Bile acid structure–activity relationship: evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC

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Abstract Two independent methods have been developed and compared to determine the lipophilicity of a representative series of naturally occurring bile acids (BA) in relation to their structure. The BA included cholic acid (CA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), ursocholic acid (UCA), hyocholic acid (HCA), as well as their glycine and taurine amidates. Lipophilicity was determined using a 1-octanol/water shake-flask procedure and the experiments were performed at different pH and ionic strengths and at initial BA concentrations below their critical micellar concentrations (CMC) and the water solubility of the protonated form. The experimental data show that both the protonated (HA) and ionized (A^-) forms of BA can distribute in 1-octanol, and consequently a partition coefficient for HA (logP' HA) and for A^- (logP' A^-) must be defined. An equation to predict a weighted apparent distribution coefficient (D) value as a function of pH and pKa has been developed and fits well with the experimental data. Differences between logP for protonated and ionized species for unconjugated BA were in the order of 1 log unit, which increased to 2 for glycine-amidated BA. The partition coefficient of the A^- form increased with Na^+ concentration and total ionic strength, suggesting an ion-pair mechanism for its partition into 1-octanol. Lipophilicity was also assessed using reverse phase chromatography (C-18-HPLC), and a capacity factor (K') for ionized species was determined. Despite a broad correlation with the logP data, some BA behaved differently. The logP values showed that the order of lipophilicity was DCA>CDCA>UDCA>HDCA>HCA>CA>UCA for both the protonated and ionized unconjugated and glycine-amidated BA, while the K' data showed an inversion for some BA, i.e., DCA>CDCA>CA>HCA>UDCA>HDCA>UCA. The logP data fitted well with other indirect measurements of BA monomeric lipophilicity such as albumin binding or accessible total hydrophobic surface area data calculated by energy minimization and molecular computer graphics. Differences between unconjugated and amidated BA are consistent with the presence of an amide bond and a lower pKa when pH dependence was studied. Capacity factors, on the other hand, were related to properties of BA micelles such as cholesterol-solubilizing capacity and membrane disruption, reflecting the BA detergency. The extrapolation of these data to biological phenomena must carefully consider the experimental conditions in which the interaction occurs, i.e., total BA concentration, ionic strength, Na^+ concentration, and pH, which in turn determine the BA species existing in solution that could interact with biological membranes or the lipid environment. —**Roda, A., A. Minutello, M. A. Angellotti, and A. Fini.** Bile acid structure-activity relationship: evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC. *J. Lipid Res.* 1990. **31:** 1433–1443.

Supplementary key words bile acid structure • ionization • interaction with membranes

Since the introduction of naturally occurring bile acids (BA) such as ursodeoxycholic (UDCA) and chenodeoxycholic acid (CDCA) as drugs to dissolve cholesterol gallstones, interest in these acids has increased in the last decade (1–3). UDCA now is widely used for gallstone dissolution and additional therapeutic indications in liver disease and gastroduodenal reflux are under investigation (4–7).

Despite numerous studies, the exact mechanism of action of these drugs is still unclear and many hypotheses have been put forward. Common BA possess a rigid nonplanar steroid nucleus with a short aliphatic side chain terminating in a carboxyl group. During their enterohepatic circulation, BA undergo several structural modifications, particularly side chain amidation with glycine or taurine and steroid dehydroxylation which significantly modifies the physicochemical properties in aqueous solution (8–14). The possible toxic effects of some BA such as LCA or DCA have been invoked to account for pathophysiological conditions during cholestasis (15–17). Moreover, the lipophilicity of a given BA may determine its effects on cholesterol transport by altering the detergency of the BA pool and the physicochemical state of bile (micelles and vesicles).

Abbreviations: BA, bile acids; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid; UCA, ursocholic acid; HCA, hyocholic acid; CMC, critical micellar concentration; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; BS, bile salts.

Defining the degree of lipophilicity of this class of acid steroids is therefore of interest in structure-activity relationship studies since few systematic accurate data are yet available and are limited to only some BA (18, 19).

Indirect evaluation of BA hydrophobicity such as albumin binding studies (20, 21), interaction with membranes (22), or reverse phase HPLC retention factors (11-13) strongly suggest that hydrophobicity depends on number, position, and orientation of the hydroxy group and amidation at the C-24 position. Hydrophobicity data indirectly determined chromatographically using C-18 stationary phase are often in good agreement with partition coefficients determined by static methods such as the 1-octanol/water shake-flask procedure (23, 24). Nevertheless, chromatography is a dynamic method and many rate processes can affect the validity of a thermodynamic measurement (association-dissociation, mass transfer, and elution process) (25). In addition, previous studies have been performed using an alcholic mobile phase of different composition and at apparent pH values ranging from 5 to 7, thus rendering any comparative evaluation method-dependent. The 1-octanol/water partition coefficient (log P) is the most widely used parameter in medicinal chemistry and can give accurate predictions of activity in a complex biological system such as membranes, keeping in mind the obvious limitation that the activity of the drug depends on its lipophilic nature (25, 26). In this study we report 1-octanol/water partition coefficients for a series of unconjugated and glycine- and taurine-conjugated BA using a shake-flask procedure. The experiments were carried out at BA concentrations below their CMC and below the aqueous solubility of the protonated species; the distribution coefficients were determined at different pH values (from 1 to 9), ionic strengths, and different counterion (Na^+) concentrations.

A model for the description of BA partition equilibrium as a function of pH and pKa is described to give the best fit for the experimental data. The data have been compared with results obtained using the reverse phase C-18 HPLC capacity factors (K') obtained under different conditions of the mobile phase. The differences between the two methods, as well as correlation with both in vitro and in vivo biological properties such as albumin binding and liver uptake, are discussed.

