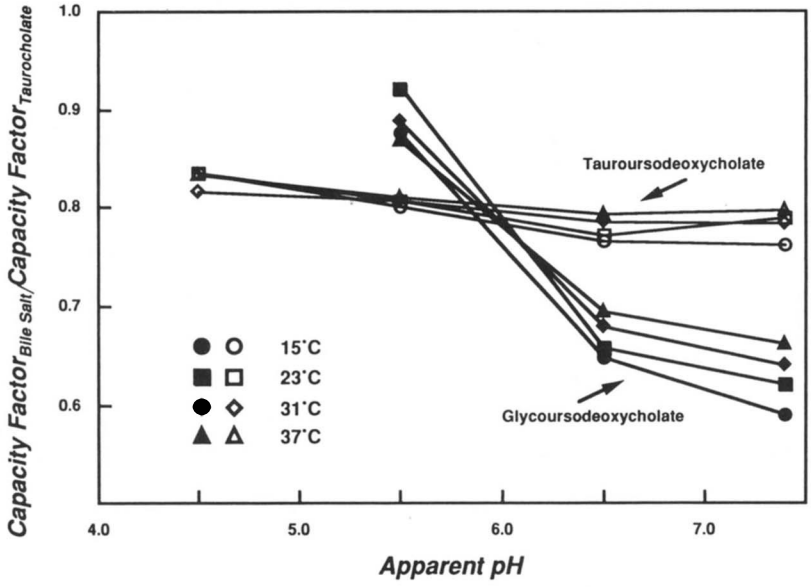

Fig. 4. Influence of apparent pH on IAM-HPLC capacity factors of glycoursodeoxycholate (closed symbols) and tauroursodeoxycholate (open symbols) normalized to capacity factors of taurocholate eluted under identical conditions (see Fig. 3). Standard deviations lie within the symbol sizes.

TABLE 1. IAM-HPLC capacity factors and interaction enthalpies (ΔH) for bile salts and fusidates

	Normalized Capacity Factors[a]				ΔH[b]
	37°C	31°C	23°C	15°C	(kcal/mol)
Bile salt					
Glycodehydrocholate	0.060 ± 0.003	0.051 ± 0.001	0.045 ± 0.001	0.038 ± 0.002	-3.06
Taurodehydrocholate	0.067 ± 0.005	0.058 ± 0.004	0.051 ± 0.001	0.042 ± 0.002	-2.86
Glycoursocholate	0.152 ± 0.002	0.139 ± 0.002	0.124 ± 0.001	0.109 ± 0.003	-3.86
Tauroursocholate	0.174 ± 0.004	0.159 ± 0.002	0.144 ± 0.001	0.129 ± 0.003	-4.14
Glyco-ϖ-muricholate	0.272 ± 0.006	0.255 ± 0.003	0.234 ± 0.001	0.216 ± 0.004	-4.70
Tauro-ϖ-muricholate	0.328 ± 0.005	0.312 ± 0.003	0.290 ± 0.002	0.272 ± 0.004	-5.02
Glyco-β-muricholate	0.358 ± 0.008	0.345 ± 0.005	0.328 ± 0.002	0.319 ± 0.007	-5.59
Glyco-α-muricholate	0.379 ± 0.008	0.364 ± 0.005	0.347 ± 0.006	0.333 ± 0.005	-5.48
Tauro-β-muricholate	0.441 ± 0.006	0.429 ± 0.004	0.418 ± 0.001	0.413 ± 0.004	-6.01
Tauro-α-muricholate	0.452 ± 0.005	0.440 ± 0.001	0.428 ± 0.003	0.424 ± 0.006	-6.00
Glycohyocholate	0.626 ± 0.019	0.610 ± 0.017	0.595 ± 0.015	0.585 ± 0.018	-5.99
Glycoursodeoxycholate	0.662 ± 0.021	0.640 ± 0.016	0.620 ± 0.017	0.589 ± 0.015	-5.62
Taurohyocholate	0.775 ± 0.011	0.765 ± 0.001	0.758 ± 0.006	0.750 ± 0.007	-6.28
Tauroursodeoxycholate	0.797 ± 0.009	0.784 ± 0.009	0.788 ± 0.004	0.761 ± 0.010	-6.20
Glycocholate	0.860 ± 0.011	0.856 ± 0.009	0.842 ± 0.002	0.821 ± 0.009	-6.15
Taurocholate	1.000	1.000	1.000	1.000	-6.53
Glycohyodeoxycholate	1.106 ± 0.005	1.112 ± 0.005	1.119 ± 0.007	1.160 ± 0.019	-6.89
Taurohyodeoxycholate	1.363 ± 0.024	1.390 ± 0.032	1.426 ± 0.029	1.467 ± 0.019	-7.12
Glycochenodeoxycholate	2.675 ± 0.032	2.733 ± 0.027	2.820 ± 0.045	2.900 ± 0.052	-7.18
Glycodeoxycholate	2.702 ± 0.034	2.739 ± 0.031	2.766 ± 0.061	2.809 ± 0.075	-6.83
Taurochenodeoxycholate	3.078 ± 0.050	3.224 ± 0.039	3.399 ± 0.022	3.547 ± 0.038	-7.67
Taurodeoxycholate	3.155 ± 0.009	3.227 ± 0.039	3.313 ± 0.023	3.473 ± 0.028	-7.21
Fusidate					
Glycofusidate	2.702 ± 0.016	2.637 ± 0.021	2.579 ± 0.015	2.482 ± 0.033	-5.86
Taurofusidate	3.032 ± 0.044	2.984 ± 0.042	2.864 ± 0.012	2.773 ± 0.025	-5.78
Glycodihydrofusidate	3.813 ± 0.024	3.739 ± 0.039	3.652 ± 0.013	3.485 ± 0.006	-5.82
Taurodihydrofusidate	4.295 ± 0.034	4.214 ± 0.044	4.117 ± 0.020	3.997 ± 0.032	-5.96

