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COMPARATIVE STUDY OF SEVERAL PHASE SYSTEMS FOR THE SEPARATION OF ESTROGEN CONJUGATES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A number of liquid–solid and liquid–liquid phase systems with aqueous mobile phases were evaluated for the chromatographic separation of estrogen conjugates. The dependence of retention, selectivity and efficiency on the type of stationary phase, the composition, pH and viscosity of the mobile phase and the temperature was investigated.

Polar chemically bonded stationary phases on silica, except LiChrosorb AN, were found to be inferior for the chromatography of steroid conjugates, and so were pellicular and superficially porous anion exchangers and anion exchangers with a polystyrene matrix.

Microparticulate octadecyl- and methyl-silica and octadecyl-silica coated with liquid anion exchanger were found to be suitable stationary phases for chromatographic profiling of complex estrogen conjugate mixtures, and were compared with anion-exchange cellulose.

INTRODUCTION

An important metabolic pathway of drugs and endogenic compounds in body fluids is conjugation with glucuronic or sulphuric acid, which increases their polarity and hence the tendency of the compounds to be excreted. A group of compounds metabolized and excreted by conjugation that provides information on the physical condition of the organism are the steroids. The methods of analysis applied to steroids generally involve hydrolysis of the conjugates prior to profiling, so that subtle changes in type or site of conjugation will be overlooked. A method that permits the direct determination of steroid conjugates without hydrolysis would probably give additional information on the pathological state.

Recently, high-pressure liquid chromatography on anion-exchange cellulose

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and mixed anion-exchange cellulose–diatomite columns has been studied for the separation of mixtures of estrogen conjugates and other closely related ionogenic and hydrophilic compounds[1–3]. The excretion level of estrogen conjugates in female urine during pregnancy gives information on the physical condition of the foetus[4]. Other stationary phases used to separate estrogen glucuronides or sulphates by high-performance liquid chromatography were polystyrene anion exchangers and ion-pair forming liquids[6,7]. Conjugates of four 17-ketosteroids were separated on a hydrophobic adsorbent that showed poor efficiency, however[8].

This study was undertaken in order to evaluate the merits of column liquid chromatography on mixed-bed anion-exchange cellulose–diatomite columns for the separation of estrogen glucuronides and sulphates[2,3] relative to other phase systems with an aqueous mobile phase and suitable for high-pressure liquid chromatography. The restriction to an aqueous mobile phase precludes the use of adsorption and ion-pair chromatography with polar stationary phases. This choice was made in order to avoid a complicated and time-consuming extraction from urine into an organic phase.

Although this work was restricted to estrogen conjugates, it might provide guidelines in the development and optimization of the separation of other complex mixtures of polar compounds.

EXPERIMENTAL

Apparatus

In most of the experiments a commercial liquid chromatograph (Hewlett-Packard Model 1010A) and stainless-steel columns (250 × 3, 150 × 3, 110 × 4 and 80 × 3 mm) were used. The detector was a variable-wavelength UV spectrophotometer (Hewlett-Packard 1030 B). Estrogen conjugates were measured at 220 or 275 nm. The chromatograms were recorded with a linear potentiometric flat-bed recorder (Servogor RE 514.9).

The experiments with Aminex A-28, Zipax SAX and Pellionex WAX were carried out with a custom-made high-pressure liquid chromatograph consisting of a high-pressure reciprocating membrane pump (Orlita DMP 1515) equipped with a flow-through Bourdon-type manometer as damping device, a septum injection port, a thermostated stainless-steel separation column (500 × 2 or 250 × 3 mm), a variable-wavelength UV detector (Zeiss PM 2 D) and a flat-bed potentiometric recorder (Servogor RE 511).

Column materials

The following materials were used as column packings:

(1) LiChrosorb RP 2, dimethyl-silica, 5 μm (4–7 μm);

(2) LiChrosorb RP 8, octyl-silica, 5 μm (4–7 μm);

(3) LiChrosorb RP 18, octadecyl-silica, 5 μm (3–8 μm);

(4) LiChrosorb AN, strong quaternary ammonium anion exchanger chemically bonded to microparticulate silica, 10 μm;

(5) LiChrosorb NH_2, polar chemically bonded amino phase on silica, 10 μm;

(6) LiChrosorb DIOL, polar chemically bonded glycophase on silica, 10 μm;

(7) Nucleosil N-$(CH_3)_2$, polar chemically bonded dimethylamino phase on silica, 10 μm;

(8) Nucleosil SB, strong $-N^+(CH_3)_3I^-$ anion exchanger chemically bonded on silica, 10 μm;

(9) Aminex A-28, strongly basic quaternary ammonium anion exchanger with polystyrene–divinylbenzene matrix (8% crosslinked), 8–12 μm, ion exchange capacity 3.2 mequiv./g;

(10) Zipax SAX, superficially porous anion exchanger with strongly basic groups and a coated polymer matrix, 25–37 μm, ion-exchange capacity 12 μequiv./g;

(11) AL Pellionex WAX, pellicular weakly basic anion exchanger with an aliphatic matrix, 44–53 μm.

LiChrosorb materials are products of E. Merck (Darmstadt, G.F.R.), Nucleosil of Macherey, Nagel & Co. (Düren, G.F.R.), Aminex A-28 of Bio-Rad Labs. (Richmond, Calif., U.S.A.), Zipax SAX of DuPont (Wilmington, Del., U.S.A.) and AL Pellionex WAX of Whatman (Maidstone, Great Britain).

Non-polar chemically bonded phases were packed into a column by a balanced-density slurry method in chloroform–tetrabromethane at a constant pressure of 5 bar per millimetre of total column length. Polar bonded phases were packed in chloroform at a constant pressure of 3 bar per millimetre of column length from a well stirred slurry in a mixing vessel at the bottom of the column. Aminex A-28 was packed into a column using a pressurized slurry technique in eluent at 0.3 bar per millimetre of total column length; Zipax SAX and AL Pellionex WAX were dry packed.

All chemicals were of pro analisi quality (E. Merck). Buffers were made up with deionized water. The specified pH was adjusted with either orthophosphoric acid or sodium hydroxide. The composition of the mobile phases is expressed in per cent (v/v). Kinematic viscosities of the mobile phases at 25 $\pm$ 0.1° were determined by means of an Ubbelohde viscosimeter and their densities by aerometry. After a change of mobile phase, the column was equilibrated until a stable non-drifting baseline was obtained. Capacity ratios were determined at least in duplicate, the standard deviation of the measurements being less than 3%.

Samples

The estrogen conjugates used were the sodium salts of testosterone β-D-glucuronide (T-G), estriol 3-β-D-glucuronide (E_3-3G), estrone β-D-glucuronide (E_1-3G), 17β-estradiol 3-β-D-glucuronide (E_2-3G), estriol 17-β-D-glucuronide (E_3-17G), estriol 16-β-D-glucuronide (E_3-16G) and 17β-estradiol 17-β-D-glucuronide (E_2-17G), all obtained from Sigma (St. Louis, Mo., U.S.A.); estriol 3-phosphate (E_3-3P), estrone 3-phosphate (E_1-3P), 17β-estradiol 3-phosphate (E_2-3P), 17β-estradiol 17-phosphate (E_2-17P), testosterone sulphate (T-S), estriol 3-sulphate (E_3-3S), estrone 3-sulphate (E_1-3S), estriol 17-sulphate (E_3-17S), 17β-estradiol 3-sulphate (E_2-3S), 17β-estradiol 17-sulphate (E_2-17S) and 17β-estradiol 3,17-β-D-glucuronide (E_2-diG), all obtained from Steraloids (Pawling, N.J., U.S.A.); equilin 3-sulphate (Eq-3S), 17α-estradiol 3-sulphate (17αE_2-3S), 17α-dihydroequilin 3-sulphate (17αEq-3S), equilenin 3-sulphate (Eqe-3S) and 17α-dihydroequilenin 3-sulphate (17αEqe-3S) were a gift from Diosynth (Oss, The Netherlands).

Cytosine was used as an unretarded tracer to determine the average residence time of the mobile phase in the column.

RESULTS AND DISCUSSION

The degree of chromatographic separation of two components j and i can be described by the resolution R_{ji}, which is related to the chromatographic process parameters by

$$R_{ji} = (r_{ji} - 1)\frac{\kappa_i}{\kappa_i + 1}\cdot\sqrt{N_i} \quad (1)$$

where

r_{ji} = ratio of the capacity factors of components j and i = selectivity coefficient;
κ_i = capacity factor (mass distribution coefficient) of component i;
$N_i = L/H_i$ = number of theoretical plates for component i;
H_i = theoretical plate height of component i;
L = column length.

