Extraction of Steroidal Glucosiduronic Acids from Aqueous Solutions by Anionic Liquid Ion-Exchangers

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A pilot study on the extraction of three steroidal glucosiduronic acids from water into organic solutions of liquid ion-exchangers is reported. A single extraction of a 0.5 mM aqueous solution of either 11-deoxycorticosterone 21-glucosiduronic acid or cortisone 21-glucosiduronic acid with 0.1 M-tetraheptylammonium chloride in chloroform took more than 99% of the conjugate into the organic phase; under the same conditions, the very polar conjugate, β -cortol 3-glucosiduronic acid, was extracted to the extent of 43%. The presence of a small amount of chloride, acetate, or sulphate ion in the aqueous phase inhibited extraction, but making the aqueous phase 4.0 M with ammonium sulphate promoted extraction strongly. An increase in the concentration of ion-exchanger in the organic phase also promoted extraction. The amount of cortisone 21-glucosiduronic acid extracted by tetraheptylammonium chloride over the pH range of 3.9 to 10.7 was essentially constant. Chloroform solutions of a tertiary, a secondary, or a primary amine hydrochloride also will extract cortisone 21-glucosiduronic acid from water. The various liquid ion exchangers will extract steroidal glucosiduronic acid methyl esters from water into chloroform, although less completely than the corresponding free acids. The extraction of the glucosiduronic acids from water by tetraheptylammonium chloride occurs by an ion-exchange process; extraction of the esters does not involve ion exchange.

Smith & Page (1948) demonstrated that long-chain aliphatic amines can participate in ion-exchange reactions in a manner analogous to that which occurs with solid ion-exchange resins. Since then, studies of ion exchange between two liquid phases have been reported by numerous investigators (reviewed by Coleman *et al.*, 1962; Kunin & Winger, 1962; Marcus, 1963). Compounds, such as the long-chain aliphatic amines, which can participate in ion exchange between two liquid phases are frequently called liquid ion-exchangers. This class of compounds has been used extensively (Cerrai & Ghersini, 1970) over the past decade for the extraction of various inorganic ions from aqueous media and for the chromatography of inorganic substances by several techniques. They have received little use in the extraction and chromatography of organic electrolytes.

Our attention was drawn to liquid ion-exchangers by Hofmann (1967), who showed that tetraheptylammonium chloride in various organic solvents would extract bile acid conjugates from aqueous media and who suggested that it would be a useful reagent for extracting steroidal glucosiduronates from urine. Kushinsky & Tang (1963a) had previously observed that a chloroform solution of the liquid ion-exchanger Amberlite LA-2 would remove conjugates of oestrogens from urine; in addition, they found that if the solution was made alkaline with ammonium hydroxide the conjugates would

pass from the chloroform into the aqueous phase. Further, they achieved partial success in attempting to chromatograph mixtures of conjugates in the presence of the ion-exchanger (Kushinsky & Tang, 1963b). Jiang & Albert (1969) reported the use of Aliquat 336 (methyltricaprylammonium chloride) to extract oestrogen conjugates from urine into an organic phase and the use of sodium dodecyl sulphate at pH 8.5 to remove the conjugates from the organic solution.

The present paper describes a pilot study with a limited number of steroidal glucosiduronic acids and esters and ion-exchangers to define the influence of selected variables, such as structure and concentration of exchanger, concentration of inorganic anion, pH and nature of organic solvent, on the extraction of this small group of conjugates from water into an organic solution. The findings should indicate whether it may be feasible to use liquid ion-exchangers for the extraction of a much larger variety of glucosiduronic acids from human urine. The findings should also be useful in applying liquid ion exchange to the chromatographic separation of steroidal glucosiduronic acids on a micro or preparative scale.

Simple mathematical equations have been derived to describe approximately the partition phenomena that occur with ionizable substances on paper impregnated with ion-exchange resins (Lederer & Kertes, 1956), on columns containing complexing agents

(Pierce & Peck, 1961), on paper strips impregnated with liquid ion-exchangers (Cerrai & Testa, 1962), and in separation funnels containing two phases and an ionic extractant (Stary, 1964; Higuchi *et al.*, 1967). The equations are based on the law of mass action in association with the Nernst distribution law, and all of them can be simplified to the same general form. The reaction of a liquid ion-exchanger with an organic acid may be represented (with symbols applicable to the work described in this paper) by the equation:

in which R_4NX is a quaternary ammonium compound, $R'CO_2^-$ is the anion of an organic acid, $R_4N \cdot R'CO_2$ is a complex between the quaternary ammonium cation and the organic anion, and X^- is an anion such as Cl^- , NO_3^- , or $CH_3CO_2^-$. The subscripts *org.* and *aq.* indicate the organic and the aqueous phase respectively. The equilibrium constant may be defined as:

$$K_{eq.} = \frac{[R_4N \cdot R'CO_2]_{org.} [X^-]_{aq.}}{[R_4NX]_{org.} [R'CO_2^-]_{aq.}} \quad (2)$$

