Journal of Chromatography, 108 (1975) 23–35

CHROM. 8168

LIQUID ION EXCHANGERS IN PAPER CHROMATOGRAPHY OF STEROIDAL GLUCOSIDURONIC ACIDS, GLUCOSIDURONIC ESTERS AND FREE STEROIDS

INFLUENCE OF CONCENTRATION OF EXCHANGER AND COUNTERION

V. R. MATTOX, JUNE E. GOODRICH and ROBERT D. LITWILLER
Mayo Clinic and Mayo Foundation, Rochester, Minn. 55901 (U.S.A.)
(First received September 30th, 1974; revised manuscript received December 30th, 1974)

SUMMARY

The chromatographic mobility of steroidal glucosiduronic acids on paper in chloroform–formamide increases as the concentration of ion exchanger in the chloroform phase increases; mobility decreases as the concentration of counterion in formamide increases. Mobility of glucosiduronic esters and of hydroxylated free steroids increases with an increase in concentration of exchanger; small changes in concentration of counterion in the stationary phase do not alter the migration of these nonionizable compounds. Data are presented which suggest that partition of the glucosiduronic acids between the two phases occurs predominantly by an ion-exchange process and that hydrogen bonding plays a secondary role. Partition of the glucosiduronic esters and hydroxylated free steroids appears to occur primarily by a hydrogen-bonding process.

INTRODUCTION

In a previous paper[1] we demonstrated that various solvent systems which are commonly used to chromatograph free steroids become suitable for chromatographing the corresponding steroidal glucosiduronic acids if a liquid ion exchanger* is added to increase the solubility of the conjugate in the less polar (mobile) phase. In the present paper** we describe the influence of changes in concentration of exchanger and counterion on the mobility of glucosiduronic acids, esters, and free steroids in a solvent system of chloroform–formamide.

* The organic amines and amine salts employed in this paper are referred to as "liquid ion exchangers" even though these substances may participate in the interchange of neutral compounds between the phases of the chromatography system.

** A subsequent paper[10] will give the chromatographic mobilities of a group of steroidal glucosiduronic acids in the presence of various liquid ion exchangers.

THEORETICAL CONSIDERATIONS

Cerrai and Testa[2] have applied a mathematical equation to express the partition phenomena that occur when ionizable substances are chromatographed on strips of paper that are impregnated with a liquid ion exchanger which serves as the stationary phase. An analogous equation may be applied to chromatography systems in which the liquid ion exchanger is present in the mobile phase.
Consider the following reaction

$$n\mathrm{R_4NX} + \mathrm{R'(CO_2^-)}_n = (\mathrm{R_4N})_n\mathrm{R'(CO_2)}_n + n\mathrm{X^-} \quad (1)$$

$\mathrm{R_4NX}$ represents a quaternary, tertiary, secondary, or primary ammonium compound, $\mathrm{R'CO_2^-}$ stands for a glucosiduronate ion, n is equivalent to the number of carboxyl groups in the glucosiduronate, $(\mathrm{R_4N})_n\mathrm{R'(CO_2)}_n$ is a complex formed from the ion exchanger and glucosiduronate, and $\mathrm{X^-}$ is an anion ($\mathrm{Cl^-}$, $\mathrm{AcO^-}$, etc.). From eqn. 1 the following relationship, analogous to that of Cerrai and Testa[2], may be derived:

$$R_M = -n \log [\mathrm{R_4NX}]_m + n \log [\mathrm{X^-}]_s + \text{const.} \quad (2)$$

R_M is defined[3] as $\log [(1/R_F) - 1]$, and subscripts m and s refer to the mobile and stationary phases, respectively. If the concentration of anion in the stationary phase is held constant, the line resulting from a plot of R_M *vs.* $\log [\mathrm{R_4NX}]_m$ should have a slope of $-n$. Conversely, if the concentration of exchanger in the mobile phase is held constant, a plot of R_M *vs.* $[\mathrm{X^-}]_s$ should produce a slope equal to n.

For nonionizable compounds such as glucosiduronic esters and free steroids, in which the exchanger forms a hydrogen bond with one or more hydroxyl groups, the following equation may be used to relate mobility of solute to concentration of organic extractant.

$$R_M = -n \log [\mathrm{R_4NX}]_m^{\beta} + \text{const.} \quad (3)$$

Application of the foregoing equations to interpretation of the experimental results is given in the discussion.

MATERIALS AND METHODS*

Chemicals

Tetraheptylammonium halides were obtained from Eastman Organic Chemi-

* Abbreviations: ALA-2 = Amberlite LA-2; Aliquat 336 = methyltricaprylylammonium chloride; β-cortol 3-GA = 11β,17,20β,21-tetrahydroxy-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid; EGA = 17-hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; FGA = 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; GA = β-D-glucopyranosyluronic acid group; GMe = β-D-glucopyranosyluronic methyl ester; QGA = 3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; SGA = 17-hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; TA·Br = tetraheptylammonium bromide; TA·Cl = tetraheptylammonium chloride; TA·I = tetraheptylammonium iodide; TA·NO_3 = tetraheptylammonium nitrate; TA·OAc = tetraheptylammonium acetate; $(TA)_2 \cdot SO_4$ = tetraheptylammonium sulfate; THE 3-GA = 17,21-dihydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid; THF 3-GA = 11β,17,21-trihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid; THA 3,21-diGA = 11,20-dioxo-5β-pregnan-3α,21-ylene di(β-D-glucopyranosiduronic acid); TOA = tri-*n*-octylamine; XLA-3 = Amberlite XLA-3.

cals (Rochester, N.Y., U.S.A.). Other tetraheptylammonium salts were prepared by washing a chloroform solution of TA·Cl with an aqueous solution which contained an excess of the appropriate silver salt. The organic phase was filtered, washed with water, concentrated *in vacuo* to remove most of the solvent, titrated[1] with sodium dodecyl sulfate, and diluted to the appropriate volume with chloroform. Analysis[4] of $(TA)_2 \cdot SO_4$, prepared by this method, indicated two tetraheptylammonium radicals per sulfate group.

