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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF STEROID GLUCURONIDES

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SUMMARY

Methods are presented for the separation of steroid glucuronides as acids and ion pairs by reversed-phase liquid chromatography using an aqueous mobile phase and LiChrosorb RP-2 and RP-18 as support for a liquid stationary phase.

When the glucuronides are separated as acids, a mixture of 1-pentanol and hydrophobic acid is used as the liquid stationary phase and when separated as ion pairs 1-pentanol is used as the stationary phase with a quaternary ammonium ion in the mobile phase.

INTRODUCTION

Conjugation with glucuronic acid is an important pathway in the metabolism of estrogenic steroids. This metabolic change increases their hydrophilic character and their tendency to be excreted in the urine. Fransson *et al.*[1] used ion-pair partition chromatography to isolate sulphate and glucuronide conjugates. A straight-phase system was used with an organic solvent as the mobile phase, which made the direct injection of a biological fluid impossible. Wahlund[2] isolated nicotinic acid from human plasma by direct injection of the biological fluid using reversed-phase ion-pair partition chromatographic systems.

This paper reports on the separation of four estrogenic steroid glucuronides by reversed-phase liquid chromatography. The study was concentrated on the investigation of the chromatographic behaviour of the substances with the purpose of designing a system suitable for direct analysis of steroid glucuronides in human urine.

EXPERIMENTAL

Apparatus

The detector was an LDC Model 1205 UV monitor, wavelength 254 nm, with an 8-μl cell cooled with water. The pump was an Altex Model 100 solvent delivery system. The columns (150 × 3.2 mm I.D.) were made of 316 stainless steel. A high-pressure injection port was used (Rheodyne, 5000 p.s.i.). A HETO Type 02 PT 923

TC water-bath (HETO, Birkeröd, Denmark), was used to thermostat the solvent reservoir and the column. pH was measured with an Orion Research Model 801 A digital pH meter equipped with an Ingold Type 401 combined electrode.

Chemicals and reagents

1-Pentanol was of Fisher Scientific A.C.S. quality (Pittsburgh, Pa., U.S.A.). Tetrapropylammonium bromide of analytical-reagent grade was obtained from East-man-Kodak (Rochester, N.Y., U.S.A.), and tetrapropylammonium hydroxide was prepared by reaction of tetrapropylammonium iodide (Eastman-Kodak) with silver oxide (Kebo-Grave Labcenter, Stockholm, Sweden). The hydroxide was purified by repeated extractions with chloroform.

The steroid glucuronides were obtained from Sigma (St. Louis, Mo., U.S.A.) and were used without further purification. Their structures are given in Table I.

TABLE I

STRUCTURES OF STEROID GLUCURONIDES
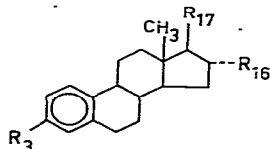
Compound	R_3	R_{16}	R_{17}
Estradiol 17β-glucuronide	OH	H	Glucuronic acid
Estradiol 3β-glucuronide	Glucuronic acid	H	OH
Estriol 17β-glucuronide	OH	OH	Glucuronic acid
Estrone 3β-glucuronide	Glucuronic acid	H	O

All other substances used were of analytical-reagent or laboratory grade. The sodium phosphate buffers used had an ionic strength of 0.1 and the magnesium phosphate buffers an ionic strength of 0.05. The chromatographic supports were LiChrosorb RP-2, with a mean particle diameter of 5 μm, and RP-18, with a mean particle diameter of 10 μm (E. Merck, Darmstadt, G.F.R.).

Column packing

The columns were packed by a balanced-density slurry technique[3]. The support RP-2 was suspended in methanol–water (60:40) and the RP-18 support in tetrabromoethane–tetrachloroethylene (3:2). After packing, the columns were washed with *n*-hexane, acetone and methanol–water (60:40).

Coating of the columns

Two coating methods were used, as described below.

Dynamic coating. The support was coated directly with the stationary phase according to Wahlund[4] by passing the mobile phase, saturated with the stationary phase through the column.