MATERIALS AND METHODS

Chemicals

The bile acids include seven unconjugated BA and their corresponding glycine and taurine amidates. Only taurocholic acid was studied for logP measurements (Table 1). The BA were obtained from Calbiochem, La Jolla, CA and some of them were gifts from Giuliani Spa, Milan, Italy (UDCA, UCA, CDCA). [24-^{14}C]Ursocholic acid was a gift from Alan F. Hofmann (UCSD, San Diego, CA).

Labeled BA were obtained from Amersham, UK and New England Nuclear, Boston, MA. The labeled glycine-amidated BA were synthesized using [1-^{14}C]glycine (sp act 40-60 mCi/mol) and were purified by TLC. Radiopurity was assessed by TLC radiochromatography using a Berthold LB-282 TLC Linear Analyzer and BA were found to be >99% pure. The solvent system used for the separation of conjugated BAs was propionic acid-isoamyl acetate-water-N-propanol 3:4:1:2 (v/v) and acetic acid-carbon tetrachloride-isopropylether-isoamyl acetate-N-propanol-benzene 1:4:6:8:2:2 (v/v) for the unconjugated BA.

BA were recrystallized as appropriate and purified by preparative TLC on silica gel G (2 mm thick) and the final purity was assessed by comparing melting points with those of reference standards, by HPLC, and TLC.

The BA were separated using a C-18 reversed-phase column (5 μm × 10 cm long). The HPLC analysis was performed using a Waters (Milford, MA) liquid chromatograph equipped with a column-thermostated system. The mobile phase consisted of methanol-0.01 M potassium phosphate (monobasic) 130:70 (v/v) pH 5.75, at a flow rate of 0.2 ml/min; the separated BA were recorded using a UV detector set at 200 nm.

In addition, a reversed phase TLC on C-18 plates, using methanol-water 3:2 (v/v) pH 4.5, as a solvent system was performed to further assess the purity.

1-Octanol/water partition coefficient

The 1-octanol/water partition coefficients were determined using both ^{14}C-labeled BA and unlabeled BA (**Table 1**). When the ^{14}C label was not available, a weighed solution of unlabeled BA was used.

The measurements of the 1-octanol/water partition coefficients were carried out starting from an initial concentration of a BA in the aqueous phase below the CMC of the ionized species and the water solubility of the protonated species (Table 1). Usually the initial concentrations ranged from 5 to 100 μmol/l when labeled or unlabeled bile acids were used (Table 2). If labeled BA were used, unlabeled BA were added to reach the same initial concentration. The initial radioactivity was approximately 10^6 dpm/ml. BA were dissolved in 10 ml of a buffer solution at pH values from 1 to 9. The aqueous buffer was previously presaturated with 1-octanol. The ionic strength in all experiments was kept constant. Na citrate buffer (0.1 M) was used from pH 1 to 5 and 0.1 M Na phosphate buffer from pH 5 to 9. Ten ml of 1-octanol presaturated with water was then added and the samples were left to equilibrate for 2 weeks under continuous stirring at a controlled temperature of 25 ± 0.1°C. After centrifugation the two phases were carefully separated. BA concentra-

TABLE 1. Physicochemical properties of the bile acids

Bile Acid (Trivial Name)	Symbol	Position and Orientation of Hydroxyls	Specific Activity of ^{14}C-Labeled Bile Acid	CMC (Ref. 9)		Water Solubility[a] (Ref. 10)	K_{aff} (Ref. 21)
				Water	0.15 M Na^+		
			Ci/mmol	*mM*		*μM*	*L/mol · 10⁴*
Cholic acid	CA	3α7α12α	25	13	11	273	0.33
Taurocholic acid	TCA		0.05	10	6		0.18
Glycocholic acid	GCA		0.05	12	10	32	0.26
Ursocholic acid	UCA	3α7β12α	0.5	60	39	1670	0.04
Glycoursocholic acid	GUCA		0.08	35	30	150	
Hyocholic acid	HCA	3α6α7α		17	8	45	0.15
Glycohyocholic acid	GHCA		0.05				
Chenodeoxycholic acid	CDCA	3α7α	10	9	4	27	5.5
Glycochenodeoxycholic acid	GCDCA		0.02	6	2	7	4.9
Ursodeoxycholic acid	UDCA	3α7β	5	19	7	9	3.8
Glycoursodeoxycholic acid	GUDCA		0.03	12	4	3	
Hyodeoxycholic acid	HDCA	3α6α		14	6	15	3.5
Glycohyodeoxycholic acid	GHDCA		0.06				
Deoxycholic acid	DCA	3α12α	0.05	10	3	28	4
Glycodeoxycholic acid	GDCA		0.04	6	2	6	3.5

[a]The solubility has been measured at pH 3 for unconjugated BA and at pH 2 for glycine-conjugated BA. The solubility of the protonated species of tauro-conjugated BA could not be measured.

tion in the two phases was measured using appropriate analytical methodologies according to the expected concentrations in the two phases (see below).

Radiochemical analysis

The concentration of radioactivity in both phases was measured. Since many BA were predominantly distributed in the 1-octanol phase, a large amount of the aqueous phase was required to achieve adequate accuracy in determining radioactivity. Usually 2 ml of the aqueous phase and 100 μl of 1-octanol were required. In order to control quenching, 100 μl of pure 1-octanol was added to the aqueous solution counting vials and 2 ml of water to 1-octanol counting vials. Ten ml of water-compatible liquid scintillation cocktail (Unisolve®, Kook Light) was added and the radioactivity was measured.

