Recovery of Steroidal Glucosiduronic Acids from Organic Solvents containing Anionic Liquid Ion-Exchangers

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(Received 23 August 1971)

Solutions of anionic liquid ion-exchangers in organic solvents are potentially useful for extracting steroidal glucosiduronic acids from biological fluids and for purifying mixtures of these acids by chromatography. If a glucosiduronic acid is to be isolated in pure form after either of these procedures, it is necessary to separate it from the ion-exchanger. Separation from an organic solution of tetraheptylammonium chloride may be accomplished by extraction with water under the following conditions, which promote transfer of a glucosiduronate to the aqueous phase: (1) an appropriate solvent (diluent) as the organic phase, (2) the presence in the two-phase mixture of an anion such as myristate or dodecyl sulphate to combine with the tetraheptylammonium ion, and (3) an increase of the pH of the aqueous phase in association with the presence of myristate or dodecyl sulphate. The foregoing factors apply also to removal of glucosiduronates from organic solutions of ion exchangers that are hydrochlorides of tertiary, secondary, or primary amines. Since these amines exert progressively less solubilizing effect for glucosiduronates as the pH of the aqueous phase is increased, the conjugates can be released from the organic phase by adjusting the pH to 10 and omitting the myristate or dodecyl sulphate.

In the preceding paper (Mattox *et al.*, 1972) we reported that certain steroidal glucosiduronic acids can be extracted almost quantitatively from aqueous solutions into organic solutions of liquid ion-exchanger. In other studies, liquid ion-exchangers have been used for chromatography of glucosiduronic acids on micro (Mattox & Goodrich, 1968; Mattox *et al.*, 1970) and preparative scales (V. R. Mattox & J. E. Goodrich, unpublished work). A simple widely applicable batch procedure for removing glucosiduronic acids from an organic solution of exchanger is needed to make it possible to utilize liquid ion-exchangers for the recovery of steroidal glucosiduronic acids from urine and after chromatography. In the present paper we describe the displacement of the glucosiduronate anion from exchangers by the anion of a long-chain fatty acid, myristic acid (tetradecanoic acid).

The stoichiometric relationship for removal of a glucosiduronate ion from a solution of ion-exchanger may be written as follows:

in which $R_4N \cdot X$ represents the liquid ion-exchanger, $R_4N \cdot R'CO_2$ is the complex between the exchanger and the glucosiduronate anion, $R'CO_2^-$ is the glucosiduronate anion, and X^- is an anion (Cl^- , $CH_3CO_2^-$ etc.). The subscripts org. and aq. indicate that the constituent is soluble in the organic phase or aqueous phase respectively. If certain assumptions and simplifications are made (Pierce & Hobbs, 1963), the

following approximate relationships can be derived (Mattox *et al.*, 1972) from eqn. (1):

$$\log D = \log [R_4N \cdot X]_{org} - \log [X^-]_{aq} + \text{constant} \quad (2)$$

in which:

$$D = [R_4N \cdot R'CO_2]_{org} / [R'CO_2^-]_{aq} \quad (3)$$

If an organic acid that is soluble in the organic phase but insoluble in the aqueous phase is used to displace $R'CO_2^-$ from the ion-exchanger, eqn. (1) becomes:

From eqn. (4) the following expression can be derived:

$$\log D = \log [R_4N \cdot X]_{org} - \log [HX]_{org} - pH + \text{constant} \quad (5)$$

According to eqn. (5) the removal of the glucosiduronate anion from the organic phase, expressed as $\log D$, should increase linearly as $\log [HX]$ in the organic phase increases and as the pH value in the aqueous phase increases. Therefore the use of a base in the aqueous phase should promote removal of glucosiduronate.

Salts of amines can act as ion-exchangers over a much narrower pH range than can a quaternary ammonium salt. In the pH range where tertiary, secondary and primary amines exist almost entirely

as salts, their behaviour as ion-exchangers is analogous to that of a quaternary ammonium salt and it may be expressed by eqns. (2) and (5). However, as the pH of the aqueous phase is increased, progressively more of the amine salt is converted into free amine, and anion is liberated. This relationship may be expressed by:

$$\log D = -pH + \text{constant} \quad (7)$$

Thus an increase in the pH of the aqueous phase should promote removal of glucosiduronates from an organic solution of a tertiary, secondary, or primary amine hydrochloride. Golumbic *et al.* (1949) have developed an equation equivalent to eqn. (7) for describing the removal of phenols from organic solvents by extraction with aqueous alkali.

