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SEPARATION OF STEROID CONJUGATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

1.1. Types of steroid conjugates

Steroid conjugates contain, in addition to the characteristic cyclopentanephenanthrene skeleton, a conjugating moiety, *e.g.*, glucuronide, sulfate, phosphate, N-acetylglucosaminide, glycine, taurine or (poly)dextrose.

Interest in steroid conjugates has been increasing since their discovery, as their biological and medical importance has been assessed. Cardiac glycosides, corticosteroid conjugates, progestines and androgen and estrogen conjugates are examples of physiologically active steroid conjugates.

Usually a large number of structurally related steroid conjugates in widely different concentrations are present in biological fluids, so an efficient separation technique is needed. High-voltage electrophoresis and paper chromatography have been used with success but are time consuming. For steroid conjugates paper chromatography is superior to thin-layer chromatography because of larger polar group selectivity[1]. Although the reproducibility, quantitation and peak capacity that can be obtained in gas chromatography are better than those with the other methods men-

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tioned, the lack of volatility of steroid conjugates and their general instability at higher temperatures makes derivatization for gas chromatographic analysis necessary. High-performance liquid chromatography (HPLC), being a highly selective, rapid, non-destructive and automatable method, is thus the obvious choice for the analysis or isolation of steroid conjugates.

The number of investigations of the separation of steroid conjugates by HPLC has been limited mainly because the polar character of the compounds makes them less suited for separation in systems with bare silica as a stationary phase. The recent widespread use of liquid ion exchangers and non-polar chemically bonded stationary phases on silica has brought about a profound change.

In this review, which covers the literature up to the beginning of 1980, the achievements with HPLC as an analytical tool in the determination of steroid conjugates thus far are discussed. Further papers that have appeared more recently[2–9] may also be consulted. A gas chromatographic method for steroid conjugate derivatives has also been published[10].

1.2. High-performance liquid chromatography

1.2.1. Separation

The degree of chromatographic separation of two components, j and i, is described by the resolution, R_{ji}, which depends on the chromatographic parameters as follows:

$$R_{ji} = (r_{ji} - 1) \cdot \frac{\kappa_i}{1 + \kappa_i} \cdot \sqrt{\frac{L}{H_i}} \tag{1}$$

where

$r_{ji} = \kappa_j/\kappa_i$ = selectivity coefficient;

κ_i, κ_j = capacity factors (mass distribution coefficients) of components i and j, respectively;

L = length of the column;

$H_i = L/N_i$ = theoretical plate height for component i;

N_i = number of theoretical plates for component i.

The main progress in column liquid chromatography in the last decade concerns the increase in the speed of separation. The time in which the separation of a mixture of n components is performed with a minimum resolution, R_{ji}, for the least resolved pair, i and j, of successively eluting compounds is given by the equation

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

where

t_{Rn} = retention time of the last eluting component, n, in the sample consisting of the components 1,2,..., $(n - 1)$, n;

$u = L/t_{R0}$ = linear flow velocity of the mobile phase;

t_{R0} = hold-up time of the eluent;

κ_1 = capacity factor of the last eluted component.

The development of microparticulate column packings requiring high operating pressures led to a 100-fold decrease in the H_i/u value and therefore a 100-fold increase in the speed of separation. In practice, the minimum value of H_i/u obtainable is set by the pressure limit of the equipment and the compressibility of the column packing. H_i/u values of less than 10 msec can be achieved with high-quality packings.

In liquid–solid chromatography on non-polar adsorbents, the eluents generally consist of water and an organic compound. In addition to low toxicity and UV transparency, three properties of the organic modifier are of importance: (1) its interaction with the solid surface; (2) its interaction with the solutes in the liquid bulk phase; and (3) its viscosity. The following modifiers are commonly used: lower alkanols, tetrahydrofuran (THF), acetonitrile, dioxan, chloroform and dichloromethane[11]. All of these organic solvents have low column hydrodynamic resistance and promote diffusion in the mobile phase, giving high efficiencies.