The following additional ion exchangers were used: Aliquat 336, from General Mills (Minneapolis, Minn., U.S.A.); TOA, from K & K Labs. (Plainview, N.Y., U.S.A.); ALA-2 and XLA-3, from Rohm & Haas (Philadelphia, Pa., U.S.A.). The hydrochlorides of the latter three amines were prepared by the previously described procedure[5].

TA·Cl from the manufacturer contains material that absorbs strongly in the hydroxyl region of the infrared (IR) spectrum. To prepare TA·Cl for use in determining IR spectra in this region a solution of the substance in chloroform was washed repeatedly with water, concentrated to an oil *in vacuo* and diluted to the appropriate volume with alcohol-free chloroform. TA·I and TA·Br from the manufacturer and the $TA \cdot NO_3$ which we prepared required no further purification. TA·OAc and $(TA)_2 \cdot SO_4$ appeared to hold water avidly. The former was dried by dissolving 3 mmoles of it in 45 ml benzene, distilling off 30 ml at atmospheric pressure, removing the remainder *in vacuo*, and diluting with alcohol-free chloroform. A solution of $(TA)_2 \cdot SO_4$ in chloroform was dried over anhydrous calcium sulfate.

Sodium (cholest-5-en-3β-yl β-D-glucopyranosid)uronate[6] (sodium cholesterol 3-glucosiduronate) was kindly supplied by Dr. John J. Schneider (Jefferson Medical College, Philadelphia, Pa., U.S.A.). Cholesterol 3-glucosiduronic acid was prepared by acidifying an aqueous ethanolic (80:20) solution of the sodium salt to a pH of 3.0 with HCl, concentrating *in vacuo* and filtering. All other steroidal glucosiduronates were synthesized in our laboratory[7,8]. The structures of these substances are shown in Fig. 1.

Chromatography

Procedures given previously[1] for preparation of reagents and for chromatography of steroidal glucosiduronates were followed. Chromatograms were run in a system of chloroform–formamide to which was added ion exchanger and, in some cases, salt for counterion (KCl, KOAc, etc.). The mobile phase (chloroform) contained ion exchanger of the concentration indicated, and the stationary phase (formamide) contained, where indicated, a salt which had an anion in common with the ion exchanger being employed (KCl with TA·Cl, KOAc with TA·OAc, etc.). A sheet of paper was impregnated by drawing it through a mixture of formamide–methanol (30:70) and allowing it to dry in air for about 5 min. When a salt was used, it was dissolved in formamide to give the desired concentration and then the solution was diluted with methanol (30:70). A value for concentration of salt in the formamide (stationary) phase is actually the concentration of salt in the formamide before it was diluted with methanol.

For detection of a 17,20β,21-trihydroxy compound on a chromatogram a modification of the procedure of De Courcy and Schneider[9] was used. The paper, which had been dried to remove the stationary phase, was sprayed with a 1% periodic

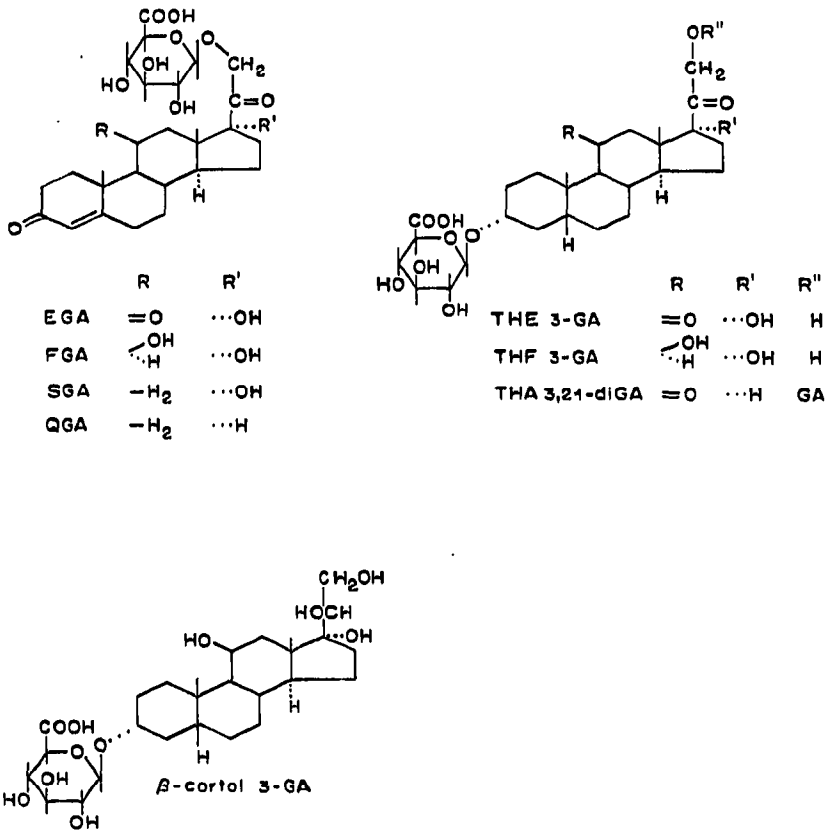

Fig. 1. Structures of steroidal glucosiduronates.