Enzymatic analysis

To correctly apply an enzymatic method to measure BA concentration in the 1-octanol phase, the sample must be freeze-dried to completely remove the 1-octanol which interferes in the assay. Moreover, the freeze-drying procedure concentrates the aqueous phase since the final concentration of bile acids is very low. Usually the aqueous phase is concentrated 10–100 times while the 1-octanol phase is only removed and reconstituted with aqueous 0.1 M phosphate buffer, pH 7.4. Bile acid concentrations were determined using an enzymatic bioluminescent assay, previously described (27). In particular, we used a specific 3α-hydroxysteroid dehydrogenase coupled with FMN oxidoreductase and bacterial luciferase.

The enzymes were immobilized on nylon tubes (1 mm id, 1.5 m long). The method is based on a continuous air flow in a segmented system; the overall analytical performances have been previously published in detail (28). The detection limit is 10 pmol/tube, allowing an accurate detection of all BA in both phases. The coefficient of variation of the method in both the inter- and intra-assay studies was less than 8%.

Enzyme immunoassay

The concentration of glycine-amidated cholic, chenodeoxycholic, and ursodeoxycholic acids was also assessed using a competitive solid phase enzyme immunoassay (29, 30). The method is a microtiter format in which the BA specific antibody is immobilized on the polystyrene support. Horseradish peroxidase-labeled BA were used as a tracer. The method acts directly on the sample, requires 1 h of incubation, followed by a washing step and the reading of the antibody-bound tracer using *o*- phenylendiamine/H_2O_2 as a chromogen/substrate at 490 nm. The method provides all the requisite standards of accuracy, precision, and the detection limit at pmolar levels. The intra-assay and inter-assay studies showed coefficients of variation less than 2% at both high and low concentrations. Since the method is extremely sensitive, the aqueous sample had to be diluted 1/100 to 1/1000. The 1-octanol sample had to be freeze-dried and reconstituted with water as appropriate.

Reverse phase HPLC method

The mobility of BA (K' retention factor) on a C-18 reverse phase column was determined using 0.01 M phosphate buffer–methanol 70:130 (v/v) pH 7.0, as a mobile phase. The apparent pH was maintained at 7.0 to ensure complete ionization of both classes of unconjugated and

amidated BA. The standard BA were dissolved in the mobile phase to give a final concentration of 1 mg/ml; the injection volumes were kept constant (0.5 μl) and differences within 0.5–1 μl had a negligible effect on retention time. Isocratic flow rate was 1 ml/min and the column temperature was kept constant 25 ± 0.1°C.

Additional experiments were performed either lowering the percentage of methanol in the mobile phase from 65 to 35% or increasing the [Na^+] to 0.15 M.

The HPLC capacity factor (K') was calculated from the eluted peak retention time (t):

$$K' = \frac{t_x - t_0}{t_0} \qquad Eq.\ 1)$$

where t_x and t_0 are the retention times of the bile acids and the unretained solvent front, respectively.

Expression of the data

Definitions. The partition properties in two immiscible phases are usually described by the partition coefficient (P) which refers to a single chemical species, e.g., one electrical form of an ionizable compound, and is distinct from the distribution coefficient (D) which is the ratio between the total concentration (protonated and ionized form) in the two phases.

Solutes that display some preference for the nonpolar phase (logP > 0) are termed lipophilic or hydrophobic, while solutes are labeled as hydrophilic (lipophobic) when logP <0. The terms lipophilicity and hydrophobicity are often considered synonymous. More recently it has been proposed to differentiate the two words. Lipophilicity can be expressed as a function of bulk (excluded volume) and polarity of the solute. "Hydrophobicity" refers to the first of these two parameters and is due to Van der Waals and hydrophobic forces. Hence, lipophilicity = hydrophobicity – polarity (25).

Model for BA 1-octanol/water distribution. The calculation of the intrinsic partition coefficient from the experimentally determined distribution coefficient requires accurate knowledge of the dissociation constants of the BA studied (31, 32). Usually, to simplify the calculations, the partitioning of the ionic form into 1-octanol is neglected, and this is a fair assumption, considering that the partition coefficient of the ionized form is 10^3–10^4 times smaller than that of the un-ionized form.

The general equation used for the calculation of logP from logD using this simplification is

$$\log P = \log D + \log(1 + 10^{pH - pKa}). \qquad Eq.\ 2)$$

The logP values calculated from this formula using experimental data obtained at pH = pKa ± 1 values, and also at very low pH, are inaccurate. This suggests that for BA molecules and, particularly, for relatively hydrophobic BA, such as dihydroxylated BA, the ionic species may also contribute markedly to the observed distribution coefficient, i.e., a large amount of ionized species partitioned into 1-octanol, thus decreasing the accuracy of the calculated partition coefficients.

These observations suggest that the simple assumption used above does not fit for BA, and the calculation of logP must also consider that ionized BA can distribute in 1-octanol. The BA structure accounts for this phenomenon: a strongly hydrophobic steroid bulk mainly determines the lipophilic character of these molecules and the ionization of the C-24 carboxy group plays a minor role.