The required concentration of modifier is strongly dependent on its polarity, the nature of the solid surface and the type of conjugate. The capacity factors for estrogen conjugates on LiChrosorb RP-18 with 15% of acetonitrile as modifier are two to four times larger than on LiChrosorb RP-2[12]. The greater retention and selectivity are, however, strongly dependent on the batch and brand of packing material owing to the variation of the degree of surface modification, *e.g.*, the number of residual silanol groups.

The cardenolide glycosides, like the bile acid conjugates, and the bufotoxines, require about 30–40% of acetonitrile[13–15], 60–70% of methanol[15–17], 40–50% of dioxane[13] or 30–40% of THF[13,16] in the mobile phase. This shows the following order of modifier strength in hydrophobic adsorption chromatography: THF > acetonitrile > dioxane > methanol. More polar conjugates are corticosteroid phosphates (50% methanol[18]), 17-ketosteroid sulfates (20% methanol[19,20]) and glucuronides (30% methanol[19,20]) and estrogen sulfates, phosphates and glucuronides (20–40% methanol[12]).

Although selectivity in HPLC is still difficult to predict with complex molecules, some trends with non-polar adsorption chromatography that hold essentially for all steroid conjugates can be discerned, *e.g.*, the interaction of surface silanol groups with electron-rich groups such as keto and hydroxy. Hydrophilic adsorption chromatography employing a non-polar mobile phase and silica as the stationary phase has been hampered by the strong interactions of the steroid conjugates with the polar silanol groups.

Anion exchange is a mode of chromatography that uses the acidic character of components of the sample to accomplish retention. Several types of anion-exchange columns exist that contain quaternary ammonium groups covalently bonded to a polystyrene–divinylbenzene resin or to a cellulosic matrix, as well as columns in which a layer of anion-exchange resin is coated on rigid particles, making the column packing mechanically more stable.

High selectivity (different from hydrophobic adsorption systems) and high efficiency are characteristics of solvent-induced anion-exchange systems in which the alkyl-substituted silica stationary phase is covered with a quaternary ammonium ion by adding a low concentration of the latter to the mobile phase.

1.2.2. Detection

Not only does the diversity of the steroid conjugates necessitate an efficient and selective separation, but their usually low concentration also imposes the problem of detection. The low sensitivity and specificity of refractive index detectors make them of limited use with biological mixtures. The most important principles for steroid conjugates are UV absorbance, fluorescence, electrochemical and reaction detection.

Many steroid conjugates have appreciable UV absorbance below 210 nm. The conjugating glucuronide, sulfate, phosphate, taurine or glycine groups hardly contribute to the UV absorbance.

Electrochemical detectors are being increasingly used in HPLC. Electrochemical detection was recently applied in the analysis of estrogen conjugates[21].

For samples that contain much extraneous material, one is often obliged to carry out extensive purification procedures. An alternative is to use a chemical reaction. Pre-column reactions[22] are considered outside the scope of this review because (like hydrolysis) they change the chromatographic characteristics of the steroid conjugates.

For post-column reaction detection, three types of reactors can be distinguished: tubular, packed-bed and segmented-flow reactors. The choice of reactor depends on the reaction time and the reagents used. Aggressive reagents such as strong acids and bases lead to special problems. For short reaction times (less than 3 min) the packed-bed reactor is the optimal choice, whereas for slow reactions the segmented-flow reactor is to be preferred[23]. Extra-column peak-broadening can be limited to negligible levels under these conditions.

Fluorimetry in 60–80 % sulfuric acid or concentrated hydrochloric acid can be sensitive and specific[24–26], but the reaction rates are slow.

Few publications[27] mention reaction rates that are sufficiently large for tubular or packed-bed reactors. A clear exception are the reactions in packed-bed reactors that contain immobilized enzymes, which can be highly specific and sensitive[28].