acid solution (0.40 *N* H_2SO_4 in 75% ethanol), the damp paper was kept between two glass plates for 30 min and then dried in air; subsequently, it was dipped into a freshly prepared mixture (1:1) of 4 *N* KOH in methanol and 2% *m*-dinitrobenzene in ethanol, blotted between two sheets of paper and placed between glass plates for viewing. 17-Ketosteroids formed by this treatment are revealed as violet spots.

When quantitative determinations of TA·Cl and formamide were to be made on a finished chromatogram, the paper was premarked with a lead pencil so that it could be divided into strips 2.0 cm wide. Immediately after development, the chloroform front was marked and a 2.0-cm-wide vertical strip was removed from the center of the paper for detection of the TA·Cl front[1]. The remainder of the chromatogram was cut into horizontal strips as marked, each strip having a surface area of 32 sq. cm. The strips were cut into small pieces, placed in a glass-stoppered flask containing water and disintegrated into a pulp by shaking vigorously. TA·Cl was extracted from the pulp with 1,2-dichloroethane and titrated with sodium dodecyl sulfate[1]. For determination of formamide, the pulp was filtered and rinsed with water; an aliquot of the filtrate was analyzed by the Kjeldahl procedure[10].

Infrared spectra

Spectra were taken on a Beckman IR-18 instrument. For solutions, matched 0.10-mm cells were used with an appropriate glucosiduronate concentration (0.10–0.20 *M*).

RESULTS

During the development of a paper chromatogram there is normally a gradient in weight of solvent per unit area of paper as the solvent flows from the starting-line toward the end of the paper[11]. Since TA·Cl is relatively non-volatile, it was possible to measure this gradient on a chromatogram which had been developed in a chloroform–formamide system (0.10 *M* TA·Cl in the mobile phase, 0.10 *M* KCl in the stationary phase). As shown in Fig. 2, there was a gradual decrease in the amount of TA·Cl per unit area of paper on moving from the origin toward the chloroform front; at a point equivalent to an R_F value of about 0.85 there was a marked decrease in the amount of TA·Cl per unit area of paper. Variations in the amount of formamide over this same area were much smaller.

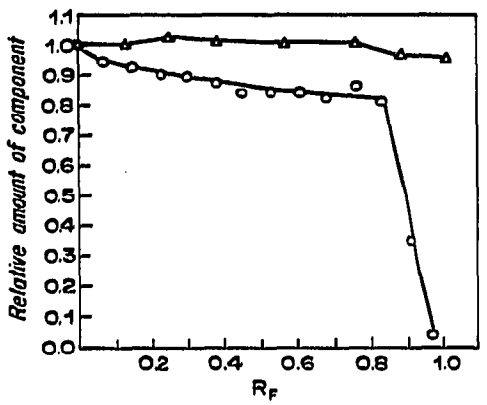

Fig. 2. Amounts of TA·Cl (○—○) and formamide (△—△) at various distances from the origin on a finished chromatogram. Values on the ordinate are relative to the amount of component per unit area of paper at $R_F = 0$. R_F = (distance to center of strip)/(distance to $CHCl_3$ front).

Fig. 3 shows the mobility of a series of steroidal glucosiduronic acids, glucosiduronic esters and free steroids as a function of the concentration of TA·Cl in chloroform. Except for acids E, F, and G at the highest concentration of TA·Cl, there was a linear relationship between the mobility (R_M) and the concentration of TA·Cl (as log concentration in moles per liter) for all of the compounds. The slope* n of the line for diglucosiduronic acid X (left panel) was −1.6; slopes of the lines for monoglucosiduronic acids (A to G) ranged from −1.2 to −1.0. Slopes of the lines for the esters (center panel) extended from −1.0 to −0.5; those for the free steroids

* In the ideal case, the slope n should indicate the number of molecules of ion exchanger combined with one molecule of solute.

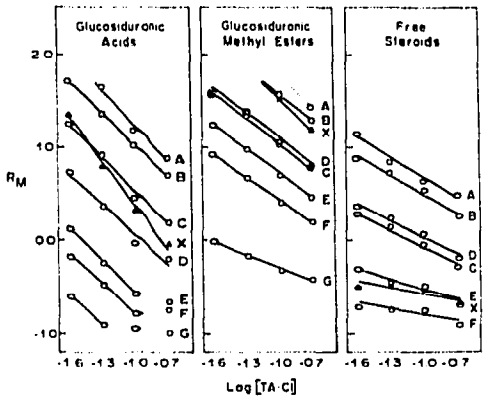

Fig. 3. R_M values of steroidal glucosiduronic acids, glucosiduronic methyl esters, and free steroids *vs.* log concentration of TA·Cl in the mobile phase. A, B, C etc. designate the same steroid nucleus in each panel. For the left panel: A = β-cortol 3-GA; B = THF 3-GA; C = THE 3-GA; D = FGA; E = EGA; F = SGA; G = QGA; X = THA 3,21-diGA. For formulas and abbreviations, see Fig. 1 and footnote on pages 26 and 24, respectively.