The capacity factor can be calculated from the measurements of the retention time, t_{Ri}, of a component i and the retention time t_{R0} of a non-retarded component:

$$\kappa_i = \frac{t_{Ri} - t_{R0}}{t_{R0}} \quad (2)$$

The time required for a given resolution is described by the following equation, which can be derived from eqns. 1 and 2:

$$t_{Rn} = \left(\frac{R_{ji}}{r_{ji} - 1}\cdot\frac{\kappa_i + 1}{\kappa_i}\right)^2\cdot\frac{H_i}{u}\cdot(\kappa_n + 1) \quad (3)$$

where

i and j = the most difficult to separate pair of successive components in the sample;
n = the last eluting component of the sample consisting of n species;
u = L/t_{R0} = flow velocity of the mobile phase.

Non-polar chemically bonded stationary phases on silica

In liquid–solid chromatography, the magnitude of the capacity factor is determined by the energy balance of the solute–stationary phase, the eluent–stationary phase and the solute–eluent interactions. The last two interactions are assumed to dominate with non-polar adsorbents, for which the solute–solvent interactions solely account for the selectivity coefficient of successive components[9]. A deviation from this ideal behaviour has been observed only for molecules with widely different rigidities, sizes or shapes[10], which is not the case with estrogen conjugates. Estrogen conjugates are molecules with a large hydrocarbon moiety and a polar ionogenic group.

Four features of the solute can be discerned: (i) the number of double bonds in the steroid skeleton; (ii) the number of hydroxyl groups of the aglycone; (iii) the type of conjugation partner; and (iv) the site of conjugation. The hydrocarbon moiety causes retention in chromatography on alkylated stationary phases. In this solvophobic effect, the surface tension plays a paramount role[11]. It necessitates the addition

of an organic solvent as a modifier to the aqueous eluent. The polar ionogenic group and the hydroxyl substituents on the steroid skeleton will contribute mainly to a decrease in retention by specific interaction with the eluent.

The effect of the pH, the salt concentration and the addition of a base or an acid to the mixture of methanol and aqueous buffer solution used as the mobile phase on the capacity factor and selectivity coefficient were investigated (see Table I). The dependence of the selectivity coefficient on the pH of the eluent was found to be minor except for the separation of estriol 17-glucuronide and estriol 16-glucuronide. The addition of sodium perchlorate to the aqueous eluent has a distinct effect on the selectivity

TABLE I

DEPENDENCE OF CAPACITY FACTORS OF ESTROGEN GLUCURONIDES AND SULPHATES ON THE COMPOSITION OF METHANOL–BUFFER MOBILE PHASES WITH HYDROPHOBIC ADSORBENTS

Phase systems: LiChrosorb RP 8. 5 μm; 25°. (A) 40% methanol + 60% 0.08 *M* phosphate, pH 4.5; (B) 40% methanol + 60% 0.05 *M* phosphate, pH 7.7; (C) 40% methanol + 60% (0.05 *M* phosphate, pH 7.7 + 0.5 *M* $NaClO_4$); (D) 40% methanol + 60% (0.07 *M* phosphate, pH 4.5 + 0.1 *M* TMA); (E) 40% methanol + 60% (0.07 *M* phosphate, pH 4.5 + 0.1 *M* TMA + 0.05 *M* PSA); (F) 40% methanol + 60% (0.07 *M* phosphate, pH 4.5 + 0.05 *M* PSA). TMA = trimethylamine; PSA = pentanesulphonic acid).

Compound	*A*	*B*	*C*	*D*	*E*	*F*
E_3-3G	0.75	0.45	0.45	0.55	0.25	0.2
E_3-17G	4.5	2.6	2.8	3.4	1.9	1.5
E_3-16G	4.6	2.8	2.9	3.7	1.9	1.55
E_1-3G	6.4	3.8	3.7	5.1	2.8	2.2
E_2-3G	8.2	4.6	4.6	6.1	3.5	2.7
E_2-17G	11.8	6.2	6.5	8.6	4.6	3.7
T-G	16.5	8.2	7.4	11.2	6.2	4.8
E_3-3S	1.8	1.2	1.2	1.7	0.7	0.55
E_3-17S	6.2	3.9	3.9	5.2	2.6	2.0
Eqe-3S	9.5	5.5	5.2	7.5	3.8	2.9
17αEqe-3S	10.5	6.2	6.1	8.4	4.4	3.4
Eq-3S	11.5	6.8	6.3	9.3	4.7	3.5
E_1-3S	13.1	7.7	7.2	10.3	5.4	4.0
17αEq-3S	13.1	7.7	7.9	10.5	5.7	4.3
E_2-3S	15.1	9.1	8.9	12.3	6.5	4.8
E_2-17S	16.6	10.0	10.5	13.6	7.3	5.3
17αE_2-3S	17.2	10.2	10.0	13.8	7.2	5.3
T-S	22.8	13.3	12.0	18.0	9.5	6.7

(*e.g.*, reversal of the elution order of E_2-17S and 17αE_2-3S and possible separation of 17αEq-3S and E_1-3S) but the capacity factors are not increased to a large extent, indicating that the increased solvophobicity is balanced and that the mechanism of separation of these steroid conjugates is complex. Chromatograms on LiChrosorb RP8 show tailing peaks. Deactivation of the strongest sites by pentanesulphonic acid naturally decreases the capacity ratio but also leads to some difference in selectivity. On the other hand, trimethylamine increases the capacity ratios non-selectively, taking into account the resulting higher pH of the eluent. Unfortunately, the addition of an acid, base or salt did not alter the tailing appreciably (see Fig. 1). To suppress ioni-

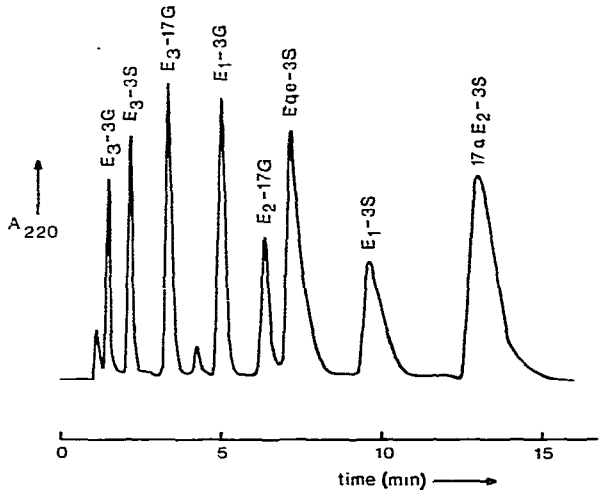

Fig. 1. Chromatogram of estrogen conjugates on microparticulate octyl-silica. Column: 100 × 4 mm; LiChrosorb RP 8, 5 μm; 40% methanol + 60% (0.1 *M* $NaClO_4$ + 0.05 *M* phosphate, pH 8.0); temperature, 70°; pressure drop, 210 bar.

zation of the conjugates, methanol–0.01 *M* perchloric acid (2:3), pH 2.3, was tried as an eluent on LiChrosorb RP 8. Chromatograms in this phase system show very broad peaks.

Three main qualities of a constituent of the mobile phase acting as a modifier are of interest: (i) its interaction with the stationary phase; (ii) its interaction with the solutes; and (iii) its viscosity at the required concentration. As representatives of

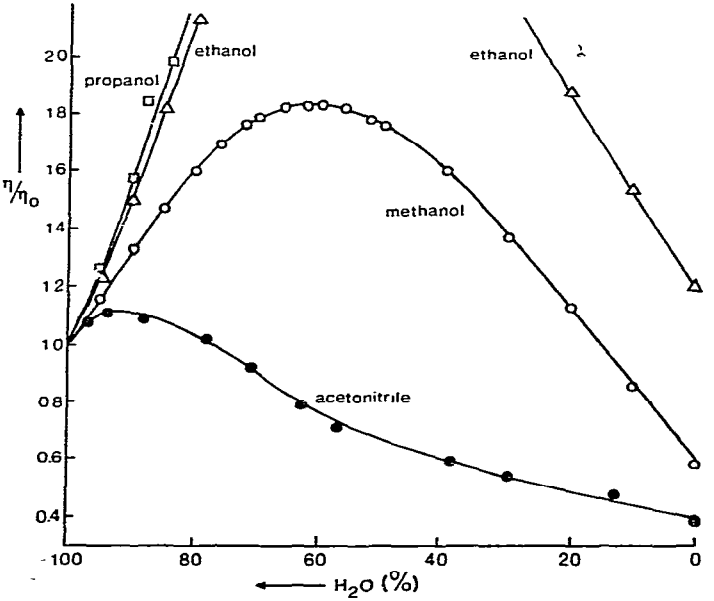

Fig. 2. Viscosity relative to water of *n*-alkanol–water[13] and acetonitrile–water[14] mixtures at 20°.

modifiers with proton donor and acceptor properties the lower *n*-alkanols were chosen, chloroform was selected for its quality as a proton donor and acetonitrile and dichloromethane mainly for their dipole moments; all of these compounds are highly polar and have low viscosity at the required concentration[12]. Lower viscosity promotes diffusion in the mobile phase, leading to higher efficiencies and a decrease in the column hydrodynamic resistance. The relative viscosity of *n*-alkanol–water and acetonitrile–water mixtures is shown in Fig. 2. The required concentration of modifier is strongly dependent on its polarity and also on the nature of the stationary phase.

