LIQUID ION EXCHANGERS IN REVERSED-PHASE SYSTEMS FOR CHROMATOGRAPHY OF STEROIDAL GLUCOSIDURONIC ACIDS

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(Received November 24th, 1975)

SUMMARY

Steroidal glucosiduronic acids were chromatographed on paper by the reversed-phase technique using five different liquid ion exchangers as stationary phase and aqueous KCl as mobile phase. The relationship of mobility of the acids (R_M) to both the amount of exchanger on the paper and the concentration of KCl in the mobile phase is discussed: the relationships may be expressed as $R_M = n \cdot \log$ [exchanger] + const. and $R_M = -n \cdot \log$ [KCl] + const., respectively. Migration of the acids in the presence of different exchangers is correlated by use of the equation R_M (exchanger Y) $= a \cdot R_M$ (exchanger X) $+ b$. The lack of appreciable correlation between migration of the acids in a reversed-phase system and a corresponding straight-phase system is discussed and expressed by means of regression equations. The correlation coefficients and standard errors of estimate from these equations provide useful indices for selecting two solvent systems that are to be used sequentially to obtain maximal resolution of a group of compounds. ΔR_M values obtained for various functional groups with reversed-phase and straight-phase techniques are compared.

INTRODUCTION

In previous papers[1-3] we reported the use of liquid ion exchangers* in the mobile phase of various solvent systems for chromatographing a group of steroidal glucosiduronic acids. In the present paper, we describe the use of five different exchangers in the stationary phase for chromatographing these acids: aqueous KCl is employed as the mobile phase. The exchangers consist of two quaternary ammonium chlorides, a tertiary, a secondary, and a primary amine hydrochloride. The general technique which we used has been employed previously[4,5] for chromatographing various inorganic cations.

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* 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 a chromatography system.

METHODS AND MATERIALS

Exchangers used are: tetraheptylammonium chloride (TA·Cl), methyltricaprylylammonium chloride (Aliquat), tri-*n*-octylamine hydrochloride (TOA·HCl), Amberlite LA-2 hydrochloride (ALA-2·HCl), and Amberlite XLA-3 hydrochloride (XLA-3·HCl); the first two substances are quaternary salts, the latter three are tertiary, secondary, and primary amine hydrochlorides, respectively. Sources of exchangers, glucosiduronic acids, and other chemicals have been given[1-3] previously along with methods used for the preparation of solutions used in the chromatographic procedures. Sheets of paper (18 × 56 cm; Schleicher and Schuell, 2043A) were drawn slowly through a chloroform solution that contained the appropriate concentration of exchanger and hung in air to dry for about 5 min. Compounds to be chromatographed were then applied on 1-cm lines ($\approx$ 20 μg in 10 μl methanol) and the papers were placed in the solvent troughs of a jar that was lined with paper and contained about 500 ml of water. Without allowing the papers to equilibrate, 40 ml of an aqueous KCl solution of a specified strength was added to the solvent troughs; the jar was

TABLE I

STEROIDAL GLUCOSIDURONIC ACIDS

The numbers assigned to the acids are the same as those used in a previous paper[3].

Compound No.	Name
1	3,20-Dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid
2	17-Hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid
3	21-Hydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
4	3α-Hydroxy-20-oxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
5	3,11,20-Trioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid
6	11β-Hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid
7	17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid
8	3α,17-Dihydroxy-20-oxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
9	(18R)11β,18-Epoxy-21-hydroxy-3,20-dioxopregn-4-en-18-yl α-D-glucopyranosiduronic acid
10	3α-Hydroxy-11,20-dioxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
11	21-Hydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
12	11β,17-Dihydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid
13	17,21-Dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
14	3α,11β-Dihydroxy-20-oxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
15	3α,17-Dihydroxy-11,20-dioxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
16	17,21-Dihydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
17	20β,21-Dihydroxy-11-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
18	11β,21-Dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
19	20α,21-Dihydroxy-11-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
20	3α,11β,17-Trihydroxy-20-oxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
21	17,20β,21-Trihydroxy-11-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
22	11β,17,21-Trihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
23	17,20α,21-Trihydroxy-11-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
24	11β,17,20β,21-Tetrahydroxy-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
25	11β,17,20α,21-Tetrahydroxy-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid
26	20-Oxo-5β-pregnan-3α,21-ylene di(β-D-glucopyranosiduronic acid)
27	11,20-Dioxo-5β-pregnan-3α,21-ylene di(β-D-glucopyranosiduronic acid)
28	11β-Hydroxy-20-oxo-5β-pregnan-3α,21-ylene di(β-D-glucopyranosiduronic acid)

sealed with silicone paste and the mobile phase was allowed to descend to 30–35 cm beyond the origin (5–6 h). The chromatograms were removed from the jar, dried, and treated[1-3] to reveal the location of the solutes.

The procedure for determining the distribution of TA · Cl on a finished chromatogram was described[2] previously. Chloride ion was eluted from a finished chromatogram with water and determined by the Volhard procedure as described[1] previously.