In situ coating with 1-pentanol + hydrophobic acid. A solution of the acid in

1-pentanol was equilibrated with an equal volume of the mobile phase. The separated 1-pentanol phase was diluted with acetone (7:3) and 25 ml of the mixture was passed through the column followed by the mobile phase until injected test samples showed constant capacity ratios.

The changes in k' values were less than 10% during 6 months' use of the columns.

Chromatographic technique

The mobile phase reservoir was immersed in a water-bath at $25 \pm 0.1°$ and the column was equipped with a water-jacket with circulating water at the same temperature. A small volume of stationary phase was present in the mobile phase reservoir, which was well filled and carefully tightened in order to prevent changes in the mobile phase composition.

The samples (10 μl) were dissolved in mobile phase. The hold-up volume of the column (V_m) was assumed to be equal to the retention volume of potassium dichromate when not otherwise stated.

RESULTS AND DISCUSSION

Phosphate buffer as mobile phase and 1-pentanol as stationary phase

In liquid chromatography, the capacity factor (k') is given by the equation

$$k' = (V_s/V_m)D \tag{1}$$

were V_s/V_m is the phase volume ratio and D the distribution ratio of the compound. When the sample, HX, is retained as an acid and the retention is due to distribution between the two liquid phases only, and protolysis in the aqueous phase is the only side-reaction, D is given by

$$D = [HX]_0/([HX] + [X^-]) \tag{2}$$

When the distribution constant

$$K_d = [HX]_0/[HX] \tag{3}$$

and the acid dissociation constant (K'_{HX}) are introduced, eqn. 1 becomes

$$k' = (V_s/V_m)K_d (1 + K'_{HX}/a_{H}^+)^{-1} \tag{4}$$

When $K'_{HX}/a_{H}^+ \gg 1$, eqn. 4 can be transformed to

$$\log k' = \log V_s/V_m + \log K_d - \log K'_{HX} - pH \tag{5}$$

According to eqn. 5, a plot of $\log k'$ against pH should give a straight line with a slope of -1 if the retention is due to distribution as acid.

Chromatography of the steroid glucuronides ($pK' \approx 3$) at pH 6–8 with sodium buffer as the mobile phase gave the results shown in Fig. 1. The curves are non-linear
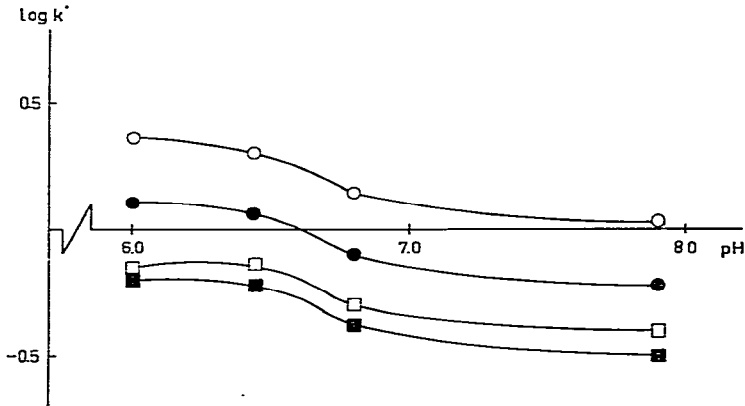
Fig. 1. Capacity ratios at different pH values. Mobile phase: sodium phosphate buffers saturated with 1-pentanol. Stationary phase: 1-pentanol. Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.28 ml/min. Samples: ○, estradiol 17β-glucuronide; ●, estradiol 3β-glucuronide; □, estriol 17β-glucuronide; ▣, estrone 3β-glucuronide.

and the slopes are greater than −1, which indicates retention of the glucuronides in an anionic form.

The peaks also showed considerable tailing, with asymmetry factors of 2.5–3 (Fig. 2). k' also decreased with increasing concentration of the sample, as demonstrated in Fig. 3.