In a general way, the magnitude of the coefficients for partition of a steroidal glucosiduronate between an aqueous solution and a solution of tetraheptylammonium chloride in chloroform should parallel the oxygen content of the steroidal moiety; the less oxygenated the steroidal moiety, the larger should be *D*. Conditions satisfactory for transfer of a weakly polar steroidal glucosiduronic acid, for example 11-deoxycorticosterone 21-glucosiduronic acid (3,20-dioxopregn-4-en-21-yl β -D-glucopyranosiduronic acid) (Scheme 1), from an organic solution of an ion-exchanger into water should be adequate for removal from the ion-exchanger of any compound that is even more polar, for example cortisone 21-glucosiduronic acid (17-hydroxy-3,11,20-trioxopregn-4-en-21-yl β -D-glucopyranosiduronic acid). In the present paper we describe various conditions that

promote the transfer of 11-deoxycorticosterone 21-glucosiduronic acid and cortisone 21-glucosiduronic acid from organic solutions of ion-exchangers into water.

According to eqn. (5), a high pH should promote transfer of glucosiduronate from an organic into an aqueous phase; however, the ketolic steroids that bear a free hydroxyl group at C-21 are oxidized readily (Velluz *et al.*, 1947; Brouillet & Mattox, 1966) by air to carboxylic acids in strong alkali. In addition, the steroidal 21-glucosiduronates that contain a 17 α -hydroxyl group and a 20-oxo function are unstable (Mattox *et al.*, 1971) in water at a pH of 12 to 13. Consequently, an appropriate combination of organic solvent, anion to combine with R_4N^+ and pH of aqueous solution is needed to achieve recovery of steroidal glucosiduronic acids from ion-exchangers and at the same time prevent formation of artifacts.

Methods

The techniques and reagents have been described by Mattox *et al.* (1972). Myristic acid was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Partitions were done in duplicate in glass-stoppered centrifuge tubes. Except where indicated otherwise, the concentration of the glucosiduronic acid being partitioned was equivalent to approximately 0.25 μ mol/ml of organic phase; the volumes of the phases were equal. Myristic acid was introduced in the organic phase; sodium dodecyl sulphate was in the aqueous phase. When myristic acid was present, formation of emulsions was minimized by bringing the phases into mutual contact by inverting the tubes 100 times, a technique shown to be adequate to establish equilibrium. When sodium dodecyl sulphate was used, more vigorous shaking was required to achieve equilibrium, and the tubes were shaken manually for 2 min. After the phases had been equilibrated the mixtures were centrifuged as previously described; the extinction and pH were determined on the aqueous phases.

Results

According to eqn. (2), an increase in the concentration of anion X^- in the aqueous phase of a mixture containing ion-exchanger and glucosiduronate should promote transfer of glucosiduronate ion from the organic to the aqueous phase. Whereas 0.1–1.0M aqueous solutions of NaCl, acetic acid, or HCl removed about 10–50% of the conjugate from an equal volume of a chloroform solution that contained 0.5mM-cortisone 21-glucosiduronic acid conjugate and 0.1M-tetraheptylammonium chloride, these aqueous solutions gave very poor recovery of 11-deoxycorticosterone 21-glucosiduronic acid. Of

Scheme 1. Structures of the steroid glucosiduronic acid conjugates

Table 1. *Effect of myristic acid on partition of 11-deoxycorticosterone 21-glucosiduronic acid conjugate*

The phases consisted of equal volumes of chloroform-carbon tetrachloride (containing 0.05 M-tetraheptylammonium chloride) and aqueous solutions; the concentration of the conjugate was equivalent to 0.25 $\mu\text{mol/ml}$ of organic phase.