A representative group of the acids listed in Table I was chromatographed in duplicate using each of five exchangers as stationary phase under the following conditions: (1) the concentration of exchanger in the impregnating solution for the stationary phase was varied stepwise while the concentration of KCl in the mobile phase was kept constant, and (2) the concentration of KCl in the mobile phase was varied stepwise while the concentration of exchanger in the solution used for impregnating the paper was held constant. In addition, the entire group of acids listed in Table I was chromatographed in the presence of each exchanger in duplicate using a 0.050 M solution of exchanger in chloroform as impregnating solution for the paper and 0.20 M aqueous KCl as the mobile phase.

RESULTS

The distribution of TA · Cl on a finished chromatogram that had been impregnated with 0.025 M TA · Cl and developed with 0.80 M aqueous KCl was found to be uniform. However, the amount of chloride ion per unit area on a finished chromatogram decreased linearly from the origin until, at an R_F value of about 0.7, it corresponded to approximately 80% of that present at the origin. From that point on, the amount decreased sharply, and at the aqueous front amounted to only about 5% of that at the origin.

Change in mobility with change in concentration of exchanger and counterion

The change in migration of various glucosiduronic acids with change in concentration of XLA-3 · HCl and TA · Cl in the respective impregnating solutions for the
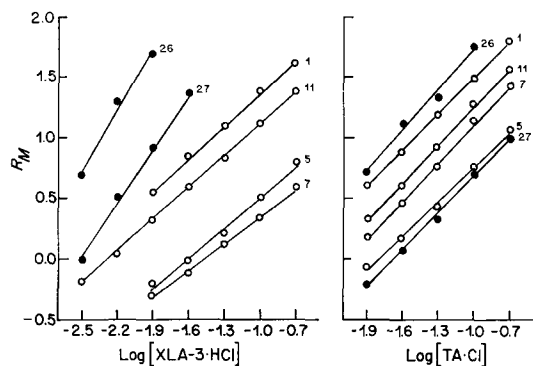

Fig. 1. R_M values of glucosiduronic acids *vs.* log [ion exchanger] in the impregnating solution for the stationary phase. KCl in the mobile phase was 0.20 M. Slopes with XLA-3 · HCl (left panel) are 1.62, 1.49, 0.90, 0.87, 0.84, and 0.74 proceeding from top to bottom. Slopes with TA · Cl (right panel) are 1.11, 0.99, 1.04, 1.05, 0.95, and 1.01 proceeding from top to bottom. See Table I for identification of acids.

stationary phase is shown in Fig. 1; the concentration of KCl in the mobile phase was held constant. The relationship between mobility and concentration of exchanger may be expressed as $R_M = n \cdot \log$ [exchanger] $+$ const.; R_M is defined[6] as $\log (\frac{1}{R_F} - 1)$. The relationship is analogous to that observed[2] in systems which employ an exchanger in the mobile phase except that mobility decreases with an increase in concentration of exchanger. With Aliquat, TOA·HCl, or ALA-2·HCl as stationary phase, slopes of lines from a plot of R_M vs. log [exchanger] are similar to those obtained with TA·Cl. Only with XLA-3·HCl do slopes for the dibasic acids (26 and 27) approach a value which is twice that obtained for the monobasic acids.

If the amount of exchanger on the paper is kept constant, migration of the acids responds to a change in the concentration of KCl in the mobile phase in the following manner: $R_M = -n \cdot \log$ [KCl] $+$ const. Chromatography of four mono-carboxylic acids and two dicarboxylic acids as a function of KCl concentration is shown in Fig. 2. Similar results are obtained with Aliquat, TOA·HCl, and ALA-2·HCl as exchanger. With each exchanger, a given change in concentration of KCl produces a change in R_M for a dibasic acid which is about twice the change found for a mono-basic acid. An eight fold change in concentration of KCl in the mobile phase does not cause an appreciable change in the migration of methyl esters of compounds 1, 5, 26, and 27 on paper that has been impregnated with 0.025 M TA·Cl.

The foregoing linear relationships between R_M and log [exchanger] and between R_M and log [KCl] do not generally hold for R_M values smaller than about -0.30 (R_F greater than about 0.7); the divergence may be associated with the marked change in the gradient in KCl at a mobility corresponding to $R_F \approx 0.7$ which is mentioned in the first paragraph under results.

Strength of exchangers

The order of strength of tetraheptylammonium salts for retaining the acids in the nonpolar phase is essentially the same as reported previously for straight-phase
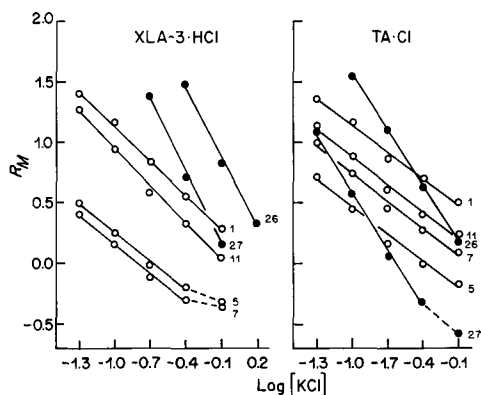

Fig. 2. R_M values of glucosiduronic acids vs. log [KCl] in the mobile phase. The concentration of XLA-3·HCl (left panel) and TA·Cl (right panel) in the impregnating solution for the stationary phase was 0.025 M. Slopes with XLA-3·HCl are -1.91, -2.03, -0.94, -1.02, -0.78, and -0.79 for acids 26, 27, 1, 11, 5, and 7, respectively. Slopes with TA·Cl are -1.53, -1.57, -0.73, -0.76, -0.75, and -0.75 for acids 26, 27, 1, 11, 7, and 5, respectively.

