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BILE ACIDS

LXIII. RELATIONSHIP BETWEEN THE MOBILITY ON REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THE STRUCTURE OF BILE ACIDS*

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SUMMARY

The mobility of bile acids, as defined by the term "relative capacity factor", was determined on a μBondapak C_{18} column, eluted with a mixture of 2-propanol and 10 m*M* potassium phosphate (pH 7.0). The contributions to mobility due to substituents such as hydroxyl, oxo, olefin, and side-chain methylene and amino acid substituents were calculated. From these values, the relative capacity factors of polyfunctionalized bile acids were computed. The agreements were good between the theoretical and observed values, provided that only hydroxyl substituents were present on the α-surface and that they were spaced sufficiently apart. When β-hydroxyl or oxo groups were also attached to the steroid nucleus, substantial disagreements were noted which could be rationalized on the basis of conformational analysis. Thus, the mobility of a compound is not necessarily correlated to the sum of the polarity contributions of each substituent.

INTRODUCTION

The ability to predict the chromatographic mobility of a compound under fixed conditions, based on its structure, offers many advantages to the analyst. Several studies have been reported on the correlation of these two parameters in gas–liquid[1–3] and thin-layer chromatography (TLC)[4] with steroids. Recently, attempts have been made with small non-rigid molecules to determine the relationship between mobility

* Common bile acids are derivatives of 5β-cholan-24-oic acid with hydroxyl substituents; their trivial names, and the positions and orientations of the hydroxyl substituents (–OH) are: cholic acid, 3α,7α,12α-$(OH)_3$; deoxycholic acid, 3α,12α-$(OH)_2$; chenodeoxycholic acid, 3α,7α-$(OH)_2$; lithocholic acid, 3α-(OH); ursodeoxycholic acid, 3α,7β-$(OH)_2$; hyocholic acid, 3α,6α,7α-$(OH)_3$; hyodeoxycholic acid, 3α,6α-$(OH)_2$; α-muricholic acid, 3α,6β,7α-$(OH)_3$; β-muricholic acid, 3α,6β,7β-$(OH)_3$; ω-muricholic acid, 3α,6α,7β-$(OH)_3$. Bile acids also occur in the 5α-(*allo*-) configuration in small amounts in mammals; *e.g.* *allo*-cholic acid is 3α,7α,12α-trihydroxy-5α-cholan-24-oic acid.

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and structure in reversed-phase high-performance liquid chromatography (HPLC) by the use of concepts such as hydrocarbonaceous surface area[5], number of carbon atoms[6], and the polarities of the substituent groups[7].

However, the effect of the stereochemical configuration of a substituent on mobility is unknown and can be obtained with rigid molecules, such as steroids. Such investigations are particularly relevant in regard to the bile acids (Fig. 1), because the latter function biologically as detergents: the mobility in reversed-phase HPLC and the critical micellar concentration are related, as expounded in a separate paper[8]. Development of a method to separate and quantitate fecal bile acids, many of which[9] are included in this study, will be useful in this endeavor. The following text presents a rational approach to the determination of the contribution due to individual polar substituents.

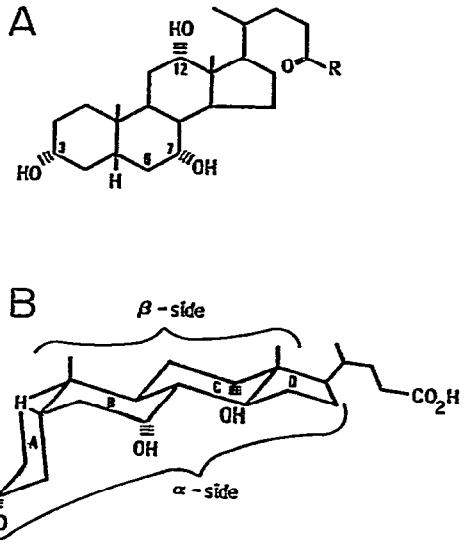


Fig. 1. Basic structure of a bile acid. (A) Substituents encountered in this study are located at the numbered carbon atoms. Free bile acid, R=OH; glycine-conjugated bile acid, R=$NHCH_2CO_2H$; taurine-conjugated bile acid, R=$NH(CH_2)_2SO_3H$; δ-aminovaleryl conjugate, R=$NH(CH_2)_4CO_2H$. (B) Side view of cholic acid showing the plane of the steroid nucleus, and the β- and α-surfaces.

EXPERIMENTAL

The analytical instrument was a Waters Assoc. Model ALC 201 liquid chromatograph, housing a U6K loop injector, a μBondapak C_{18} column (30 × 0.4 cm I.D.), a Model 6000 pump, and a R401 differential refractometer. The eluent consisted of 2-propanol–10 m*M* potassium phosphate, pH 7.0 (8:17) at a flow-rate of 1 ml/min. The composition was varied occasionally, as indicated. At the end of each day of use, the column was washed with methanol–water (63:37), as recommended by the manufacturer. The amount of each bile acid injected in 10 μl of methanol was kept at a minimum without affecting the visualization of the peak, while the differential refractometer was set at 8×. For the determination of the relative capacity factor, the com-

pound of interest was injected in a solution of 10 μl of methanol containing the standard, which was usually deoxycholic acid. In instances where deoxycholic acid was unsuitable as a standard because of inappropriate elution times, other bile acids were used as references during chromatography. The mobilities were then calculated in relation to deoxycholic acid.

Bile acids were pure substances, prepared and characterized previously in this laboratory. 3α-Hydroxy-5β-chol-11-enoic acid, 3α-hydroxy-12-oxo-5β-chol-9(11)-enoic acid, 23-*nor*-3α,12α-dihydroxy-5β-cholanoic acid, glycohyodeoxycholate and taurohyodeoxycholate were obtained from Steraloids (Wilton, NH, U.S.A.). Glycoursodeoxycholate and tauroursodeoxycholate were products of Calbiochem (La Jolla, CA, U.S.A.). Methyl esters were hydrolyzed overnight at room temperature with aqueous methanolic KOH to generate the free bile acids, which were collected after precipitation with mineral acid. 2-Propanol and methanol were Fisher HPLC grade; potassium hydrogen phosphate was Mallinckrodt analytical grade, and distilled water was de-ionized.