Distribution of the acids as an ion pair with sodium, in connection with dis-
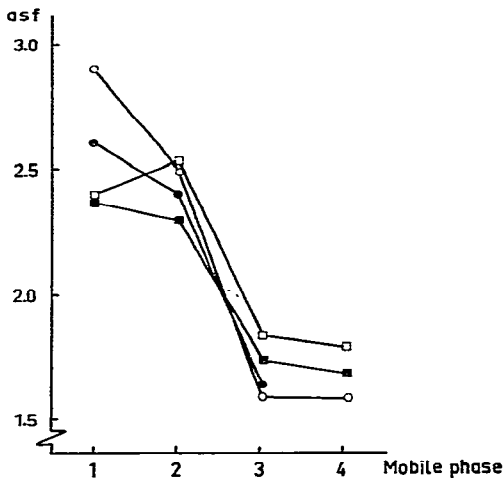
Fig. 2. Asymmetry factors (asf) with different stationary liquid phases. Mobile phases: 1, sodium phosphate buffer (pH 6.36) saturated with 1-pentanol; 2, sodium phosphate buffer (pH 6.35) saturated with 1-pentanol + 5.0 · 10^{-3} M lauric acid; 3, sodium phosphate buffer (pH 6.40) saturated with 1-pentanol + 2.9 · 10^{-2} M cholic acid; 4, sodium phosphate buffer (pH 6.17) saturated with 1-pentanol + 5.0 · 10^{-3} M bis-(2-ethylhexyl)phosphoric acid. Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.26 ml/min. Samples: ○, estradiol 17β-glucuronide (4.25 nmole); ●, estradiol 3β-glucuronide (2.96 nmole); □, estriol 17β-glucuronide (1.79 nmole); ▣, estrone 3β-glucuronide (2.18 nmole).

$k^{\cdot}$

1.8

1.6

1.4

1.2

1.0

5   10   15   20

1

2

4

3
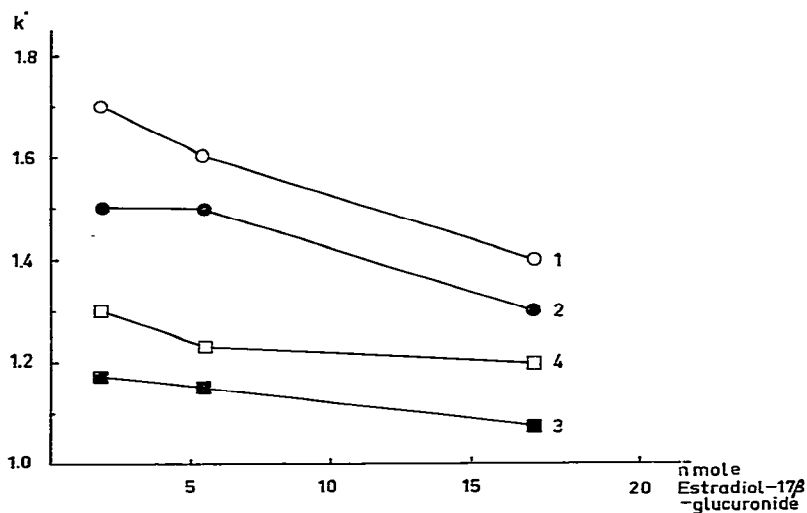
ñmole
Estradiol–17β
–glucuronide

Fig. 3. Capacity ratios with different liquid stationary phases. Mobile phases as in Fig. 2. Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.26 ml/min. $V_s/V_m = 0.47$.

sociation of the ion pair in the organic phase, may give rise to disturbances in chromatograms of this type. Test runs were made with mobile phases in which sodium in the buffer was replaced with magnesium. No significant improvement in the chromatographic behaviour was obtained.

Support effects

Interaction with the support is a more likely cause of the deviating chromatographic behaviour of the steroid glucuronides. This effect was investigated according to Wahlund and Beijersten[5] by measuring k' when V_s increases during the dynamic coating procedure.

The retention volumes (V_R) of two glucuronides were measured, V_m being obtained from the front peak. An approximate capacity factor ($k'_{appr.}$) was calculated by using the relationship

$$k'_{appr.} = (V_R - V_m)/V_m \tag{6}$$

Plots of $k'_{appr.}$ against the phase volume ratio V_s/V_m shown in Fig. 4A indicate that $k'_{appr.}$ decreases with increasing phase volume ratio, which suggests that the retention of the steroid glucuronides to a large extent is due to adsorption to the support.