In Table II and Fig. 3 it can be seen that the influence of the nature of the modifier on the selectivity coefficients of successive components is considerable. The estrone and equine estrogenic conjugates and T-G are relatively less retained on employing a modifier that contains a hydroxyl moiety. This trend cannot be explained by the magnitude of the proton acceptor solubility parameter (δ_a) alone, because

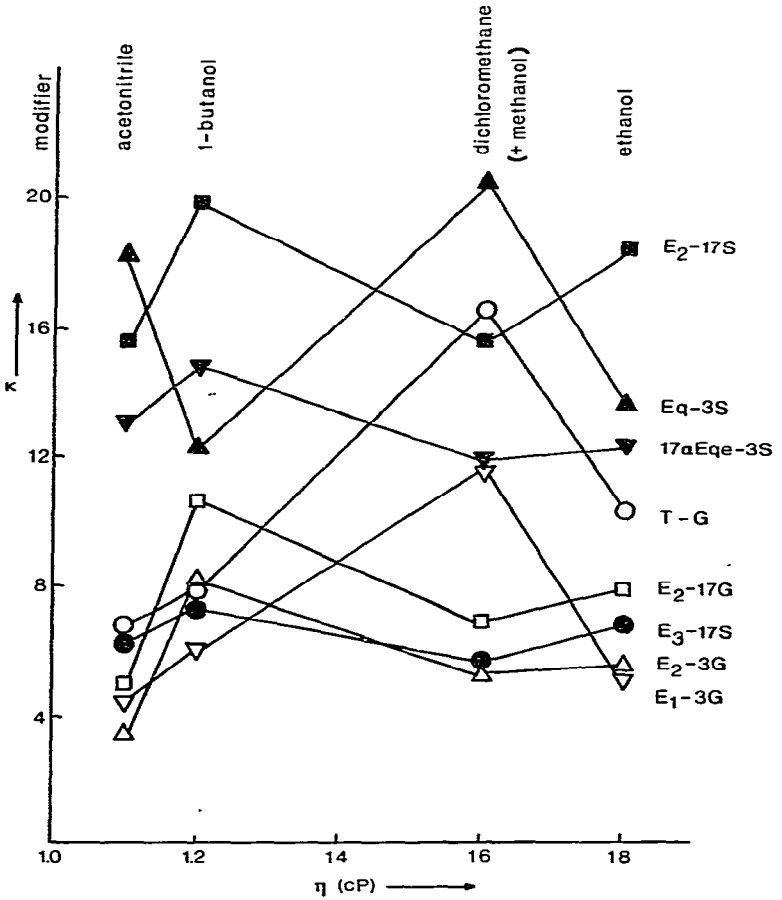

Fig. 3. Relationship between the capacity factor of a hydrophobic adsorbent (LiChrosorb RP 2) for estrogen conjugates and the viscosity of the mobile phase under approximately time-normalized conditions. Phase systems correspond to system E, D, F and B in Table II.

TABLE II

DEPENDENCE OF CAPACITY FACTORS OF ESTROGEN GLUCURONIDES AND SULPHATES ON THE ORGANIC MODIFIER OF THE MOBILE PHASE AND THE TEMPERATURE WITH HYDROPHOBIC ADSORBENTS

Phase systems: (A)-(L), LiChrosorb RP 2, 5 μm. (A) 30% methanol + 70% 0.05 *M* phosphate, pH 8.0, 25°; (B) 20% ethanol + 80% 0.05 *M* phosphate, pH 8.0, 25°; (C) 12% 1-propanol + 88% 0.05 *M* phosphate, pH 8.0, 25°; (D) 7% 1-butanol + 93% 0.05 *M* phosphate, pH 8.0, 25°; (E) 15% acetonitrile + 85% 0.05 *M* phosphate, pH 8.0, 25°; (F) 2% dichloromethane + 28% methanol + 70% 0.05 *M* phosphate, pH 8.0, 25°; (G) 7% 1-butanol + 93% 0.05 *M* phosphate, pH 8.0, 70°; (H) 15% acetonitrile + 85% 0.05 *M* phosphate, pH 8.0, 70°; (I) 2% dichloromethane + 28% methanol + 70% 0.05 *M* phosphate, pH 8.0, 70°; (K) 2% chloroform + 35% methanol + 63% 0.05 *M* phosphate, pH 8.0, 70°. (L), LiChrosorb RP 8, 5 μm; (L) 20% methanol + 20% acetonitrile + 60% 0.05 *M* phosphate, pH 5.0.

Compound	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*		
											25°	*50°*	*75°*
E_3-3G	0.8	0.7	0.7	1.0	0.6	0.6	0.4	0.55	0.5	0.1	0.17	0.14	0.13
E_3-17G	3.0	3.5	3.4	4.3	2.7	2.7	1.8	2.4	1.8	0.7	1.28	1.12	0.86
E_3-16G	3.0	3.6	3.5	4.3	2.7	2.7	1.8	2.4	1.8	0.7	1.28	1.12	0.86
E_1-3G	4.0	5.2	5.6	6.1	4.5	11.9	2.0	4.2	5.2	3.3	3.0	2.7	2.0
E_2-3G	4.5	5.7	6.4	8.2	3.5	5.3	2.8	3.4	4.5	1.5	2.1	1.89	1.47
E_2-17G	6.6	7.9	8.1	10.6	5.0	7.0	3.7	4.4	4.75	1.5	2.5	2.2	1.61
T-G	9.0	10.4	9.5	7.9	6.8	16.7	2.6	6.1	8.1	4.2	4.0	3.5	2.7
E_3-3S	2.3	2.6	2.2	2.3	2.5	2.0	1.2	2.1	1.25	0.4	1.32	1.14	0.90
E_3-17S	5.8	6.9	6.0	7.3	6.3	5.8	3.4	5.0	3.1	1.1		3.2	2.4
Eqe-3S	9.4	12.0	10.7	11.9	16.9	19.2	4.5	11.9	8.1	5.1			
17αEqe-3S	9.5	12.5	12.1	14.9	13.1	12.0	6.2	9.9	5.8	2.4			
Eq-3S	10.7	13.7	12.1	12.3	18.3	20.6	4.7	12.6	9.7	5.5			
E_1-3S	11.8	15.4	13.5	15.5	21.4	38	5.5	15.2	12.5	7.2			
E_2-3S	12.4	16.9	15.5	18.3	16.6	16.1	8.0	12.0	8.1	3.3			
E_2-17S	13.7	18.6	17.3	19.9	15.6	15.7	8.9	10.8	7.6	2.9			
17αE_2-3S	14.7	20.2	18.6	21.6	18.0	25.3	9.4	13.7	9.9	3.85			
η (cP)	1.6	1.8	1.5	1.2	1.1	1.6							

acetonitrile and 1-butanol have approximately equal values[12]. Also, the dipole orientation solubility parameter (δ_0) alone cannot be responsible as the absolute values for dichloromethane and ethanol are similar and dichloromethane is mixed with methanol. A satisfactory correlation is obtained, however, on comparing selectivity and δ_0/δ_a. Fig. 3 indicates that a viscous modifier such as ethanol can be