A detection method that is not based on a steroid moiety reaction is complexation of steroid sulfates with methylene blue and extraction into chloroform[29].

Laser-desorption mass spectrometry has been applied successfully to the identification of several steroid conjugates in the aqueous effluent of a high-performance liquid chromatograph[30]. This requires, however, an expensive off-line instrument which is not yet used as a routine liquid chromatographic detector.

In the following sections the present state of the application of HPLC in the analysis of steroid conjugates is reviewed.

2. ESTROGEN CONJUGATES

Hermansson[31] found with 1-pentanol-saturated phosphate buffer as the mobile phase and 1-pentanol on LiChrosorb RP-18 as the stationary phase that the capacity factors of estrogen glucuronides decrease with increasing phase ratio. This indicates that residual silanol groups on the packing cause retention of the conjugates. Also, the observed elution order estrone (E_1), estriol (E_3), estradiol (E_2) compared with the expected order E_3, E_2, E_1 (the remaining hydroxy group of E_2-monoconjugates is very accessible to polar interactions, as was shown on anion exchangers)[12] in-

TABLE I

SELECTIVITY COEFFICIENTS, $r_{((n-1)n)}$, FOR CONSECUTIVE ESTROGEN CONJUGATES WITH DIFFERENT ORGANIC MODIFIERS[12]

n	*Organic modifier*							
	Methanol	*Butanol*		*Acetonitrile*		*Dichloromethane*		*Chloroform,*
	25°C	*25°C*	*70°C*	*25°C*	*70°C*	*25°C*	*70°C*	*70°C*
E_3-3G	5.6	8.2	7.0	5.7	6.2	8.8	9.0	15
E_2-3G	0.9	0.7	0.7	1.3	1.2	2.2	1.2	2.2
E_1-3G								
E_3-17G	2.2	2.5	2.1	1.9	1.8	2.6	2.6	2.1
E_2-17G	1.4	0.8	0.7	1.4	1.4	2.4	1.7	2.8
T-G								
E_3-3S	5.4	7.9	6.7	6.8	5.7	8.1	6.5	8.2
E_2-3S	1.0	0.8	0.7	1.3	1.3	2.4	1.5	2.2
E_1-3S								
E_3-17S	2.4	2.7	2.6	2.4	2.2	2.7	2.5	2.6
E_2-17S								
17α-Eqe-3S	1.0	0.8	0.7	1.3	1.2	1.6	1.4	2.1
Eqe-3S								

dicates this. In this system, asymmetric peaks and a decrease in the capacity factor with increasing amount of estrogen conjugate injected were found, which are characteristics of a non-linear isotherm, highly probably owing to the silanol adsorption sites. Addition of a strong hydrophobic acid reduced the tailing and the retention.

It is observed with estrogen conjugates that an additional hydroxy group on the solute leads to a higher relative retention when using alkanols as modifiers in the eluent while the hydrophobic surface area of the solute decreases. The true hydrophobic effect, a decrease in retention, is seen when using acetonitrile as organic modifier (Table I).

The organic modifier adsorbs on non-polar stationary phases and competes with the solutes. This adsorption and competition increase with increasing chain length of lower alkanols and causes a larger selectivity coefficient between E_1-3-conjugates and E_2-3-conjugates and between equilenin (Eqe)- and 17α-dihydroequilenin (17αEqe)-3-sulfates. The equilibrium distribution of the organic modifier between the hydrophobic adsorbent and the aqueous stationary phase is expected to change with temperature. The silanol groups on the packing material then become more accessible. This could explain the large decrease in selectivity coefficients between E_1-3- and E_2-3-conjugates in a dichloromethane-containing mobile phase whereas the mixed mechanism keeps these selectivity coefficients virtually constant in a mobile phase with 1-butanol as modifier[12]. A practical consequence of the retention characteristics resulting from the use of different modifiers is that it allows one to minimize the viscosity of the mobile phase while maintaining a constant retention range. Fig. 1 shows this relationship for some estrogen conjugates. For a given capacity factor range the most viscous eluent containing ethanol can be replaced by less viscous eluents, with modifiers such as acetonitrile and 1-butanol. These eluents have different selectivities, however. An illustration of the separation of estrogen con-

