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LIQUID ION EXCHANGERS FOR CHROMATOGRAPHY OF STEROIDAL GLUCOSIDURONIC ACIDS AND OTHER POLAR COMPOUNDS*

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SUMMARY

Many of the solvent systems which are commonly used to chromatograph corticosteroids can be used to chromatograph the corresponding glucosiduronic acids if the mobile phase is made about 0.1 *M* with tetraheptylammonium chloride and the stationary phase is made about 0.1 *M* with potassium chloride. The rates of movement of the glucosiduronates relative to one another are different in the various solvent systems which are described.

INTRODUCTION

Although liquid ion exchangers[2] have been used extensively in the extraction and chromatography of inorganic ions[3,4], they have received scant attention in the chromatography of organic compounds. In the steroid field, KUSHINSKY AND TANG[5] achieved a partial resolution of estrogen glucosiduronates on a column of Amberlite LA-1 using the reversed-phase technique. Oleic acid[6] has been used as a liquid ion exchanger in the chromatography of alkaloids.

In a paper on the extraction of bile acid conjugates from aqueous media, HOFMANN[7] suggested that a chloroform or ethyl acetate solution of tetraheptylammonium chloride (THAC) might be useful for extracting steroidal glucosiduronic acids from urine. We have found[8] that solutions of THAC in chloroform or other organic solvents are in fact very satisfactory for extracting glucosiduronic acids from aqueous media. It seemed logical to expect that steroidal glucosiduronic acids would be soluble in formamide and that they could be chromatographed on paper in a chloroform–formamide system, provided the chloroform contained an appropriate concentration of THAC. We therefore explored the use of THAC for the paper chromatography of

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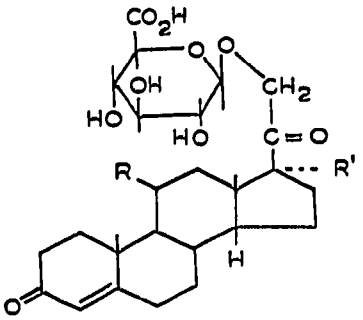
various steroidal glucosiduronic acids and esters with solvent systems such as chloroform–formamide, toluene–ethylene glycol, and benzene–methanol–water[9].

This is the first of a series of papers which will describe the results we have obtained through the use of anionic liquid ion exchangers*.

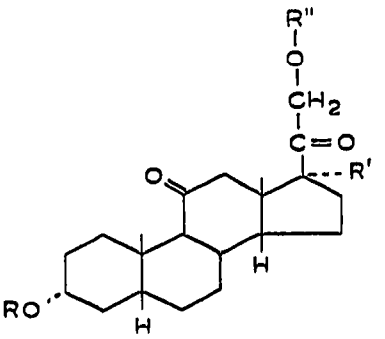
METHODS AND MATERIALS

Chemicals

Tetraheptylammonium chloride was purchased from Eastman Organic Chemicals; sodium dodecyl sulfate was purchased from Mann Research Laboratories. Other chemicals were of reagent grade; solvents were either of reagent grade or were redistilled before use. Steroidal glucosiduronates (Fig. 1) were synthesized in our laboratory[10].



	R	R′
EGA	O	OH
FGA	H, OH	OH
SGA	H_2	OH
BGA	H, OH	H
AGA	O	H
QGA	H_2	H



	R	R′	R″
THA 3-GA	GA	H	H
THA 21-GA	H	H	GA
THA 3,21-diGA	GA	H	GA
THE 3-GA	GA	OH	H

Fig. 1. Steroidal glucosiduronates used in this study and abbreviations used to refer to them in text. E, F, B, and A are Kendall's designations for adrenal hormones; S and Q are Reichstein's. THA = 3α,21-dihydroxy-5β-pregnane-11,20-dione; THE = 3α,17,21-trihydroxy-5β-pregnane-11,20-dione; GA = β-D-glucopyranosyluronic acid group; GMe = β-D-glucopyranosyluronic methyl ester.