RESULTS

Bile acids with dissociation constants ranging between pH 5 and 6.5[10] were analyzed as anions in a buffer maintained at pH 7.0. Poor solubility of these compounds in the elution mixture did not pose a problem if they were introduced into the chromatograph as a solution in methanol. Within the range of 2–12 μl the volume of methanol had a negligible effect on mobility denoted as capacity factor (k') (Fig. 2). Snyder and Kirkland[11] have indicated that the capacity factor is unaffected by small fluctuations (1–3°C) in room temperature. However, several other parameters did influence the capacity factor.

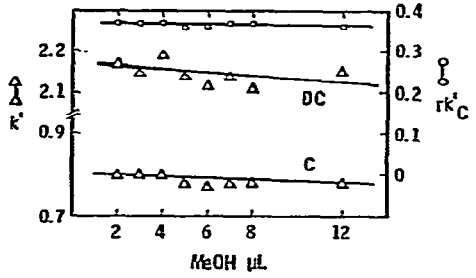


Fig. 2. Effect of methanol on the k' and rk' values. C=cholate; DC=deoxycholate.

After repeated injections of several concentrations of bile acids during the course of a day, the capacity factor of a bile acid decreased slowly (Fig. 3A). This change was observed consistently over a period of fourteen days for cholic acid, deoxycholic acid (Fig. 3B), lithocholic acid, and hyodeoxycholic acid. These observations suggest that the continuous use of the column leads to blocking of some of the binding sites on the column. Subsequent studies showed that the capactity factor could be almost completely reproduced each day after washing the column with 60 ml of methanol–water (63:37).

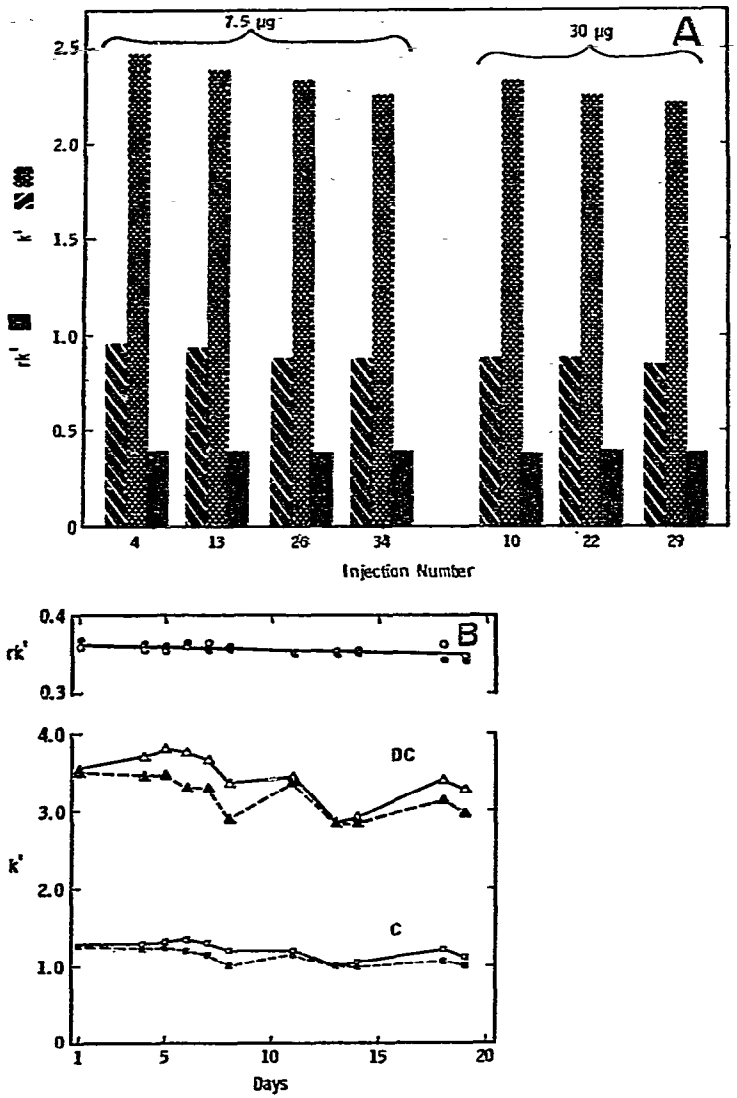


Fig. 3. Changes in k' and rk' values after multiple injections during each work-day. (A) During the course of a work-day, 34 injections were made in which the weight of the solute dissolved in 10 μl of methanol was varied at random. Data for only two amounts (7.5 μg and 30 μg) of each solute are given. The solutes are: cholic acid (C), the k' values of which are represented by the striped left-hand bar, and deoxycholic acid (DC), the k' values of which are represented by the crossed center bar. The solid bar shows the rk' values. The specific injection numbers of these concentrations are given on the abscissa. (B) The k' values are consistently lower at the end of the day (closed squares and triangles) than at the beginning of the day (open squares and triangles) over 3 weeks. The rk' values are fairly constant.