(right panel) ranged from −0.8 to −0.1. As the R_M values of the glucosiduronic esters and the free steroids decrease, there is a marked decrease in the slopes of the lines; this subject is discussed below.

In none of the foregoing chromatography systems was KCl used. This salt, when present in the formamide phase, decreases migration of the glucosiduronic acids; migration of the esters and free steroids is not changed. For monoglucosiduronic acids in 0.1 *M* TA·Cl the presence of 0.05 *M* KCl decreased mobility by about 0.45 R_M units. The slopes of the lines representing R_M *vs.* log [TA·Cl] for the glucosiduronic acids were more negative by 0.1 to 0.2 unit than in the absence of KCl.

From the plots in Fig. 3, there appears to be a general trend toward decreasing slope with increasing mobility (decreasing R_M) for the three classes of compounds. In Table I, the relationship between the chromatographic mobility of a compound in 0.10 *M* exchanger and the slope of the line representing R_M *vs.* log [exchanger] is summarized for the acids when chromatographed in the presence of three different ion exchangers. With the exception of the diglucosiduronic acid, which is atypical, the acids are arranged in order of increasing mobility. When TA·OAc or TA·Cl was used as exchanger, the slopes of the lines representing R_M *vs.* log [exchanger] decreased as the mobility of the compounds in the series increased; slopes obtained when Aliquat 336 was used as exchanger were similar to those obtained with TA·Cl. There was no significant change in slope for the group of compounds when the primary amine hydrochloride XLA-3·HCl was employed. The tertiary amine hydrochloride (TOA·HCl) and the secondary amine hydrochloride (ALA-2·HCl) gave slopes which were nearly identical with those observed with XLA-3·HCl. Glucusiduronic acids tend to streak on paper chromatograms employing TOA·HCl, ALA-2·HCl and XLA-3·HCl when no KCl is employed in the stationary phase.

For none of the foregoing solvent systems did the linear relationship between R_M and log [exchanger] persist when the concentration of exchanger in the mobile phase was higher than 0.20 *M*. In addition, in most cases the linear relationship did not

TABLE I

RELATIONSHIP BETWEEN MOBILITY OF STEROIDAL GLUCOSIDURONIC ACIDS AND THE SLOPE OF A PLOT OF R_M *vs.* log [Exchanger]

R_F values in the table were obtained with 0.10 *M* exchanger in the mobile phase and with 0.10 *M* counterion in the stationary phase. For counterion, KOAc was used with TA·OAc, KCl with TA·Cl and XLA-3·HCl. For determining slopes, R_M values were obtained using 0.20, 0.10, 0.05, 0.025, 0.0125, and 0.0063 *M* exchanger while holding the concentration of counterion constant. For the first four compounds, 0.10 *M* KOAc was used as counterion with TA·OAc; 0.05 *M* KCl was used with the other exchangers. For the last four compounds, 0.40 *M* KOAc was used with TA·OAc; 0.20 *M* KCl with the other exchangers. R_M values which exceeded 1.7 were not used for determining slopes.

Compound	*Exchanger*					
	TA·OAc		*TA·Cl*		*XLA-3·HCl*	
	R_F	*Slope*	R_F	*Slope*	R_F	*Slope*
THA 3,21-diGA	0.51	−2.2	0.028	−1.7	0.013	−1.6
β-Cortol 3-GA	0.18	−1.7	0.018	—	<0.010	—
THF 3-GA	0.21	−1.6	0.026	−1.3	0.011	—
THE 3-GA	0.52	−1.6	0.089	−1.3	0.022	−0.8
FGA	0.71	−1.6	0.20	−1.3	0.070	−0.9
EGA	0.81	−1.4	0.47	−1.2	0.27	−0.9
SGA	0.87	−1.4	0.61	−1.2	0.52	−0.8
QGA	0.91	−1.1	0.78	−1.1	0.76	−0.8

persist to mobilities corresponding to R_F values greater than about 0.60; this type of behavior is not restricted to solvent systems which contain liquid ion exchangers but is characteristic of partition chromatography on paper in tanks[12,13].

Table I also shows that for a particular solute there is a marked difference in the slope of the line representing R_M *vs.* log [exchanger] when different exchangers are employed. For all of the compounds the order of effect on mobility is TA·OAc > TA·Cl > XLA-3·HCl. The slopes of the lines representing R_M *vs.* log [exchanger] decrease in the same order.

Table II summarizes the relationships between chromatographic mobilities and slopes for the methyl esters. The observations for the esters are analogous to those made with the acids: slope is directly dependent on the polarity of the solute and on the intrinsic strength of exchanger.

Fig. 4 illustrates the effect of a sixteen-fold change in the concentration of counterion on the chromatographic mobility of a series of glucosiduronic acids; in these experiments the concentration of exchanger in the chloroform phase was held constant. For each of the exchangers there is a segment of line with a slope of about 0.8 for monoglucosiduronic acids D, E, F, and G. With TA·Cl as exchanger the rectilinear portion extended from 0.0125–0.20 *M* counterion; for TA·OAc and XLA-3·HCl linearity extended from 0.10–0.80 *M* counterion.