The equivalent weight of THAC was determined by titrating a weighed amount for chloride ion by the Volhard procedure: about 500 mg of THAC in 15 ml of chloroform was added to 15.00 ml of 0.100 *N* silver nitrate and 5 ml of 6 *N* nitric acid. The solution was shaken vigorously and filtered. Saturated ferric ammonium sulfate (1 ml) was added to the filtrate, and the mixture was titrated with 0.100 *N* ammonium thiocyanate. Different batches of THAC contained 93–95% of the calculated amount of Cl^-. In addition, the tetraheptylammonium ion was assayed[11] by titrating THAC

* In this paper we are using the term "liquid ion exchanger" to designate a class of compounds which, under appropriate conditions, can react with ionic substances by an ion-exchange mechanism. We do not wish to imply that ion exchange is the only mechanism responsible for the properties of this class of compounds.

with sodium dodecyl sulfate using eosin Y as an indicator. Results from assays by the two different methods agreed within 2%. The concentrations of THAC given throughout the paper are based on the Cl^- determination.

Chromatographic technique

Chromatograms were run by descending flow on 18 × 56 cm sheets of Schleicher & Schüll 2043A paper in the direction in which the paper was machined. This paper is sufficiently strong so that it can be handled without tearing, even when wet with aqueous solutions. Compounds to be chromatographed (20 μg in 10 μl of methanol) were applied on 1-cm lines which were 1 cm apart and 12 cm from the top of the paper. Jars were lined with paper and were allowed to equilibrate with the appropriate phases for at least 24 h before being used.

In general, the chromatographic technique was the same as has been used for conventional ZAFFARONI-type systems except that the new chromatography systems contain liquid ion exchanger, *viz.* THAC, in the mobile phase and potassium chloride (KCl) in the stationary phase.

Chromatography systems

Conventional system. The conventional chloroform–formamide system[12] was run in the same general manner as described below for system II except that neither THAC nor KCl was present.

Modified Zaffaroni-type systems

System I. The technique was as described for system II except that the mobile phase was 0.025 *M* with THAC and the stationary phase contained no KCl.

System II. A jar was lined with sheets of paper which had previously been impregnated with formamide–methanol (3:7) and dried (about 15 min) until the methanol had evaporated. Enough mobile phase was added to the jar to bring the liquid level above the bottom of the liner.

Preparation of mobile phase: To 100 ml of 0.10 *M* THAC in chloroform was added 10 ml of 0.10 *M* KCl in formamide. The mixture was shaken vigorously and allowed to separate, and the bottom phase was used as the mobile phase for developing the chromatogram. The stationary phase consisted of 0.10 *M* KCl in formamide, prepared just before use. The papers were impregnated by drawing them through the mixture: stationary phase–methanol (3:7). They were then hung in air for about 5 min to dry. The compounds to be chromatographed were applied, and the papers were placed in the solvent troughs. About 50 ml of mobile phase was added immediately to each trough, the paper liner around the jar was wetted with the formamide-saturated chloroform, and the lid was placed on the jar. The solvent was allowed to advance 30–35 cm beyond the origin. The chloroform front was marked immediately after the paper was removed from the jar.

System III. Chromatograms were run in system III in an analogous manner. The mobile phase was 0.10 *M* THAC in toluene, the stationary phase was 0.10 *M* KCl in ethylene glycol (papers were impregnated by drawing them through a mixture of stationary phase and methanol (3:7)).

Chloroform–ethyl acetate–THAC–water–KCl (system IV)

The liner in the chromatography jar was in contact with a mixture of chloro-

form–ethyl acetate–water (1:1:2). The chromatography paper strip was weighed, drawn through a solution of 0.20 *M* KCl in water, and blotted between two sheets of paper. The compounds to be chromatographed were then applied. The paper was reweighed periodically[6]; when the solvent had evaporated to the point such that the paper weighed approximately 1.5 times the original weight (about 15 min), it was placed in a chromatography jar. A 0.10 *M* solution of THAC in chloroform–ethyl acetate (1:1) was saturated with 0.20 *M* KCl and used as the mobile phase. The mobile phase was added to the chromatography trough as soon as the paper to be chromatographed was in place.