The distribution ratio of $R'CO_2^-$ between the two phases may be designated as:

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

Substituting D in equation (2) and writing the expression in logarithmic form yields:

$$\log D = \log [R_4NX]_{org.} - \log [X^-]_{aq.} + \text{constant} \quad (4)$$

From eqn. (4) it is apparent that there should be a linear relationship between $\log D$ and $\log [R_4NX]_{org.}$ if $[X^-]_{aq.}$ is kept constant. If $[R_4NX]_{org.}$ is kept constant, there should be a direct dependence of $\log D$ on $-\log [X^-]$. When salts of tertiary, secondary, or primary amines are used as ion-exchangers, R_3NHX , R_2NH_2X and RNH_3X are considered to be analogous to R_4NX in the foregoing equations. We have attempted to interpret the partition of steroidal glucosiduronic acids between aqueous solutions and organic solutions of liquid ion-exchangers by means of these relationships.

Materials and Methods

Spectra were read with a Beckman DU spectrophotometer. Fluorimetric determinations were made with a Farrand photoelectric fluorimeter (model A-3) by using a Wratten 47B primary and a Corning 2A-12 secondary filter. Readings of pH values were made with a glass electrode and a Beckman Expando-matic pH-meter. Partitions were done in glass-stoppered centrifuge tubes.

The steroidal glucosiduronic acids and the methyl

ester were synthesized in our laboratory (Mattox *et al.*, 1969).

$(NH_4)_2SO_4$ and NaCl were of reagent grade. Sodium dodecyl sulphate, 99% pure, was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Organic solvents were redistilled before use. The following liquid ion-exchangers were used without further purification unless otherwise indicated: tetraheptylammonium chloride (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.); methyltricaprylammonium chloride (Aliquat 336) (General Mills, Minneapolis, Minn., U.S.A.; trioctylamine (K & K Laboratories, Plainview, N.Y., U.S.A.); Amberlite LA-2, a branched-chain aliphatic secondary amine with an average mol.wt. of 374 (Rohm and Haas, Philadelphia, Pa., U.S.A.); Amberlite XLA-3, a branched-chain aliphatic primary amine with an average mol.wt. of 353 (Rohm and Haas).

The exchangers have negligible solubility in water. The hydrochlorides of trioctylamine and the two Amberlites were made by washing a chloroform solution of the free amine twice with an excess of 0.2M-HCl. The organic phase was concentrated *in vacuo* to about one-third its volume and the residue was diluted to the appropriate volume with redistilled chloroform. The Cl^- concentration of the amine hydrochlorides, as well as that of the quaternary ammonium chlorides, was determined by the Volhard procedure. Also, the concentration of the amine salts was determined by titrating the amine moiety with sodium dodecyl sulphate as described by Milun & Moyer (1956). Values found for the Cl^- concentration were 93 to 99% of those obtained for the amine concentration.

Unless indicated otherwise, the compounds under investigation were partitioned in duplicate between equal volumes of aqueous and organic phases. The substance to be partitioned was introduced into the partition system either in aqueous solution or as a dry residue (a methanol solution of the substance was concentrated to dryness *in vacuo* and the components of the partition system were added to it). Except in instances indicated otherwise, the concentration of the compound being partitioned was equivalent to approximately $0.50 \mu\text{mol/ml}$ of aqueous phase. Any salt or acid used either to promote extraction or to provide a competing anion was introduced in the aqueous phase. The phases were shaken together manually in glass-stoppered test tubes for 2 min at $27 \pm 1^\circ\text{C}$, a technique that was found to be adequate for the establishment of equilibrium between phases. The mixture was centrifuged at 1000 g for 2 min to ensure complete separation of the phases, and measurements were performed on the clear solutions.

Concentrations of the conjugates that contained the Δ^4 -3-oxo structure were determined on the basis of ϵ 16000 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$. The aqueous phases and standards were read against appropriate blanks at the

wavelength giving maximum extinction: 244-nm for cortisone 21-glucosiduronic acid (17-hydroxy-3,11,20-trioxopregn-4-en-21-yl β -D-glucopyranosiduronic acid) and methyl (cortisone 21-glucosid)uronate [methyl (17-hydroxy-3,11,20-trioxopregn-4-en-21-yl β -D-glucopyranosid)uronate]; 248 nm for 11-deoxycorticosterone 21-glucosiduronic acid (3,20-dioxopregn-4-en-21-yl β -D-glucopyranosiduronic acid). For 3 α -hydroxy-12-oxochol-9-enic acid (McKenzie *et al.*, 1948) ϵ 11 300 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (at 241 nm) was used. When necessary, solutions were diluted with appropriate solvents before the extinction was read. The amount of conjugate in the organic phase was determined by difference.