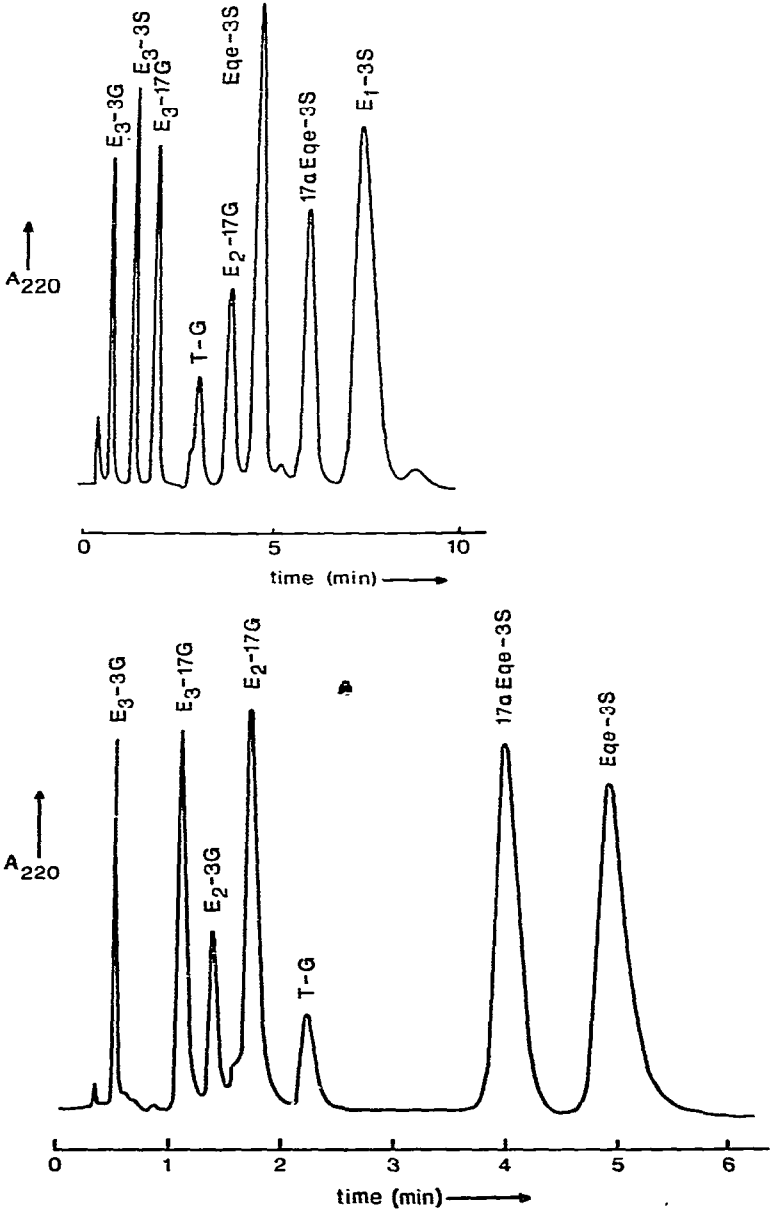

Fig. 4. Chromatograms of estrogen conjugates on dimethyl-silica. Column: 150 × 3 mm; LiChrosorb RP 2, 5 μm; temperature, 70°; pressure drop, 250 bar. (A) 7% 1-butanol + 93% 0.05 *M* phosphate, pH 8.0; (B) 15% acetonitrile + 85% 0.05 *M* phosphate, pH 8.0.

replaced with a mixture of less viscous modifiers such as acetonitrile, 1-butanol and dichloromethane. The dependence of the selectivity coefficient on the type of modifier is also illustrated in Fig. 4: the elution order of T-G and E_2-17G, and 17αEqe-3S and Eqe-3S, can be reversed. Also within the group of *n*-alkanols as modifiers large selectivity differences occur, *e.g.*, the elution order of Eq-3S and 17αEqe-3S reverses (see Fig. 5).

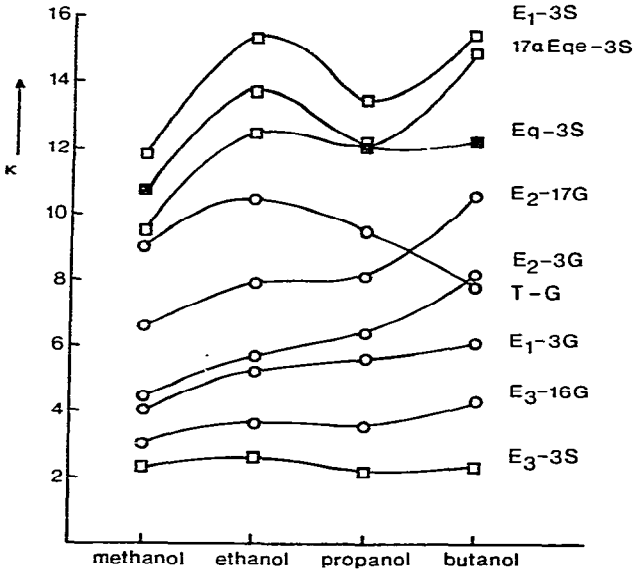

Fig. 5. Influence of the length of the alkyl chain of *n*-alkanols as modifiers under approximately time-normalized conditions on the capacity factor of a hydrophobic adsorbent system for estrogen conjugates Phase systems correspond to systems A, B, C and D in Table II.

The decrease in capacity factor with temperature (see Table II and Fig. 6) is generally accompanied by a slight decrease in selectivity coefficient[15], which counteracts the effect of the decrease in theoretical plate height on resolution; in order to compensate for the decrease in capacity factor and selectivity coefficient at higher temperatures one can decrease the concentration of the modifier (Table III).

Table III shows that the capacity factors of estrogen glucuronides and sulphates on LiChrosorb RP 18 as a stationary phase and 15% acetonitrile as modifier are two to four times greater than those on LiChrosorb RP 2. To obtain equal capacity factors, approximately 20% of acetonitrile must be used on LiChrosorb RP 18. It is of advantage to use LiChrosorb RP 2 with *n*-alkanols as modifiers in order to combine high selectivity with high efficiency. Even with acetonitrile as modifier, the columns of LiChrosorb RP 2 appear to be more efficient than those packed with LiChrosorb RP 18 (see Fig. 7), which is a combined effect of a wider particle size distribution of the batch and slower mass transfer with RP 18. Because of a larger selectivity, however, RP 18 can well be used for the separation of several estrogen conjugates, as is illustrated in Fig. 8.

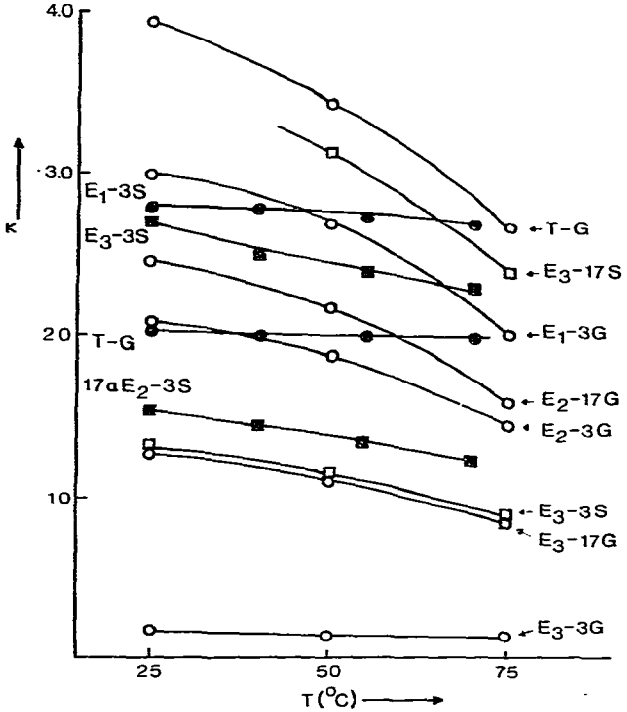

Fig. 6. Dependence of the capacity factors of estrogen conjugates on temperature in a hydrophobic adsorption system and a system with a surfactant adsorbed to a hydrophobic adsorbent. Columns: ●, ■, LiChrosorb RP 18, 5 μm; 50% acetonitrile + 50% (0.02 *M* phosphate, pH 5.0 + 1% CTMABr); ○, □, LiChrosorb RP 8, 5 μm; 20% methanol + 20% acetonitrile + 60% 0.05 *M* phosphate, pH 5.0.

Solvent-induced anion-exchange stationary phases

A phase system with a liquid anion exchanger coated on diatomite was used in this laboratory for high-pressure liquid chromatography[16]. Recently, an ion-exchange layer was generated on a hydrophobic adsorbent using a mobile phase with a surfactant ("soap chromatography")[17]. Both processes are akin to ion-pair partition chromatography with a non-polar stationary liquid[18]. As the selectivity of solvent-induced ion-exchange systems is different from that of hydrophobic adsorption systems, and on account of their potential high efficiency, we were prompted to investigate this type of phase system for the chromatographic separation of estrogen conjugates.

A weak ion-pair-forming surfactant, tri-*n*-octylamine (TOA), and a strong ion-pair-forming surfactant, cetyltrimethylammonium bromide (CTMABr), were used. Table III shows the main changes in capacity factor on addition of the ion pair former to the eluent, concurrently increasing the concentration of the modifier to keep the retention times within reasonable limits. CTMABr at pH 8.0 and pH 3.8 and TOA at pH 3.8 decrease the capacity factor of 17-conjugates relative to 3-conjugates; estrone-conjugates and T-G are relatively less retarded than estradiol and estriol conjugates and equine estrogenic conjugates relatively more. At pH 8.0, LiChrosorb

TABLE III

DEPENDENCE OF CAPACITY FACTORS OF ESTROGEN GLUCURONIDES AND SULPHATES ON THE LENGTH OF THE ALKYL BRISTLE OF THE ADSORBENT, THE CONCENTRATION OF THE MODIFIER AND THE TYPE OF SURFACTANT IN THE MOBILE PHASE

Phase systems: (A) LiChrosorb RP 2, 5 μm; 15% acetonitrile + 85% 0.05 *M* phosphate, pH 8.0; 70°; (B) LiChrosorb RP 18, 5 μm; 15% acetonitrile + 85% 0.05 *M* phosphate, pH 8.0; 70°; (C) LiChrosorb RP 18, 5 μm; 20% acetonitrile + 80% 0.05 *M* phosphate, pH 8.0; 70°; (D) LiChrosorb RP 18, 5 μm; 40% acetonitrile + 60% (0.1 *M* phosphate, pH 3.8, TOA saturated); 70°; (E) LiChrosorb RP 18, 5 μm; 20% acetonitrile + 80% (0.05 *M* phosphate, pH 8.0, TOA saturated); 70°; (F) LiChrosorb RP 18, 5 μm; 40% acetonitrile + 60% (0.1 *M* phosphate, pH 3.8 + 0.1% CTMABr); 70°; (G) LiChrosorb RP 18, 5 μm; 40% acetonitrile + 60% (0.05 *M* phosphate, pH 8.0 + 0.1% CTMABr); 70°. (TOA = trioctylamine; CTMABr = cetyltrimethylammonium bromide).