TABLE II

R_F AND R_M VALUES OF STEROIDAL GLUCOSIDURONIC ACIDS IN REVERSED-PHASE CHROMATOGRAPHY

Paper was impregnated with 0.050 M exchanger in chloroform; mobile phase, 0.20 M aqueous KCl. Numbers refer to compounds as listed in Table I.

Compound	Exchanger used as stationary phase									
	TA·Cl		Aliquat		TOA·HCl		ALA-2·HCl		XLA-3·HCl	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
1	0.062	1.18	0.052	1.26	0.13	0.83	0.28	0.41	0.075	1.09
2	0.041	1.37	0.043	1.35	0.095	0.98	0.28	0.41	0.15	0.75
3	0.010	—	0.015	—	0.018	1.74	0.043	1.35	0.019	1.71
4	0.010	—	0.014	—	0.018	1.74	0.040	1.38	0.016	1.79
5	0.27	0.43	0.20	0.60	0.45	0.09	0.61	−0.19	0.38	0.21
6	0.085	1.03	0.065	1.16	0.16	0.72	0.39	0.19	0.18	0.66
7	0.15	0.75	0.16	0.72	0.34	0.29	0.58	−0.14	0.43	0.12
8	0.012	—	0.012	—	0.019	1.71	0.051	1.27	0.023	1.63
9	0.57	−0.12	0.54	−0.07	0.67	−0.31	0.75	−0.48	0.52	−0.04
10	0.062	1.18	0.041	1.37	0.14	0.79	0.24	0.50	0.065	1.16
11	0.11	0.91	0.086	1.03	0.21	0.58	0.36	0.25	0.13	0.83
12	0.061	1.19	0.061	1.19	0.12	0.87	0.38	0.21	0.31	0.35
13	0.015	—	0.016	1.79	0.020	1.69	0.064	1.17	0.035	1.44
14	0.039	1.39	0.034	1.45	0.072	1.11	0.23	0.53	0.079	1.07
15	0.049	1.29	0.054	1.24	0.13	0.83	0.32	0.33	0.11	0.91
16	0.065	1.16	0.10	0.95	0.17	0.69	0.38	0.21	0.24	0.50
17	0.081	1.06	0.076	1.08	0.18	0.66	0.38	0.21	0.071	1.12
18	0.062	1.18	0.083	1.04	0.13	0.83	0.34	0.29	0.16	0.72
19	0.099	0.96	0.10	0.95	0.23	0.53	0.47	0.05	0.12	0.87
20	0.053	1.25	0.045	1.33	0.079	1.07	0.30	0.37	0.15	0.75
21	0.14	0.79	0.18	0.66	0.38	0.21	0.60	−0.18	0.30	0.37
22	0.089	1.01	0.15	0.75	0.18	0.66	0.45	0.09	0.33	0.31
23	0.20	0.60	0.27	0.43	0.47	0.05	0.65	−0.27	0.37	0.23
24	0.20	0.60	0.25	0.48	0.42	0.14	0.65	−0.27	0.36	0.25
25	0.25	0.48	0.38	0.21	0.49	0.02	0.70	−0.37	0.46	0.07
26	0.045	1.33	0.024	1.61	0.058	1.21	0.15	0.75	—	—
27	0.32	0.33	0.10	0.95	0.38	0.21	0.51	−0.02	0.021	1.67
28	0.20	0.60	0.10	0.95	0.26	0.45	0.51	−0.02	0.028	1.54

chromatography[3], namely $(TA)_2 \cdot SO_4 \approx TA \cdot OAc > TA \cdot Cl > TA \cdot Br > TA \cdot I$. On the average, using 0.20 M aqueous KCl as mobile phase, the migration of glucosiduronic acids was retarded by only 0.14 R_M unit more on a paper impregnated with 0.050 M tri-n-octylamine than on paper alone; migration of the acids on paper impregnated with 0.050 M TOA · HCl was retarded by 1.35 R_M units more than on paper alone. The order of strength of various salts of tri-n-octylamine for retaining the acids is $(TOA \cdot H)_2SO_4 > TOA \cdot HCl > TOA \cdot HNO_3 \approx TOA \cdot HBr > TOA \cdot HOAc$.