This phenomenon affected the interpretation of the observed effect of solute loading on the k' value. The value of k' was reduced as solute weight was increased (Fig. 4). The reduction was more significant at lower solute load (open symbols; Fig. 4A) than at higher load. However, the slope of the decline at low solute load

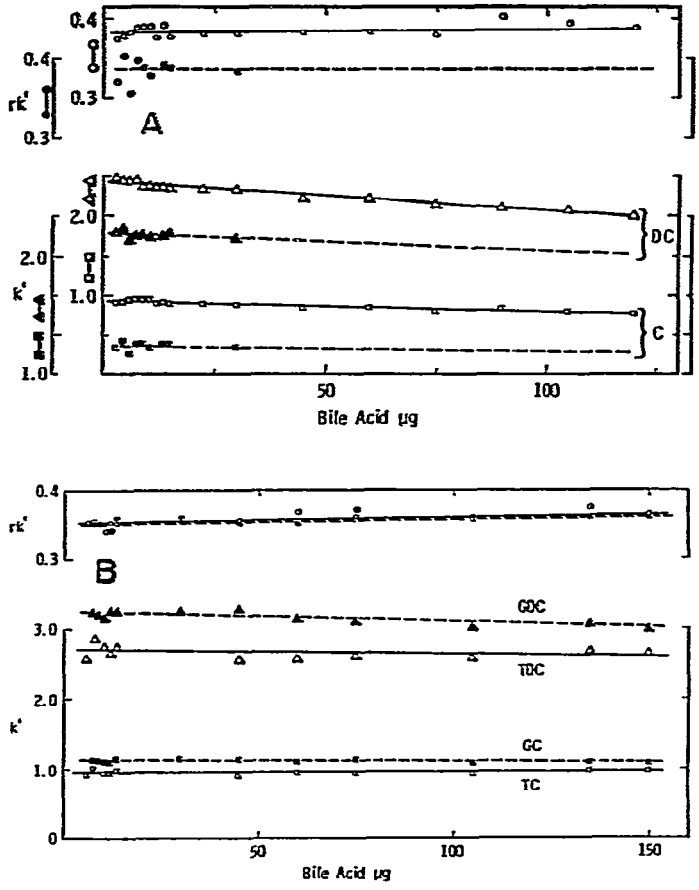
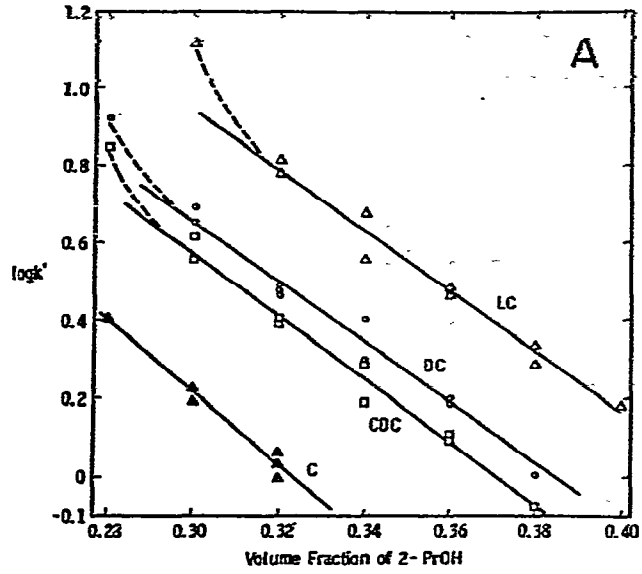


Fig. 4. Effect of sample load on k' and rk' values. The effect is generally more pronounced on k' than on rk'. (A) Closed symbols represent values re-determined at the end of the day for cholate (C) and deoxycholate (DC). They show a smaller decrease in k' values as the solute load is increased, when compared with open symbols which are obtained earlier during the work-day. (B) Similar plot for conjugated biles acids. TC = Taurocholate; GC = glycocholate; TDC = taurodeoxycholate; GDC = glycodeoxycholate. Open circles give the ratio of the k' values of TDC/TC and closed circles GDC/GC. Only the lowest k' values of multiple determinations are given.

became less pronounced if k' values obtained near the end of this experiment were used (closed symbols, Fig. 4A).

The parameter which affected k' most significantly was the solvent composition. Occasionally the composition of the solvent was changed intentionally to obtain reasonable k' values for some bile acids. The changes of k' in response to variations in the solvent composition are illustrated in Fig. 5. A semi-log plot gave straight lines which were almost parallel (Fig. 5); the values for free bile acids at log $k' > 0.8$ deviated from linearity (Fig. 5A), but no deviation was observed with taurine-conjugated bile acids (Fig. 5B).

Difficulties in achieving reproducible k' values led to the adoption of the con-



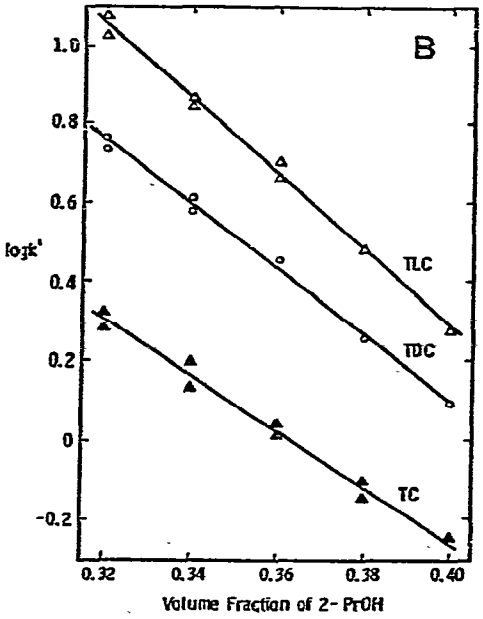


Fig. 5. Relationship between k' and solvent composition. (A) For free bile acids. For cholate (C): b (intercept), 2.9800; m (slope), −9.2100; cr (correlation coefficient), −0.9888. CDC, chenodeoxycholate; b, 2.9588; m, −7.9726; cr, −0.9886. For deoxycholate (DC): b, 2.9367; m, −7.6237; cr, −0.9870. For lithocholate (LC): b, 3.2566; m, −7.72737; cr, −0.9867. (B) For taurine-conjugated bile acids. For taurocholate (TC): b, 2.5642; m, −7.0624; cr, −0.9923. For taurodeoxycholate (TDC): b, 3.3852; m, −8.2121; cr, −0.9975. For taurolithocholate (TLC): b, 4.1211; m, −9.5860; cr, −0.9979.