Addition of hydrophobic acids to the stationary phase

Columns coated with 1-pentanol were equilibrated with mobile phases of sodium phosphate buffer (pH 6.5) saturated with 1-pentanol and containing cholic acid in two different concentrations (7.0 · 10^{-5} and 4.8 · 10^{-4} M). $k'_{appr.}$ values were determined during the equilibration process. Plots of $k'_{appr.}$ against volume of eluate are shown in Fig. 5. The $k'_{appr.}$ values decreased by 10% and 35%, respectively, for the two concentrations of cholic acid stated. Tailing was also reduced during the equilibration.
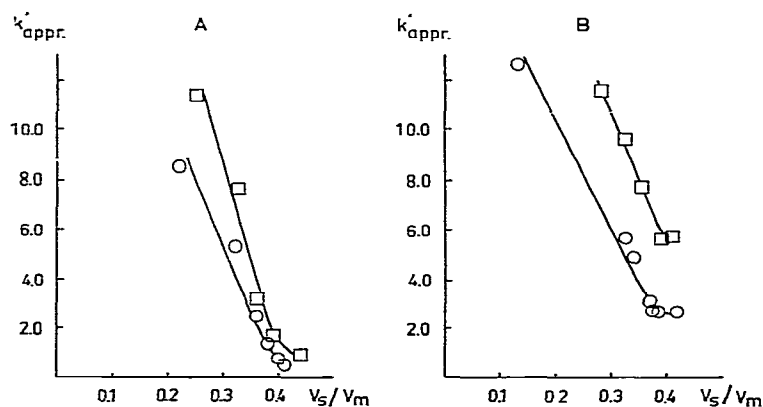
Fig. 4. Capacity ratios of steroid glucuronides during the coating procedure. Mobile phases: (A) sodium phosphate buffer (pH 7.5) saturated with 1-pentanol; (B) sodium phosphate buffer (pH 7.5) saturated with 1-pentanol containing 0.0399 M tetrapropylammonium bromide. Stationary phase: 1-pentanol. Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.26 ml/min. Samples: ○, estradiol 3β-glucuronide; □, estrone 3β-glucuronide.

Further tests were made with columns containing higher concentrations of acid in the stationary phase prepared by *in situ* coating. The capacity ratios obtained with three different acids, bis-(2-ethylhexyl)phosphoric acid, cholic acid and lauric acid, in the stationary phase are demonstrated in Fig. 3. Results obtained with 1-pentanol as stationary phase are included for comparison.

Bis-(2-ethylhexyl)phosphoric acid and cholic acid gave a larger decrease in k' than lauric acid and the decrease in k' with increasing sample concentration is also
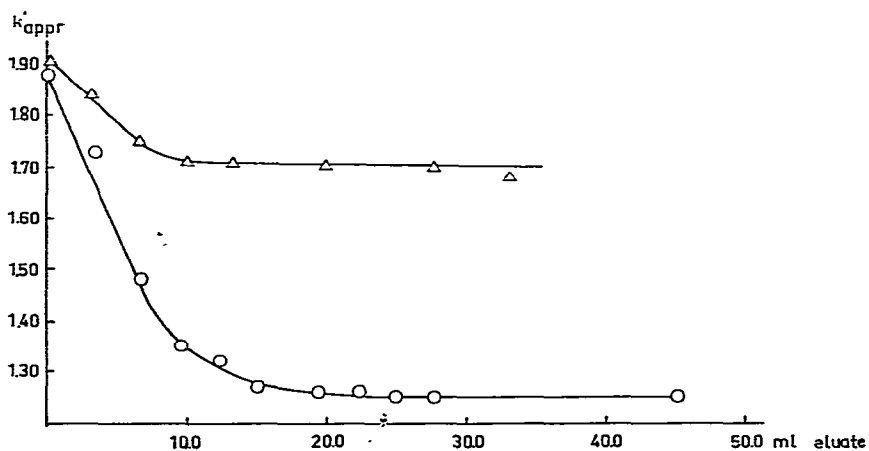