[a]Capacity factors (mean ± SD, n ≥ 3) were determined at apparent pH of 7.4 as described in Experimental Procedures and normalized to capacity factor determined for taurocholate at the same temperature and apparent pH (Fig. 3).
[b]Calculated from van't Hoff plots (Fig. 5) according to text.

formly higher magnitudes of ΔH than corresponding glycine conjugates at a pH_{app} 7.4.

Because capacity factors for each bile salt species varied uniquely as functions of temperature, values of ΔH did not scale in direct proportion to bile salt retention times for a given temperature (Table 1). Nevertheless, **Fig. 6** demonstrates a highly significant linear relationship between ΔH values of bile salts and natural logarithms of normalized capacity factors at 23°C. Similar relationships were observed at 15, 31, and 37°C (data not shown). Also represented in Fig. 6 are the conjugated fusidates that plot apart from the linear regression line established for bile salts. Although a shallower slope is suggested for the fusidates, a sufficiently broad variation in fusidate molecular structures was not available to establish a significant relationship as was observed for bile salts.

To assess whether ΔH values determined by IAM-HPLC (Table 1) predict actual bile salt–membrane binding, we measured equilibrium partitioning coefficients of ^{14}C-labeled bile salts into sonicated small unilamellar vesicles composed of egg yolk phosphatidylcholine. **Figure 7** demonstrates correlations between ln(partition coefficient) and ΔH values (R=0.997), natural logarithms of normalized capacity factors measured at 23°C (R= 0.949), or literature values for bile salt hydrophobic index by ODS-HPLC (R= 0.882) (7). Analysis of covariance (14) demonstrated that the correlation between ln(partition coefficient) and ΔH (Fig. 7A) is significantly better ($P < 0.0001$) than correlations between ln(partition coefficient) and either IAM capacity factors (Fig. 7B) or hydrophobic indices (Fig. 7C). Whereas ln(partition coefficient) correlated better with IAM capacity factors (Fig. 7B) than did hydrophobic indices, (Fig. 7C), statistical significance was marginal ($P = 0.05$) presumably because of the limited number of data points.

DISCUSSION

Bile salt interactions with membranes within the enterohepatic circulation are central to their pathophysiology and xenobiotic pharmacology. Whereas hydrophilic–hydrophobic balance of these biological amphiphiles is a critical determinant of membrane association, current quantitative measures of bile salt hydrophobicity are based on partitioning between polar and hydrocarbon phases. Such determinations omit complex and potentially important contributions of phospholipid head groups that comprise membrane surfaces. To overcome this potential shortcoming, we have exploited newly developed IAM technology to evaluate