Migration of the diglucosiduronic acid (X) was retarded even more strongly by counterion, the slopes being 1.7, 1.3, and 1.6 in TA·OAc, TA·Cl and XLA-3·HCl, respectively. When the tertiary amine salt (TOA·HCl) and secondary amine salt (ALA-2·HCl) were used as exchangers in association with KCl the findings (not shown) for monoglucosiduronic acids were very similar to those with XLA-3·HCl and KCl.

TABLE II

RELATIONSHIP BETWEEN MOBILITY OF STEROIDAL GLUCOSIDURONIC METHYL ESTERS AND THE SLOPE FROM A PLOT OF R_M *vs.* log [exchanger]

R_F values in the table were obtained with 0.10 *M* exchanger in the mobile phase; no counterion was added to the stationary phase. For obtaining slopes, R_M values were determined for a series of concentrations of exchanger, each concentration being half that of the previous run. The ranges over which the concentrations were varied were: for TA·OAc, 0.10–0.0125 *M*; for TA·Cl (see Fig. 3) and XLA-3·HCl, 0.20–0.025 *M*. R_M values which exceeded 1.7 were not used for determining slopes.

*Compound**	*Hydroxyl groups*	*Exchanger*					
		TA·OAc		*TA·Cl*		*XLA-3·HCl*	
		R_F	*Slope*	R_F	*Slope*	R_F	*Slope*
THA 3,21-diGMe	6	0.37	−1.6	0.029	−1.1	—	—
β-Cortol 3-GMe	7	0.16	−1.4	0.018	—	—	—
THF 3-GMe	6	0.18	−1.3	0.027	−0.9	—	—
FGMe	5	0.40	−1.3	0.080	−0.9	—	—
THE 3-GMe	5	0.45	−1.3	0.087	−0.9	—	—
EGMe	4	0.60	−1.2	0.17	−0.9	0.046	−0.4
SGMe	4	0.72	−1.2	0.29	−0.8	0.089	−0.4
QGMe	3	0.85	−0.9	0.68	−0.5	0.43	−0.1

* GMe = Methyl β-D-glucopyranosiduronate

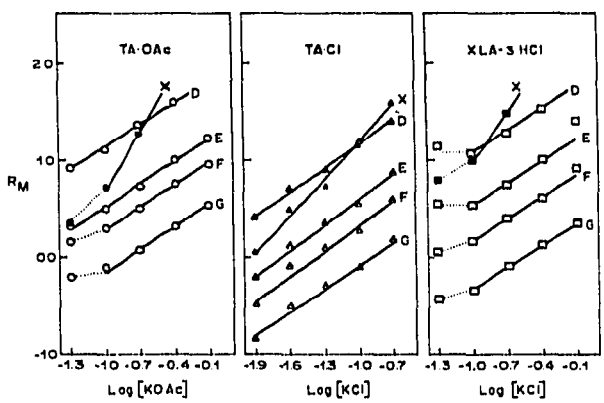

Fig. 4. R_M Values of glucosiduronic acids *vs.* log concentration of counterion in the formamide phase. The concentration of exchanger in the mobile phase was held constant (○—○, 0.0125 *M* TA·OAc; ●—●, 0.05 *M* TA·OAc; △—△, 0.05 *M* TA·Cl; ▲—▲, 0.20 *M* TA·Cl; □—□, 0.05 *M* XLA-3·HCl; ■—■, 0.20 *M* XLA-3·HCl). X = THA 3,21-diGA; D = FGA; E = EGA; F = SGA; G = QGA. For formulas and abbreviations, see Fig. 1 and footnote on pages 26 and 24, respectively.

In contrast to the foregoing results, variation of the KCl concentration from 0.025 to 0.20 *M* did not change the mobility of a group of free steroids, monoglucosiduronic esters, or the diglucosiduronic ester (THA 3,21-diGMe) in the presence of 0.20 *M* TA·Cl.

Evidence that the carboxyl group of a glucosiduronic acid exists as a salt-like structure in a chloroform solution of ion exchanger was provided by comparison of the IR spectra of alkylammonium cholesterol 3-glucosiduronates in chloroform with the spectra of cholesterol 3-glucosiduronic acid and sodium cholesterol 3-glucosid-

uronate in KBr. These and other relevant data are summarized in Table III. Cholesterol 3-glucosiduronic acid was employed because (1) it has no function that interferes with detection of the carboxyl absorption in the range of 1800–1600 cm^{-1}, and (2) it is sufficiently soluble in a chloroform solution of ion exchanger to give a strong carboxylate band.