Correlation of data on reversed-phase systems

The R_F and R_M values obtained for 28 glucosiduronic acids under a standard set of conditions in five reversed-phase systems are given in Table II. Properties of these systems may be compared by choosing one system as a reference (TA · Cl, Table

II) and relating mobilities of the acids in the other systems to the reference system by a linear regression equation.[*]

		n	r	$sy \cdot x$	
R_M(Aliquat)	$= 1.04\ R_M(\text{TA} \cdot \text{Cl}) - 0.06$	21	0.944	0.139	(1)
R_M(XLA-3·HCl)	$= 0.77\ R_M(\text{TA} \cdot \text{Cl}) - 0.14$	21	0.763	0.251	(2)
R_M(TOA·HCl)	$= 1.01\ R_M(\text{TA} \cdot \text{Cl}) - 0.39$	21	0.966	0.104	(3)
R_M(ALA-2·HCl)	$= 0.75\ R_M(\text{TA} \cdot \text{Cl}) - 0.60$	21	0.926	0.117	(4)

These equations are of the general form R_M (exchanger Y) $= a \cdot R_M$ (reference exchanger) $+ b$. Symbols a, b, n, r, and $sy \cdot x$ designate slope, intercept, number of compounds involved in the comparison, correlation coefficient, and standard error of estimate, respectively[3]. Values for a/r from the above equations are 1.10, 1,01, 1,05, and 0.81, respectively; these values express the extent of resolution of the group of acids in each system relative to the reference system (TA·Cl = 1.00). Thus, the system with ALA-2·HCl spreads the acids over a somewhat smaller R_M range than the other systems.
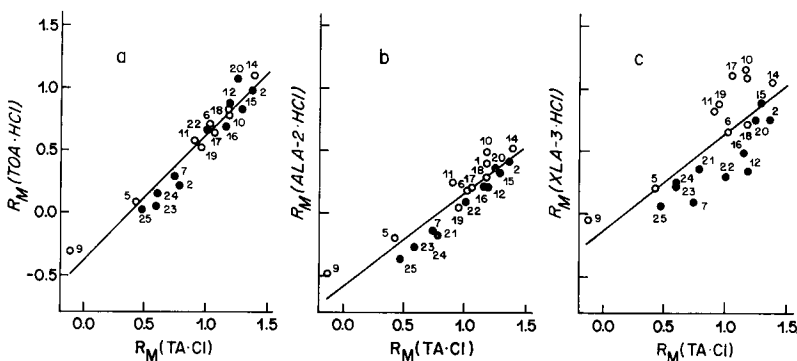

Fig. 3. Comparison of the chromatographic migration (R_M scale) of glucosiduronic acids in systems which contain (a) TOA·HCl, (b) ALA-2·HCl, and (c) XLA-3·HCl to migration in the system which contains TA·Cl. Reversed-phase technique is used. Data are from Table II; see eqns. 3, 4, and 2 in text. Acids having a 17-hydroxyl group are represented by solid circles, those with a hydrogen at C-17 by open circles.

The comparisons represented by eqns. 3, 4 and 2 are illustrated graphically in Fig. 3. The degree of linear association between the R_M values in these systems and the reference system decreases in the order TOA·HCl (tertiary amine) > ALA-2·HCl (secondary amine) > XLA-3·HCl (primary amine). This decrease is equivalent to saying that the systems which contain the tertiary, the secondary, and the primary amine hydrochlorides are progressively less like the reference system in chromatographic properties. The magnitude of values for r and $sy \cdot x$ in the equations indicate the same phenomenon. Compounds having a 17-hydroxyl group (solid circles) tend to

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[*] For a discussion of the use of regression equations for correlating chromatographic data, see ref. 3.

fall below the line of best fit and those with a 17-hydrogen (open circles) tend to fall above the line. This trend becomes progressively prominent as the degree of linear association diminishes.

Comparison of straight-phase and reversed-phase systems

Previously, the R_M values of the acids listed in Table I were obtained[3] in chloroform–formamide* using the exchangers employed in this paper and the straight-phase technique. Because different polar phases are employed, these straight-phase systems are not truly analogous to the reversed-phase systems discussed in this paper; however, since the same exchangers are used, the R_M values obtained with each straight-phase system have been correlated with those found with the corresponding reversed-phase system (Table II). The correlations are summarized by the following equations; subscripts SP and RP denote straight-phase and reversed-phase, respectively.

			n	r	$sy \cdot x$	
$R_M(\text{TA} \cdot \text{Cl}_{RP})$	$= -0.0091$	$R_M(\text{TA} \cdot \text{Cl}_{SP})$	$+ 0.99$	19	-0.015	0.379 (5)
$R_M(\text{Aliquat}_{RP})$	$= -0.16$	$R_M(\text{Aliquat}_{SP})$	$+ 1.14$	20	-0.214	0.417 (6)
$R_M(\text{TOA} \cdot \text{HCl}_{RP})$	$= -0.13$	$R_M(\text{TOA} \cdot \text{HCl}_{SP})$	$+ 1.00$	18	-0.135	0.580 (7)
$R_M(\text{ALA-2} \cdot \text{HCl}_{RP})$	$= -0.18$	$R_M(\text{ALA-2} \cdot \text{HCl}_{SP})$	$+ 0.60$	17	-0.177	0.555 (8)
$R_M(\text{XLA-3} \cdot \text{HCl}_{RP})$	$= -0.11$	$R_M(\text{XLA-3} \cdot \text{HCl}_{SP})$	$+ 0.94$	18	-0.118	0.553 (9)