Compound	*A*	*B*	*C*	*D*	*E*	*F*	*G*
E_3-3G	0.55	0.65	0.3	0.35	0.3	0.2	0.2
E_3-17G	2.4	5.0	1.5	1.35	1.85	0.8	0.95
E_3-16G	2.4	5.0	1.5	1.25	1.8	0.8	0.95
E_1-3G	4.2	17.8	4.8	3.4	5.3	1.9	2.7
E_2-3G	3.4	11.6	2.8	2.3	3.2	1.2	1.6
E_2-17G	4.4	12.5	3.2	1.8	3.7	1.1	1.5
T-G	6.1	29.0	5.9	1.85	6.0	1.5	1.9
E_3-3S	2.1	4.2	1.1	2.6	1.4	1.8	2.3
E_3-17S	5.0	12.7	3.7	6.4	4.7	3.6	5.0
Eqe-3S	11.9	58	14.1	24.3	17.8	13.1	20.9
17αEqe-3S	9.9	48	10.5	18.2	12.7	10.2	16.1
Eq-3S	12.6	68	15.3	25.8	18.6	13.8	22.4
E_1-3S	15.2		19.2	25.2	21.8	15.5	24.1
E_2-3S	12.0	56	12.0	19.6	14.0	9.9	15.4
E_2-17S	10.8	40	9.6	15.1	12.6	7.2	10.4
17αE_2-3S	13.7	77	16.2	24.8	21.2	12.7	20.8

RP 18 modified with TOA ($pK_a^{eff} = 8$) behaves like a non-polar bonded stationary phase.

In Table IV, it can be seen that the capacity factor increases with decreasing pH until the pK_a value of the estrogen conjugates is reached. Below these pH values, the capacity factor decreases sharply. At pH 2.5 the glucuronides are almost neutral[19] whereas the more acidic sulphates are still present as anions, which is reflected in the magnitude of the capacity factors given in Table IV.

The dependence of the capacity factor of estrogen conjugates on the concentration of the surfactant in the mobile phase at pH 5.0 roughly follows the equation

$$\kappa_i = \alpha[\text{CTMABr}]^{0.4} \quad (4)$$

The coefficient 0.4 originates from the adsorption of CTMA to the surface of the adsorbent[17], simultaneously increasing the counter ion concentration[20]. Because the capacity factor increases upon addition of surfactant to the mobile phase, the concentration of modifier must be increased to keep retention about constant. In contrast to hydrophobic adsorption systems, this does not merely lead to smaller selectivity coefficients but results in a mobile phase with low viscosity (Fig. 2). The improvement

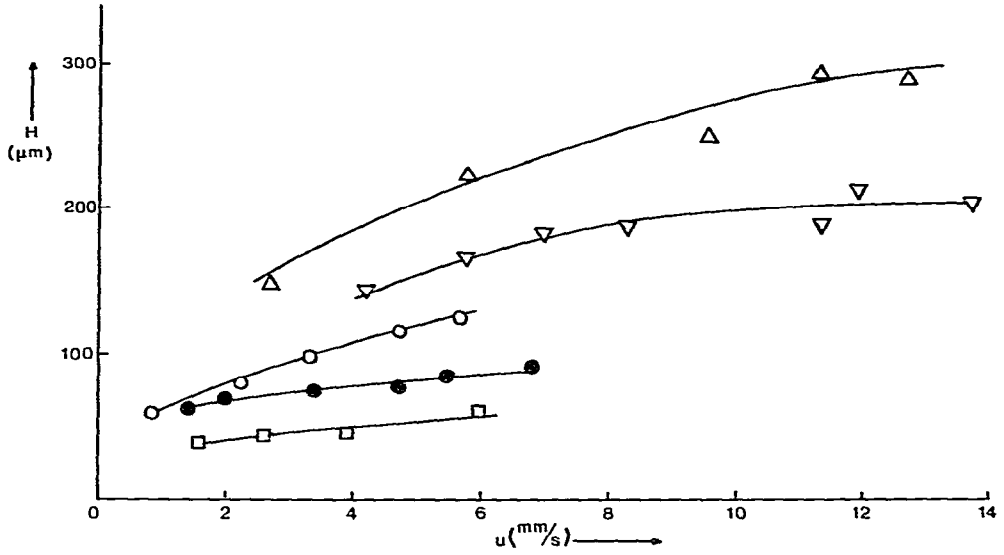

Fig. 7. Theoretical plate height *versus* mobile phase velocity for several phase systems.

Symbol	*Stationary phase*	*Mobile phase*				*Temperature (°C)*	*Test compound*	κ
		CTMA (%)	*Methanol (%)*	*Acetonitrile (%)*	*Aqueous buffer (%)*			
□	LiChrosorb RP 2	0	0	15	85	70	17αEqe-3S	9.9
○	LiChrosorb RP 18	0	0	20	80	70	17αEqe-3S	10.5
●	LiChrosorb RP 18	0.1	0	50	50	70	E_1-3S	8.5
▽	LiChrosorb AN	0	50	40	10	70	17αEqe-3S	20.9
△	LiChrosorb AN	0	50	40	10	25	17αEqe-3S	24.1

in efficiency is demonstrated in Fig. 7 by the smaller increase in the theoretical plate height with increasing flow velocity. Rapid separations of both estrogen glucuronides and sulphates in these systems are illustrated in Fig. 9.

The layer of surfactant on the adsorbent determines the capacity factor to a large extent but the modifier retains its influence on the selectivity, as is demonstrated in Table IV, which renders this technique very versatile.

The dependence of the capacity factor on temperature for a phase system with CTMABr is much smaller than for a hydrophobic adsorption system (Table IV) and is illustrated in Fig. 6.

The usefulness of solvent-induced ion exchange has also been demonstrated in the separation of amino acids[20].

Polar chemically bonded stationary phases on silica

Hydrolytically stable, more or less polar siloxane phases chemically bonded to siliceous supports of small particle size having high sample capacity have been developed[21]. Systematic investigations employing this type of material have been

TABLE IV

CAPACITY FACTORS OF ESTROGEN GLUCURONIDES AND SULPHATES ON OCTADECYL-SILICA WITH CETYLTRIMETHYLAMMONIUM BROMIDE IN THE MOBILE PHASE

Phase systems: LiChrosorb RP 18, 5 μm. (A) 50% acetonitrile + 50% 0.02 *M* phosphate, pH 5.0, 70°; (B) 5% chloroform + 60% methanol + 35% 0.02 *M* phosphate, pH 5.0, 70°; (C) 2% dichloromethane + 58% methanol + 40% 0.02 *M* phosphate, pH 5.0, 70°; (D) 70% methanol + 30% 0.02 *M* phosphate, pH 5.0, 70°; (E) 70% methanol + 30% 0.02 *M* phosphate, pH 8.0, 70°; (F) 70% methanol + 30% 0.02 *M* phosphate, pH 2.5, 70°; (G)-(M) 50% acetonitrile + 50% 0.02 *M* phosphate, pH 5.0, 25°; (N) 50% acetonitrile + 50% 0.02 *M* phosphate, pH 5.0, 40°; (P) 50% acetonitrile + 50% 0.02 *M* phosphate, pH 5.0, 55°; (Q) 50% acetonitrile + 50% 0.02 *M* phosphate, pH 5.0, 70°. Figures in parentheses are CTMABr concentrations (%).