TABLE III

WAVE NUMBER OF CARBOXYL BAND AND STATUS OF CARBOXYL GROUP

Compound	*Concentration of solute*	*($\bar{\nu}_{max}$ cm^{-1})*	*Status of carboxyl group*
Steroidal glucosiduronic acids (10 compounds)*	1% in KBr	1730–1790	acid
Cholesterol 3-glucosiduronic acid	1% in KBr	1750	acid
Steroidal glucosiduronate salts (10 compounds)*	1% in KBr	1590–1610	salt
Sodium cholesterol 3-glucosiduronate	1% in KBr	1610	salt
Tetraheptylammonium cholesterol 3-glucosiduronate	0.20 *M* in $CHCl_3$**	1615	salt
Tri-*n*-octylammonium cholesterol 3-glucosiduronate	0.13 *M* in $CHCl_3$***	1600	salt
Tetraheptylammonium acetate*	0.20 *M* in $CHCl_3$	1570	salt
Tetraheptylammonium lactate*	0.20 *M* in $CHCl_3$	1600	salt

* Synthesized and characterized in our laboratory[7,8], and unpublished results.
** Solution 1.0 *M* with TA·Cl.
*** Solution 2.3 *M* with TOA·HCl.

The ability of alkylammonium salts to form hydrogen bonds with hydroxyl groups of alcohols and phenols in various solvents is well known[14,15]. Even in the absence of exchanger, the hydroxyl groups of glucosiduronates are associated and the IR spectra show a broad, intense general hydroxyl band at about 3450 cm^{-1}. Consequently, free steroids having a single hydroxyl group were chosen as models for demonstrating the influence of liquid ion exchangers on the wave number of hydroxyl absorption. The effect which three concentrations of TA·Cl had on the hydroxyl absorption of cholesterol (3β-OH), corticosterone 21-acetate (11β-OH), 17α-hydroxyprogesterone (17α-OH), and 11-deoxycorticosterone (21-OH) in chloroform is shown in Table IV. TA·Cl had almost no effect on the intensity or wavenumber of the 3-oxo band (1660 cm^{-1}) or the Δ^4 band (1616 cm^{-1}) of corticosterone 21-acetate, 17α-hydroxyprogesterone and 11-deoxycorticosterone.

When different tetraheptylammonium salts were added to a chloroform solution of cholesterol, displacement of the hydroxyl band varied, as shown in Table V. The influence of these tetraheptylammonium salts on the chromatographic mobility of methyl glucosiduronates in chloroform–formamide is shown in Table VI. With the exception of the order of Br^- and NO_3^- the further an exchanger displaces the OH band of cholesterol from 3610 cm^{-1} (Table V) the greater effect it exerts in increasing the R_F value of a glucosiduronic ester during chromatography (Table VI). The hydrochlorides of TOA, ALA-2, and XLA-3 also decrease the intensity of the unassociated OH band of cholesterol in chloroform; however, they produce smaller effects than the same concentration of TA·Cl.

TABLE IV

APPARENT OPTICAL DENSITIES OF THE HYDROXYL BANDS OF 0.10 *M* SOLUTIONS OF STEROIDS IN CHLOROFORM IN THE PRESENCE OF 0.00, 0.20, 0.40, AND 0.60 *M* TA·Cl

Compound	*Molarity of TA·Cl*	*Wavenumber (cm^{-1}) of hydroxyl band*						
		Monomeric band	*Self-associated band*			*TA·Cl-associated band*		
		3610	*3500*	*3490*	*3480*	*3300*	*3240*	*3230*
Cholesterol	0.00	0.074	—	—	—	0.000	—	—
	0.20	0.044	—	—	—	0.068	—	—
	0.40	0.022	—	—	—	0.102	—	—
	0.60	0.009	—	—	—	0.137	—	—
Corticosterone 21-acetate	0.00	0.069	—	—	0.019	0.000	—	—
	0.20	0.030	—	—	masked	0.104	—	—
	0.40	0.015	—	—	masked	0.160	—	—
	0.60	0.004	—	—	masked	0.192	—	—
17α-Hydroxy-progesterone	0.00	0.025	0.035	—	—	—	0.002	—
	0.20	0.012	0.023	—	—	—	0.090	—
	0.40	0.003	0.013	—	—	—	0.140	—
	0.60	0.000	0.010	—	—	—	0.175	—
11-Deoxy-corticosterone	0.00	—	—	0.051	—	—	—	0.002
	0.20	—	—	0.045	—	—	—	0.038
	0.40	—	—	0.033	—	—	—	0.068
	0.60	—	—	0.029	—	—	—	0.105

TABLE V

EFFECT OF TETRAHEPTYLAMMONIUM SALTS ON THE WAVE NUMBER OF THE HYDROXYL BAND OF CHOLESTEROL IN CHLOROFORM

The tetraheptylammonium salt solutions were 0.6 *M* with respect to the tetraheptylammonium group; cholesterol was 0.2 *M*.

Tetraheptylammonium salt employed	$\tilde{\nu}_{max.}$ *(cm^{-1}) of hydroxyl band*	$\Delta\tilde{\nu}$ *(cm^{-1})*
None	3610	—
Iodide	3395	215
Nitrate	3385	225
Bromide	3345	265
Chloride	3300	310
Acetate	3240	370
Sulfate	3200	410

DISCUSSION

The influence of concentration of ion exchanger and counterion on the chromatographic mobility of steroidal glucosiduronic acids and of nonionizable steroids may be interpreted by use of eqns. 2 and 3. We postulate that in the chromatography

TABLE VI

R_F VALUES OF GLUCOSIDURONATE ESTERS IN THE CHLOROFORM–FORMAMIDE SYSTEM IN THE PRESENCE OF VARIOUS TETRAHEPTYLAMMONIUM SALTS

The mobile phase was 0.10 *M* with respect to the tetraheptylammonium group.