According to these findings, both ionized and protonated species must be considered to partition in the 1-octanol phase and, consequently, the distribution coefficient D which takes this into account, must be defined:

$$D = \frac{[HA]_{oct} + [A^-]_{oct}}{[HA]_w + [A^-]_w} \qquad Eq.\ 3)$$

where [HA] and [A^-] are the concentrations of protonated and ionized species in 1-octanol (oct) and water (w), respectively. Combining this equation with the dissociation constant expression and with the intrinsic partition coefficient of ionized and protonated species gives:

$$D = \frac{P'_{HA}\,[H^+] + P'_A\,K_a}{[H^+] + K_a} \qquad Eq.\ 4)$$

where P'_{HA} and P'_A are the intrinsic partition coefficients or HA and A^-, respectively, and K_a the dissociation constant of the BA. Thus D is a weighted average of P'_A and P'_{HA}, which are measured at pH values at least 2 units below and above the pKa, respectively.

RESULTS

Analytical considerations

Accurate measurements of the 1-octanol/water partition coefficient require the use of highly specific and precise analytical methods. The necessity to carry out the experiments below the CMC of the anion and the water solubility of the protonated species limited the choice of analytical methods. The initial concentration of the BA as reported in **Table 2** ranged from 5 to 100 μM. The relatively high differences in BA lipophilicity greatly affect the equilibrium concentration in the two phases, with a final concentration in one of the two phases very low (usually water). Different methods have been used as reported in Table 2 according to the analytical facilities available commercially or in house.

TABLE 2. Experimentally determined partition coefficients of the protonated (P'_{HA} and $logP'_{HA}$) and ionized (P'_A and $logP'_A$) bile acids (mean values of five experiments ± SD)

Bile Acids	Initial Concentration	Method	P'_{HA}	$logP'_{HA}$	P'_A	$logP'_A$
	μM					
CA	100	R/En	100 ± 5	2.02	12.5 ± 0.6	1.1
TCA	100	R/EIA	nd	nd	0.32 ± 0.01	-0.50
GCA	10	R/EIA	40.7 ± 2.8	1.65	0.40 ± 0.02	-0.40
UCA	100	R/En	7.9 ± 0.4	0.92	1.00 ± 0.04	0
GUCA	100	En	4.8 ± 0.2	0.68	0.07 ± 10^{-3}	-1.15
HCA	100	En	630 ± 12	2.80	70 ± 2	1.84
GHCA	100	En	79.4 ± 4	1.90	1.25 ± 0.01	0.10
CDCA	10	R/En	1905 ± 10	3.28	178 ± 2	2.25
GCDCA	10	R/EIA	132 ± 2	2.12	2.8 ± 0.01	0.45
UDCA	5	R/EIA	1000 ± 15	3.0	158 ± 1	2.20
GUDCA	5	EIA	105 ± 5	2.02	1.58 ± 0.01	0.20
HDCA	5	EIA	1202 ± 10	3.08	190 ± 2	2.28
GHDCA	5	EIA	141 ± 9	2.15	1.70 ± 0.01	0.23
DCA	5	R	3160 ± 20	3.50	446 ± 4	2.65
GDCA	5	R	178 ± 8	2.25	6.3 ± 0.02	0.80

R, radiochemical method; EIA, enzyme immunoassay; En, enzymatic method; nd, not determinable.

All the methods gave accurate results when compared, and the coefficients of variation of the enzymatic method or enzyme immunoassay were always less than 3–5%. The use of biospecific methodologies limited the effect of impurities of the matrix thanks to the high specificity of the enzymes or antibodies used, particularly when the water samples were concentrated.

The capacity factors, on the other hand, were less affected by the above-mentioned problems; the only limitations derive from the high percentage of organic content in the mobile phase, low pH range, and the column characteristics that limited interlaboratory comparison.

Determination of BA lipophilicity by 1-octanol/water partition coefficient

The ionization of an acid changes with the pH. Experimentally, and under specified conditions of pH and ionic strength, one obtains the distribution coefficient (D) of the ionizable solute. The distribution coefficient is strongly affected by the BA structure with differences of a factor 10^3 between UCA and DCA (**Fig. 1**). The logD versus pH plot for some unconjugated and glycine-conjugated BA are reported in Fig. 1 and **Fig. 2.** The plot is a sigmoid curve with an inflection point at a pH equal to the pKa of the BA, i.e., 5 for unconjugated BA (pKa = 5) and 4 for glycine conjugates (pKa = 3.9). The logD of the taurocholate slightly increases as the pH is lowered, but never reaches a plateau.

P'_A and P'_{HA} values and the corresponding log data for the studied BA are reported in Table 2. The calculated D values at different pH using equation 4 fit well with the experimentally measured data, suggesting the validity of the proposed model (Table 2).

Trihydroxy BA in the order of 3α7β12α, 3α7α12α, and 3α6α7α present the lowest P'_A and P'_{HA} values, followed by dihydroxy BA in the order 3α6α, 3α7β, 3α7α, and 3α12α. Amidation with glycine modifies the pH behavior, and the P'_A and P'_{HA} values are systematically lower than those of the corresponding unconjugated BA.

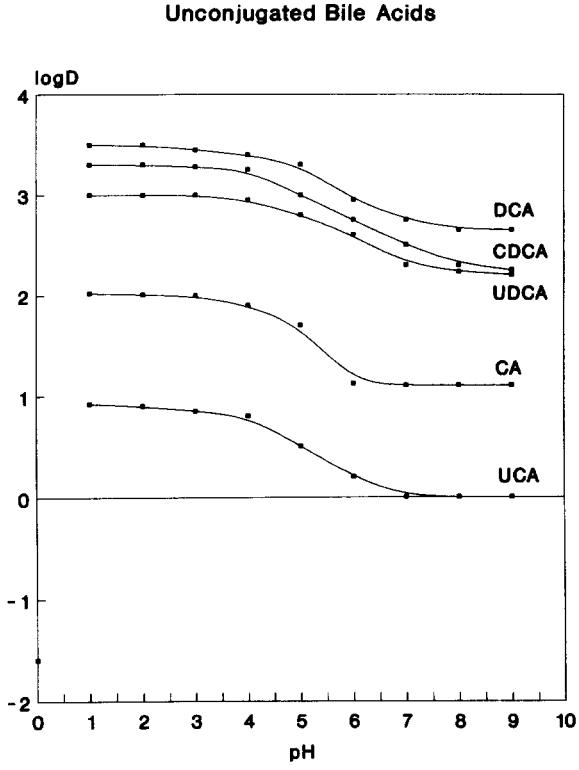

Fig. 1. Effect of pH on 1-octanol/water distribution coefficient for unconjugated bile acids.