Myristic acid (initial molarity)	Molar ratio, myristic acid: tetraheptylammonium chloride	% of conjugate in aqueous phase	
		0.5M-NaHCO ₃	0.25M-NH ₄ OH
0.15	3.00	94.8	
0.1375	2.75	90.2	
0.125	2.50	86.6	
0.1125	2.25	75.4	
0.1	2.00	51.5	95.2
0.0875	1.75	32.4	90.0
0.075	1.50	16.9	81.6
0.0625	1.25	8.9	57.9

several substances investigated briefly, the long-chain carboxylic acids and aliphatic sulphates (Epton, 1948) appeared most promising. Myristic acid was chosen for detailed study because it has adequate solubility in organic solvents and low solubility in water and does not interfere with the measurement of the Δ^4 -3-oxo conjugates in the aqueous phase by spectral techniques.

Myristic acid

In preliminary experiments it was found that the extent to which a given amount of myristic acid would displace either glucosiduronate from an organic phase into aq. NH₄OH was strongly dependent on the solvent that was used for the organic phase; transfer of the glucosiduronates to the aqueous phase increased progressively as the organic phase was changed from chloroform to chloroform-carbon tetrachloride (1:1, v/v) to carbon tetrachloride.

The effect of various amounts of myristic acid on the partition of 11-deoxycorticosterone 21-glucosiduronic acid between aqueous solutions and 0.05M-tetraheptylammonium chloride in chloroform-carbon tetrachloride (1:1, v/v) is shown in Table 1. When 0.25M-NH₄OH was the aqueous phase (pH 9.8–10.1), it was not feasible to use concentrations of myristic acid higher than about 0.1M (2 equivalents based on ion-exchanger) because formation of turbidity in the aqueous phase precluded satisfactory readings of extinction. When 0.5M-NaHCO₃ was the aqueous phase, the pH was in the range 8.2–8.6; 3 equivalents of myristic acid could be used without the appearance of turbidity.

The results (Table 1) are shown in Fig. 1 as a plot of $\log D$ against $\log(\text{initial concentration of myristic acid in the organic phase})$. Approximately linear relationships are obtained with slopes approaching -6 , indicating a very favourable effect of high concentrations of myristic acid on the displacement of glucosiduronate from the ion-exchanger.

Fig. 1. *Partition of 11-deoxycorticosterone 21-glucosiduronic acid between chloroform-carbon tetrachloride (1:1, v/v) (containing 0.05M-tetraheptylammonium chloride) and water as a function of the initial myristic acid concentration in the organic phase*
The results are from Table 1. O, Aqueous phase, 0.5M-NaHCO₃; Δ, aqueous phase, 0.25M-NH₄OH.

Studies by Mattox *et al.* (1972) have shown that the partition of cortisone 21-glucosiduronic acid between water and a solution of tetraheptylammonium

Table 2. *Effect of pH on partition of chloride and glucosiduronates*

The phases consisted of equal volumes of carbon tetrachloride (containing 0.05M-tetraheptylammonium chloride and 0.0625M-myristic acid) and aqueous solutions; the concentrations of 11-deoxycorticosterone 21-glucosiduronic acid (Q-GA) and cortisone 21-glucosiduronic acid (E-GA) were equivalent to 0.25 μ mol/ml of organic phase. The Cl^- distribution was based on the initial tetraheptylammonium chloride concentration and was determined by Volhard titration.

NH ₄ OH in aqueous phase (M)	% of anion and pH in aqueous phase after equilibration					
	Cl ⁻	pH	E-GA	pH	Q-GA	pH
0.5			96.9	10.2	92.6	10.2
0.25			93.6	9.7	88.4	9.7
0.125			87.7	9.4	75.9	9.4
0.1	99.0	9.4				
0.08	97.5	9.2				
0.075			79.0	9.1	55.9	9.0
0.0625	96.0	8.9				
0.025			56.7*	8.6*	34.0*	8.5*
0.055			66.3	8.5	43.0	8.5
0.05	89.0	8.3	60.3	8.3	33.7	8.3
0.0375	72.5	7.4				
0.035			49.5	7.2	23.8	7.1
0.025	51.3	6.8	49.6	6.4	18.9	6.2
0.0125	25.0	5.6	42.9	4.3	9.9	4.3
0.000	10.0	2.7	48.6	2.7	13.4	2.6

* NaHCO_3 was used for these experiments.