Compound	*A* (0.10)	*B* (0.10)	*C* (0.10)	*D* (0.10)	*E* (0.10)	*F* (0.10)	*G* (0.00)	*H* (0.01)	*I* (0.03)	*K* (0.10)	*L* (0.30)	*M* (1.00)	*N* (1.00)	*P* (1.00)	*Q* (1.00)
E_3-3G		1.3	0.9	0.8	0.4	0.2	0.0	0.05	0.15	0.3	0.55	0.55	0.55	0.55	0.5
E_3-17G		1.7	2.3	1.3	0.9	0.6	0.0	0.2	0.4	0.7	1.0	1.25	1.2	1.15	1.1
E_3-16G		1.7	2.3	1.3	0.9	0.6	0.0	0.2	0.4	0.7	1.0	1.25	1.2	1.15	1.1
E_1-3G		5.5	5.5	2.4	1.45	0.8	0.0	0.2	0.5	1.05	1.8	2.8	2.8	2.8	2.7
E_2-3G		2.7	4.1	2.3	1.2	0.75	0.0	0.15	0.4	0.75	1.2	1.9	1.9	1.8	1.7
E_2-17G		2.2	4.0	2.1	1.4	0.7	0.0	0.2	0.4	0.75	1.2	1.7	1.6	1.6	1.6
T-G		5.1	6.0	3.1	1.6	1.0	0.0	0.25	0.45	0.85	1.4	2.1	2.0	2.0	2.0
E_3-3S	1.5	1.8	3.1	1.6	0.8	1.45	0.0	0.25	0.55	1.1	1.9	2.7	2.5	2.4	2.3
E_3-17S	2.9	3.4	5.3	2.6	1.3	2.1	0.0	0.5	1.0	2.2	3.4	4.8	4.4	4.3	4.0
Eqe-3S	7.4	11.7	14.3	5.5	2.4	4.2	0.0	1.2	2.6	6.4	10.2	15.7	14.6	13.4	12.2
17αEqe-3S	6.3	7.0	12.8	5.4	2.6	4.1	0.05	0.9	1.9	4.6	7.7	12.4	12.3	11.0	10.0
Eq-3S	8.0	13.4	16.1	6.3	2.7	4.6	0.1	1.25		6.7	11.1	16.2	15.4	14.6	13.0
E_1-3S	8.5	14.9	17.3	6.3	2.9	4.9	0.1	1.3	3.0	7.1	11.8	16.7	16.3	14.9	13.8
E_2-3S	5.8	7.5	14.1	6.1	2.8	4.6	0.05	0.75	1.8	4.5	7.3	11.3	11.0	10.1	9.3
E_2-17S	4.4	5.7	11.6	5.6	2.5	4.2	0.05	0.5	1.3	3.5	5.4	8.4	8.3	7.4	6.7
17αE_2-3S	7.2	9.4	17.4	7.2	3.2	5.3	0.1	1.0	2.1	5.6	9.5	15.4	14.7	13.7	12.5

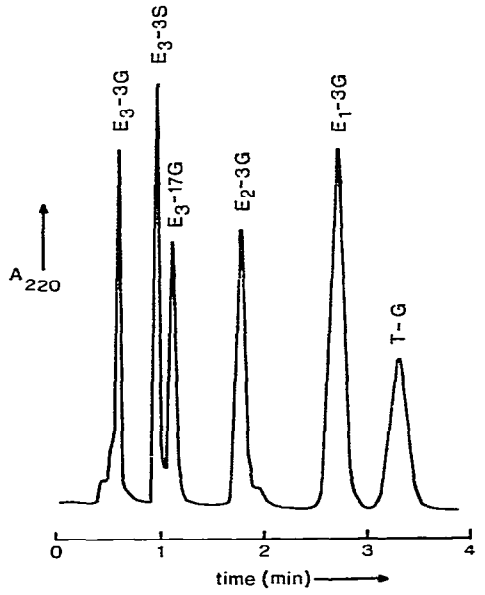

Fig. 8. Rapid separation of estrogen conjugates on octadecyl-silica. Column: 150 × 3 mm; LiChrosorb RP 18, 5 μm; 20% acetonitrile + 80%-0.05 *M* phosphate, pH 8.0; temperature, 70°; pressure drop, 250 bar.

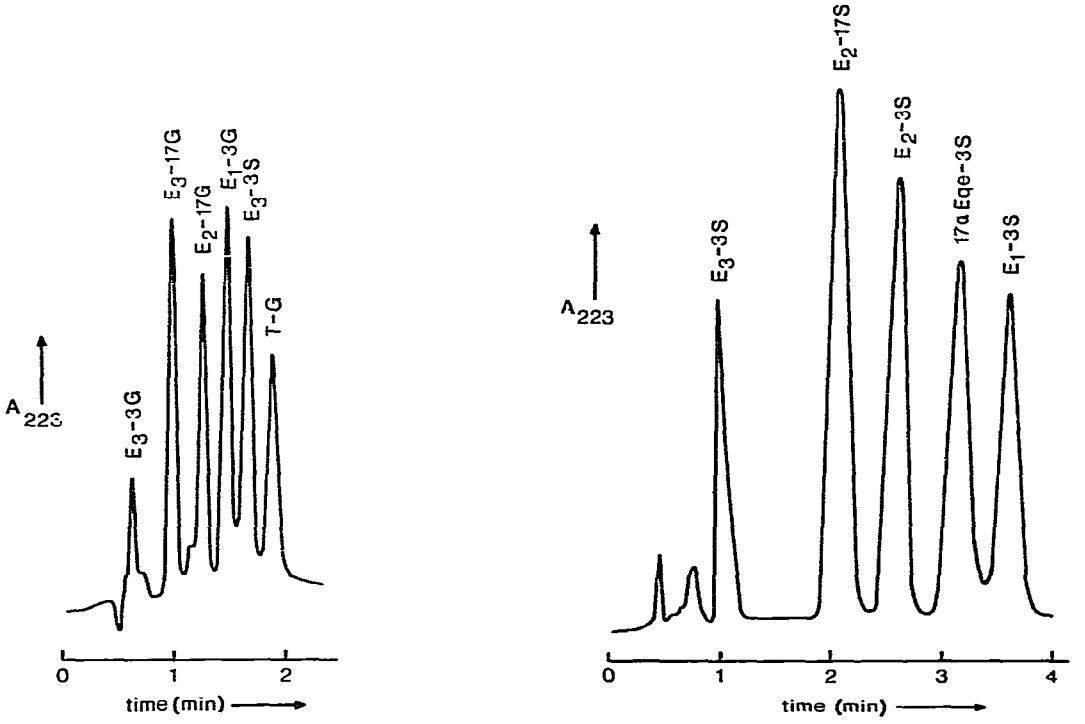

Fig. 9. Rapid separation of estrogen conjugates on octadecyl-silica with a solvent-induced ion-exchange coating. Column: 150 × 3 mm; LiChrosorb RP 18, 5 μm; temperature, 70°; pressure drop, 250 bar. (A) 40% acetonitrile + 60% (0.05 *M* phosphate, pH 8.0 + 0.1% CTMABr); (B) 70% methanol + 30% (0.02 *M* phosphate, pH 5.0 + 0.1% CTMABr).

TABLE V

CAPACITY FACTORS OF ESTROGEN CONJUGATES ON LICHROSORB AN

Phase systems: LiChrosorb AN, 10 μm. (A) 25% 1-propanol + 75% 0.01 *M* phosphate, pH 9, 60°; (B) 50% ethanol + 50% 0.01 *M* phosphate, pH 9, 70°; (C) 70% ethanol + 30% 0.01 *M* phosphate, pH 9, 70°; (D) 80% methanol + 20% 0.01 *M* phosphate, pH 9, 70°; (E) 80% methanol + 20% 0.01 *M* phosphate, pH 6, 70°; (F) 60% methanol + 40% 0.01 *M* phosphate, pH 9, 60°; (G) 60% methanol + 40% (1.0 *M* $NaClO_4$ + 0.01 *M* phosphate, pH 9), 70°; (H) 60% methanol + 40% (0.01 *M* phosphate, pH 9–0.2% propylamine), 70°; (I) 71% methanol + 9% acetonitrile + 20% 0.01 *M* phosphate, pH 9, 60°; (K) 60% methanol + 20% acetonitrile + 20% 0.01 *M* phosphate, pH 9, 60°; (L) 60% methanol + 20% acetonitrile + 20% 0.01 *M* phosphate, pH 6, 70°; (M) 60% methanol + 20% acetonitrile + 20% 0.01 *M* phosphate, pH 3, 70°; (N) 48% methanol + 24% acetonitrile + 24% 0.01 *M* phosphate, pH 3, 70°.

Compound	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*	*M*	*N*
T-G	1.86	0.92	0.66	0.71	2.2	1.20	0.71	0.75	0.74	0.40	1.57	1.70	1.6
E_3-3G	1.31	0.92	0.96	0.99	2.7	1.02	0.11	0.66	1.04	0.53	2.0	2.4	2.7
E_2-17G	3.3	1.55	1.09	1.31	3.1	2.4	0.28	1.35	1.24	0.72	2.2	2.0	1.8
T-S	5.8	2.1	1.12	1.22	3.3	2.5	0.30	1.55	1.42	0.63	2.0	12.7	8.0
E_3-3S	6.8	2.8	1.89	1.88	4.3	3.1	0.24	1.75	1.66	0.88	2.5	15.5	9.0
17αEqe-3S	14.4	5.0	2.2	2.7	5.9	7.4	0.47	3.6	2.5	1.17	3.1	18.3	10.2
E_2-diG	4.7	4.6	4.8	6.4	3.2	4.9	0.15	2.9	6.8	2.9		22.7	20.3
E_2-17P	16.8	8.4	7.3	7.6	5.8	10.4	0.56	3.3	4.7	3.5	4.4	14.4	16.2
E_2-3P	12.5	10.2	9.9	13.0	31.8	9.1	0.48	4.6	12.7	6.0	21.3	20.3	17.1
E_1-3P	13.3	10.2	11.3	13.6	33.6	10.0	0.54	4.7	12.7	5.8	21.7	19.6	15.6

performed with different types of samples and non-aqueous eluents[22]. Those materials with anion-exchange properties were used with aqueous buffers as the mobile phase to separate ionogenic compounds[23,24].