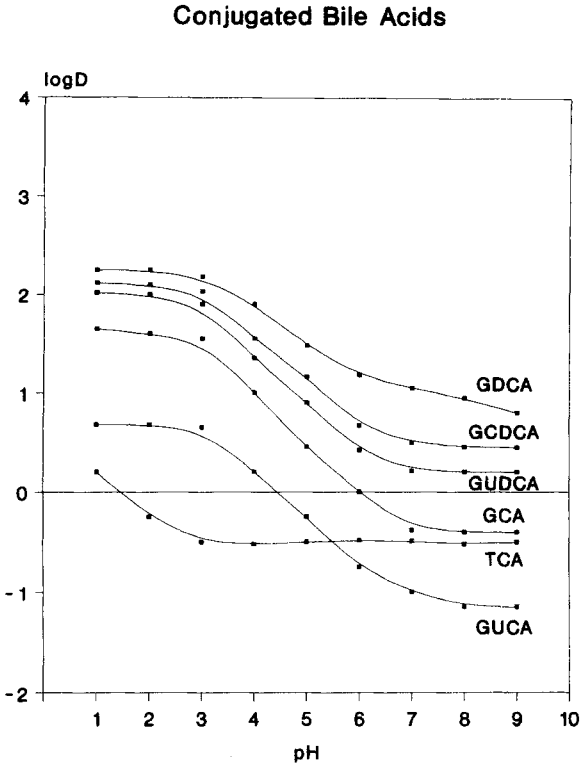

Fig. 2. Effect of pH on 1-octanol/water distribution coefficients for glycine- and taurine-conjugated bile acids.

The differences among P'_{HA} and P'_{A} for glycine-amidated BA are higher than those of the corresponding unconjugated BA, usually in the order of two log units. The P'_{HA} for the taurine amidate could not be measured while the P'_{A} was similar to those of glycine-amidated BA (Table 2), at least for taurocholic acid. It is of interest that the P'_{A} values for dihydroxy unconjugated BA such as DCA, CDCA, or UDCA were higher than the P'_{HA} of the corresponding glycine-amidated BA.

Ion-pair effect

The distribution of a bile salt at relatively high pH (7.0) is strongly affected by the Na^+ counter ion activity. Increasing the initial Na^+ concentration in the aqueous phase from 0.01 to 100 mM, increases the 1-octanol/water distribution coefficient. Data for cholic acid and glycocholic acid are reported in **Fig. 3.** The logD values begin to increase when the Na^+ concentration was similar to that of BA, i.e., 0.2 mM.

The amount of Na^+ in the 1-octanol phase is very low in respect to the initial concentration in the aqueous phase, and less than 2% distributes into 1-octanol. The anionic species of the buffers (citrate, phosphate) do not distribute into 1-octanol, as evaluated by the determination of citrate and phosphate in the aqueous phase using conventional enzymatic or chemical methods.

Determination of BA lipophilicity by reverse phase chromatography

The capacity factors of unconjugated, glycine-, and taurine-conjugated BA are reported in **Table 3.** Data are in quite good agreement with those obtained under similar analytical conditions (12, 13) or data obtained with a different composition of the mobile phase (11). The order of elution is $3\alpha7\beta12\alpha$, $3\alpha7\beta$, $3\alpha6\alpha$, $3\alpha6\alpha7\alpha$, $3\alpha7\alpha12\alpha$, $3\alpha12\alpha$, $3\alpha7\alpha$ and is the same for both unconjugated and glycine- or taurine-amidated BA.

When the experiments were performed at higher Na^+ content, i.e., 0.15 M, the elution times followed the same trend but differences between unconjugated and amidated BA were lower (Table 3). Slight differences exist among glycine- and taurine-conjugated BA and this tallies with the structure of these molecules. When the K' values obtained at different volume fractions of methanol were plotted for two representative BA such as taurocholic and tauroursodeoxycholic acid (**Fig. 4**), a linear relationship was observed only at the higher methanol volume fractions; at lower volume fractions of methanol, a marked deviation from the linearity was observed. The volume fraction of methanol cannot be lower than 0.5, since the retention time becomes too long and the BA are completely retained by the column.