chloride in chloroform is essentially constant over the pH range 3.9 to 10.7. Also, no significant amount of Cl^- passed from the organic into the aqueous phase when 0.05M-tetraheptylammonium chloride in chloroform was equilibrated with 1.0M- NH_4OH at pH 11.1. However, in the presence of myristic acid, the amount of both Cl^- and glucosiduronate transferred to the aqueous phase (Table 2) was strongly dependent on pH. Fig. 2 shows that progressively more of the Cl^- of a tetraheptylammonium chloride solution in carbon tetrachloride is replaced by myristate ion as the pH of the aqueous phase is increased. From a pH of about 5.6 to about 9.4 there was an almost linear dependence of $\log D$ of Cl^- on pH, and at pH 9.4 essentially all of the Cl^- had been transferred from the organic to the aqueous phase.

Fig. 2 also illustrates the effect of pH on the partition of cortisone 21-glucosiduronic acid and of 11-deoxycorticosterone 21-glucosiduronic acid between the organic solution of tetraheptylammonium chloride and the aqueous phase. For both glucosiduronic acids there was a linear dependence of $\log D$ on pH from about 8.2 to about 10.2; a change in pH produced a much greater change in $\log D$ at a pH between 8.2 and 10.2 than at lower pH values. Moreover, under any given condition, cortisone 21-glucosiduronic acid was transferred to the aqueous phase to a greater extent than the less oxygenated compound, 11-deoxycorticosterone 21-glucosiduronic acid. When

NaHCO_3 rather than NH_4OH was used to adjust the pH, the points representing $\log D$ for both the glucosiduronic acids were in good agreement with the results obtained by use of NH_4OH .

The influence of the organic solvent on the extraction of 11-deoxycorticosterone 21-glucosiduronic acid is illustrated in Fig. 3. At a given pH value, the conjugate was removed to a much greater extent from the carbon tetrachloride solution than from the chloroform-carbon tetrachloride mixture. When the ratio of myristic acid to tetraheptylammonium chloride was increased from 1.25 to 3.0, the line representing $\log D$ against pH for 11-deoxycorticosterone 21-glucosiduronic acid was shifted about 2.3 pH units to the left.

The extraction of a steroidal glucosiduronic acid and its methyl ester by Aliquat 336 (methyltricaprylylammonium chloride) has been reported by Mattox *et al.* (1972); a solution of this quaternary ammonium chloride in chloroform removed about the same percentage of steroidal conjugates from aqueous solutions as was extracted by a comparable concentration of tetraheptylammonium chloride. Fig. 3 shows that, over the pH range 8.2–10.0, the removal of 11-deoxycorticosterone 21-glucosiduronic acid from a solution of Aliquat 336 in carbon tetrachloride containing myristic acid occurs to the same extent as removal from a solution of tetraheptylammonium chloride in carbon tetrachloride.

Fig. 2. Partition of anions between carbon tetrachloride (containing 0.05M-tetraheptylammonium chloride and 0.0625M-myristic acid) and water as a function of pH

The results are from Table 2. $\circ$, Cl^- ; Δ , cortisone 21-glucosiduronic acid; $\square$, 11-deoxycorticosterone 21-glucosiduronic acid. $\blacktriangle$, $\blacksquare$, NaHCO_3 was used instead of NH_4OH for adjusting the pH.

Fig. 3. Partition of 11-deoxycorticosterone 21-glucosiduronic acid between equal volumes of water and organic solution of liquid ion exchangers as a function of pH

$\circ$, Organic phase, 0.05M-tetraheptylammonium chloride and 0.0625M-myristic acid in chloroform-carbon tetrachloride, 1:1 (v/v); the aqueous phase is that given in Table 2. Δ , Organic phase, 0.05M-tetraheptylammonium chloride and 0.0625M-myristic acid in carbon tetrachloride; the aqueous phase is that given in Table 2. $\blacktriangle$, Organic phase, 0.05M-Aliquat 336 and 0.0625M-myristic acid in carbon tetrachloride; the aqueous phase is that given in Table 2. $\square$, Organic phase, 0.05M-tetraheptylammonium chloride and 0.15M-myristic acid in carbon tetrachloride; the aqueous phase is NH_4OH solutions, the highest pH being achieved with 0.055M- NH_4OH .