Modified Bush-type systems (systems V, VI and VII)

After the components of the solvent system were mixed and the phases had separated, THAC and KCl were added to the mobile (less dense) phase and stationary phase, respectively, to the desired concentrations. Equal volumes of the THAC-containing mobile phase and the KCl-containing stationary phase were then mixed and allowed to separate again into two phases. These phases were used as follows. A sheet of paper was drawn through the stationary (more dense) phase and blotted briefly between two sheets of dry paper. Without delay, the compounds to be chromatographed were applied, and the paper was then placed in the trough of a chromatography jar which was lined and contained the appropriate phases in the bottom. After 30 min of equilibration, the mobile phase containing the THAC was added to the trough and the chromatogram was run in the conventional manner. The solvent front moved 30–35 cm in 3–4 h. Chromatography systems V and VI were modifications of solvent mixtures which contain *tert.*-butyl alcohol[13]; system VII was a modification of system B_5 of BUSH[14].

Detection of THAC front

In some solvent systems, the THAC front moved slower than the front of the mobile phase. The THAC front could be detected by covering the chromatogram with a glass plate extending from the origin to within 5 cm of the front of the mobile phase and then spraying the exposed area with a 0.01% (w/v) solution of eosin Y in acetone[11]. The combination of THAC and eosin Y produces a strong pink fluorescence under 365-nm radiation. The THAC front may also be detected by dipping the chromatogram in the alkaline tetrazolium blue reagent described below and placing it on a glass plate. The area of paper containing THAC gives a blue background within a few minutes, whereas the area lacking THAC turns blue much more slowly.

Detection of steroids

After chromatography, papers that had been impregnated with formamide or ethylene glycol were mounted horizontally on a rack and dried in an air current in a fume hood. Drying was facilitated by keeping the paper about 7 cm from an 18 × 36 cm metal plate that was maintained at about 150° (two hot plates placed side by side were used as a source of heat). Paper chromatograms were dipped into a freshly prepared mixture of 0.4% tetrazolium blue in water–3.0 *N* NaOH (1:9) and allowed to remain on a glass plate for observation. The compounds were revealed as blue, yellow, or bluish gray spots on a white background. Alternatively, 3-oxo-Δ^4 compounds could be detected by viewing the paper over 254-nm radiation or by treating it with

alkali to develop fluorescence[14]; for production of maximal fluorescence, tetrazolium blue should not be used in the alkaline solution.

RESULTS

When various synthetic steroidal glucosiduronic acids were chromatographed in chloroform–formamide with and without THAC, none of the glucosiduronic acids had an R_F value greater than 0.01 in the absence of THAC (Table I). However, in the presence of 0.025 M THAC in the chloroform solution, their migration increased to such an extent that several of them migrated at roughly the same rates as the corresponding free steroids in the absence of THAC. Even the very polar diglucosiduronic acid, THA 3,21-diGA (which has two ketone, six hydroxyl, and two carboxyl groups) migrated slightly, having an R_F value of 0.04.

TABLE I

R_F VALUES OF VARIOUS STEROIDAL GLUCOSIDURONATES (ACIDS AND METHYL ESTERS) AND OF FREE STEROIDS IN CHLOROFORM–FORMAMIDE WITH AND WITHOUT THAC

Without THAC, the conventional ZAFFARONI-type system[13]; paper impregnated with formamide–methanol (3:7); R_F is based on chloroform front. With THAC, system I; chloroform phase 0.025 M with THAC; paper impregnated with formamide–methanol (3:7); R_F is based on THAC front. Further details in text.