The inter-method comparison results show a correlation with the 1-octanol partition coefficients ($logP_A$) and the capacity factors (K') but some BA behave in opposite

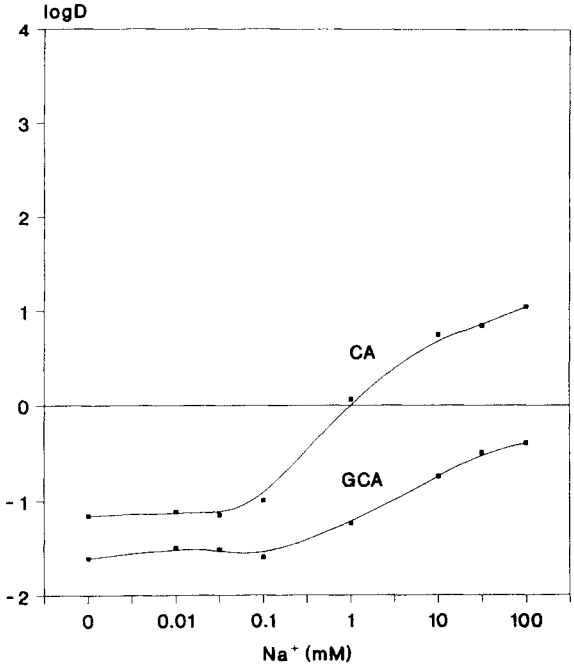

Fig. 3. Effect of $[Na^+]$ concentration on 1-octanol/water distribution coefficient at pH = 7 for cholic acid glycocholic acid (initial BA concentration = 200 μM).

TABLE 3. Capacity factors, K', and relative capacity factors, rK'

Bile Acids	K'[a] Present Work A⁻	rK'[b] Present Work A⁻	K'[a] Present Work A⁻	rK'[b] Present Work A⁻	rK' (Ref. 11) A⁻	K' (Ref. 12) A⁻	rK' (Ref. 12) A⁻	HI[c] (Ref. 13) HA	HI[c] (Ref. 13) A⁻
	0.15 M Na⁺								
CA	4.54	0.37	3.92	0.43	0.35	7.12	0.41	0.83	0.13
TCA	3.71	0.30	1.88	0.19	0.31	1.04	0.06	0.00	0.00
GCA	3.86	0.31	1.93	0.21	0.30	3.43	0.20	0.30	0.07
UCA	0.91	0.07	0.99	0.10					
TUCA			0.40	0.04					-0.94
GUCA			0.46	0.05					
HCA			3.70	0.41					
THCA			1.80	0.19					-0.45
GHCA			1.99	0.22					
CDCA	10.54	0.86	8.03	0.89	0.80	15.28	0.89	1.37	0.59
TCDCA	7.78	0.63	3.65	0.40	0.68	1.80	0.10		0.46
GCDCA	8.34	0.68	4.07	0.43	0.65	6.27	0.36	0.77	0.51
UDCA	2.44	0.20	3.66	0.40	0.25	4.75	0.27	0.49	-0.31
TUDCA	2.00	0.16	0.98	0.10	0.34	0.55	0.03		-0.47
GUDCA	2.02	0.16	1.06	0.11	0.24	1.71	0.10	0.15	-0.43
HDCA			3.60	0.39	0.36				
THDCA			1.00	0.11	0.29				-0.35
GHDCA			1.08	0.12	0.28			0.01	-0.30
DCA	12.21	1.00	9.11	1.00	1.00	17.09	1.00	1.46	0.72
TDCA	9.48	0.77	2.85	0.30	0.83	2.21	0.12		0.59
GDCA	10.21	0.83	5.05	0.54	0.83	7.89	0.46	0.94	0.65

[a] K', capacity factor calculated as reported in the text.
[b] rK', relative capacity factor calculated according to $rK' = \frac{t_{BA} - t_0}{t_D + t_0}$ where t_{BA} = retention time of the BA and t_D = retention time of deoxycholic acid.
[c] HI, hydrophobicity indices of BA protonated (HA) and ionized (A) forms from ref. 13.

ways (**Fig. 5**). This is particularly evident for UDCA and CA in which the partition coefficient shows the order log P_{UDCA} > log P_{CA} while for K': K'_{CA} > K'_{UDCA}. This is also evident for all the BA containing an equatorial hydroxy group (see 3α6α, 3α6α7α). The differences between unconjugated, glycine-, and taurine-conjugated BA are consistent with the presence of an amide bond and both logP and K' values are all systematically lower than those of unconjugated BA (Table 3).

DISCUSSION

The methodological approach used for the measurements of BA lipophilicity using either 1-octanol/water partition coefficients or capacity factors is fundamental for accurate definition of this physicochemical property.

The analytical methods used for logP experiments gave accurate values of BA concentrations in both phases. The impurities present in the matrix play a minor role since biospecific methods have been used (enzymatic or enzyme immunoassay).

Bile acids also distribute preferentially in 1-octanol when protonated, and substantial amounts of BA can partition in 1-octanol when ionized, i.e., at high pH. This is

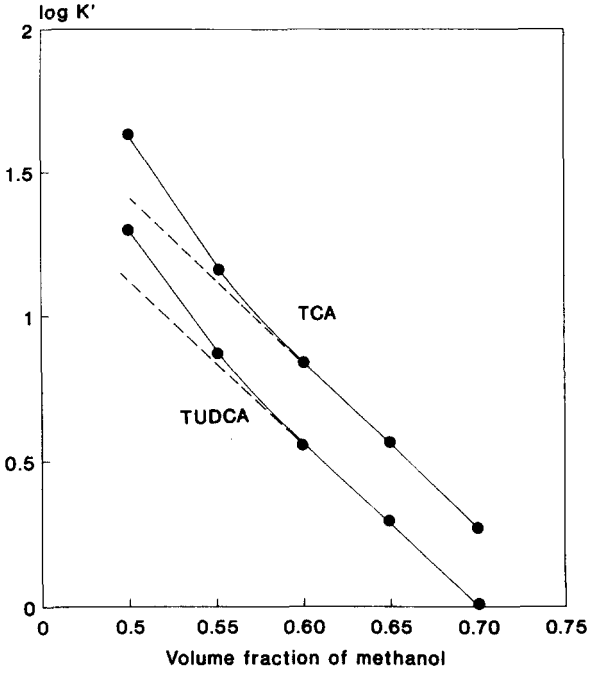

Fig. 4. Effect of volume fraction of methanol on the capacity factors for taurocholic and taurodeoxycholic acid.