The performance for the chromatographic separation of estrogen conjugates of several types of polar chemically bonded phases was evaluated in this work using aqueous eluents.

LiChrosorb AN was used with mobile phases consisting of *n*-alkanol–aqueous buffer and methanol–acetonitrile–aqueous buffer at different pH values (Tables V and VI). An eluent containing 30% of propanol is equivalent to one containing 50% of ethanol or 60% of methanol. Selectivity between the members of one type of conjugate increases as the methanol concentration decreases or the pH increases. The column tends to strip off slowly, however, at high pH values and shows increasing peak broadening with increasing water content of the mobile phase. Even with the mobile phase 50% methanol–40% acetonitrile–10% 0.01 *M* phosphate, pH 3.0, the graph of theoretical plate height *versus* flow velocity shows that considerably more peak dispersion occurs than for the dimethyl-(RP 2) and octadecyl-(RP 18) silica (see Fig. 7). The shape of this curve suggests that this difference is caused not by the larger particle size but by the less uniform packing geometry. This was confirmed by studies with nucleotide monophosphates with this type of ion exchanger.

TABLE VI

CAPACITY FACTORS OF ESTROGEN CONJUGATES ON LICHROSORB AN

Phase systems: LiChrosorb AN, 10 μm; 70°. (A) 70% ethanol + 30% 0.01 *M* phosphate, pH 9; (B) 50% methanol + 40% acetonitrile + 10% 0.01 *M* phosphate, pH 3; (C) 60% methanol + 40% (0.01 *M* phosphate + 0.33% propylamine, pH 9.8).

Compound	*A*	*B*	*C*
T-G	0.66	4.5	1 54
E_3-3G	0.96	10.5	1.70
E_3-17G	0.98	14.7	3.0
E_3-16G	1.04	16.1	3.0
E_1-3G	1.08	7.6	2 7
E_2-3G	1.08	7.0	2.7
E_2-17G	1.09	7.7	4.0
T-S	1.12	12.2	4.2
E_3-3S	1.89	24.6	6.5
Eqe-3S	2.4	19.2	11.3
17αEqe-3S	2.5	24.1	12.2
Eq-3S	2.0	17.7	10.4
E_1-3S	2.0	16.5	10.2
17αEq-3S	1.92	20.9	10.5
E_2-3S	1.89	19.4	10.2
E_2-17S	2.1	24.1	11.2
17αE_2-3S	2.0	19.9	9.8
E_1-3P	11.3	30.1	13.7
E_2-3P	9.9	25.0	14.6
E_2-17P	7.3	39.5	16.4
E_2-diG	4.8		9.9

TABLE VII

CAPACITY FACTORS OF ESTROGEN GLUCURONIDES ON PELLICULAR AND POLYSTYRENE ANION EXCHANGERS ELUTED WITH PURE AQUEOUS BUFFERS

Phase systems: (A) Zipax SAX; 3.0 *M* acetate, pH 4.0; 20°; (B) Zipax SAX; 2.0 *M* acetate, pH 4.0; 20°; (C) Zipax SAX; 2.0 *M* acetate, pH 4.0; 40°; (D) Zipax SAX; 2.0 *M* acetate, pH 4.0; 60°; (E) Pellionex WAX; 0.005 *M* perchlorate + 0.0002 *M* phosphate, pH 4.0; 20°; (F) Pellionex WAX; 0.01 *M* perchlorate + 0.0002 *M* phosphate, pH 4.0; 20°; (G) Pellionex WAX; 0.01 *M* perchlorate + 0.0002 *M* phosphate, pH 4.0; 40°; (H) Pellionex WAX; 0.01 *M* perchlorate + 0.0002 *M* phosphate, pH 4.0; 60°; (I) Pellionex WAX; 0.1 *M* acetate, pH 4.0; 20°; (K) Aminex A-28; 6.0 *M* acetate, pH 4.0; 70°; (L) Aminex A-28; 8.3 *M* acetate, pH 4.0; 70°; (M) Aminex A-28; 8.3 *M* acetate, pH 4.0; 90°; (N) Aminex A-28; 8.3 *M* acetate, pH 5.1; 90°; (P) Aminex A-28; 10.1 *M* acetate, pH 4.0; 90°.

Compound	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*	*M*	*N*	*P*
T-G	0.00	1.19	0.94	0.60	1.37	0.78	0.77	0.76	0.86	4.8	1.61	1.51	1.75	0.65
E_3-3G	0.00	0.17	0.17	0.13	2.3	1.26	1.14	0.92	1.99	6.4	2.5	2.3	2.5	1.11
E_3-17G	0.26	1.57	1.15	0.76	3.5		1.72	1.47			7.0	5.9	6.2	2.8
E_3-16G	0.26	1.67	1.20	0.80	3.9	2.0	1.81	1.56	4.2		7.5	6.1	6.6	2.9
E_1-3G	0.29	2.4	1.45	1.02	6.4	3.6	2.6	1.95	2.2		4.9	4.1	4.9	2.0
E_2-3G	0.42	2.7	1.79	1.20	5.5	2.9	2.6	1.84	4.1		7.2	6.1	7.3	2.9
E_2-17G	1.00	6.3	3.7	2.2	6.0	3.6	2.8	2.4	9.1		14.3	11.0	14.1	5.3

Nucleosil SB stripped off, even at neutral pH, and had a non-linear adsorption isotherm (see Fig. 10).

Attempts using columns packed with LiChrosorb NH_2, LiChrosorb DIOL and Nucleosil N-$(CH_3)_2$ failed to yield capacity factors in the useful range for estrogen conjugates, except with acidic aqueous eluents without an organic solvent; peak broadening was excessive in these phase systems.

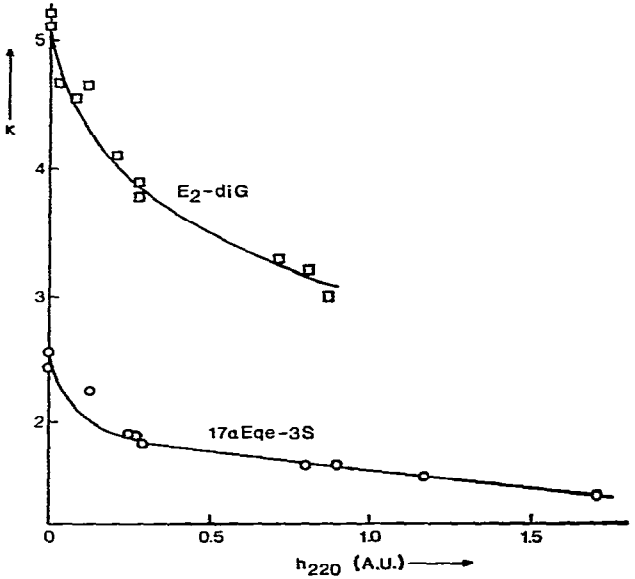

Fig. 10. Dependence of the capacity factor calculated from the retention time of the peak maximum on the peak height at 220 nm (h_{220}). Column: 250 × 3 mm; Nucleosil SB, 10 μm; 0.05 *M* $NaClO_4$ + 0.01 *M* phosphate, pH 6.8; temperature, 25°.

Anion exchangers with a hydrocarbon matrix

Disappointing results in the chromatographic separation of steroid conjugates have been reported for anion-exchange resins with a hydrophobic polystyrene–divinylbenzene matrix[25], but a successful separation with unusual selectivity on these anion exchangers has been described[5].

To re-assess these results, experiments with Aminex A-28 were performed. The concentration of acetate, pH and temperature were varied (see Table VII). In agreement with the theory of ion exchange, the estrogen glucuronides are less retarded at higher counter ion concentrations and lower pH values. The selectivity coefficient hardly decreased as the temperature increased. It was not possible to reproduce the previous reported selectivity[5]. Moreover, the concentration of acetate in the eluent has to be impractically high and the maximal eluent velocity attainable is low, leading to long separation times, as demonstrated by the chromatogram in Fig. 11.