Values for a/r from these equations are 0.61, 0.75, 0.96, 1.02, and 0.93, respectively. When quaternary exchangers (TA·Cl and Aliquat) are used in the two techniques, resolution is considerably poorer for the reversed-phase procedure; with the amine hydrochlorides, essentially the same degree of resolution is obtained with the two techniques. The negative value of r for each comparison indicates an inverse relation-
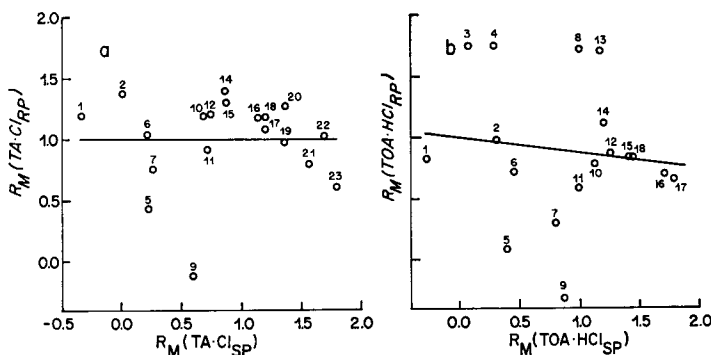

Fig. 4. Comparison of the chromatographic migration (R_M scale) of glucosiduronic acids in reversed-phase systems to migration in straight-phase systems: (a) TA·Cl$_{RP}$ vs. TA·Cl$_{SP}$, (b) TOA·HCl$_{RP}$ vs. TOA·HCl$_{SP}$. Data are from Table II and ref. 3; see eqns. 5 and 7 in text.

* Mobile phase was 0.10 M exchanger in chloroform; stationary phase was 0.10 M KCl in formamide.

ship between values of R_M in a straight-phase and reversed-phase system. Since each value for r is relatively near zero, there is little linear association between the migration of the acids in the different modes of chromatography. The large values for $sy \cdot x$ also illustrate that the actual values found in each reversed-phase system deviate markedly from those predicted from the corresponding values in the straight-phase system. The comparisons represented by eqns. 5 and 7 are illustrated graphically in Fig. 4.

ΔR_M values in straight-phase and reversed-phase systems

ΔR_{Mg} and ΔR_{Mr} values[*] obtained with different exchangers when used in reversed-phase chromatography are given in Tables III and IV. Compounds are grouped according to position of conjugation (C-3 or C-21) and a mean ΔR_M value[**] is given for each grouping. Also given are mean ΔR_M values which were obtained[3] in the aforementioned straight-phase systems.

In the straight-phase systems, all ΔR_M values are positive; this finding indicates that oxygenated compounds migrate more slowly than deoxy analogs and that conjugates with a hydroxyl group migrate more slowly than the corresponding oxo compounds.

In the reversed-phase systems, compounds with either a hydroxyl or an oxo group at C-11 migrate faster than the 11-deoxy analog and all ΔR_{Mg} values for these functions are negative. Except for the system employing XLA-3·HCl, there are several instances in which a compound with a 17-hydroxyl group migrates slower than its 17-deoxy analog (positive ΔR_{Mg} values). This occurrence is most frequent in C-21 glucosiduronic acids.

Conjugates with the 3α-hydroxy-5β-pregnane structure migrate slower than their 3-oxo-Δ^4 analogs; 11β-hydroxy compounds which are conjugated at C-21 tend to migrate slower than their 11-oxo analogs.

Based on mean ΔR_M values, a reversed-phase system is generally less effective than the corresponding straight-phase system for separating a compound with a hydroxyl function from its deoxy analog; a reversed-phase system is generally better for separating an 11-oxo compound from the corresponding 11-deoxy substance.

DISCUSSION

Previous studies have provided evidence that both ion exchange and hydrogen bonding are involved when liquid ion exchangers are employed to extract[8] or to chromatograph[2] steroidal glucosiduronic acids. It seems probable that both of these mechanisms are involved in chromatography of the acids discussed in this paper.

Evidence to support ion exchange is as follows: Addition of counterion to the aqueous phase of a chromatography system decreases the retention of the glucosiduronic acids by the exchangers and the effect of a given increment of counterion on

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[*] The change in R_M caused by substitution of hydrogen by a group is designated ΔR_{Mg}; change in R_M caused by a more complicated change in substituents is designated ΔR_{Mr}.[7] ΔR_{Mg} values are calculated by subtracting the R_M of a reference compound from the R_M of a compound in which a group has been substituted for hydrogen; ΔR_{Mr} values are calculated in an analogous manner.

[**] Where both negative and positive values appear in a column, the absolute mean of ΔR_M is given. This mean indicates the average separation found for compounds which differ by the function(s) being compared.

TABLE III

ΔR_{M_g} VALUES FOR ACIDS IN REVERSED-PHASE SYSTEMS

Values are from data in Table II. Numbers of compounds: see Table I.