Compound[a]	*As acid*		*As ester*		*As free steroid*
	Without THAC	*With THAC*	*Without THAC*	*With THAC*	*Without THAC*
EGA	<0.01	0.43	0.01	0.05	0.47
FGA	<0.01	0.16	<0.01	0.03	0.19
SGA	<0.01	0.60	0.04	0.11	0.72
BGA	<0.01	0.46	0.03	0.12	0.69
AGA	<0.01	0.49	0.09	0.21	0.82
QGA	<0.01	0.80	0.32	0.49	0.88
THA 3-GA	<0.01	0.19	0.05	0.11	0.68
THA 21-GA	<0.01	0.20	0.03	0.07	—
THA 3,21-diGA	<0.01	0.04	<0.01	0.01	—
THE 3-GA	<0.01	0.06	0.01	0.03	0.19

[a] See Fig. 1 for key to structure of compounds.

Comparison of the relative rates of movement of a group of compounds during chromatography in two solvent systems is facilitated by use of ΔR_{M8}, a value defined[15] as the difference between the mobility (R_M) of a compound in system I and the mobility of the same compound in another system. If, for a series of compounds, all of the ΔR_{M8} values are equal, the compounds then move at the same relative rates in the two solvent systems. If the differences in the ΔR_{M8} values are larger than the experimental errors encountered in determining mobility, the compounds do not move at the same relative rates. The larger the differences in ΔR_{M8} values, the greater the differences in relative rates of migration of the compounds in the two systems. Such differences[15,16] can be used as a figure of merit in determining how really different two solvent systems are in separating a pair of compounds.

By converting the R_F values of columns 2 and 3 of Table I into R_M values[17] and subtracting each R_M value of column 3 from the corresponding R_M value of column 2, we obtain ΔR_{Ms} values. Assuming that there is no appreciable adsorption of the conjugates on the paper, these ΔR_{Ms} values correspond approximately to the logarithms of the ratios of the partition coefficients of the compounds in chloroform–formamide (conc. in chloroform phase/conc. in formamide phase) in the absence and presence of THAC. From the magnitude of these values, it is apparent that the presence of 0.025 *M* THAC in the chloroform phase effectively increases the partition coefficients of the conjugates roughly one hundred-fold.

When the methyl esters of the glucosiduronic acids were chromatographed on paper in chloroform–formamide, the least polar one, QGM, migrated with an R_F of 0.32 whereas the other esters had R_F values in the range of <0.01–0.09 (see Table I). With 0.025 *M* THAC in the same system, the migration of the methyl esters increased markedly, although not to the same extent that the mobilities of the acids had been increased by THAC. Table I also shows that, in the presence of THAC, the glucosiduronic methyl esters migrate more slowly than the parent steroids in the absence of THAC. If the ΔR_{Ms} values from columns 4 and 5 are obtained, it becomes apparent that the presence of THAC in the chloroform phase increases the partition coefficients of the esters from seven- to ten-fold.

THAC also produces small but detectable increases in the mobility of the free compounds E, F, and THE, but has an insignificant effect on the mobility of the less polar substances S, B, A, Q, and THA.

When the concentration of THAC in the chloroform phase was increased to 0.10 *M*, the R_F values of a few of the conjugates were too large to be meaningful. However, when the formamide-impregnating solution was made 0.10 *M* with KCl

TABLE II

R_F VALUES OF STEROIDAL GLUCOSIDURONIC ACIDS IN VARIOUS CHROMATOGRAPHY SYSTEMS WHICH CONTAIN THAC IN MOBILE PHASE AND KCl IN STATIONARY PHASE

Solvent systems: II = 0.10 *M* THAC in chloroform, 0.10 *M* KCl in formamide; III = 0.10 *M* THAC in toluene, 0.10 *M* KCl in ethylene glycol; IV = 0.10 *M* THAC in chloroform–ethyl acetate (1:1), 0.20 *M* KCl in water; V (E_2B) = isooctane–*tert.*-butanol–water (50:25:45), 0.10 *M* THAC in top phase, 0.10 *M* KCl in bottom phase; VI = (NE_{10}) benzene–*tert.*-butanol–methanol–water (100:10:40:60), 0.10 *M* THAC in top phase, 0.20 *M* KCl in bottom phase; VII (B_5) = benzene–methanol–water (10:5:5), 0.10 *M* THAC in top phase, 0.25 *M* KCl in bottom phase. Further details in text.