Pellicular and superficially porous anion exchangers have a much lower ion-exchange capacity and do not need such high concentrations of counter ion in the mobile phase. The selectivity of these anion exchangers is excellent at 20° but decreases

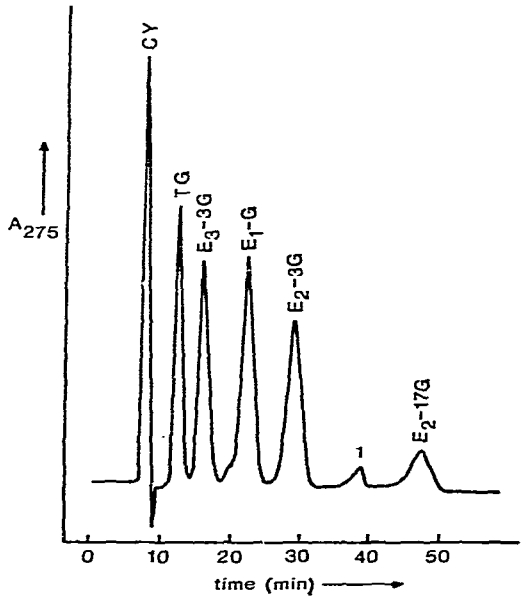

Fig. 11. Separation of a test mixture of estrogen glucuronides on Aminex A-28. Column: 250 × 3 mm; Aminex A-28, 8–12 μm; 10.1 *M* acetate, pH 4.0; temperature, 90°; pressure drop, 100 bar.

considerably with increasing temperature (see Table VII). The chromatograms in Figs. 12 and 13 illustrate that these materials are not suitable for separating a large number of compounds because of the low column efficiency.

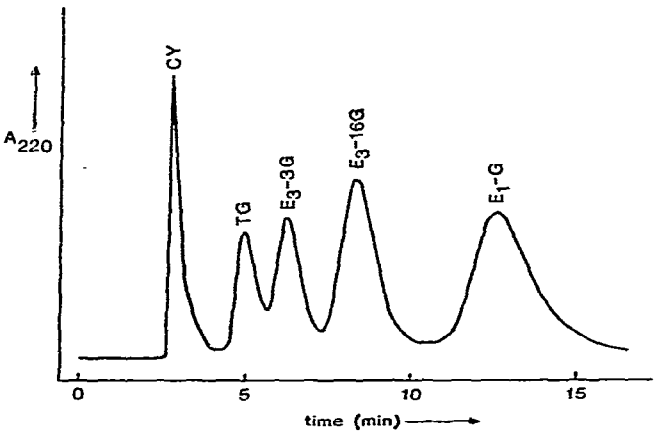

Fig. 12. Separation of estrogen glucuronides on a superficially porous anion exchanger. Column: 500 × 2 mm; Zipax SAX, 25–37 μm; 2.0 *M* acetate, pH 4.0; temperature, 20°; pressure drop, 100 bar.

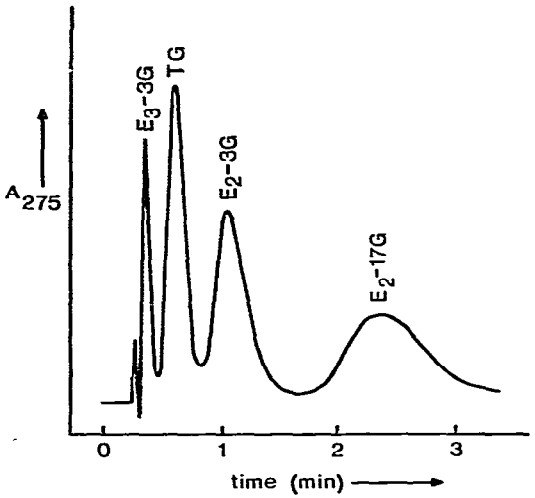

Fig. 13. Separation of estrogen glucuronides on a pellicular anion exchanger. Column: 500 × 2 mm; AL Pellionex WAX, 44–53 μm; 0.01 *M* perchlorate + 0.0002 *M* phosphate, pH 4.0; temperature, 20°; pressure drop, 21 bar.

CONCLUSION

The final choice of a phase system for the chromatographic separation of steroid conjugates depends on several factors. In addition to the phase system selectivity and column efficiency, which are discussed here, load capacity, stability and convenience are also important.

In profiling complex mixtures such as body fluids, high selectivity and high efficiency are needed. Our results show that hydrophobic adsorption chromatography on octadecyl- or dimethyl-silica, chromatography on octadecyl-silica with a surfactant adsorption layer and chromatography on a high-performance cellulose anion exchanger[3] are all suitable for this purpose, depending on the specific composition of the sample. For example, the separation of five estrogen sulphates occurring in pregnant mare's urine requires slightly less time when chromatographed on ECTEOLA-cellulose than in one of the phase systems described in this paper, because its lower efficiency is over-compensated for by its higher selectivity (see table VIII). None of the phase systems is optimized, however, for this particular separation.

The choice of the chromatographic system is strongly dependent on the specific composition of the sample because the order of elution of the components is different in the respective phase systems. The cellulose and polystyrene anion exchangers show the elution order T, E_3, E_1, Eq, E_2, Eqe, 17αEqe; as an exception, on Zipax SAX, T-G is more retained than E_3-3G. On LiChrosorb AN and AL Pellionex WAX, T-G is eluted before the estrogen glucuronides. The elution order on RP 2, RP 8 and RP 18 and on these materials coated with a surfactant is E_3, Eqe, 17αEqe, Eq, E_1, E_2, T, employing a modifier with a large δ_a/δ_0 ratio. E_2 conjugates and to some extent 17αEqe-3S are relatively less retained when a modifier with a small δ_a/δ_0 ratio is used. The cellulose and polystyrene anion exchangers show a large

TABLE VIII

COMPARISON OF THE SPEED OF SEPARATION OF A MIXTURE OF ESTROGEN·SULPHATES ON DIFFERENT CHROMATOGRAPHIC PHASE SYSTEMS

Phase systems: (I D) LiChrosorb RP 8, 5 μm; 40% methanol + 60% (0.07 *M* phosphate, pH 4.5 + 0.1 *M* trimethylamine); 25°; (II F) LiChrosorb RP 2, 5 μm; 2% dichloromethane + 28% methanol + 70% 0.05 *M* phosphate, pH 8.0; 25°; (II K) LiChrosorb RP 2, 5 μm; 2% chloroform + 35% methanol + 63% 0.05 *M* phosphate, pH 8.0; 70°; (III C) LiChrosorb RP 18, 5 μm; 20% acetonitrile + 80% 0.05 *M* phosphate, pH 8.0; 70°; (P) ECTEOLA-cellulose B 300, 19 μm; 0.10 *M* $NaHSO_4$ + 0.01 *M* phosphate, pH 6.8; 70°; (Q) ECTEOLA-cellulose B 300, 19 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 8.4; 70°.

Compound	*Parameter*	*System*					
		II F	*II K*	*I D*	*III C*	*P*	*Q*
Eqe-3S	Capacity factor	19.2	5.1	7.5	14.1	6.6	2.7
17αEqe-3S		12.0	2.4	8.4	10.5	9.9	3.9
Eq-3S		20.6	5.5	9.3	15.3	3.7	1.53
E_1-3S		38	7.2	10.3	19.2	2.8	1.12
E_2-3S		16.1	3.3	12.3	12.0	4.7	1.99
—	$(r_{ji})_{min}$	1.19	1.08	1.11	1.09	1.29	1.30
—	$(H_i/u)_{min}$ (sec)	0.03	0.02	0.04	0.03	0.2	0.2
—	$t_{Rn}^{(4)}$* (sec)	580	590	880	1370	760	480

* $t_{Rn}^{(4)}$ is the time needed for the separation of the total mixture with a resolution of at least 4.

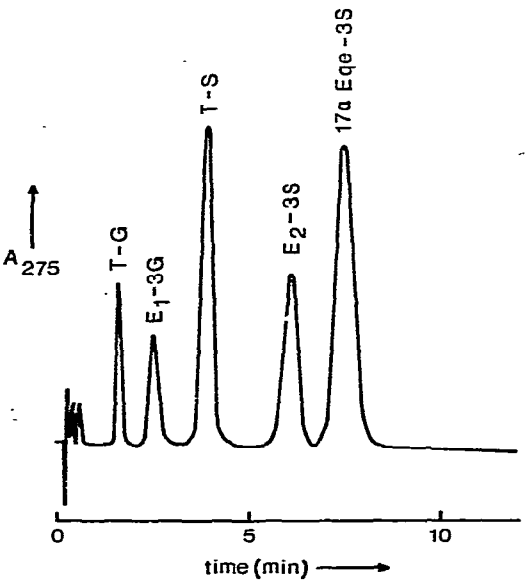

Fig. 14. Separation of steroid conjugates on LiChrosorb AN. Column: 250 × 3 mm; LiChrosorb AN, 10 μm; 50% methanol + 40% acetonitrile + 10% 0.01 *M* phosphate, pH 3.0; temperature, 70°; pressure drop, 170 bar.

selectivity for the site of conjugation; with other phase systems this selectivity is much smaller.

Despite the systematic investigation of the influence of eluent composition, no phase system consisting of a polar chemically bonded phase and an aqueous eluent was found to be suitable for separating individual estrogen conjugates. LiChrosorb AN has a limited but important scope of applicability in the separation of estrogen conjugates by type (Fig. 14).

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