Compound pair	Exchanger used as stationary phase				
	TA·Cl	Aliquat	TOA·HCl	ALA-2·HCl	XLA-3·HCl
11β-Hydroxyl					
Conjugated at C-3					
18–3	—	—	−0.91	−1.06	−0.99
22–13	—	−1.04	−1.03	−1.08	−1.13
Mean ΔR_M in reversed-phase system	—	—	−0.97	−1.07	−1.06
Mean ΔR_M in straight-phase system	0.99	0.97	1.37	1.33	1.31
Conjugated at C-21					
6–1	−0.15	−0.10	−0.11	−0.22	−0.43
12–2	−0.18	−0.16	−0.11	−0.20	−0.40
14–4	—	—	−0.63	−0.85	−0.72
20–8	—	—	−0.64	−0.90	−0.88
Mean ΔR_M in reversed-phase system	—	—	−0.37	−0.54	−0.61
Mean ΔR_M in straight-phase system	0.71	0.66	0.86	0.97	0.88
Conjugated at C-3 and C-21					
28–26	−0.73	−0.66	−0.76	−0.77	—
17α-Hydroxyl					
Conjugated at C-3					
13– 3	—	—	−0.05	−0.18	−0.27
16–11	0.25	−0.08	0.11	−0.04	−0.33
22–18	−0.17	−0.29	−0.17	−0.20	−0.41
21–17	−0.27	−0.42	−0.45	−0.39	−0.75
23–19	−0.36	−0.52	−0.48	−0.32	−0.64
Mean ΔR_M in reversed-phase system	0.26[*]	−0.33	0.25[*]	−0.23	−0.48
Mean ΔR_M in straight-phase system	0.48	0.42	0.92	0.81	0.73
Conjugated at C-21					
2– 1	0.19	0.09	0.15	0.00	−0.34
8– 4	—	—	−0.03	−0.11	−0.16
7– 5	0.32	0.12	0.20	0.05	−0.09
12– 6	0.16	0.03	0.15	0.02	−0.31
15–10	0.11	−0.13	0.04	−0.17	−0.25
20–14	−0.14	−0.12	−0.04	−0.16	−0.32
Mean ΔR_M in reversed-phase system	0.18[*]	0.10[*]	0.10[*]	0.09[*]	−0.25
Mean ΔR_M in straight-phase system	0.32	0.26	0.56	0.55	0.45
11-Oxo					
Conjugated at C-3					
11– 3	—	—	−1.16	−1.10	−0.88
16–13	—	−0.84	−1.00	−0.96	−0.94
Mean ΔR_M in reversed-phase system	—	—	−1.08	−1.03	−0.91
Mean ΔR_M in straight-phase system	0.48	0.48	0.73	0.70	0.61
Conjugated at C-21					
5–1	−0.75	−0.66	−0.74	−0.60	−0.88
7–2	−0.62	−0.63	−0.69	−0.55	−0.63
10–4	—	—	−0.95	−0.88	−0.63
15–8	—	—	−0.88	−0.94	−0.72
Mean ΔR_M in reversed-phase system	—	—	−0.82	−0.74	−0.72
Mean ΔR_M in straight-phase system	0.36	0.40	0.60	0.57	0.55
Conjugated at C-3 and C-21					
27–26	−1.00	−0.66	−1.00	−0.77	—

[*]   Mean of absolute ΔR_M values.

TABLE IV

ΔR_{Mr} VALUES FOR ACIDS IN REVERSED-PHASE SYSTEMS

Values are from data in Table II. Numbers of compounds: see Table I.

Compound pair	Exchanger used as stationary phase				
	TA·Cl	Aliquat	TOA·HCl	ALA-2·HCl	XLA-3·HCl
11β-Hydroxyl vs. 11-oxo					
Conjugated at C-3					
18–11	0.27	0.01	0.25	0.04	−0.11
22–16	−0.15	−0.20	−0.03	−0.12	−0.19
24–21	−0.19	−0.18	−0.07	−0.09	−0.12
25–23	−0.12	−0.22	−0.03	−0.10	−0.16
Mean ΔR_M in reversed-phase system	0.18*	0.15*	0.10*	0.09*	−0.15
Mean ΔR_M in straight-phase system	0.51	0.50	0.46	0.41	0.44
Conjugated at C-21					
6– 5	0.60	0.56	0.63	0.38	0.45
12– 7	0.44	0.47	0.58	0.35	0.23
14–10	0.21	0.08	0.32	0.03	−0.09
20–15	−0.04	0.09	0.24	0.04	−0.16
Mean ΔR_M in reversed-phase system	0.32*	0.30	0.44	0.20	0.23*
Mean ΔR_M in straight-phase system	0.29	0.26	0.20	0.35	0.34
Conjugated at C-3 and C-21					
28–27	0.27	0.00	0.24	0.00	−0.13
3α-Hydroxy-5β- vs. 3-oxo-Δ^4					
Conjugated at C-21					
4– 1	—	—	0.91	0.97	0.70
10– 5	0.75	0.77	0.70	0.69	0.95
14– 6	0.36	0.29	0.39	0.34	0.41
8– 2	—	—	0.73	0.86	0.88
15– 7	0.54	0.52	0.54	0.47	0.79
20–12	0.06	0.14	0.20	0.16	0.40
Mean ΔR_M in reversed-phase system	0.43	0.43	0.58	0.58	0.69
Mean ΔR_M in straight-phase system	0.56	0.50	0.67	0.68	0.66
20β-Hydroxyl vs. 20-oxo					
Conjugated at C-3					
17–11	0.15	0.05	0.08	−0.04	0.29
21–16	−0.37	−0.29	−0.48	−0.39	−0.13
24–22	−0.41	−0.27	−0.52	−0.36	−0.06
Mean ΔR_M in reversed-phase system	0.31*	0.20*	0.36*	−0.26	0.16*
Mean ΔR_M in straight-phase system	0.45	0.41	0.81	—	—
20α-Hydroxyl vs. 20-oxo					
Conjugated at C-3					
19–11	0.05	−0.08	−0.05	−0.20	0.04
23–16	−0.56	−0.52	−0.64	−0.48	−0.27
25–22	−0.53	−0.54	−0.64	−0.46	−0.24
Mean ΔR_M in reversed-phase system	0.38*	−0.38	−0.44	−0.38	0.18*
Mean ΔR_M in straight-phase system	0.64	0.57	—	—	—