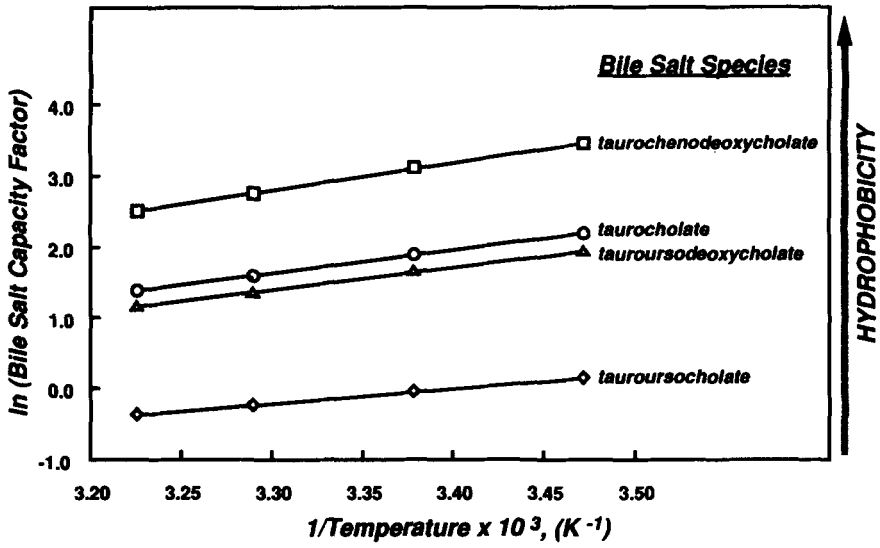

Fig. 5. van't Hoff plots demonstrating influence of temperature on IAM-HPLC capacity factors for representative taurine-conjugated bile salt species at pH_{app} 7.4. Increasing bile salt hydrophobicity according to literature values by ODS-HPLC (7) is indicated schematically on the right. Standard deviations lie within the symbol sizes.

amphiphilicity of bile salts with the specific objective of better predicting pathophysiologically important physical–chemical interactions with membranes.

Ionization states of glycine and taurine, the common amino acid conjugates of bile salts in mammals, contribute fundamentally to physical properties of bile salts in solution (28). To quantitate these effects by reverse-phase HPLC, Heuman (7) systematically examined bile salt hydrophobicity as functions of pH_{app}. Due to protonation of glycine-conjugated bile salts, hydrophobic indices increased sharply with decreases in pH_{app} values in the vicinity of the pK_as. Due to the very low pK_as of ~1 of taurine conjugates (28), no pH_{app}-dependent variations in hydrophobicity were demonstrated for these bile salts. We observed similar effects of pH_{app} using IAM-HPLC (Figs. 3 and 4), with the exception that bile salt capacity factors increased as functions of pH_{app} for taurine- as well as glycine-conjugated bile salt species. In each case, pH_{app}-induced increases occurred at values substantially higher than the pK_a values of the same bile salts in aqueous solutions. Cabral, Hamilton, and Small (29) demonstrated increases in apparent pK_a values of approximately 2 log-units for unconjugated bile salts in the presence of egg lecithin vesicles. Strong increases in

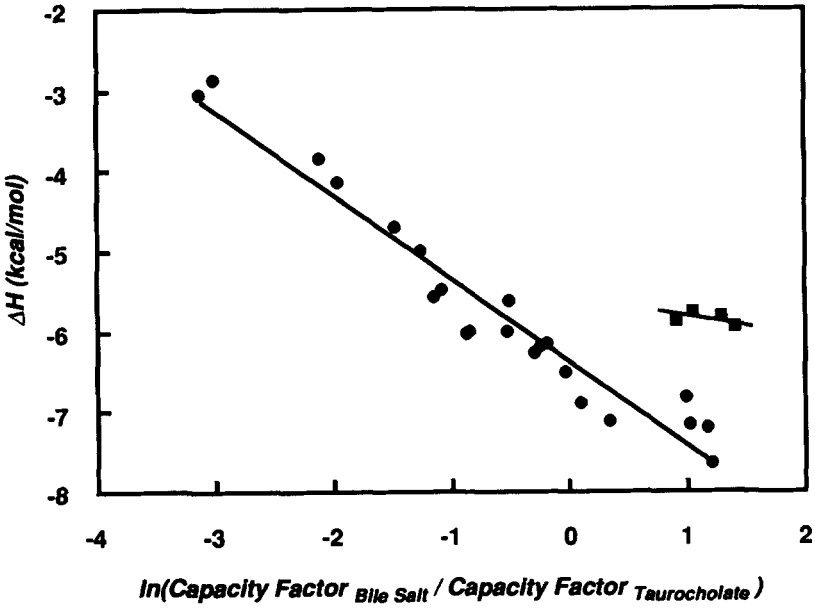

Fig. 6. Inverse relationship between values of ΔH for bile salt (●) and fusidate (■) binding to the IAM stationary phase, and IAM-HPLC capacity factors of individual bile salts normalized to capacity factors of taurocholate at 23°C (values from Table 1). The tight negative correlation for bile salts ($R<-0.97$) by linear least-squares analysis provides a standard curve from which ΔH values for other bile salts may be estimated from determinations of their capacity factors at room temperature (23°C).