β -Cortol 3-glucosiduronic acid (11 β ,17,20 β ,21-tetrahydroxy-5 β -pregnan-3 α -yl β -D-glucopyranosiduronic acid) was measured by a fluorimetric procedure. In establishing a standard curve, a linear relationship was observed between the weight of β -cortol 3-glucosiduronic acid used and the amount of fluorescence produced when the conjugate was treated with H₂SO₄-ethanol (4:1, v/v) (Sweet, 1954) at 27°C. In applying the procedure, the phase in which the initial concentration of all the reactants was held constant was analysed; the concentration in the phase not analysed was determined by difference. Samples (20 μ l, Hamilton syringe) of the appropriate phase, along with suitable standards and blanks, were taken to dryness under a stream of air; 2 ml of fluorescing reagent was added to the residue and the fluorescence was measured 20 min later.

Results and Discussion

Scheme 1 shows the structures of the three steroidal glucosiduronic acids (Mattox *et al.*, 1969) and the

steroidal glucosiduronic ester used in this study. β -Cortol 3-glucosiduronic acid is very polar, with one carboxyl and seven hydroxyl groups; cortisone 21-glucosiduronic acid is of intermediate polarity with one carboxyl, four hydroxyl and three carbonyl groups; 11-deoxycorticosterone 21-glucosiduronic acid is weakly polar with one carboxyl, three hydroxyl and two carbonyl groups. If the partition of these compounds between an ion-exchanger in a non-polar solvent and water parallels that ordinarily observed for oxygenated organic substances, β -cortol 3-glucosiduronic acid should be the most difficult of the three substances to extract from aqueous media into an organic solution of liquid ion-exchanger, and 11-deoxycorticosterone 21-glucosiduronic acid should be the easiest.

Concentration of exchanger

Extraction of approx. 0.5 mm aq. cortisone 21-glucosiduronic acid and 11-deoxycorticosterone 21-glucosiduronic acid with equal volumes of 0.1 M-tetraheptylammonium chloride in chloroform removed more than 99% of these conjugates from the aqueous phase; only 43% of the β -cortol 3-glucosiduronic acid was extracted under these conditions. However, 74% of the β -cortol 3-glucosiduronic acid was extracted when 1,2-dichloroethane was used as solvent. When each glucosiduronic acid was extracted with progressively smaller concentrations of tetraheptylammonium chloride in the organic phase, the percentage of glucosiduronic acid that was removed decreased (Table 1); 11-deoxycorticosterone 21-glucosiduronic acid was extracted much more readily than cortisone 21-glucosiduronic acid.

There was a direct correlation between log *D* and

Scheme 1. Structures of the steroid glucosiduronic acid conjugates

Table 1. *Effect of concentration of tetraheptylammonium chloride on partition of glucosiduronic acids*

Glucosiduronic acids were partitioned between equal volumes of water and organic solutions of tetraheptylammonium chloride. The concentration of glucosiduronic acid was equivalent to $0.5 \mu\text{mol/ml}$ of aqueous phase.

Tetraheptylammonium chloride (initial molarity)	Glucosiduronic acid, in organic phase (%)		
	11-Deoxycorticosterone 21-glucosiduronic acid in chloroform	Cortisone 21-glucosiduronic acid in chloroform	β -Cortol 3-glucosiduronic acid in 1,2-dichloroethane
0.2			89.5
0.1	approx. 100	approx. 100	73.8
0.05	approx. 100	99.6	56.2
0.02	approx. 100	99.0	32.7
0.01	approx. 100	97.6	22.2
0.005	approx. 100	95.1	
0.002	99.7	87.8	
0.001	98.8	76.4	
0.0005	94.3	61.8	
0.0000	13.4	1.8

Fig. 1. *Partition of conjugates between equal volumes of organic solvent and water as function of tetraheptylammonium chloride concentration*

Results are taken from Table 1. The equilibrium concentration of tetraheptylammonium chloride, $[\text{tetraheptylammonium chloride}]_{\text{eq.}}$, is calculated by subtracting the molar amount of the conjugate in the organic phase from initial molar amount of tetraheptylammonium chloride in the organic phase. $\circ$, 11-Deoxycorticosterone 21-glucosiduronic acid in chloroform; Δ , cortisone 21-glucosiduronic acid in chloroform; $\square$, β -cortol 3-glucosiduronic acid in 1,2-dichloroethane.

$\log [R_4NX]$ in the organic phase (Fig. 1). The slope of each line is approx. 1.0, a value which, from eqn. (4), indicates that one molecule of tetraheptylammonium ion binds one molecule of glucosiduronate ion in the process of transferring a conjugated steroid from the aqueous phase to the organic phase.