Compound[a]	*Solvent systems*					
	II	*III*	*IV*	*V*	*VI*	*VII*
EGA	0.38	0.33	0.45	0.22	0.31	0.17
FGA	0.14	0.23	0.28	0.25	0.20	0.11
SGA	0.55	0.48	0.71	0.48	0.53	0.35
BGA	0.41	0.44	0.50	0.37	0.39	0.25
AGA	0.41	0.31	0.36	0.22	0.31	0.17
QGA	0.73	0.54	0.74	0.50	0.60	0.47
THA 3-GA	0.19	0.20	0.24	0.22	0.20	0.11
THA 21-GA	0.18	0.23	0.36	0.41	0.26	0.14
THA 3,21-diGA	0.017	0.057	<0.01	0.019	<0.01	<0.01
THE 3-GA	0.066	0.15	0.17	0.21	0.11	0.051

[a] See Fig. 1 for key to structure of compounds.

(system II), the mobilities of the glucosiduronic acids were of the same order of magnitude as in 0.025 *M* THAC in chloroform–formamide in the absence of KCl.

There are numerous solvent systems which give significantly different relative rates of movement of various free steroids during chromatography. It was of interest to determine whether, in the presence of THAC, a series of steroidal glucosiduronic acids also would have different relative rates of movement in a selected group of these same chromatography systems. In Table II are given the chromatographic mobilities of the conjugates in systems II–VII which contained 0.10 *M* THAC in the mobile phase and 0.10–0.25 *M* KCl in the solvents used for impregnating the paper with the stationary phase.

TABLE III

R_M VALUES OF STEROIDAL GLUCOSIDURONIC ACIDS IN VARIOUS CHROMATOGRAPHY SYSTEMS
Values from data in Table II.

Compound[a]	*Solvent system*					
	II	*III*	*IV*	*V*	*VI*	*VII*
EGA	0.21	0.31	0.09	0.55	0.35	0.69
FGA	0.79	0.53	0.41	0.48	0.60	0.91
SGA	−0.09	0.04	−0.39	0.04	−0.05	0.27
BGA	0.16	0.10	0.00	0.23	0.19	0.48
AGA	0.16	0.35	0.25	0.55	0.35	0.69
QGA	−0.43	−0.07	−0.45	0.00	−0.18	0.05
THA 3-GA	0.63	0.60	0.50	0.55	0.60	0.91
THA 21-GA	0.66	0.53	0.25	0.16	0.45	0.79
THA 3,21-diGA	1.76	1.22	>1.99	1.71	>1.99	>1.99
THE 3-GA	1.15	0.75	0.69	0.58	0.91	1.27

[a] See Fig. 1 for key to structure of compounds.

In Table III are shown the R_M values for the glucosiduronic acids in the various solvent systems. In Fig. 2, the ΔR_{Ms} values for the group of glucosiduronic acids are plotted for five pairs of solvent systems (THA 3,21-diGA, which is exceptional because it is a diglucosiduronate, is not shown). The ΔR_{Ms} values for the individual glucosiduronic acids differ little for systems VI and VII. This indicates that the

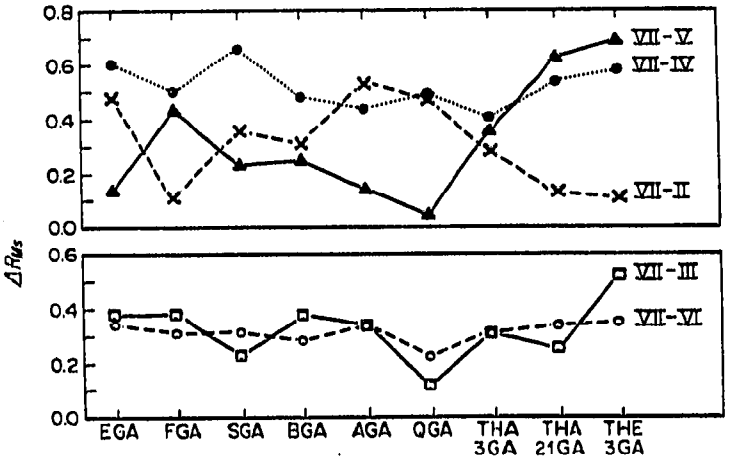


Fig. 2. ΔR_{Ms} values of glucosiduronic acids for five pairs of solvent systems; data from Table III, all compared to R_M in system VII.

compounds move at similar relative rates in these systems. However, for each of the other pairs of solvent systems, the ΔR_{Ms} values are markedly different.