Compound	R_F					
	Tetraheptylammonium salt employed					
	Iodide	*Bromide*	*Nitrate*	*Chloride*	*Acetate*	*Sulfate*
THF 3-GMe	0.010	0.012	0.016	0.032	0.19	0.24
FGMe	0.015	0.035	0.043	0.088	0.40	0.49
THE 3-GMe	0.022	0.047	0.056	0.12	0.46	0.47
EGMe	0.048	0.096	0.12	0.22	0.60	0.62
SGMe	0.11	0.21	0.23	0.38	0.72	0.74
QGMe	0.54	0.66	0.66	0.78	0.85	0.82

of the glucosiduronic acids in chloroform–formamide in the presence of an ion exchanger the transfer of glucosiduronate between phases occurs predominately by an exchange of ions (as defined by eqns. 1 and 2) but that formation of hydrogen bonds between the anions of exchanger and hydroxyl groups of the solute (eqn. 3) plays an ancillary role. We assume that the effect which liquid ion exchangers have on the partition of neutral hydroxy compounds (glucosiduronic esters and free steroids) between chloroform and formamide is produced chiefly by formation of hydrogen bonds.

For chromatography systems which obey the law of mass action and in which the active solvent is not associated, it is generally considered[16,17] that n (the slope of R_M *vs.* log [extractant]) represents the number of molecules of extractant which combine with one molecule of solute. It may be observed that for a particular ion exchanger the slope of R_M *vs.* log [exchanger] for the diglucosiduronic acid is from 1.5–2.0 times as great as the slope for QGA, a monoglucosiduronic acid which has no hydroxyl group other than in the glucosiduronate moiety. The mobility of all of the glucosiduronic acids is decreased by the addition of counterion, the slope of R_M *vs.* log [counterion] for monocarboxylic acids (Fig. 4) being 0.7–0.8 and that for the dicarboxylic acid being 1.3–1.7. The IR spectrum of the product formed when cholesterol glucosiduronic acid is extracted from water into a chloroform solution of liquid ion exchanger indicates that the carboxyl function is present in a salt-like structure (ion pair) rather than as COOH. These findings concerning ion exchange are in harmony with the observation that an exchange of ions actually occurs in the extraction of a glucosiduronic acid[5] from water by a solution of TA·Cl in chloroform.

The free amines (TOA, ALA-2 and XLA-3) have an insignificant influence on the chromatographic mobility of glucosiduronic esters in the chloroform–formamide system; the corresponding hydrochlorides have a detectable but weak effect, the slopes of R_M *vs.* log [exchanger] being −0.1 to −0.4 (Table II). The quaternary exchanger TA·Cl has a strong effect on the mobility of the esters (the slope being −0.5 to −1.1) and its acetate analogue (TA·OAc) has a still greater effect (the slope being −0.9 to −1.6). In Table II it is apparent that as the number of hydroxyl groups of a glucosiduronic ester increases the slope of R_M *vs.* log [exchanger] increases. Changes in concentration of counterion in the stationary phase over a range of sixteenfold produces a negligible effect on the mobility of nonionizable steroids.

Data in Tables IV and V indicate that the anions of quaternary exchangers can form hydrogen bonds with the hydroxyl groups of cholesterol and 17α-hydroxyprogesterone and analogous compounds. The general correlation between magnitude of shift of a hydroxyl band by an ion exchanger and the effect that the exchanger produces on the chromatographic mobility of glucosiduronic esters (Tables V and VI) implies that formation of hydrogen bonds contributes importantly to the chromatographic mobility of the esters. These results are in accord with the previous finding[18] that for a series of proton acceptors which are very similar in structure commonly there is a close relationship between magnitude of shift in frequency of a hydroxyl band and strength of the hydrogen bond which is formed.

For the primary amine hydrochloride (XLA-3·HCl) the slopes for the various acids in a plot of R_M *vs.* log [exchanger] are smaller (Table I) than the number of carboxyl groups present in the substance. For the quaternary exchanger (TA·Cl) the slopes for the monoglucosiduronic acids are from 0.1–0.3 unit greater than the number of carboxyl groups; for TA·OAc, which is strongest relative to hydrogen bonds, the slopes are from 0.1–0.7 unit greater than the number of carboxyl groups present. In view of these observations it seems very probable that both ion exchange and hydrogen bonding are involved in the chromatography of glucosiduronic acids in the presence of the quaternary exchangers and that hydrogen bonding plays a minor role when the amine hydrochlorides are employed.