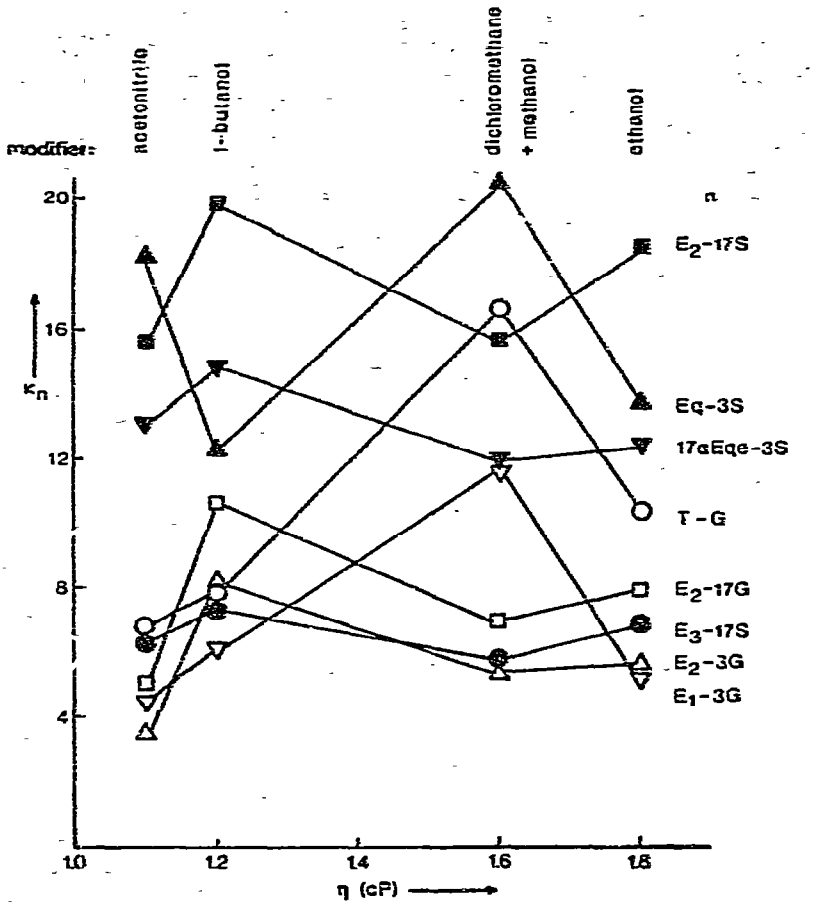


Fig. 1. Relationship between the selectivity of a hydrophobic adsorbent (LiChrosorb RP-2) for estrogen conjugates and the viscosity of the mobile phase under approximately time normalized conditions. From ref. 12.

jugates is given in Fig. 2. Note the reversal in the elution order of T-glucuronide and E_2-17-glucuronide and of Eqe-3-sulfate and 17α-Eqe-3-sulfate.

The high selectivity and high efficiency of solvent-induced anion-exchange systems prompted the investigation of their usefulness for the chromatographic separation of steroid conjugates. A study of the selectivity effects of solvent-induced anion-exchange systems for the separation of estrogen conjugates was made[12]. Cetyltrimethylammonium bromide (CTAB) decreases the capacity factor of the 17-conjugates relative to the 3-conjugates, and E_1-conjugates relatively more than others. The capacity factors increase with decreasing pH until the pK_a value of the estrogen conjugates is reached. Below these pH values the capacity factors decrease sharply. At pH 5 the relationship between capacity factor and concentration of the liquid anion exchanger follows roughly the equation