Concentration of counter-ion

According to eqn. (4), an increase of the concentration of anion X^- in the aqueous phase should decrease the partition coefficient D . When tetraheptylammonium chloride is being used as the ion-exchanger, X^- is Cl^- ; however, anions such as Br^- , NO_3^- , and CH_3CO_2^- could participate in the reaction in the same general manner as Cl^- . The results in Fig. 2 show that addition of NaCl to the aqueous solutions decreases the extraction of cortisone 21-glucosiduronic acid by 0.1 and 0.025M solutions of tetraheptylammonium chloride in chloroform. Over the range of concentration investigated (0.0625–0.5M) there was a linear dependence of $\log D$ on $-\log [\text{Cl}^-]$. The slope of the lines is about -0.75 , a value which approaches the theoretical value of 1.0, which would be required by eqns. (1) and (4), if activities were used instead of concentrations and if all factors other than Cl^- concentration were held constant.

In Fig. 3 the effect of various concentrations of tetraheptylammonium chloride on the extraction of cortisone 21-glucosiduronic acid from 0.0625 and 0.25M- NaCl is shown. With NaCl present in the aqueous phase there was a linear dependence of $\log D$ on $\log [R_4NX]$, with a slope of about 1.0, just as when no NaCl was present.

Fig. 2. Partition of cortisone 21-glucosiduronic acid between equal volumes of water and tetraheptylammonium chloride in chloroform as a function of NaCl concentration.

For details see the text. The concentrations of tetraheptylammonium chloride were 0.1M (○) and 0.025M (Δ).

These results show that increases in the concentration of tetraheptylammonium chloride in the organic phase promote extraction of cortisone 21-glucosiduronic acid whereas increases in the salt concentration in the aqueous phase decrease the extraction. Thus the NaCl in human urine, present in concentrations that may range up to 0.25M, should have a detrimental influence on the extraction of glucosiduronic acids by anionic liquid ion exchangers.

Effect of pH

In the ion-exchange systems studied in the present work it is not feasible to determine partition coefficients as a function of pH by using conventional buffer systems (such as acetic acid-sodium acetate). As can be shown by the Henderson-Hasselbalch equation, the anion concentration of a buffer changes as the pH changes, and the partition coefficient is influenced strongly by changes in anion concentration. Consequently, partition experiments were performed with the first two aqueous phases shown in

Fig. 3. Partition of cortisone 21-glucosiduronic acid between equal volumes of chloroform and water as a function of tetraheptylammonium chloride concentration, with and without a supplementary counter-ion added to the aqueous phase.

For details see the text and Fig. 1. The concentrations of NaCl were 0M (○), 0.0625M (Δ) and 0.25M (□).

Table 2. When the concentration of Cl^- in the aqueous phase was kept constant, the percentage of cortisone 21-glucosiduronic acid extracted decreased markedly when the pH was decreased from 3.6 to 1.3. This decrease could be due to the effect of H^+ in depressing the ionization of the conjugate (converting anion $\text{R}'\text{CO}_2^-$ into $\text{R}'\text{CO}_2\text{H}$); such a condition should make the reaction in eqn. (1) proceed to the left by removing the glucosiduronate anion. This hypothesis seems plausible because a pH of 1.3 is about 2 units lower than the pK range found ($\text{pK} = 3.0\text{--}4.0$) for a group of glucosiduronic acids (Williams, 1959).

The remainder of Table 2 shows the partition of cortisone 21-glucosiduronic acid between an organic solution of tetraheptylammonium chloride and water in the presence of increasing concentrations of alkali. The percentage of cortisone 21-glucosiduronic acid extracted into chloroform did not change significantly over the pH range 3.9–10.7. At a pH of 12.4, with 0.1M-NaOH as the aqueous phase, a smaller amount of cortisone 21-glucosiduronic acid was in the

Table 2. *Partition of cortisone 21-glucosiduronic acid conjugate between equal volumes of water and organic solvent containing tetraheptylammonium chloride as function of pH*

The concentration of cortisone 21-glucosiduronic acid was equivalent to 0.5 μ mol/ml of aqueous phase.

	Aqueous phase					
	0.1M-HCl	0.1M-NaCl	Water	0.001M-NH ₄ OH	0.1M-NH ₄ OH	0.1M-NaOH
Chloroform as organic phase						
pH, aqueous phase	1.3	3.6	3.9	8.2	10.7	
Conjugate in organic phase (%)	27.3*	66.6*	68.1†	69.7†	67.8†	
Benzene as organic phase						
pH, aqueous phase			3.6	8.8	10.5	12.3
Conjugate in organic phase (%)			40.9†	44.5†	48.2†	50.5†

* Organic phase initially contained 0.05M-tetraheptylammonium chloride.

† Organic phase initially contained 0.0005M-tetraheptylammonium chloride.