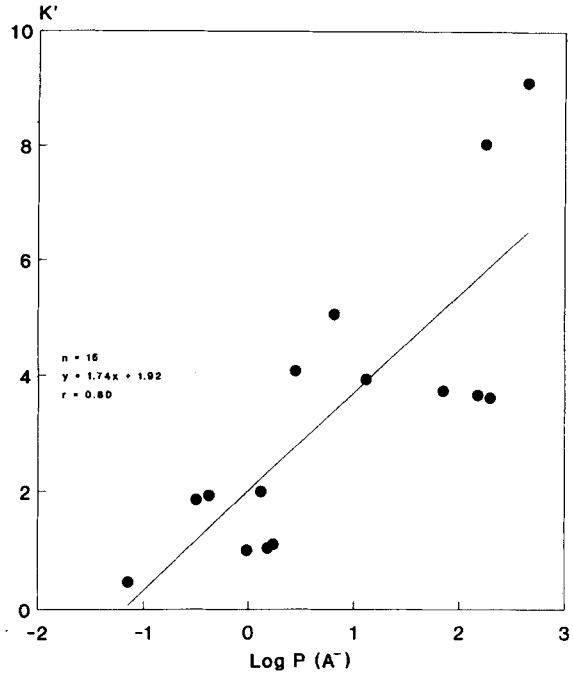

Fig. 5. Relationship between capacity factors (K') and the bile salt (anion) partition coefficients.

particularly true for unconjugated dihydroxy bile acids such as CDCA, DCA, and UDCA, and the data showed slight differences in the partition coefficients P'_A and P_{HA} of the two species, i.e., protonated and ionized.

The variation of the distribution coefficient with the pH shows a sigmoidal curve typical of ionizable acid molecules and the inflection occurs at the pKa of the BA. As the lipophilic character of the bile acid increases, the inflection point becomes less pronounced and the difference between the partition coefficients of the two species decreases as the hydrophobicity of the molecule predominates over the effect due to ionization.

The distribution of ionized BA into nonpolar amphiprotic 1-octanol can be attributed both to the lipophilic character of the molecule and to an evident ion-pair phenomenon. The ion-pair partition of the complex bile acid anion and Na^+ into 1-octanol is demonstrated by the strong effect of Na^+ on the distribution coefficient measured at pH 7. This effect further explains the behavior of the distribution coefficient with pH and the high logP values obtained even at high pH.

As far as the relationship between partition data and BA structure is concerned, the data show that the order of lipophilicity is related mainly to the number of hydroxy groups $3\alpha,12\alpha > 3\alpha7\alpha > 3\alpha,7\beta > 3\alpha,6\alpha > 3\alpha6\alpha7\alpha > 3\alpha,7\alpha,12\alpha > 3\alpha,7\beta,12\alpha$ in the steroid nucleus. The presence of an equatorial hydroxy group ($6\alpha,7\beta$), i.e., inserted in the hydrophobic back of the steroid, slightly decreases the lipophilicity over its axial epimer. The position of the hydroxy group slightly modifies the lipophilicity (see $3\alpha7\alpha$ vs $3\alpha12\alpha$).

The logP data suggest that among unconjugated or glycine-amidated BA, it is the hydrophobic surface area of the steroid nucleus that influences the overall lipophilicity of the BA molecules and that the topographical arrangement of the hydroxy group is less relevant. This is also consistent with the partition theory which refers to a property of a molecule as a monomer independent of movement and orientation in the bulk solution.

Comparisons with logP data measured by others (18, 19) using a similar 1-octanol procedure are misleading. Vadnere and Lindenbaum (18) reported logP data for BA, but the partition was performed using an equilibrium between a micellar BA solution at pH 7. The logP data collected using a premicellar BA concentration are, however, in good agreement with ours.

The accuracy of the 1-octanol/water determination was further confirmed by the correlation between the log P data and the BA molecular surface area obtained by energy minimization and molecular computer graphics (33, 34).

If we correlate the total hydrophobic surface areas of the α and β sides of the BA molecule, as calculated by Koichiro et al. (34) with log P data, a good correlation is observed (**Fig. 6**). On the other hand, the logP data do not fit well with the hydrophobic surface area of only the β side of the BA.

As far as the effect of amidation on 1-octanol water partition coefficient is concerned, the presence of an amide bond significantly lowers the partition coefficient. The increase in the length of the side chain is not compensated

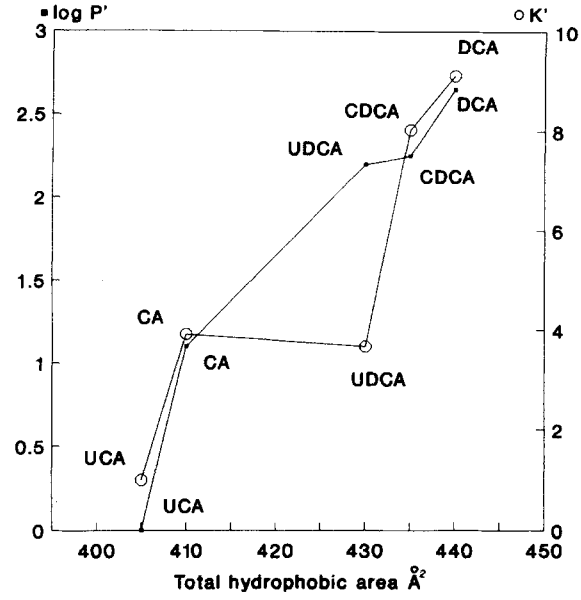

Fig. 6. Comparison between capacity factors (K'), the bile salt (anion) partition coefficients, and total hydrophobic area of bile acids.