$$\kappa_i \propto [\text{CTAB}]^{0.4}$$

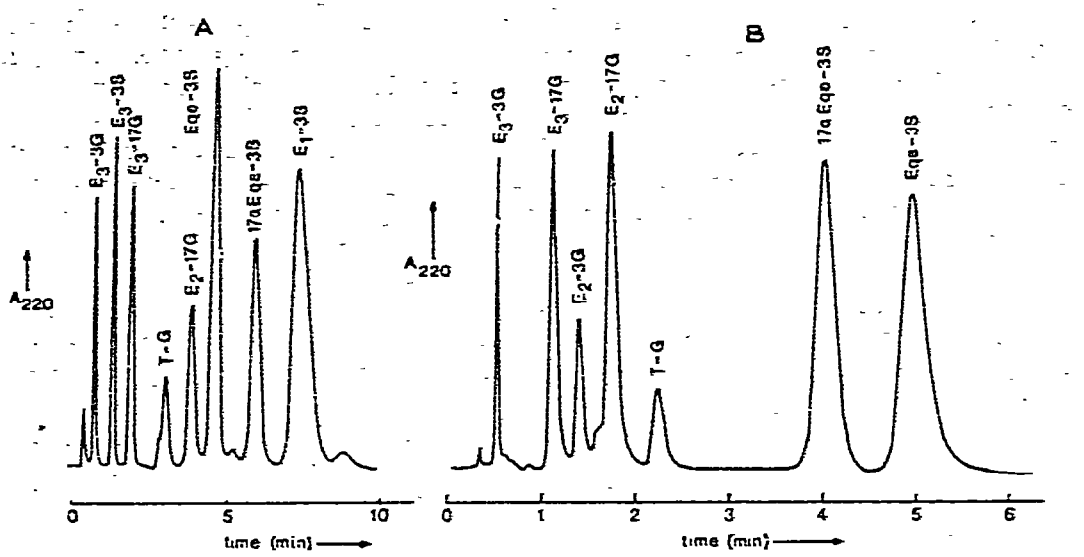


Fig. 2. Chromatograms of estrogen conjugates on dimethylsilica. Column: 150 × 3 mm I.D.; LiChrosorb RP-2, 5 μm; temperature, 70°C; pressure drop, 250 bar. (A) 7% 1-butanol–93% 0.05 *M* phosphate (pH 8.0); (B) 15% acetonitrile–85% 0.05 *M* phosphate (pH 8.0). From ref. 12.