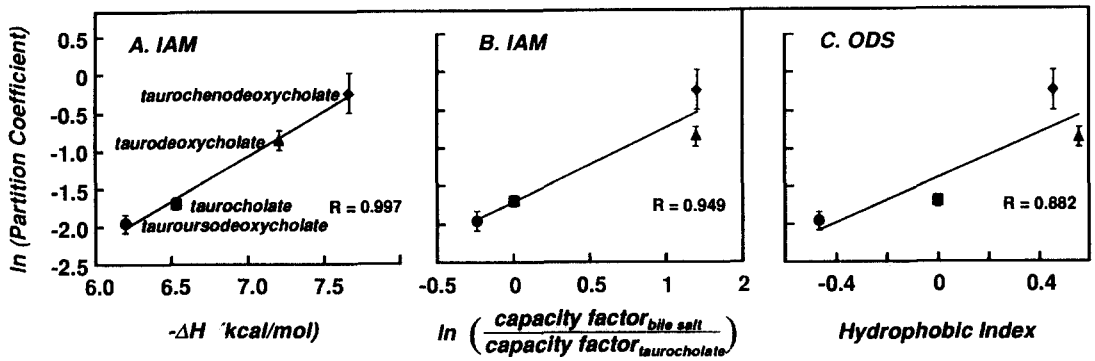

Fig. 7. Correlation between natural logarithms of equilibrium partition coefficients for bile salt binding to small unilamellar vesicles composed of egg yolk phosphatidylcholine and A) ΔH values determined by IAM-HPLC (Table 1); B) natural logarithms of IAM-HPLC bile salt capacity factors normalized to capacity factors of taurocholate at 23°C (Table 1); and C) literature values for bile salt hydrophobic indices as determined by ODS-HPLC (7). Values of ΔH best predict bulk bile salt–membrane interactions.

IAM capacity factors reflecting tighter binding of taurine conjugates beginning at pH_{app} 4.5 (Fig. 3) and glycine conjugates beginning at pH_{app} 6.5 (Fig. 4) are consistent with similar interfacial effects of immobilized phosphatidylcholine on the pK_as of conjugated bile salts. A consequence of these pH effects is that glycine-conjugated bile salts elute after their taurine-conjugated counterparts at pH_{app} 5.5, but that this order reverses at pH_{app} 6.5 (Fig. 4). Therefore, in contrast to bile salt hydrophobicity as defined by ODS-HPLC (6–8), IAM-HPLC at physiological pH demonstrates that taurine- compared with glycine-conjugated bile salts bind more avidly to membranes.

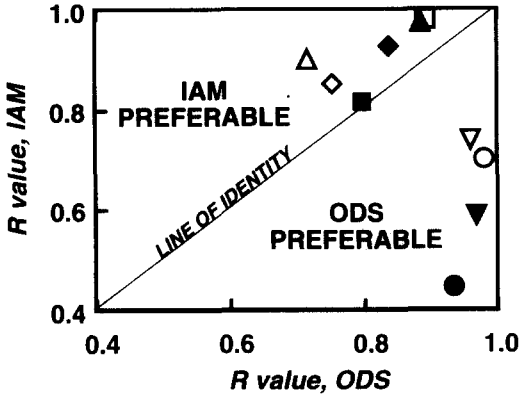

Fig. 8. Comparison of IAM- versus ODS-HPLC for predicting bile salt structure–activity relationships. Each point plots correlation coefficients r for a bile salt activity with IAM-HPLC determinations of ΔH on the vertical axis and with values of ODS-HPLC bile salt hydrophobic indices (7) on the horizontal axis. The line of identity represents points that are equally well correlated with IAM- and ODS-HPLC measures of bile salt hydrophobicity. Bile salt functions that are better correlated with values of ΔH (IAM Preferable) include: bile salt binding to small unilamellar vesicles (□) (Fig. 7), biliary phosphatidylcholine secretion rates in bile fistula hamsters (▲) (15), and in bile fistula prairie dogs (△) (14), stimulation of phosphatidylcholine transfer protein activity due to membrane perturbation by bile salts (◆) (26), bile salt dissolution rates of immobilized phosphatidylcholine membranes (■) (30), and intervesicular/intermicellar bile salt concentrations (◇) (Donovan, J. M., personal communication). Bile salt functions that are better correlated with bile salt hydrophobic index (ODS Preferable): solubility of cholesterol in simple bile salt micelles (i.e., no phosphatidylcholine) (●) (6), bile salt stimulation of cholesterol transfer from hepatocyte plasma membranes to high density lipoproteins (○) (32) and from high density lipoproteins to hepatocyte plasma membranes (▼) (32), bile salt stimulation of bile flow in bile fistula guinea pigs (▽) (31).