EBERLEIN AND BONGIOVANNI[13] noted that in a solvent system based on *tert.*-butanol–hydrocarbon–water (their system E_2B) the relative polarities of hydroxyl and ketone groups at C-3 and C-11 were usually the reverse of the polarities found in a system based on methanol–hydrocarbon–water (system B_5 of BUSH[14]). A corresponding difference in polarities for hydroxyl and ketone groups at C-11 in steroidal glucosiduronic acids was also observed with these systems in the presence of THAC (*cf.* systems V and VII in Table II).

The chromatographic mobilities of the methyl esters of the glucosiduronic acids were determined in three solvent systems (Table IV). As found for the acids, there were differences in the relative rates of movement of the esters in the various systems. In addition, the ΔR_{Mr} values corresponding to conversion of each acid to its methyl ester are not constant for the three systems studied. FGA and THA 3-GA are separable in system II but not in systems III–VII. However, the corresponding esters, FGM and THA 3-GM, are readily separable in systems II, III and V. In addition, acids EGA and AGA are separable only in system IV, whereas the corresponding methyl esters are readily separable in systems II and III.

TABLE IV

R_F AND R_M VALUES OF STEROIDAL GLUCOSIDURONIC ACID METHYL ESTERS

Solvent systems II, III and V as described in text and Table II.

Compound[a]	*Solvent systems*					
	II		*III*		*V*	
	R_F	R_M	R_F	R_M	R_F	R_M
EGMe	0.18	0.66	0.20	0.60	0.33	0.31
FGMe	0.064	1.17	0.13	0.83	0.38	0.21
SGMe	0.33	0.31	0.32	0.33	0.52	−0.04
BGMe	0.29	0.39	0.36	0.25	0.50	0.00
AGMe	0.44	0.10	0.37	0.23	0.35	0.27
QGMe	0.77	−0.53	0.64	−0.25	0.68	−0.33
THA 3-GMe	0.34	0.29	0.42	0.14	0.49	0.02
THA 21-GMe	0.22	0.55	0.30	0.37	0.61	−0.19
THA 3,21-diGMe	0.026	1.57	0.12	0.87	0.11	0.91
THE 3-GMe	0.098	0.96	0.29	0.39	0.46	0.07

[a] See Fig. 1 for key to structure of compounds.

Color reactions

The presence of THAC on the finished chromatograms does not affect appreciably the detection of 3-oxo-Δ^4 steroids by observation over 254-nm illumination. The strong tetrazolium blue solution that is used gives satisfactory results for α-ketolic steroids even in the presence of formamide. In the presence of THAC, the alkaline tetrazolium blue solution gives an intense blue color with 3-oxo steroids and 3-oxo-Δ^4 steroids. The 11-oxo group is not chromogenic. Alkaline tetrazolium blue produces a yellow or bluish gray color with BGA, QGA, THA 21-GA, THA 3,21-diGA, and the corresponding methyl esters; the solvent system used for chromatography has some effect on the color produced.

Two fronts

When a solution of THAC in chloroform flows down a sheet of paper which is impregnated with KCl in formamide, the chloroform front migrates significantly faster than the THAC front. Because the glucosiduronic acids owe their migration almost exclusively to the THAC in the mobile phase, the THAC front was used in calculating the R_F values[18]. The mobility of the THAC front is usually about 0.94 that of the chloroform front when 0.10 *M* KCl is used in the formamide phase. With system IV, the THAC front migrates 0.98 as far as does the mobile phase. The THAC front also moves more slowly than the toluene front in system III. In the other solvent systems, the THAC front coincides with the front of the mobile phase.