* Mean of absolute ΔR_M values.

dibasic acids (Fig. 2) is twice as great as that on monobasic acids. In chromatography of non-ionic compounds (free steroids and glucosiduronic esters) in the presence of $TA \cdot Cl$, mobility does not change with a change in concentration of counterion.

$TA \cdot Cl$ in chloroform[2] forms hydrogen bonds with hydroxyl groups at C-3, C-11, C-17, and C-21 in substituted steroids. XLA-3·HCl is significantly less effective than $TA \cdot Cl$ in abolishing the unassociated hydroxyl band in the infrared spectrum[9] of these steroids in chloroform. With $TA \cdot Cl$ as stationary phase, many of the ΔR_{Mg} values for the 17-hydroxyl group in glucosiduronic acids are positive (Table III), which implies that $TA \cdot Cl$ had considerable affinity for the 17-hydroxyl group; all of the corresponding values with XLA-3·HCl are negative. Amine hydrochlorides are much more effective than the corresponding free amines in retarding the migration of glucosiduronic esters; this difference is attributable to the greater tendency of the hydrochlorides to form hydrogen bonds. These findings imply that hydrogen bonding plays a significant role in chromatography by the reversed-phase procedure.

It would be expected that hydrophobic bonding[10] should contribute to retention of the glucosiduronates by reversed-phase chromatography since water is the principal component of the mobile phase and both the steroid nucleus and the alkyl chains of the exchanger are non-polar in nature. However, we have no data to confirm this expectation; if hydrophobic bonding plays a major role, it would seem that tri-n-octylamine should retard migration of the acids to a greater extent than is observed.

Bush[11] has stated that with any one solvent system the range of ΔR_{Mg} values for a specific substituent in different steroids is usually $\leqslant \pm 15\%$ of the mean value. Variability of the ΔR_{Mg} values for groups in glucosiduronic acids in reversed-phase systems which employ ion exchangers as stationary phase (Table III) is often greater. The agreement of the ΔR_{Mg} values is generally greatly improved if the conjugates are grouped according to various structural differences; this finding is illustrated in Table V. Irregularities in ΔR_{Mg} values have been attributed[12] principally to experimental errors and to intramolecular interactions; it is probable that some of the inconsistencies in the ΔR_{Mg} values for the glucosiduronic acids stem from these causes. In compounds conjugated at C-3, differences in the ΔR_{Mg} values for the 17α-hydroxyl group for compounds with the 20-oxo function relative to values for analogs with a 20-hydroxyl group may be due to vicinal effects (Table V, lines 2 and 3 under 17α-hydroxyl). However, it seems likely that some of the variations in the ΔR_{Mg} values for the glucosiduronic acids may be attributed in part to the large size of the exchanger molecule and the manner in which it exists in combination with the conjugate.

The ΔR_{Mg} values for an 11β-hydroxyl group in compounds conjugated at C-21 having the 3α-hydroxy-5β-pregnane structure are significantly more negative than in analogs having the 3-oxopregn-4-ene structure (Table V, lines 3 and 4 under 11β-hydroxyl). In addition to having one molecule of exchanger form an ion-pair with one molecule of glucosiduronic acid, it may be spatially possible for another molecule of exchanger to form a hydrogen bond with a hydroxyl group of the steroid nucleus. In compounds with both the 3α-hydroxyl group and the 11β-hydroxyl group, it seems unlikely that both of these groups can be bonded at the same time because of the size of the exchanger molecule. If the 3α-hydroxyl group is bonded more readily than the 11β-hydroxyl group, the latter group will probably not be bonded at all and will, therefore, retain most of its usual polarity although the 11β-hydroxyl group may be slightly less polar than normal due to shielding by the alkyl groups of the exchanger molecule.

TABLE V

MEAN AND AVERAGE DEVIATION OF ΔR_{Mg} VALUES FOR ACIDS IN REVERSED-PHASE SYSTEMS

Values are from data in Table III. N designates the number of pairs of compounds from which the mean was determined.