Capacity factors of bile salts increase strongly as functions of decreasing temperature (Fig. 3 and Table 1). The linearity of van't Hoff plots in Fig. 5 permitted calculations of ΔH values, which represent intrinsic quantitations of affinity of bile salt binding to the IAM stationary phase. Values of ΔH were negative for bile salts and fusidates, demonstrating membrane binding to be an exothermic process. Assuming that bile salt–membrane interactions are dominated by a large negative ΔH term (27), calculated values of ΔG should also be negative, as would be expected for spontaneous binding of bile salts to the IAM surface. Indeed, this is supported by a very tight positive correlation in Fig. 7A between ΔH values and membrane partition coefficients.

Measurements of capacity factors at multiple column temperatures (Table 1) in order to determine ΔH values are time and labor intensive. However, as illustrated for the data set at 23°C, there was a tight correlation ($R < -0.97$) between values of ΔH and normalized capacity factors for bile salts (Fig. 6). This relationship can be used conveniently as a standard curve for direct estimation of ΔH for a new bile salt based on a determination of its capacity factor at room temperature alone. In contrast, the same relationship did not apply for fusidates because measured values of ΔH plotted away from the regression line for bile salts (Fig. 6). Rather, ΔH values for fusidates appear to form a different line, suggesting the possibility that structurally related families of amphiphiles may generate distinct standard curves that reflect fundamental differences in physical–chemical mechanisms of binding to membrane lipids.

Values of ΔH for bile salts were correlated best with direct measurements of bile salt binding to model phosphatidylcholine membranes (Fig. 7A) whereas, by comparison, literature values for bile salt hydrophobic indices by ODS-HPLC (7) did not correlate nearly as well (Fig. 7C). These data imply that IAM-HPLC may be more suitable for predicting physiologically important bile salt–membrane interactions. To more fully assess this possibility, **Fig. 8** compares correlation of literature values for structure–activity relationships of bile salts with IAM- versus ODS-HPLC. Each point in Fig. 8 represents a pair of correlation coefficients in which points that fall on the line of identity are equally well correlated with IAM- and ODS-HPLC. Points that fall above the line of identity are better correlated with ΔH values by IAM-HPLC, whereas points that fall below are better correlated with bile salt hydrophobic indices by ODS-HPLC. IAM-HPLC of bile salts is more highly correlated with biliary secretion of phosphatidylcholine in hamsters (15) and prairie dogs (14), bile salt binding to model membranes (Fig. 7A), bile salt perturbation of membranes that results in stimulation of phosphatidylcholine transfer protein activity (26), and intermicellar/intervesicular concentration of bile salts (Donovan, J. M., personal communication). Whereas bile salt dissolution of membranes immobilized on Millipore filters (30) is slightly better correlated with IAM-HPLC, this data point falls very close to the line of identity in Fig. 8. A possible explanation is that detergent solubilization of membranes by micellar bile salt concentrations depends less upon interfacial interactions that are influenced by phosphatidylcholine head groups and more upon bile salt interactions with the hydrophobic hydrocarbon sides chains.

Figure 8 further demonstrates that ODS-HPLC is better correlated with solubility of cholesterol in simple micelles (6) and bile salt-stimulated bile flow (31). Interestingly, bile salt stimulation of cholesterol exchange between hepatocytes and high density lipoproteins (32) is better predicted by ODS- compared with IAM-HPLC despite high phospholipid contents of hepatocyte membranes. This may indicate that bile salt–phosphatidylcholine interactions are less important for promoting cholesterol transfer than other possible hydrophobic interactions such as between bile salts and a less abundant phospholipid class (e.g., sphingomyelin) or membrane cholesterol itself. Taken together, these results support the hypothesis that IAM-HPLC of bile salts more accurately predicts bile salt interactions with the principal phospholipid component of membranes, but that bile salt functions that do not require phosphatidylcholines are better predicted by conventional ODS-HPLC.

We have presented a new method for HPLC of bile salts using a recently developed IAM stationary phase and a principally aqueous mobile phase. Compared with previous measures of hydrophobicity, this technique includes interactions of both phosphatidylcholine head groups and hydrocarbon side chains in determining capacity factors of bile salts within the IAM column. Because phosphatidylcholines are the principal lipid component of most biological membranes and the major phospholipid class found in bile, this technique should prove valuable for predicting pathophysiologically important bile salt–membrane interactions as well as testing the therapeutic potential of bile salts and closely related amphiphiles that may be available in quantities too small to be evaluated by conventional physical–chemical studies.

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