Table 3. *Effect of acetic acid on partition of β -cortol 3-glucosiduronic acid*

The phases consisted of equal volumes of water and 0.1M-tetraheptylammonium chloride in carbon tetrachloride. The concentration of β -cortol 3-glucosiduronic acid was equivalent to 0.5 μ mol/ml of aqueous phase.

Initial concn. of acetic acid in water (M)	β -Cortol 3-glucosiduronic acid in organic phase (%)	pH of aqueous phase
0.0000	69.4	3.4
0.0625	59.0	2.8
0.125	36.8	2.7
0.25	18.5	2.5
0.5	10.5	2.3
1.0	4.3	2.1
2.0	2.2	1.9

organic phase. At this pH, NaOH reacts with chloroform to liberate Cl^- , and presumably the increased concentration of this anion in the aqueous phase was largely responsible for the decreased percentage of cortisone 21-glucosiduronic acid in the organic phase. With benzene as the organic phase, the percentage of cortisone 21-glucosiduronic acid in the organic phase increased slightly as the pH of the aqueous phase was increased.

It was of interest also to determine the effect of acetic acid on the extraction of glucosiduronate from aqueous solutions because this acid is used to prevent bacterial growth in samples of urine that are to be stored before being analysed. The results for the ex-

Table 4. *Effect of ammonium sulphate on the partition of β -cortol 3-glucosiduronic acid*

The phases consisted of equal volumes of water and 0.1M-tetraheptylammonium chloride in carbon tetrachloride. The concentration of β -cortol 3-glucosiduronic acid was equivalent to 0.5 μ mol/ml of aqueous phase.

(NH ₄) ₂ SO ₄ (M)	β -Cortol 3-glucosiduronic acid in organic phase (%)	pH of aqueous phase
0.00	69.4	3.4
0.25	64.7	4.4
0.5	73.4	4.5
1.0	79.6	4.7
2.0	91.0	4.8
4.0	93.0	4.9

traction of β -cortol 3-glucosiduronic acid by a solution of tetraheptylammonium chloride in carbon tetrachloride are shown in Table 3. The graph of $\log D$ against $\log$ (initial concentration of acetic acid in the aqueous phase) was rectilinear with an indicated slope of 1.2 (not shown). When $\log D$ was plotted against the pH of the aqueous phase after equilibration with the organic phase, a linear relationship with a slope of 1.9 was obtained (not shown). The concentration of acetic acid in the solution had a marked effect on the partition of β -cortol 3-glucosiduronic acid between the two phases.

Salting out

Ammonium sulphate is commonly added to aqueous solutions to promote the extraction of con-

Table 5. *Effect of organic solvent on partition of glucosiduronic acids*

The phases consisted of equal volumes of aqueous and organic solutions of tetraheptylammonium chloride. The concentration of the conjugates was equivalent to 0.5 $\mu\text{mol/ml}$. A lower concentration of tetraheptylammonium chloride was used for cortisone 21-glucosiduronic acid than for β -cortol 3-glucosiduronic acid.

Concn. of tetraheptylammonium chloride ... Conjugate ... Solvent	% of conjugate in organic phase	
	0.001 M Cortisone 21-glucosiduronic acid*	0.1 M β -Cortol 3-glucosiduronic acid
Methylene chloride	90.7	69.2*
Ethylene chloride	87.8	73.8*
Chloroform	79.2	43.7*
Ethyl acetate†	75.1	84.5* 81.4‡
Benzene	59.5	93.6* 91.7‡
Carbon tetrachloride	19.0	63.2*

* The concentration of the conjugate was measured in the aqueous phase.

† The ethyl acetate and water were mutually saturated before being used.

‡ The concentration of the conjugate was measured in the organic phase.

jugates into an organic phase. Table 4 shows the effect of various concentrations of this salt on the extraction of the very polar conjugate, β -cortol 3-glucosiduronic acid, into a solution of tetraheptylammonium chloride in carbon tetrachloride. At a concentration of 0.25M this salt inhibited extraction of the conjugate; however, at higher concentrations it promoted extraction greatly. The extraction of cortisone 21-glucosiduronic acid into a chloroform solution of Amberlite XLA-3·HCl also was increased greatly by making the aqueous phase 1–4M with $(\text{NH}_4)_2\text{SO}_4$.

Solvents for ion-exchangers

The solvent used as diluent for the tetraheptylammonium chloride has a large effect on the partition of cortisone 21-glucosiduronic acid or β -cortol 3-glucosiduronic acid between the organic solution of exchanger and water (Table 5). Carbon tetrachloride was much less effective for extracting cortisone 21-glucosiduronic acid than was chloroform, ethylene chloride, or methylene chloride. However, the order of effectiveness of the solvents for extracting these two glucosiduronic acids from water was different.