cept "relative capacity factor", in analogy to the term "relative retention time" commonly used in gas chromatography[2,3]. Relative capacity factor, rk', is defined as

$$rk' = \frac{k'_x}{k'_s} = \frac{t_x - t_0}{t_s - t_0} \quad (1)$$

where t is the retention time, x the bile acid of interest, and s the standard. In accordance with methodology developed in gas chromatography, deoxycholic acid (DC) was chosen as the standard. Relative k' is identical to the term "resolution" as denoted by "α"[11]; but in order to avoid conceptual confusions, we shall use rk' for our purpose here. As expected, rk' values fluctuated much less than k' values from day to day (Fig. 3), at different solute concentrations (Fig. 4), and apparently in various solvent compositions, since the plots were virtually parallel (Fig. 5).

Capacity factor is related to the structure of an organic molecule by the following equation[12]:

$$\log k' = mA + b + \Sigma F(J) \quad (2)$$

where A = hydrocarbonaceous surface area of the molecule, m = slope, b = constant, and $F(J)$ = the contribution of each substituent. Normally, the C_{24} bile acids have a constant surface area and only hydroxyl or ketonic substituents. The relative capacity factor is therefore given by:

$$\log rk' = \log \frac{k'_x}{k'_s} = \log k'_x - \log k'_s$$

$$= [mA + b + \Sigma F(\mathrm{O})_x] - [mA + b + \Sigma F(\mathrm{O})_s]$$

$$= \Sigma F(\mathrm{O})_x - \Sigma F(\mathrm{O})_s \quad (3)$$

where $F(J)$ is replaced by $F(\mathrm{O})$, the contribution due to the oxygenated substituent.

From eqn. 3, the $F(\mathrm{O})$ for each substituent was calculated (Table I), as described in the Appendix. The rk' values of 7α- and 12α-hydroxyl groups were obtained at 38% 2-propanol rather than at 32%, so that the k' values of these compounds were within the acceptable range of 1–4 (Fig. 5A). The contributions due to substituents in 5α-(*allo*-) bile acids could be determined (Table I) by a slight modification of eqn. 3:

$$\log rk'_{Ax} = \log \frac{k'_{Ax}}{k'_{ADC}} \cdot \frac{k'_{ADC}}{k'_s}$$

$$= [\Sigma F(\mathrm{O})_{Ax} - \Sigma F(\mathrm{O})_{ADC}] + \log \frac{k'_{ADC}}{k'_s}$$

$$= \Sigma F(\mathrm{O})_{Ax} - \Sigma F(\mathrm{O})_{ADC} - 0.038 \quad (4)$$

where A = *allo* and $\log rk'$ of *allo*-deoxycholate (ADC) in 32% propanol was −0.038. But this somewhat cumbersome procedure can be replaced by the use of eqn. 3, since

TABLE I

CONTRIBUTIONS DUE TO SUBSTITUENTS AS CALCULATED FROM MONO-OXYGENATED CHOLANOIC ACIDS

Substituent	*5β*		*5α*	
	rk'	*F(O)*	*rk'*	*F(O)*
3α-OH	2.15	−0.73	2.13	−0.73
3β-OH	1.47	−0.89	1.26	−0.96
3=O	1.74	−0.82	1.62	−0.85
6α-OH	2.05	−0.75	2.03	−0.75
6β-OH	2.49	−0.66	3.30	−0.54
6=O	2.00	−0.76	2.65	−0.64
7α-OH*	4.26	−0.43	4.28	−0.43
7β-OH	3.51	−0.51	4.20**	−0.44
7=O	2.93	−0.59	—	—
12β-OH*	5.43	−0.33	5.35	−0.33

* Determined in 38% 2-propanol.
** Determined in 40% 2-propanol.

the log *rk'* values for the hydroxyl groups at 3α- and 12α-positions of 5β- and 5α-cholanic acids are almost identical (Table I). The *F*(O) values for the monohydroxyl groups, calculated by both methods, are similar, and only those based on eqn. 3 are included in Table I.

From the contribution function of a substituent, it was possible to calculate the expected *rk'* of a polysubstituted bile acid. Table II lists the values of some of the common bile acids and their experimental values. It appears that compounds having only α-substituted hydroxyl groups which are spaced sufficiently apart, *e.g.* at C-3, C-7 and C-12 positions, provide excellent agreement between the calculated and experimental values. Those in which the α-hydroxyl groups are adjacent (*e.g.* C-6 and C-7) and in which both α- and β-hydroxyl groups, or oxo and hydroxyl groups are

TABLE II

RELATIVE CAPACITY FACTORS OF SOME POLYOXYGENATED BILE ACIDS

Substituents	*5β*			*5α*		
	Calculated		*Observed rk'*	*Calculated*		*Observed rk'*
	log rk'	*rk'*		*log rk'*	*rk'*	
3α,6α-$(OH)_2$	−0.42	0.38	0.36	−0.42	0.38	0.46
3α,7α-$(OH)_2$	−0.10	0.79	0.80	−0.10	0.79	0.82
3α,12α-$(OH)_2$	0	1.00	1.00	0	1.00	0.92
7α,12α-$(OH)_2$	0.30	2.00	1.88	0.3	2.00	1.81
3α,6β-$(OH)_2$	−0.33	0.47	0.24	−0.21	0.62	0.33
3α,7β-$(OH)_2$	−0.18	0.66	0.34	—	—	—
3=O,7α-(OH)	−0.19	0.64	0.61	−0.22	0.60	0.41
3=O,12α-(OH)	−0.09	0.81	0.69	−0.12	0.76	0.48
3α,7α,12α-$(OH)_3$	−0.43	0.37	0.35	−0.43	0.37	0.36
3α,6α,7α-$(OH)_3$	−0.85	0.14	0.26	−0.85	0.14	0.32

present, did not follow the simple additivity rule closely. Therefore, the contributions of β-hydroxyl, oxo, and other functional groups were obtained by a different approach:

$$F(J) = \log rk'_J - \log rk'_p \quad (5)$$

where p = parent compound and J = parent compound containing substituent J. These rk' and $F(J)$ values are given in Table III. Table IV provides the calculated effects of *cis*- and *trans*-glycols. These are rationalized in the Discussion section. From eqn. 5, the effects of double bonds at C-4, 5, 6, 9(11), and 11, and of side-chain length, and of taurine, glycine, and δ-aminovaleryl groups were also obtained (Tables V and VI).