From Table 3 it is apparent that the concentration of 11-deoxycorticosterone 21-glucosiduronic acid initially present has a negligible effect on the percentage of it extracted by the aqueous phase but that the nature of the organic solvent has a considerable effect. The most favourable condition found for removing the conjugate from an organic solvent containing tetraheptylammonium chloride by the use of myristic acid utilized: (1) 3.0 equivalents of myristic acid, (2) a pH of about 8.2 achieved by using aq. 0.5M- NaHCO_3 , and (3) carbon tetrachloride as solvent. Under these conditions, no emulsion or precipitate formed and more than 99% of the conjugate passed into the aqueous phase in a single extraction. The same final pH may be achieved by the use of NH_4OH but separation of phases is much quicker and cleaner when NaHCO_3 is used.

Sodium dodecyl sulphate

Table 4 shows that, for the removal of 11-deoxycorticosterone 21-glucosiduronic acid from a chloro-

form solution of tetraheptylammonium chloride, 1.10 equivalents of sodium dodecyl sulphate (Jiang & Albert, 1969) in water was somewhat more effective at pH 8.6–10.8 than at pH about 5. Also, when using the same molar quantities of reactants over the pH range 4.5–10.5, sodium dodecyl sulphate

Table 3. *Effect of amount of 11-deoxycorticosterone 21-glucosiduronic acid conjugate on partition*

The phases consisted of 4.00ml of organic solvent (containing 0.05M-tetraheptylammonium chloride and 0.150M-myristic acid) and 4.00ml of aq. 0.5M-NaHCO₃. After equilibration, the pH was 8.1 to 8.2.

Solvent	Amount of conjugate	% of conjugate in aqueous phase	
		140 μ g	1400 μ g
Chloroform	...	77.4	76.8
Chloroform-carbon tetrachloride (1:1, v/v)		94.8	95.2
Carbon tetrachloride		>99	97.4

Table 4. *Effect of pH on partition of 11-deoxycorticosterone 21-glucosiduronic acid conjugate*

The phases consisted of equal volumes of 0.055M-sodium dodecyl sulphate in water and 0.05M-tetraheptylammonium chloride in chloroform; the concentration of the conjugate was equivalent to 0.25 μ mol/ml of organic phase.

Aqueous phase	% of conjugate in aqueous phase	pH in aqueous phase
Water	74.0	4.9
0.25M-NaHCO ₃	98.5	8.6
0.001M-NH ₄ OH	97.8	8.8
0.1M-NH ₄ OH	96.7	10.8

was much more effective than myristic acid in promoting removal of the conjugate from organic solutions of tetraheptylammonium chloride. However, it caused formation of troublesome emulsions in chloroform and in carbon tetrachloride; in addition, it is readily soluble in water and if any excess is used, it remains in the aqueous phase. We have not investigated the removal of sodium dodecyl sulphate from aqueous solutions of steroidal glucosiduronates.

Recovery from amine salts

Kushinsky & Tang (1963) observed that oestrogen conjugates could be removed from an organic solution of amine salt by extraction with alkali. Previous studies (Kunin & Winger, 1962) had shown that anions could be 'stripped' from amine salts by contact with basic solutions. These findings implied that, if the pH of a solution was high enough to convert essentially all of the amine hydrochloride into free amine and all of the carboxyl group of the glucosiduronic acid into the salt (eqn. 6) the conjugate would pass into the aqueous phase. In Table 5, values for the partition of 11-deoxycorticosterone 21-glucosiduronic acid between water and a 0.05M solution of a tertiary, a secondary, or a primary amine hydrochloride in chloroform are shown as a function of pH. These results, if converted into log *D* values, show a linear dependence of log *D* on pH. For each ion-exchanger the slope of the line of a graph of log *D*

versus pH approaches 1.0, a value predicted by eqn. (7).