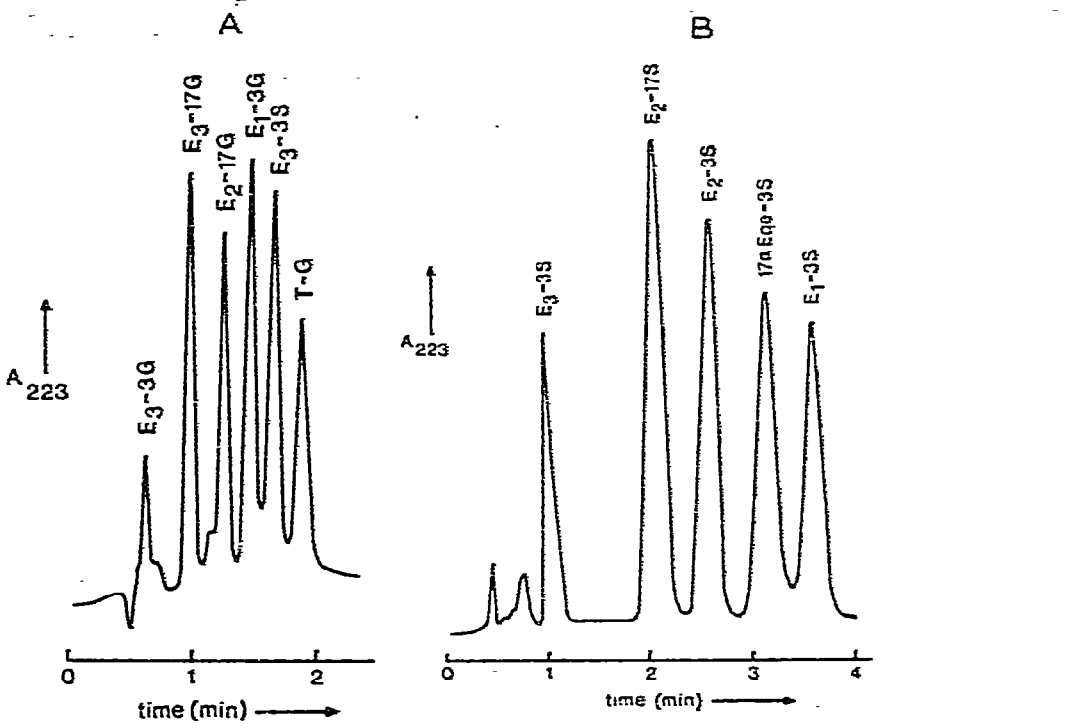


Fig. 3. Rapid separation of estrogen conjugates on octadecylsilica using solvent-induced anion exchange. Column: 150 × 3 mm I.D.; LiChrosorb RP-18, 5 μm; temperature, 70°C; pressure drop, 250 bar. (A) 40% acetonitrile–60% 0.05 *M* phosphate (pH 8.0) + 0.1% CTAB; (B) 70% methanol–30% 0.02 *M* phosphate (pH 5.0) + 0.1% CTAB. From ref. 12.

The modifier concentration must be increased upon addition of CTAB to keep the retention constant. This results in a mobile phase with low viscosity and thus a higher column efficiency at lower hydrodynamic resistance. The ion-exchanger additive determines the capacity factor to a large extent but the modifier retains its influence on the selectivity, which renders this technique even more versatile. Different selectivities are obtained at different temperatures, but a much smaller overall decrease in capacity factor than in non-polar adsorption chromatography is observed, which obviates the need for accurate thermostatting of the columns to obtain good reproducibility[12]. Some rapid separations are shown in Fig. 3.

This mode of separation can also be used with a polar stationary phase and a (relatively) non-polar mobile phase[32]. Although this is less convenient and involves a complex mixed mechanism because the separation implicates intentionally the silanol groups of the stationary phase, it offers a selectivity alternative.

Attempts to separate estrogen conjugates on polar chemically bonded phases on silica using a mixed organic–aqueous eluent were only moderately successful[12]. One problem is the non-linear adsorption isotherm (due to non-homogeneous adsorption site energy distribution), another stripping of the stationary phase from the column at neutral pH. Separations with reasonable efficiency and low selectivity can be performed in almost non-aqueous mobile phases at low pH. The selectivity increases when the organic modifier concentration is decreased or the pH is increased. Decreased organic modifier concentrations give drastically lower efficiencies, and increased pH values induce stripping of the stationary phase. This suggests that novel types of packing material in which not only moderately polar groups are attached to