TABLE III

CONTRIBUTION DUE TO SUBSTITUENTS, AS CALCULATED FROM POLYOXYGENATED CHOLANIC ACIDS

Substituent	*Derivative (J)*	rk'_J	*Parent compound*	rk'	$F(J)^{*}_{calc}$	$F(J)^{**}_{exp}$	C^{***}	*Av. C*
(3β-OH)$_{5\beta}$	3β,12α-(OH)$_2$	0.48	12α-(OH)	5.43	−1.05	−0.89	−0.16	−0.21
	3β,7α,12α-(OH)$_3$	0.13	7α,12α-(OH)$_2$	1.88	−1.15		−0.26	
(3=O)$_{5\beta}$	3=O-7α-(OH)	0.61	7α-(OH)	4.26	−0.84	−0.82	−0.02	−0.10
	3=O-12α-(OH)	0.69	12α-(OH)	5.43	−0.89		−0.07	
	3=O-7α,12α-(OH)$_2$	0.19	7α,12α-(OH)$_2$	1.88	−0.99		−0.17	
	nor-3=O-7α,12α-(OH)$_2$	0.07	[*nor*-3α,7α,12α-(OH)$_3$]-[*F*(3α)]	0.12 2.15	−0.96		−0.14	
(6β-OH)$_{5\beta}$	3α,6β-(OH)$_2$	0.24	3α-(OH)	2.15	−0.95	−0.66	−0.29	−0.36
	6β,12α-(OH)$_2$	0.40	12α-(OH)	5.43	−1.13		−0.47	
	6β,7α,12α-(OH)$_3$	0.19	7α,12α-(OH)$_2$	1.88	−0.99		−0.33	
(7β-OH)$_{5\beta}$	3α,7β-(OH)$_2$	0.34	3α-(OH)	2.15	−0.80	−0.51	−0.29	−0.29
(7=O)$_{5\beta}$	3α-(OH)-7=O	0.36	3α-(OH)	2.15	−0.77	−0.59	−0.18	−0.26
	3α,12α-(OH)$_2$-7=O	0.12	3α,12α-(OH)$_2$	1.00	−0.92		−0.33	
(12=O)$_{5\beta}$	3α-(OH)-12=O	0.43	3α-(OH)	2.15	−0.70	—	—	—
	3α,7α-(OH)$_2$-12=O	0.12	3α,7α-(OH)$_2$	0.80	−0.82	—	—	—
(3β-OH)$_{5\alpha}$	3β,7α-(OH)$_2$	0.31	7α-(OH)	4.28	−1.14	−0.96	−0.18	−0.25
	3β,12α-(OH)$_2$	0.35	12α-(OH)	5.35	−1.19		−0.23	
	3β,7α,12α-(OH)$_3$	0.09	7α,12α-(OH)$_2$	1.81	−1.31		−0.35	
(3=O)$_{5\alpha}$	3=O-7α-(OH)	0.41	7α-(OH)	4.28	−1.02	−0.85	−0.17	−0.19
	3=O-12α-(OH)	0.48	12α-(OH)	5.35	−1.05		−0.20	
(6β-OH)$_{5\alpha}$	3α,6β-(OH)$_2$	0.33	3α-(OH)	2.13	−0.81	−0.54	−0.27	−0.20
	3β,6β-(OH)$_2$	0.27	3β-(OH)	1.26	−0.67		−0.13	
(6=O)$_{5\alpha}$	3,6(=O)$_2$	0.23	3=O	1.62	−0.85	−0.64	−0.21	−0.21
(7β-OH)$_{5\alpha}$	3=O-7β-(OH)	0.26	3=O	1.62	−0.80	−0.44	−0.36	−0.33
	3β,7β-(OH)$_2$	0.23	3β-(OH)	1.26	−0.74		−0.30	

* Values obtained using eqn. 5.
** Values from Table II.
*** Correction factor $C = F(J)_{calc} - F(J)_{exp}$.

A few qualitative generalizations can be made concerning the structure–mobility relationship. Retention time increased in the order: 3-OH=6-OH < 7-OH < 12-OH (Table I), in concert with a decrease in polarity, based on TLC mobility[13,14]. However, 3-oxo bile acids were frequently eluted ahead of their 3α-hydroxy analogues (Table I), even though the latter were more polar than the former[15]. Among mono-