Capacity

Chromatography was run in several of the solvent mixtures in the absence of KCl but with 0.025 *M* THAC in the mobile phase as described for system I. Under these conditions the spots were less compact and the capacity of the system was considerably less than in the presence of 0.10 *M* THAC. Systems II, III and VII (which contain both THAC and KCl) will accommodate at least 40 μg of EGA, SGA, QGA, or THA 3-GA on a 1-cm line without being overloaded.

DISCUSSION

BUSH[15] stated that it is generally more difficult to separate a given pair of steroidal glucosiduronic acids by partition chromatography than it is to separate the respective pair of parent steroids. A chromatography system which gives a satisfactory partition coefficient with a free steroid will need to have the polarity of the mobile phase increased before it will give a useful partition coefficient with the corresponding steroidal glucosiduronic acid; this increase of the polarity of the mobile phase[15,16] decreases the difference in polarities of the mobile and stationary phases, a change which often decreases the selectivity of a chromatography system. The use of liquid ion exchangers in association with conventional chromatography systems offers an additional approach to the chromatographic separation of steroidal glucosiduronic acids. As shown in this paper, when the liquid ion exchanger, THAC, is used with a series of conventional solvent systems, the rates of movement of the glucosiduronic acids relative to one another change from system to system. Changes in the concentration of THAC in the mobile phase and of Cl^- in the stationary phase may be used to obtain appropriate R_F values. Forthcoming papers will show that, if different liquid ion exchangers are used with a particular solvent system, different relative rates of movement of glucosiduronic acids will be obtained. Migration of the conjugates relative to one another is dependent also on which ion exchanger is used when chromatograms are run by a reverse-phase technique. These chromatographic systems which use liquid ion exchangers should be useful also for the separation of various other classes of neutral and acidic compounds which are highly polar.

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REFERENCES

1 V. R. MATTOX, J. E. GOODRICH AND W. D. VRIEZE, *J. Chromatogr.*, 48 (1970) 123.
2 R. KUNIN AND A. G. WINGER, *Angew. Chem. Int. Ed. Engl.*, 1 (1962) 149.
3 E. CERRAI, *Chromatogr. Rev.*, 6 (1964) 129.
4 U. A. TH. BRINKMAN, G. DE VRIES AND E. VAN DALEN, *J. Chromatogr.*, 22 (1966) 407.
5 S. KUSHINSKY AND J. (WU) TANG, *Acta Endocrinol.*, 43 (1963) 361.
6 E. SOCZEWIŃSKI, G. MATYSIK AND H. SZUMILO, *Sep. Sci.*, 2 (1967) 25.
7 A. F. HOFMANN, *J. Lipid Res.*, 8 (1967) 55.
8 V. R. MATTOX, R. D. LITWILLER AND J. E. GOODRICH, *Biochem. J.*, 126 (1972) 533.
9 V. R. MATTOX AND J. E. GOODRICH, *Fed. Proc.*, 27 (1968) 822.
10 V. R. MATTOX, J. E. GOODRICH AND W. D. VRIEZE, *Biochemistry*, 8 (1969) 1188.
11 A. MILUN AND F. MOYER, *Anal. Chem.*, 28 (1956) 1204.
12 A. ZAFFARONI, *Recent. Prog. Horm. Res.*, 8 (1953) 51.
13 W. R. EBERLEIN AND A. M. BONGIOVANNI, *Arch. Biochem. Biophys.*, 59 (1955) 90.
14 I. E. BUSH, *Biochem. J.*, 50 (1952) 370.
15 I. E. BUSH, *Biochem. Soc. Symp.*, 18 (1960) 1.
16 I. E. BUSH, *Methods Biochem. Anal.*, 13 (1965) 357.
17 E. C. BATE-SMITH AND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
18 A. NIEDERWIESER AND M. BRENNER, *Experientia*, 21 (1965) 50.