The use of myristic acid to promote removal of 11-deoxycorticosterone 21-glucosiduronic acid from a solution of trioctylamine hydrochloride in chloroform-carbon tetrachloride (1:1, v/v) as a function of pH was explored. Essentially all of the conjugate was transferred to the aqueous phase at pH 8.5 when 1.25 equivalents of myristic acid (based on amine hydrochloride) were used (Table 6). If partition coefficients are derived from these results and the values of log *D* are plotted against pH, an almost linear relationship is observed over a pH range from 3.4-8.5, the slope being approx. 1.0. Almost identical results were obtained when Amberlite LA-2·HCl and Amberlite XLA-3·HCl were used as ion-exchangers.

Emulsions

When a solution of ion-exchanger in chloroform, chloroform-carbon tetrachloride (1:1, v/v) or carbon tetrachloride is equilibrated with aq. NH₄OH in the presence of myristic acid or sodium dodecyl sulphate, an emulsion may form. When myristic acid is present, both phases eventually become clear if left; sometimes a slight precipitate remains at the interface. However, when sodium dodecyl sulphate is present in greater molar quantity than tetraheptylammonium chloride, an emulsion tends to persist in the organic phase; a clear aqueous phase is obtained.

Table 5. *Effect of pH on partition of 11-deoxycorticosterone 21-glucosiduronic acid conjugate with various ion-exchangers*

The phases consisted of equal volumes of chloroform (containing 0.05M-liquid ion-exchanger) and water; the concentration of the conjugate was equivalent to 0.25 μ mol/ml of organic phase. Abbreviations: TOA·HCl, trioctylamine hydrochloride; ALA-2·HCl, Amberlite LA-2 hydrochloride; and XLA-3·HCl, Amberlite XLA-3 hydrochloride.

NH ₄ OH in aqueous phase (M)	% of conjugate and pH in aqueous phase					
	TOA·HCl	pH	ALA-2·HCl	pH	XLA-3·HCl	pH
0.000	<1.0	3.1	<1.0	3.2	<1.0	3.3
0.025	<1.0	5.4	<1.0	6.5	1.9	6.6
0.05	6.4	6.7	7.0	7.0	13.2	6.8
0.055	55.5	8.1	61.6	8.1	86.6	8.2
0.065	74.1	8.7	84.5	8.8	93.9	8.8
0.1	87.6	9.2	93.9	9.4	96.6	9.3
0.5	94.2	10.1	97.7	10.4	97.7	10.2

Table 6. *Effect of pH on partition of 11-deoxycorticosterone 21-glucosiduronic acid conjugate*

The phases consisted of equal volumes of chloroform-carbon tetrachloride (1:1, v/v) (containing 0.05M-trioctylamine hydrochloride and 0.0625M-myristic acid) and water; the concentration of the conjugate was equivalent to 0.25 μ mol/ml of organic phase.

NH ₄ OH in aqueous phase (M)	% of conjugate in aqueous phase	pH in aqueous phase
0.000	3.4	2.5
0.025	1.7	3.4
0.035	3.1	4.1
0.0425	7.1	4.6
0.05	56.6	6.2
0.0625	>99	8.5

In general, in the presence of myristate, formation of intractable emulsions can be minimized by mixing the two phases by gentle inversion of the tubes rather than by vigorous shaking. When emulsions or suspensions were formed, clear aqueous phases could be obtained in most cases by centrifuging the mixture for 10 min at 1000g.

In particular, no difficulty was encountered in achieving a quick, clean separation of the phases when using 0.0625M-myristic acid until the pH was greater than 10.2 ($[\text{NH}_4\text{OH}] > 0.5\text{M}$). When using 0.15M-myristic acid and NH_4OH some difficulty was encountered when the pH was greater than 7.1 ($[\text{NH}_4\text{OH}] > 0.05\text{M}$); when using 0.15M-myristic acid and 0.5M- NaHCO_3 (pH=8.2) no emulsion was formed. At pH 10.0 (0.25M- NH_4OH) there was no difficulty in achieving clean separation of the phases

until the myristic acid concentration was greater than 0.1M.

Recovery of glucosiduronic acids

When myristic acid in chloroform or carbon tetrachloride was used to promote transfer of a steroidal glucosiduronate from a chloroform or carbon tetrachloride solution of ion-exchanger into aq. NaHCO_3 (pH < 8.5), only a trace of myristic acid passed into the aqueous phase. As has been shown for palmitic acid (Murray, 1923), this is because myristic acid is many times more soluble in the organic phase than in water. This property increases the apparent pK value of myristic acid in this two-phase mixture. We observed that, when a 0.0625M-myristic acid in carbon tetrachloride was equilibrated with an equal volume of water that contained enough NaOH to neutralize one tenth of the acid, the pH of the aqueous solution was 10.0; when the acid was half-neutralized the pH was 10.1.