TABLE IV

VICINAL EFFECTS IN BILE ACIDS*

	Substituent	*Derivative (J)*	rk'_J	*Parent compound*	rk'_p	$F(J)_{calc}$	$F(J)_{exp}$	*C*	*Av. C*
cis-effect	$(6\alpha)_{5\beta}$	3α,6α,7α-$(OH)_3$	0.26	3α.7α-$(OH)_2$	0.80	−0.48	−0.75	0.27	0.28
	$(7\alpha)_{5\beta}$	3α 6α,7α-$(OH)_3$	0.26	3α,6α-$(OH)_2$	0.36	−0.14	−0.43	0.29	
	$(6\beta)_{5\beta}$	3α,6β,7β-$(OH)_3$	0.17	3α,7β-$(OH)_2$	0.34	−0.30	−0.66	0.36	0.36
	$(7\beta)_{5\beta}$	3α,6β,7β-$(OH)_3$	0.17	3α,6β-$(OH)_2$	0.24	−0.15	−0.51	0.36	
	$(6\alpha)_{5\alpha}$	3α,6α,7α-$(OH)_3$	0.32	3_α,7α-$(OH)_2$	0.82	−0.40	−0.75	0.35	0.32
	$(7\alpha)_{5\alpha}$	3α,6α,7α-$(OH)_3$	0.32	3α,6α-$(OH)_2$	0.46	−0.14	−0.43	0.29	
	$(7\beta)_{5\alpha}$	3α,6β,7β-$(OH)_3$	0.20	3α,6β-$(OH)_2$	0.33	−0.22	−0.44	0.22	
trans-dieq-uatorial effect	$(6\alpha)_{5\beta}$	3α,6α,7β-$(OH)_3$	0.17	3α,7β-$(OH)_2$	0.34	−0.30	−0.75	0.45	0.45
	$(7\beta)_{5\beta}$	3α,6α,7β-$(OH)_3$	0.17	3α,6α-$(OH)_2$	0.36	−0.33	−0.51	0.18	
trans-diaxial effect	$(7\alpha)_{5\beta}$	3α,6β,7α-$(OH)_3$	0.17	3α,6β-$(OH)_2$	0.24	−0.15	−0.43	0.28	0.28
	$(6\beta)_{5\beta}$	3α,6β,7α-$(OH)_3$	0.17	3α,7α-$(OH)_2$	0.80	−0.67	−0.66	−0.01	

* See footnotes of Table III for explanation.

TABLE V

CONTRIBUTION* DUE TO DOUBLE BONDS AND CHAIN LENGTHS

Substituent	*Derivative (J)*	rk'_J	*Parent compound*	rk'_p	$F(J)_{calc}$	*Av.* $F(J)_{calc}$
$(\Delta^4)_{5\alpha}$	Δ^4-3=O-7α-OH	0.27	3=O-7α-OH	0.41	−0.18	−0.18
$(\Delta^4)_{5\beta}$	Δ^4-3=O-7α-OH	0.27	3=O-7α-OH	0.61	−0.35	−0.35
$(\Delta^5)_{5\alpha}$	Δ^5-3β-OH	0.81	3β-OH	1.26	−0.19	−0.19
$(\Delta^5)_{3\beta}$	Δ^5-3β-OH	0.81	3β-OH	1.47	−0.26	−0.26
$(\Delta^6)_{3\beta}$	Δ^6-3α-OH	1.60	3α-OH	2.15	−0.13	−0.11
	Δ^6-3=O	1.46	3=O	1.74	−0.08	
$(\Delta^{9(11)})_{5\beta}$	Δ^9-3α-(OH)-12=O	0.30	3α-(OH)-12=O	0.43	−0.15	−0.17
	Δ^9-3α,7α-$(OH)_2$-12=O	0.08	3α,7α-$(OH)_2$-12=O	0.12	−0.18	
$(\Delta^{11})_{5\beta}$	Δ^{11}-3α-OH	1.59	3α-OH	2.15	−0.13	−0.13
C_{24}	5β-3α,12α-$(OH)_2$	1.00	5β-*nor*-3α,12α-$(OH)_2$	0.47	0.33	0.43
	5β-3α,7α,12α-$(OH)_3$	0.35	5β-*nor*-3α,7α,12α-$(OH)_3$	0.12	0.46	
	5β-3=O-7α,12α-$(OH)_2$	0.19	5β-*nor*-3=O-7α,12α-$(OH)_2$	0.07	0.43	
	5α-3α,7α,12α-$(OH)_3$	0.36	5α-*nor*-3α,7α,12α-$(OH)_3$	0.12	0.48	
C_{25}	5β-*homo*-3α,7α-$(OH)_2$	1.31	5β-3α,7α-$(OH)_2$	0.80	0.22	0.26
	homo-Δ^4-3=O-7α-OH	0.52	Δ^4-3=O-7α-OH	0.27	0.29	

* See footnotes of Table III for explanation

hydroxylated bile acids, the equatorial hydroxyl compounds were eluted more quickly than the axial ones, *except* the C-3 epimers of 5β-cholanoic acids (Table I). Differences in mobilities between 5α- and 5β-epimers of mono-α-hydroxylated bile acids were very small or even negligible; but 3α-hydroxyl or 3-oxo substituents delayed 5β-steroids in comparison with 5α-epimers, while the reverse was true when the substituents were at C-6 (Table I). Similar results were obtained with poly-oxygenated bile acids (Table III), with the exception of 3α,12α-dihydroxycholanic acids, in which the 5α-epimer moved slightly ahead of the 5β-analogue (Table II). With bile acids oxygenated at identical positions, the completely α-hydroxylated acids had the lowest

TABLE VI

CONTRIBUTION FUNCTIONS OF AMINO ACIDS IN CONJUGATED BILE ACIDS AT pH 7.0

*Compound**	*Conjugated bile acids*		*Free bile acids*		*F(J)***	*Av. F(J)*
	rk'_J	$\log rk'_J$	rk'_p	$\log rk'_p$		
TC	0.31	−0.51	0.35	−0.46	−0.05	
TAC	0.32	−0.50	0.36	−0.44	−0.06	
THDC	0.29	−0.54	0.36	−0.44	−0.10	
TUDC	0.25	−0.60	0.34	−0.47	−0.13	
TCDC	0.68	−0.17	0.80	−0.10	−0.07	
TACDC	0.69	−0.16	0.82	−0.09	−0.07	
TDC	0.83	−0.08	1.00	0	−0.08	taurine = −0.07
TADC	0.82	−0.09	0.92	−0.04	−0.05	
TLC	1.77	0.25	2.15	0.33	−0.08	
TALC	1.83	0.26	2.13	0.33	−0.07	
T-β-M	0.15	−0.82	0.17	−0.77	−0.05	
T-α-M	0.15	−0.82	0.17	−0.77	−0.05	
GC	0.30	−0.52	0.35	−0.46	−0.06	
GAC	0.29	−0.54	0.35	−0.44	−0.10	
GHDC	0.28	−0.55	0.36	−0.44	−0.11	
GUDC	0.24	−0.62	0.34	−0.47	−0.15	
GCDC	0.65	−0.19	0.80	−0.10	−0.09	
GACDC	0.66	−0.18	0.82	−0.09	−0.09	
GDC	0.83	−0.08	1.00	0	−0.08	glycine = −0.09
GADC	0.85	−0.07	0.92	−0.04	−0.03	
GLC	1.65	0.22	2.15	0.33	−0.11	
GALC	1.71	0.23	2.13	0.33	−0.10	
AVC	0.41	−0.39	0.35	−0.46	0.07	0.07