Types of compounds compared	N	Exchanger used as stationary phase		
		$TOA \cdot HCl$	$ALA\text{-}2 \cdot HCl$	$XLA\text{-}3 \cdot HCl$
		11β-Hydroxyl		
All conjugates	6	-0.57 ± 0.31	-0.72 ± 0.34	-0.76 ± 0.24
Compounds conjugated at C-3	2	-0.97 ± 0.06	-1.07 ± 0.01	-1.06 ± 0.07
3α-Hydroxy-5β-pregnanes conjugated at C-21	2	-0.64 ± 0.01	-0.88 ± 0.03	-0.80 ± 0.08
3-Oxopregn-4-enes conjugated at C-21	2	-0.11 ± 0.00	-0.21 ± 0.01	-0.42 ± 0.02
		17α-Hydroxyl		
All conjugates	11	-0.05 ± 0.17	-0.14 ± 0.11	-0.35 ± 0.13
20-Hydroxy compounds conjugated at C-3	2	-0.47 ± 0.02	-0.36 ± 0.04	-0.70 ± 0.06
20-Oxo compounds conjugated at C-3	3	-0.04 ± 0.10	-0.14 ± 0.07	-0.34 ± 0.05
3α-Hydroxy-5β-pregnanes conjugated at C-21	3	-0.01 ± 0.03	-0.15 ± 0.02	-0.24 ± 0.06
3-Oxopregn-4-enes conjugated at C-21	3	0.17 ± 0.02	0.02 ± 0.02	-0.25 ± 0.10
		11-Oxo		
All conjugates	6	-0.90 ± 0.12	-0.84 ± 0.18	-0.78 ± 0.12
Compounds conjugated at C-3	2	-1.08 ± 0.08	-1.03 ± 0.07	-0.91 ± 0.03
3α-Hydroxy-5β-pregnanes conjugated at C-21	2	-0.92 ± 0.04	-0.91 ± 0.03	-0.68 ± 0.05
3-Oxopregn-4-enes conjugated at C-21	2	-0.72 ± 0.03	-0.58 ± 0.03	-0.76 ± 0.13

In compounds with the 3-oxo function and the 11β-hydroxyl group, the 11β-hydroxyl group will be hydrogen bonded to some extent and thereby rendered less polar than normal. In effect, the 11β-hydroxyl group is more polar in the presence of the 3α-hydroxyl group than in the presence of the 3-oxo function, and thus the ΔR_{Mg} values for an 11β-hydroxyl group are more negative when the 3α-hydroxyl group is present.

Discriminating potential of chromatography systems

How can one select from a group of chromatography systems the two that, if used in succession, have the best chance of discriminating a pair of arbitrarily chosen compounds? The combined resolving potential of one pair of systems relative to that of another pair can be judged visually by comparing the degree of scatter of the points in plots such as those in Figs. 3 and 4. Successive chromatography of glucosiduronic acids in the systems compared in Fig. 4b is much more likely to yield separation of two randomly chosen glucosiduronic acids than is successive chromatography of the same two compounds in the systems compared in Fig. 3c.

Plots of R_M(system Y) *vs.* R_M(system X) are conveniently summarized[3] by regression equations of the type $R_M(\text{system Y}) = a \cdot R_M(\text{system X}) + b$. The scatter of points in such plots, and thus the combined resolving potential of a pair of systems, is reflected in (1) the value of correlation coefficient r from the equation, and (2) the standard deviation of the differences between R_M values found in system Y and those predicted by the equation (standard error of estimate $sy \cdot x$). The relationship[13] between r and $sy \cdot x$ is given by the equation $sy \cdot x = Sy \sqrt{1-r^2}$ where Sy designates the standard deviation from the mean of the R_M values found in system Y. As r approaches zero,

$sy \cdot x$ approaches its maximal value which is Sy; if r is constant, $sy \cdot x$ increases with Sy. Thus, in general, the probability of being able to separate a pair of arbitrarily chosen compounds* by chromatography in any two systems in sequence increases as the value for r from the equation relating the two systems approaches zero. Furthermore, if the values for r are near zero for several pairs of systems, the pair for which the value for $sy \cdot x$ is largest offers the greatest chance of separation. Consider eqns. 1, 2, 5, and 7. Values for r, which are 0.944, 0.763, -0.015, and -0.135, respectively, indicate that the combined resolving potential of the pairs of systems increases in the following order (using an equation number to designate a pair of systems): $1 < 2 < 7 \approx 5$. In addition, values for $sy \cdot x$ indicate that the combined discriminating potential of the pair of systems represented by eqn. 7 ($sy \cdot x = 0.580$) is better than that of the pair represented by eqn. 5 ($sy \cdot x = 0.379$). This type of approach can be applied to selecting three or more chromatography systems which are to be used sequentially to obtain optimal discrimination of the components of a mixture.

ACKNOWLEDGMENTS

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* If the specific substances to be discriminated are not from the group of compounds for which the equations were derived, they must be sufficiently like those substances that, if included in that group, they would not produce a significant change in the constants of the equations or in values for r and $sy \cdot x$.