Fig. 5. Capacity ratios during coating with a hydrophobic acid in the mobile phase. Mobile phase: ○, sodium phosphate buffer (pH 6.45) saturated with 1-pentanol containing 4.8 · 10⁻⁴ M cholic acid; △, sodium phosphate buffer (pH 6.45) saturated with 1-pentanol containing 6.99 · 10⁻⁵ M cholic acid. Stationary phase: 1-pentanol. Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.28 ml/min. Sample: estradiol 17β-glucuronide.

smaller. The improvement in the peak symmetry with these acids present in the stationary phase is demonstrated in Fig. 2.

Ion-pair chromatography with 1-pentanol as stationary phase

The glucuronides showed good chromatographic behaviour in systems with 1-pentanol as the stationary liquid phase when a quaternary ammonium ion was added to the mobile phase, as demonstrated in Fig. 6 for the separation of four glucuronides.
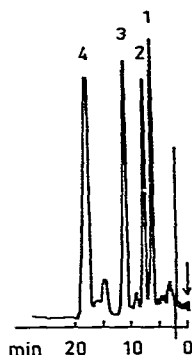

Fig. 6. Separation with tetrapropylammonium as counter ion. Mobile phase: 0.041 M tetrapropyl-ammonium bromide in sodium phosphate buffer (pH 6.40). Stationary phase: 1-pentanol ($V_s/V_m = 0.43$). Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.27 ml/min (340 psi, 0.01 a.u.f.s.). Samples: 1 = estriol 17β-glucuronide (1.24 μg); 2 = estrone 3β-glucuronide (0.99 μg); 3 = estradiol 3β-glucuronide (0.98 μg); 4 = estradiol 17β-glucuronide (2.83 μg).

The retention of the samples increased with increasing concentration of the quaternary ammonium ion (as tetrapropylammonium bromide), in the mobile phase, as demonstrated in Fig. 7. This indicates that the retention is affected by an ion-pair distribution mechanism when the following relationship is valid:

$$k' = V_s/V_m \cdot E_{QX}^* \cdot C_Q \tag{7}$$

where E_{QX}^* is the conditional extraction constant for the ion pair QX and C_Q is the counter ion concentration in the mobile phase.

The deviation from linearity in Fig. 7 may be due to ion-pair dissociation in the stationary phase and/or ion-pair formation in the aqueous mobile phase, both of which will affect the magnitude of E_{QX}^* (ref. 1). The intercepts of the curves with the ordinate in Fig. 7 indicate that the glucuronides are also retained in forms other than as ion pairs with the quaternary ammonium ion.

The retention mechanism in the ion-pair system is elucidated to some extent by Fig. 4B, which shows that k' decreases with increasing V_s/V_m, indicating that adsorption to the support has an important influence on the retention.

Comparison of retention as an acid and as an ion pair

The retention of the glucuronides in acidic form can be regulated by adjusting the pH, but the changes in the capacity ratios that can be obtained with 1-pentanol as
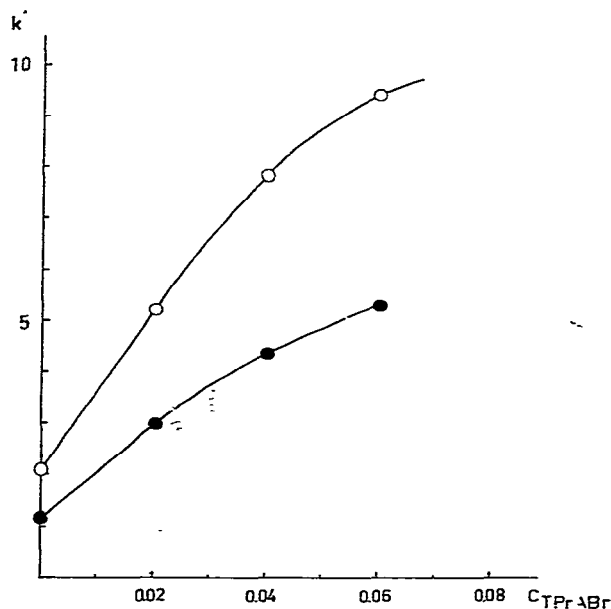
Fig. 7. Regulation of capacity factors. Mobile phase: tetrapropylammonium bromide in sodium phosphate buffer (pH 6.50). Stationary phase: 1-pentanol. Support: LiChrosorb RP-18 (10 μm). Flow-rate: 0.28 ml/min. Samples: $\bigcirc$, estradiol 17β-glucuronide; $\bullet$, estradiol 3β-glucuronide.