PRACTICAL CONSIDERATIONS

Since steroidal glucosiduronic acids differ widely in their polarity and ion exchangers vary considerably in their intrinsic strength, it may be necessary to adjust the concentration of the exchanger and/or counterion to provide appropriate mobilities for the particular compounds under study. If essentially maximal resolution of two substances is desired, the mean R_M should be about 0.0 ($R_F = 0.50$). For a particular ion exchanger there is a linear dependence of mobility (R_M) of a compound on log [exchanger] over a considerable range of concentration. Thus, by use of eqn. 2, it is possible to estimate the concentration of ion exchanger needed to obtain a particular R_M value for a monoglucosiduronic acid if R_M of the substance is known at some specific concentration, and if in addition the slope n is known. An average value of −0.9 for the amine hydrochlorides, −1.3 for TA·Cl and −1.6 for TA·OAc, can be used as a rough estimate of slope n for calculating R_M. If R_M is determined at two concentrations of exchanger, the actual value of n for a compound can be determined and this number may be employed in eqn. 2. Likewise, if the R_M of a monoglucosiduronic acid in the presence of both exchanger and counterion is known, it is possible to predict the concentration of counterion needed to give a desired R_M value by use of eqn. 2 and employing 0.8 for n. For a hypothetical substance which has a slope of −1.2 for a plot of R_M *vs.* log [exchanger] and which with 0.025 N exchanger gives $R_M = 0.90$ ($R_F = 0.11$), doubling the concentration of exchanger should change R_M by −1.2 log 2.0, *i.e.* −0.36 R_M unit, to give R_M 0.54 (R_F 0.22). Doubling the concentration once more to 0.10 N should give R_M 0.18 ($R_F = 0.40$); increasing the concentration to 0.20 N should give R_M −0.18 ($R_F = 0.60$). As an example of alterations of mobility of a monoglucosiduronic acid with changes in concentration of counterion, consider a hypothetical substance for which $R_M = -0.27$ ($R_F = 0.65$) in the presence

of 0.10 *N* TA·Cl and 0.0125 *N* KCl and for which the slope is 0.8. Increasing the concentration of KCl to 0.025 *N* should give R_M −0.03 (R_F = 0.51); 0.05 *N* KCl should give R_M 0.21 (R_F = 0.38); 0.10 *N* KCl should give R_M 0.45 (R_F = 0.26).

Whereas the mobility of a pair of monoglucosiduronic acids relative to one another is not changed appreciably by altering the concentration of the weaker exchangers (TOA·HCl, ALA-2·HCl or XLA-3·HCl) or of KCl over a relatively large range, the mobility of a monoglucosiduronic acid relative to the mobility of a diglucosiduronic acid is greatly affected by changing the concentration of ion exchanger. Furthermore, a change in concentration of KCl in the stationary phase will alter the mobility of an acid without changing the mobility of a neutral compoundsignificantly. Thus, a mixture containing a monoglucosiduronic acid, a diglucosiduronic acid and a glucosiduronic ester, all of which migrate at the same rate in a solvent system containing a liquid ion exchanger, should become separable if the concentration of counterion in the stationary phase is adjusted properly. For the stronger exchangers such as TA·OAc and TA·Cl the slopes of the lines representing R_M *vs.* log [exchanger] for monoglucosiduronic acids differ appreciably among themselves (see Table I). However, the difference in slopes for compounds of very similar mobility (*e.g.*, THF 3-GA and THE 3-GA) is small and thus the foregoing comments relative to separation of diglucosiduronic acids, monoglucosiduronic acids and glucosiduronic esters apply also to TA·OAc and TA·Cl.

ACKNOWLEDGMENTS

This investigation was supported in part by Research Grant AM-05452-10 from the National Institutes of Health, Public Health Service. We are indebted to Dr. Ian Bush and to Dr. Edward Soczewinski for reading the manuscript and making several useful suggestions. We wish to thank General Mills for supplying us with methyltricaprylylammonium chloride and Rohm & Haas for providing Amberlite LA-2 and Amberlite XLA-3.

REFERENCES

1 V. R. Mattox, J. E. Goodrich and R. D. Litwiller, *J. Chromatogr.*, 66 (1972) 337.
2 E. Cerrai and C. Testa, *J. Chromatogr.*, 8 (1962) 232.
3 E. C. Bate-Smith and R. G. C. Westall, *Biochim. Biophys. Acta*, 4 (1950) 427.
4 W. Davey and J. R. Gwilt, *J. Appl. Chem.*, 5 (1955) 474.
5 V. R. Mattox, R. D. Litwiller and J. E. Goodrich, *Biochem. J.*, 126 (1972) 533.
6 J. J. Schneider and N. S. Bhacca, *J. Org. Chem.*, 34 (1969) 1990.
7 V. R. Mattox, J. E. Goodrich and W. D. Vrieze, *Biochemistry*, 8 (1969) 1188.
8 V. R. Mattox and W. D. Vrieze, *J. Org. Chem.*, 37 (1972) 3990.
9 C. de Courcy and J. J. Schneider, *J. Biol. Chem.*, 223 (1956) 865.
10 S. Siggia, *Quantitative Analysis via Functional Groups*, Wiley, New York, N.Y., 2nd ed., 1964, p. 47.
11 J. C. Giddings, G. H. Stewart and A. L. Ruoff, *J. Chromatogr.*, 3 (1960) 239.
12 J. R. Howe, *J. Chromatogr.*, 3 (1960) 389.
13 J. Green, S. Marcinkiewicz and D. McHale, *J. Chromatogr.*, 10 (1963) 158.
14 H. Lund, *Acta Chem. Scand.*, 12 (1958) 298.
15 A. Allerhand and P. von Rague Schleyer, *J. Amer. Chem. Soc.*, 85 (1963) 1233.
16 E. Soczewinski and G. Matysik, *J. Chromatogr.*, 32 (1968) 458.
17 E. Soczewinski, *Advan. Chromatogr.*, 5 (1968) 3.
18 T. Gramstad, *Acta Chem. Scand.*, 15 (1961) 1337.
19 V. R. Mattox, R. D. Litwiller and J. E. Goodrich, *J. Chromatogr.*, 109 (1975) in press.