Volumes of phases

In studying the partition of acetylcholine between solutions of sodium tetraphenylboron in butyl ethyl ketone and NaCl in water, Fonnum (1969) found that the percentage of acetylcholine extracted into the organic phase was determined by the amount, not the concentration, of sodium tetraphenylboron in the organic phase and the amount of NaCl in the aqueous

Table 6. *Effect of phase volume on partition of cortisone 21-glucosiduronic acid conjugate between tetraheptylammonium chloride in chloroform and sodium chloride in water*

The amount of tetraheptylammonium chloride was constant at 0.3mmol and the amount of NaCl was constant at 0.75mmol; 750 μg of cortisone 21-glucosiduronic acid was used.

Aqueous phase (ml)	Organic phase (ml)	Conjugate in organic phase (%)
3.0	24.0	56.7
3.0	12.0	59.4
3.0	6.0	63.3
3.0	3.0	67.5
6.0	3.0	67.4
12.0	3.0	71.1
24.0	3.0	72.2

phase. Changes in the volumes of the organic phase and of the aqueous phase had no significant effect on the extraction. We made analogous observations concerning the partition of cortisone 21-glucosiduronic acid between 0.3mmol of tetraheptylammonium chloride in chloroform and 0.75mmol of NaCl in water. As shown in Table 6, when the volume of the organic phase was altered from 3.0 to 24.0ml, the percentage of cortisone 21-glucosiduronic acid extracted into it ranged from 67.5 to 56.7%. When the volume of the aqueous phase was altered from 3.0 to 24.0ml, the percentage of cortisone 21-glucosiduronic acid extracted increased from 67.5 to 72.2%.

Extraction of esters

As shown in Table 7, tetraheptylammonium chloride also promotes the extraction of the steroidal glucosiduronic ester methyl (cortisone 21-glucosid)uronate from water into chloroform: a 0.1 M solution of it increased the partition coefficient of the ester by about 65-fold. There was a linear dependence of $\log D$ on $\log [R_4NX]$, with a slope of about 1.0. In marked contrast with the findings with cortisone 21-glucosiduronic acid, the presence of a small amount

of NaCl (<0.5 M) in the aqueous phase had a negligible effect on the extraction.

Tertiary, secondary and primary amines

The relative effectiveness of quaternary, tertiary, secondary and primary amines for extracting cortisone 21-glucosiduronic acid and its methyl ester from water was investigated briefly. The tertiary, secondary and primary amines were used both as hydrochlorides and as free amines. Cortisone 21-glucosiduronic acid and its methyl ester were extracted to a greater extent by the quaternary ammonium chlorides than by the tertiary, secondary, or primary amine hydrochlorides (Table 8). We have not determined the effect of increasing the concentration of NaCl in the aqueous phase on the partition of cortisone 21-glucosiduronic acid between water and chloroform solutions of the tertiary, secondary, and primary amine hydrochlorides. However, when using these exchangers in paper chromatography (V. R. Mattox & J. E. Goodrich, unpublished work), addition of small amounts of KCl to the more polar phase decreased the solubility of glucosiduronic acids in the less-polar phase just as it did with tetraheptylammonium chloride.

The free amines promoted extraction of cortisone 21-glucosiduronic acid into chloroform at a pH of about 4 in the aqueous phase; however, with the exception of the primary amine, Amberlite XLA-3, they produced an insignificant effect on the extraction of methyl (cortisone 21-glucosid)uronate.

Table 7. *Effect of concentration of tetraheptylammonium chloride on partition of methyl (cortisone 21-glucosid)uronate conjugate*

The phases consisted of equal volumes of water and tetraheptylammonium chloride in chloroform; the concentration of the conjugate was equivalent to 0.5 μ mol/ml of aqueous phase.

Initial concn. of tetraheptylammonium chloride (M)	Conjugate in organic phase (%)
0.1	94.0
0.05	88.2
0.02	72.0
0.01	55.8
0.005	41.6
0.002	32.6
0.001	25.9
0.0005	22.0
0.0000	18.8

Table 8. *Effect of various amines on partition of cortisone 21-glucosiduronic acid and its methyl ester*

The phases consisted of equal volumes of water and chloroform solution of amine; the concentration of the acid and methyl ester conjugates was equivalent to 0.50 μ mol/ml of aqueous phase. A lower concentration of amine was used for the acid conjugate than for its methyl ester.

Amine	Type of amine	Acid conjugate in 0.001 M-amine in organic phase (%)	pH of aqueous phase	Methyl ester conjugate in 0.1 M-amine in organic phase (%)	pH of aqueous phase
Tetraheptylammonium chloride	Quaternary	78.0	3.7	94.0	3.8
Aliquat 336*	Quaternary	74.4	3.6	94.3	3.6
Trioctylamine hydrochloride	Tertiary	60.5	3.5	46.7	2.8
Amberlite LA-2·HCl	Secondary	60.6	3.4	33.3	2.9
Amberlite XLA-3·HCl	Primary	51.2	3.3	39.3	3.0
Trioctylamine	Tertiary	55.3	4.2	22.2	6.0
Amberlite LA-2	Secondary	44.3	4.3	19.4	6.2
Amberlite XLA-3	Primary	21.4	3.9	37.2	6.1
None		1.8	3.9	18.8	6.3

* Aliquat 336, methyltricaprylammonium chloride.