* T = taurine-conjugated; G = glycine-conjugated; AV = δ-aminovaleryl-conjugated; A = *allo*-(5α-); C = cholate; UDC = ursodeoxycholate; DC = deoxycholate; LC = lithocholate; M = muricholate.

** $F(J) = \log rk'_J - \log rk'_p$.

mobility, compounds with both oxo and α-hydroxyl groups intermediate mobility, and those with both β- and α-hydroxyl groups the highest mobility. In polyhydroxy compounds with vicinal hydroxyl groups the correction factor is large (Table IV). Olefinic bonds decreased retention while elongation of the side chain with methylene groups increased it (Table V). Conjugation with taurine or glycine resulted in decreased retention (Table VI).

DISCUSSION

This study was performed on a single column, since rk' values are presently not necessarily reproducible on other columns[14]. Data from Fig. 4 show that the capacity factor decreases with sample load. However, this change is more obvious at higher k' values (*e.g.* $k' = 3$) than at lower ones (Figs. 4A and 4B), suggesting non-ideal chromatographic behavior in the former case. This assumption is further supported by the observation that at similar k' values, changes in k' in response to load decrease as the solubility of the bile acid in water increases (Fig. 4), in the order: deoxycholate < glycodeoxycholate < taurodeoxycholate[15]. Deviation from ideal

behavior is also observed in the relationship between log k' and volume fraction of 2-propanol: at log $k' > 0.8$ for lithocholic, deoxycholic, and chenodeoxycholic acids, deviation from linearity is observed (Fig. 5A), although the more soluble taurine-conjugated bile acids remain unaffected. A plausible explanation is given below.

Ideal chromatographic behavior requires that the rate of equilibration of a solute molecule between the mobile and stationary phases be at least as rapid as the flow-rate of the mobile phase. This requirement is obviously fulfilled by the more water-soluble taurine-conjugated bile acids, which gave sharper eluted peaks than the less soluble free bile acids. When a solute is poorly soluble in the mobile phase, the rate of equilibration may be delayed, possibly because an additional process sets in: the formation of micellar aggregates of the solute molecules to enhance its solubility in the essentially aqueous mobile phase[10]. This would lead to a slower elution time than expected. Aggregation of conjugated bile acid molecules during reversed-phase HPLC has been observed[16], whether the compound was ionized (taurine-conjugated) or protonated (glycine-conjugated)[16,17]. Based on these arguments and depending on the capacity of the column and the amount of solute applied, a bile acid which is fairly soluble in the mobile phase should be eluted with little or no decrease in the retention times in response to increasing load at levels far below that of the limiting capacity of the column. Any decrease would be the result of an increase in bandwidth and peak broadening. This was true with taurine and glycine conjugates, and with free bile acids eluted with k' values of *ca.* 1. However, with free bile acids eluted with k' values of above 2, such as deoxycholic acid, the reduction in k' was more pronounced, presumably because the higher load favored molecular aggregation, allowing greater solubility in the mobile phase.

The plots of log k' *vs.* volume fraction of 2-propanol (Fig. 5) are nearly parallel, suggesting that the rk' values obtained at slightly different solvent compositions are interchangeable. This enables the comparison of suitable rk' values for bile acids of vastly different substitution patterns. Other difficulties in achieving reproducible k' determination can also be overcome by the use of rk' (Figs. 3 and 4).

With the use of eqns. 3 and 4, the contribution due to a substituent was calculable from the relative capacity factors of monosubstituted cholanoic acids (Table I). The rk' values of polyoxygenated bile acids can be calculated by summing the contribution factors. Thus, mobilities of non-vicinally α-polyoxygenated bile acids can be predicted quite well. With β-hydroxyl- or oxo-cholanoic acids, agreements are poor and correction factors must be introduced. The correction factor for a particular substituent represents the difference between expected and observed values obtained from a pair of compounds with the use of eqn. 5. Data in Table III indicate that negative correction factors are needed for bile acids containing β-dihydroxyl groups, mixed α- and β-hydroxyl groups, and oxo substituents. In other words, these compounds are eluted sooner than expected from the sum of the contributions of individual substituents. The following rationalization can be provided to explain the results. The mobility of a solute in reversed-phase partition chromatography is dependent on its solubilities in the mobile and stationary phases. Increase in polarity of the molecule will enhance solution in the former phase, while availability of a hydrophobic surface will favor solubility in the latter. With non-vicinal poly-α-hydroxylated bile acids, a constant β-surface area[18] is available for hydrophobic binding with the stationary phase. It is not unreasonable to assume that the whole β-face would interact, owing to

the vast surface area of liquid C_{18}-hydrocarbon on the column support[5]. Therefore, changes in the hydroxyl groups on the α-face would only alter the polarity of the molecule and hence its solubility in the aqueous mobile phase. On the other hand, β-hydroxyl or oxo groups in polyoxygenated cholanoic acids reduce the hydrophobic surface area, which not only changes the polarity of the molecule, but also reduces the affinity of the hydrophobic surface for the C_{18}-hydrocarbon, causing the elution time to be shorter than expected. Implicit in this argument is the assumption that even though hydrophobic interaction is possible for both the α- and β-faces of a steroid molecule, the latter is preferred[18].