for by the presence of the polar amide. Both $logP'_A$ and $logP'_{HA}$ of glycine conjugates are significantly lower and the inflection point of the curve occurs at low pH as result of their lower pKa (3.9) with respect to unconjugated BA (5.0). Differences in the $logP_{HA}$ and $logP_A$ for glycine-amidated BA higher than those for unconjugated BA. Taurine-conjugated BA have lower values even at very low pH as a result of the low pKa of the taurine moiety. Moreover, at the same degree of ionization, i.e., at high pH, the $logP'_A$ values of taurine conjugates are similar to those of glycine conjugates. These data are partially in agreement with our previously published data on the quantification of hydrophilicity using C-18 reverse phase HPLC (11-13).

Despite a good correlation between highly hydrophilic or highly hydrophobic bile acids such as UCA or DCA, the correlation fails when, for example, CA and UDCA, HDCA and HCA are compared. The HPLC data showed that UDCA or HDCA are more hydrophilic than CA according to their lower retention time on the C-18 column. In our opinion, the partition equilibrium using HPLC is mainly driven by an apolar interaction between the β face of the BA molecule and the stationary phase, since their values are well related to the accessible hydrophobic surface area of the β face (33, 34).

A recent study by Heuman (13) on quantitative estimation of the hydrophilic/hydrophobic balance of bile salts by C-18 HPLC reported data for BA consistent with previous studies. This suggests that their capacity factor can predict the properties of concentrated mixed micellar BS solutions (up to CMC), thus reflecting the detergent properties and, consequently, the related interaction with complex biological systems (solubilization of cholesterol and phospholipids, micellar transport of cholesterol and phospholipids, and cytotoxicity).

On the other hand, the 1-octanol/water partition coefficients seem to be related to the intrinsic monomeric lipophilicity of a BA which is independent of the topographical distribution of hydroxyls but related to the ratio between hydrophobic and hydrophilic area. As a consequence, the biological phenomena related to these data are those in which the BS is involved as a monomer, e.g., interaction with proteins, passive liver uptake, etc. In the light of these findings it is not surprising that CA is more hydrophilic and a better detergent than UDCA as a result of more hydroxy groups and their favorable positions on the steroid ring (all oriented toward the α-face, allowing good back-to-back self-aggregation of the molecule).

As previously reported (9) we measured the critical micellar concentration of a large number of BA and showed that not only the number of hydroxy groups may be more important, but that their position and orientation are also key factors in determining the CMC value, i.e., the detergency of the BA molecule. UDCA contains fewer hydroxy groups (less hydrophilic) but their orientation on both faces of the steroid perturbs the hydrophobic area thus rendering less favorable micelle formation, i.e., UDCA has less detergency.

On the other hand, other physicochemical properties strongly indicate that UDCA is less hydrophilic than CA, particularly the affinity constant (K_{aff}) of this BA with human serum albumin (20). In previous studies carried out in our laboratory using microcalorimetric techniques and thermodynamic calculations, we demonstrated that the interaction of BA with HSA is hydrophobic and the affinity constant values fit with the 1-octanol/water data. The K_{aff} values for CA and UDCA are 0.3×10^4 and 3.8×10^4 $dm^3 \cdot mole^{-1}$, respectively (20).

Physiological implications

Table 4 shows the best predictors of a given biological property using either 1-octanol/water partition coefficients or the capacity factors. The data clearly show that the results obtained by the two methods are different and in some way complementary because of the different chemical and thermodynamic mechanisms involved. The C-18 HPLC capacity factor values seem to reflect and to predict the detergent properties of a given BA and the related biological properties such as solubilization of cholesterol, disruption of membranes. This results from the type of in-

TABLE 4. Relationship between biological phenomena and C-18-HPLC K' and 1-octanol/water logP

Biological System	Interaction	Predictor	Ref.
Micellar cholesterol solubilizing capacity	BS (mixed) micelles	C-18 HPLC K'	12
Solubilization of lipid membranes	BS (mixed) micelles	C-18 HPLC K'	22, 37
Ability to permeate vesicles	BS (mixed) micelles	C-18 HPLC K'	16
Promote absorption of drugs	BS (mixed) micelles	C-18 HPLC K'	38
Albumin binding	BS monomer	1-octanol/water logP	20, 21
Passive hepatic uptake	BS monomer	1-octanol/water logP	36
Passive intestinal absorption	BS monomer	1-octanol/water logP	39
Lipid membrane partition	BS monomer	1-octanol/water logP	8

teraction between the stationary phase and the hydrophobic back of the BA molecule, which is similar to the BA aggregation mode, to form primary micelles (back to back).

On the other hand, the 1-octanol/water partition coefficient data are consistent with a property of the monomeric form of the BA and the distribution occurs as a result of the overall lipophilicity of the molecule. As a consequence, all the interactions with a biological system involving a BA monomer (<CMC) in aqueous solution, such as albumin binding or passive hepatic or intestinal uptake, are well predicted by the logP data.

Other physiological properties such as the choleretic effect or acute and chronic effects on transport of cholesterol and phospholipids, which gave controversial results, must be carefully evaluated considering the biotransformations that administered BA undergo, the physicochemical properties of the metabolites (40-42), and additional specific effects.

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