Table 9. *Partition of cortisone 21-glucosiduronic acid, its methyl ester and a bile acid*

The phases consisted of 5.00 ml of 0.01 M-tetraheptylammonium chloride in chloroform and 5.00 ml of water. The tetraheptylammonium chloride solution was prepared in the following manner so that a low blank for chloride would be obtained when the organic phase was equilibrated with water. A 0.1 M-tetraheptylammonium chloride solution was washed successively with five equal volumes of water. Approximately 0.7% of the Cl^- in the tetraheptylammonium chloride was lost; the first wash removed 0.55%, and the fifth removed 0.03%. After the fifth wash the volume was adjusted with chloroform (previously saturated with water) to give the 0.01 M solution that was used in the partition experiments. Appropriate blanks were used for spectral measurements and for titration of chloride ion (Cotlove Chloridometer) (Cotlove, 1964).

Compound	Amount of compound partitioned (μmol)	Amount of compound transferred to organic phase (μmol)	Amount of Cl^- transferred to aqueous phase (μmol)	Transfer ratio (μmol of Cl^- / μmol of compound)
Cortisone 21-glucosiduronic acid	4.44	4.23	4.69	1.11
	4.53	4.31	4.36	1.01
Methyl (cortisone 21-glucosid)-uronate	4.30	2.74	0.00	0.00
	4.45	2.64	0.00	0.00
3 α -Hydroxy-12-oxochol-9-enic acid	6.30	6.30	1.52	0.241
	6.42	6.42	1.57	0.245

Exchange of ions

The effect of increased concentrations of Cl^- on the partition coefficient of cortisone 21-glucosiduronic acid and the lack of significant effect on the partition coefficient of its methyl ester imply that the solubilizing effect of tetraheptylammonium chloride for the free acid is produced by a mechanism different from that for the ester. The results imply that the extraction of the conjugate, cortisone 21-glucosiduronic acid, occurs by an ion-exchange process. As shown in Table 9, the quantity of Cl^- that passed into the aqueous phase was approximately equal to the amount of glucosiduronate that passed into the organic phase. When methyl (cortisone 21-glucosid)-uronate was extracted, no detectable amount of Cl^- passed into the aqueous phase. A bile acid, 3 α -hydroxy-12-oxochol-9-enic acid (McKenzie *et al.*, 1948), which can be extracted quantitatively from water by chloroform in the absence of tetraheptylammonium chloride, caused transfer of only 24% of the theoretical amount of Cl^- .

Mechanism

The following comments about the mechanism by which tetraheptylammonium chloride and various amine salts extract glucosiduronic acids and esters from water into non-polar organic solvents should be regarded as a tentative hypothesis. Probably,

a steroid conjugate such as cortisone 21-glucosiduronic acid dissolves in water partially as a result of the hydrogen bonds that form between the water molecules and the carboxyl, hydroxyl and carbonyl groups of the conjugate. When a carboxyl group ionizes in the aqueous phase, a negative ion is formed; such an anion, at the interface between the aqueous and organic phases, should be attracted to the potential cationic centre of the liquid ion-exchanger ($\text{R}_4\text{N}^+\text{Cl}^-$, $\text{R}_3\text{NH}^+\text{Cl}^-$, $\text{R}_2\text{NH}_2^+\text{Cl}^-$, or $\text{RNH}_3^+\text{Cl}^-$), releasing an anion (Cl^-) to the aqueous phase. (For the latter three amines the same final result would be achieved if the non-ionized acid reacted with the free amine; however, the process would not be one of ion exchange.) The negative charge of the glucosiduronate anion and the positive charge on the ammonium cation should bring these centres in close proximity as an ion pair. In this association the carbon chain(s) of the ion-exchanger should prevent formation of a lattice of hydrogen bonds between the hydroxyl and carboxyl functions of the glucosiduronate moiety and molecules of water. Also, the carbon chain(s) of the ion-exchanger should inhibit intermolecular hydrogen-bonding between different molecules of glucosiduronate. The aliphatic chains, in close proximity to the hydroxyl groups, should thus partially mask the polar character of the carbohydrate moiety and thereby increase the solubility of the steroidal glucosiduronate in the chloroform solution. Possibly, hydrogen-bonding between the

hydroxyl groups of the conjugate and chloride ion of the exchanger and mutual attraction of carbon chains of the exchanger and the non-polar part of the conjugate, as discussed in the following paragraph, contribute a small additional solubilizing effect.