the stationary phase are limited, as demonstrated in Fig. 1. The possibility of regulating the retention is considerably better when the glucuronides are retained as ion pairs, as not only the concentration but also the nature of the quaternary ammonium ion will affect the capacity ratio. The separation efficiency is better in the ion-pair systems than in systems with the samples migrating as acids. Examples are shown in Fig. 8.
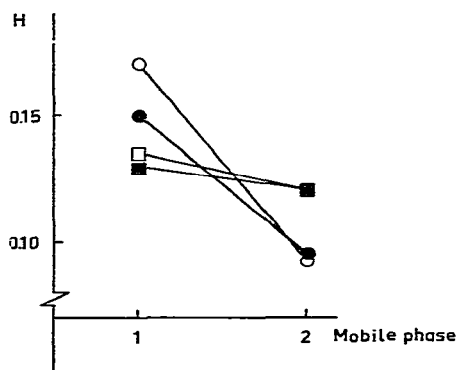

Fig. 8. Column efficiency with samples migrating as acids and ion pairs. Mobile phases: 1, sodium phosphate buffer (pH 6.44) containing 4.8 · 10^{-4} M cholic acid; 2, 4.1 · 10^{-2} M tetrapropylammonium bromide in sodium phosphate buffer (pH 6.40). Stationary phase: 1-pentanol. Support: LiChrosorb RP-18 (10 μm). Samples: $\bigcirc$, estradiol 17β-glucuronide; $\bullet$, estradiol 3β-glucuronide; $\square$, estriol 17β-glucuronide; $\blacksquare$, estrone 3β-glucuronide.

A small change in the capacity ratio with the amount of sample was observed in the ion-pair systems. This variation was, however, considerably larger when the samples were migrating as acids (Fig. 3).

The two types of system give about the same separation selectivity on RP-18, as demonstrated in Fig. 9. The use of RP-2 results in a slight increase in retention in the ion-pair system but has no effect on the selectivity.
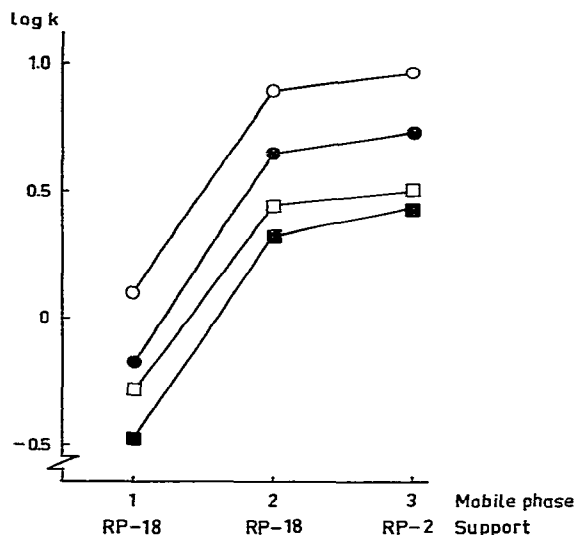

Fig. 9. Influence of support on the selectivity. Mobile phase: 1, sodium phosphate buffer (pH 6.44) containing $4.8 \cdot 10^{-4}$ M cholic acid; 2, 0.041 M tetrapropylammonium bromide in sodium phosphate buffer (pH 6.40). 3, 0.041 M tetrapropylammonium bromide in sodium phosphate buffer (pH 6.40). Stationary phase: 1-pentanol. Column: 150 × 3.2 mm I.D. Samples: as in Fig. 8. Flow-rate: 0.27 ml/min.

ACKNOWLEDGEMENTS

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