The magnitudes of the correction factors are dependent on the geometry, position and number of substituents. Oxo groups which are generally in the same plane of the steroid nucleus, cause less deviation from the expected values than β-hydroxyl groups (Table III). This is also true of β-hydroxyl substituents in β-dihydroxylated 5α-cholanoic acids (*e.g.* 3β, 6β- and 3β, 7β-) in comparison with those present in bile acids containing both α- and β-hydroxyl substituents (Table III). In regard to the position and number of substituents, the magnitudes of the correction factors increased for the three hydroxyl derivatives of 3-oxo-5β-cholanoic acids in the order 7α-OH < 12α-OH < 7α, 12α-$(OH)_2$ (Table I). The increase in the absolute value of the correction factor as a result of the increased number of other substituents is appropriately described by the term "cooperative effect". Upon careful examination, this is found even among non-vicinal poly-α-hydroxylated bile acids in which the differences between the calculated and observed *rk'* and log *rk'* values become less negative when chenodeoxycholates and cholates are compared (Table II). In these instances, the "cooperative effects" are sufficiently small to be ignored.

"Positional effects" account for the influence of the location of other substituents on the correction factors. An unusual example is illustrated by the pair, 3α,6β-dihydroxy- and 6β,12α-dihydroxy-5β-cholanoic acids, in which the correction factors for the 6β-OH groups were —0.29 and —0.47, respectively (Table III). To ascertain whether the reduction of the steroid surface for interaction with the stationary phase is much more dramatic when the substituent is situated in the middle of the steroid nucleus (12α) than at the edge (3α), more examples are needed.

The "vicinal effect" is seen with the 6,7-glycols, *e.g.* the hyocholates and muricholates. It denotes a reduction in the summed effects of two hydroxyl groups when they are adjacent because of overlap of spheres of influence. The "*cis* vicinal effect" results from the unidirectional orientation of the two hydroxyl groups with respect to the plane of the steroid nucleus. The correction factors are greater with 6,7-dihydroxy-5β-bile acids than with the *allo* analogues (Table IV). The "*trans* vicinal effect" refers to the influence of diols which are *trans* to each other with respect to the steroidal plane. The *trans*-diequatorial diol in which the hydroxyl groups are equidistant as in the *cis*-diols, nevertheless yields a correction factor of greater magnitude (Table IV). The *trans*-diaxial diol, which may share an area of influence on the side of the steroidal nucleus, also deviates from the expected value. Of interest is the similarity of the differences of the two $F(J)_{exp}$ factors from the *trans* diequatorial 5β-derivative (—0.27) and the *trans* diaxial 5β-derivative (—0.27). These values appear to reflect the contribution due to addition of β-substituents at C-6 and C-7 positions to the α-substituted bile acids. Unfortunately, the comparable 5α-*trans*-glycols were not available for these studies.

The presence of a double bond tends to increase the mobility of a compound, and the effect of the double bond is dependent on its position and other substituents. For instance, the contribution due to the 6-double bond in 3α-hydroxycholenoic acid is −0.13, but with the 3-oxocholenoic acid it is −0.08 (Table V). It is reasonable to assume that, since the contributions to mobility due to olefinic bonds are determined by the effects of these bonds on the conformations of the molecules, the mobilities should be related to the heats of hydrogenation of the double bonds[19]. The order of stability of the double bonds [$(\Delta^5)_{5\alpha} > (\Delta^4)_{5\alpha} > \Delta^{9(11)} > \Delta^{11} > \Delta^6$] as deduced from the mobilites on reversed-phase HPLC appears to agree with that derived from C=C stretching frequencies, except for the reversal of Δ^6 and Δ^{11}. The agreement may be even better if identical compounds are used in both studies.

The contribution of other functional groups to mobility can also be calculated from eqn. 5. Although an increase in length of the side chain results in an increase in rk', the contribution function, $F(J)$, due to the additional methylene group is decreased. Thus, $F(C_{24})$ is 0.43 and $F(C_{25})$ 0.26 (Table V). The effect of methylene groups is also observed in glycine, taurine and δ-aminovaleryl conjugates of bile acids. These amino acids contained one, two and four methylene groups, respectively, and as expected, the contribution due to each amino acid becomes increasingly positive as the negative contribution by the amide linkage is steadily reduced by the increasing number of methylene groups (Table VI). Deviations from the normal ranges of contributions due to taurine and glycine are observed with ADC and ursodeoxycholate (Table VI). Reasons for these abnormal observations are unclear.

The precision by which the relative capacity factor of a bile acid can be obtained enables the application of reversed-phase HPLC to conformational analysis and physicochemical investigations, as mentioned. Currently, the efforts in this laboratory are directed toward the resolution of compounds which are not separated in the system described.

APPENDIX

In determining the contribution due to each substituent, those due to 3α-hydroxyl and 12α-hydroxyl groups were calculated first, as for example:

$$\log rk' \text{ of lithocholic acid} = \Sigma F(\mathrm{O})_x - \Sigma F(\mathrm{O})_s$$
$$= F(3\alpha\text{-OH}) - [F(3\alpha\text{-OH}) + F(12\alpha\text{-OH})]$$
$$F(12\alpha\text{-OH}) = -0.33$$

Based on these values, the contributions due to other mono-oxygenated groups were calculated, *e.g.*

$$\log rk' \text{ of } 7\alpha\text{-hydroxy-}5\beta\text{-cholanic acid} = F(7\alpha\text{-OH}) - [F(3\alpha\text{-OH}) + F(12\alpha\text{-OH})]$$
$$0.63 = F(7\alpha\text{-OH}) - [-0.74 - 0.33]$$
$$F(7\alpha\text{-OH}) = -0.44$$

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