It is postulated that the methyl ester is made soluble by formation of hydrogen bonds between the hydroxyl groups of the glucosiduronate and the anion of the exchanger (for example, chloride when the exchanger is tetraheptylammonium chloride) while it is associated as an ion pair with the cationic centre of the amine. A similar hypothesis has been proposed (Taylor & Taylor, 1967) to explain the effect of α -lecithin in increasing the solubility of glucose and other carbohydrates in benzene. This concept is consistent with the following observations. (1) In contrast with their hydrochlorides, trioctylamine and Amberlite LA-2 as free amines have a very small effect in promoting transfer of methyl (cortisone 21-glucosid)uronate from an aqueous solution to a chloroform solution of exchanger. (2) It has been shown by means of n.m.r. and i.r. spectra that hydrogen bonds are formed (Relles, 1970) between substituted phenols and benzyltriphenylphosphonium chloride in anhydrous methylene chloride solution; this solution is analogous to a solution of steroidal glucosiduronic ester in a mixture of tetraheptylammonium chloride and methylene chloride. Further, there is excellent evidence for hydrogen-bond formation between tetrabutylammonium bromide and methyl alcohol (Sadek & Fuoss, 1950) in nitrobenzene solution. Probably, Van der Waals forces between the chains of the amines and the conjugate play a minor role in the solubilizing process.

A 0.001 M solution of tetraheptylammonium chloride in chloroform will extract only 6% more of the methyl (cortisone 21-glucosid)uronate from a 0.5 mM aqueous solution than is extracted by chloroform alone; however, the same chloroform solution will extract about 76% more of the cortisone 21-glucosiduronic acid from a 0.5 mM aqueous solution than is extracted by chloroform alone. These results are consistent with the concept that an ionic attraction, such as that between R_4N^+ and $R'CO_2^-$, is much stronger than that of a hydrogen bond, such as that postulated between R_4NCl and $HO-R$. This line of thought leads us to suggest that the strong coulombic force between the cation of the ion-exchanger and the anion of the glucosiduronate holds the ion-exchanger in a manner such that it neutralizes most of the polar character of both the carboxyl and hydroxyl groups of the glucosiduronate moiety. By this mechanism the hydroxyl groups of the glucosiduronic acid may be masked more fully than are the hydroxyl groups of the ester when attraction of the exchanger to the hydroxyl groups is dependent solely on formation of hydrogen bonds.

Conclusions

The foregoing findings imply that, under appropriate conditions, organic solutions of the previously mentioned liquid ion-exchangers should be satisfactory for extracting steroidal glucosiduronic acids of weak and intermediate polarity from urine. No doubt, by choosing the most favourable solvent as a carrier for the exchanger, by adding ammonium sulphate to the aqueous phase, and by extracting more than once with an equal volume of organic phase, the more polar glucosiduronic acids also could be recovered in high yield. The concentration of the ion exchanger in the organic phase could be increased to promote more efficient extraction. No anion that would compete with the glucosiduronate for the cation of the ion-exchanger should be added. Eqn. (4) should provide a rough indication of the effect of changes in concentration of ion-exchanger and of anion on the partition of a glucosiduronic acid between two phases.

In addition to this application, these ion-exchangers offer considerable potential for use in the chromatography of steroidal glucosiduronic acids and of other compounds of similar solubility characteristics. In principle, it should be possible to vary the chromatographic mobility of a compound in a particular solvent system over a considerable range merely by changing the concentration of the ion exchanger in the less-polar phase and the inorganic counter-ion in the more-polar phase.

As shown in Table 5, when tetraheptylammonium chloride is used as the exchanger, chloroform is a better solvent than carbon tetrachloride for extracting cortisone 21-glucosiduronic acid from water; however, carbon tetrachloride is a better solvent for extracting β -cortol 3-glucosiduronic acid from water. These results imply that the relative rates of chromatographic movement of a group of glucosiduronic acids in a system of chloroform-tetraheptylammonium chloride-water should be different from the relative rates of movement of the same compounds in carbon tetrachloride-tetraheptylammonium chloride-water. It is well known (Kunin & Winger, 1962; Soczewinski *et al.*, 1967) that the diluent used as a solvent for ion-exchangers influences both the solvent power and the selectivity of the solution for separating groups of compounds.

The ion-exchangers also have potential use for chromatography of glucosiduronic esters and other neutral compounds of similar solubility characteristics. They might be used for separating neutral compounds from acids. If, in a particular chromatographic system, an ester and an acid migrated at the same rate, it would be necessary only to change the concentration of the counter-ion in the more polar phase in order to obtain a system that would separate the compounds. Small changes in counter-ion con-

centration alter the partition coefficients of acids in a predictable manner but have a negligible effect on the partition coefficients of esters.

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