The myristate that passes into the aqueous phase during recovery of a steroidal glucosiduronate can be removed by extraction with carbon tetrachloride after the solution has been acidified. Finally, the glucosiduronic acids can be separated (Mattox *et al.*, 1969) from the accompanying inorganic acids and salts by using a column of Amberlite XAD-2, a resin that retains glucosiduronic acids but not the inorganic components. The glucosiduronic acids can be eluted from the column with ethanol and recovered after removal of the solvent.

Discussion

The results in Table 3 show that, in the presence of 3.0 equivalents of myristic acid, 11-deoxycorticosterone 21-glucosiduronic acid can be extracted

almost completely from a 0.05M solution of tetraheptylammonium chloride in chloroform-carbon tetrachloride (1:1, v/v) or in carbon tetrachloride by use of an equal volume of aq. NaHCO_3 . The ion-exchanger Aliquat 336, a technical grade of tri-caprylmethylammonium chloride, is very similar to tetraheptylammonium chloride in its ability to promote extraction (Mattox *et al.*, 1972) of glucosiduronic acids. We have separated a mixture of 11-deoxycorticosterone 21-glucosiduronic acid, 11-deoxycortisol 21-glucosiduronic acid and cortisone 21-glucosiduronic acid on a column by using Aliquat 336 in chloroform as the mobile phase and KCl in formamide as the stationary phase (V. R. Mattox & J. E. Goodrich, unpublished work). The individual glucosiduronic acids were recovered from the chloroform phase by adding an equal volume of carbon tetrachloride that contained 3 equivalents of myristic acid and then extracting the solution with 0.40M- NaHCO_3 . Analogous conditions, with myristate and NaHCO_3 , have been used for recovering the C-3 glucosiduronic acids of cortol, β -cortol, cortolone, and β -cortolone (Fukushima *et al.*, 1955) from organic solutions of Aliquat 336. These steroids occur in urine as conjugates with glucuronic acid.

We are indebted to Dr. Alan F. Hofmann for making several useful suggestions during the course of this work. This investigation was supported in part by Research

Grant AM-5452 from the National Institutes of Health, U.S. Public Health Service.

References

- Brouillet, J. C. & Mattox, V. R. (1966) *J. Clin. Endocrinol.* **26**, 453
- Epton, S. R. (1948) *Trans. Faraday Soc.* **44**, 226
- Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B. & Gallagher, T. F. (1955) *J. Biol. Chem.* **212**, 449
- Golumbic, C., Orchin, M. & Weller, S. (1949) *J. Amer. Chem. Soc.* **71**, 2624
- Jiang, N. S. & Albert, A. (1969) *Mayo. Clin. Proc.* **44**, 121
- Kunin, R. & Winger, A. G. (1962) *Angew. Chem. Int. Ed. Engl.* **1**, 149
- Kushinsky, S. & (Wu) Tang, J. (1963) *Acta Endocrinol. (Copenhagen)* **43**, 345
- Mattox, V. R. & Goodrich, J. E. (1968) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **27**, 822
- Mattox, V. R., Goodrich, J. E. & Vrieze, W. D. (1969) *Biochemistry* **8**, 1188
- Mattox, V. R., Goodrich, J. E. & Vrieze, W. D. (1970) *J. Chromatogr.* **48**, 123
- Mattox, V. R., Goodrich, J. E. & Vrieze, W. D. (1971) *Steroids* **18**, 147
- Mattox, V. R., Litwiller, R. D. & Goodrich, J. E. (1972) *Biochem. J.* **126**, 533
- Murray, C. D. (1923) *J. Biol. Chem.* **56**, 569
- Pierce, T. B. & Hobbs, R. S. (1963) *J. Chromatogr.* **12**, 74
- Velluz, L., Petit, A., Pesez, M. & Berret, R. (1947) *Bull. Soc. Chim. Fr. 5 Ser.* **14**, 123