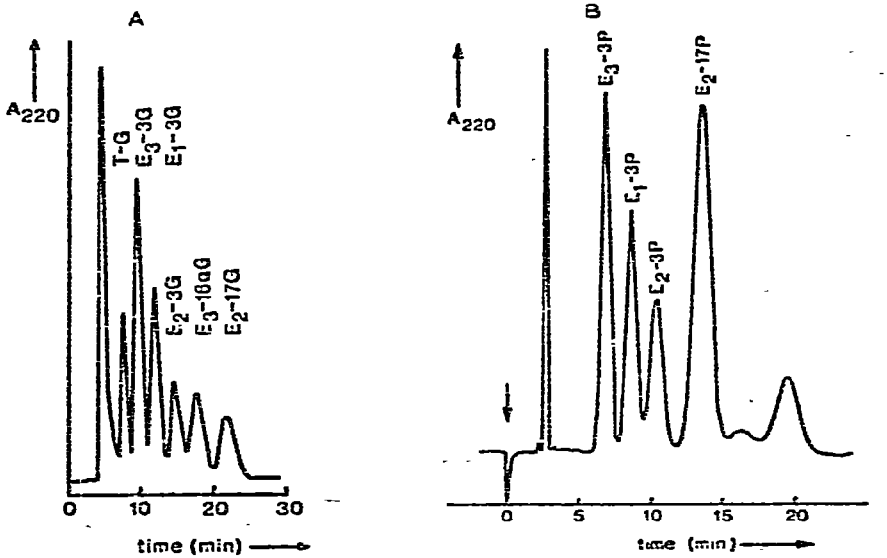


Fig. 4. (A) Separation of an estrogen glucuronide mixture. Phase system: ECTEOLA-cellulose B 300, 13 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate (pH 7.0). Temperature, 25°; pressure, 16 bar. (B) Separation of estrogen monophosphates. Phase system: ECTEOLA-cellulose ET 41, 11 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate (pH 8.5). Temperature, 70°C; pressure, 30 bar. Column: 250 × 3 mm I.D. From ref. 37.

the packing, but also accessible silanol groups are end-capped with trimethylsilane, might give much better results[33].

Separations of estrogen conjugates on Partisil SAX, an anion exchanger chemically bound to alkylsilica, with aqueous mobile phases at pH 4–5 have been described[34]. The selectivity is similar to that with solvent-induced anion-exchange systems (*e.g.*, 17-glucuronides elute before 3-glucuronides, elution order E_3, E_2, E_1), but the columns seems to lose their typical anion-exchange selectivity at the expense of an increase in retention, even during a series of five runs. Large differences in capacity factor, selectivity, peak shape and efficiency within 0.2 pH units stress that the separation mechanism is extremely complicated, possibly involving a gradient during the run (samples were applied in 20% methanol). Anion-exchange resins with a hydrophobic polystyrene–divinylbenzene matrix require an impractically high counter ion concentration[12] and separations will be slow even at an elevated temperature and pressure drop owing to the compressibility of the packing beads. Pellicular and superficially porous anion exchangers have an excellent selectivity but a low column efficiency, loadability and peak capacity[12]. They should be used only for the rapid analysis of simple mixtures that contain no trace components to be measured in addition to the main compound[35].

A type of phase system that has been studied in great detail but is unfortunately not commercially available combines a microparticulate cellulose anion exchanger stationary phase and a neat aqueous buffer mobile phase[36,37]. Its selectivity for estrogen conjugates is very high (see Fig. 4) and the capacity factors obey the equation

$$\kappa_i = \frac{qK_1}{[A^-]_m (1 + K_2[H^+]_m)} \tag{3}$$

where

q	=	mass to volume ratio of stationary and mobile phases;
$[A^-]_m$	=	anion concentration of the counter ion of the anion exchanger in the mobile phase;
$[H^+]_m$	=	hydrogen ion concentration in the mobile phase;
K_1	=	ion-exchange equilibrium constant;
K_2	=	formation constant of the sample salt.

A drawback of these phase systems is their moderate efficiency. An increase in the speed of separation is restricted by the compressibility of the cellulose. The non-availability of a rigid cellulose-like ion exchanger prompted the investigation of mixed-bed anion-exchange cellulose–diatomite columns. Mixed-bed columns containing ECTEOLA-cellulose and diatomite (average particle sizes 7 μm) in a volume ratio of 5:1 were found to be superior in almost every respect to the pure anion-exchange cellulose columns[38]. The order of elution on the cellulose–diatomite mixed-bed columns is similar to that on the less studied polystyrene–divinylbenzene anion-exchange systems but is completely different from that on hydrophobic adsorption and solvent-induced anion-exchange systems (see Fig. 5). An abundance of π-electrons in the steroid moiety causes retention on cellulose–diatomite systems whereas it reduces

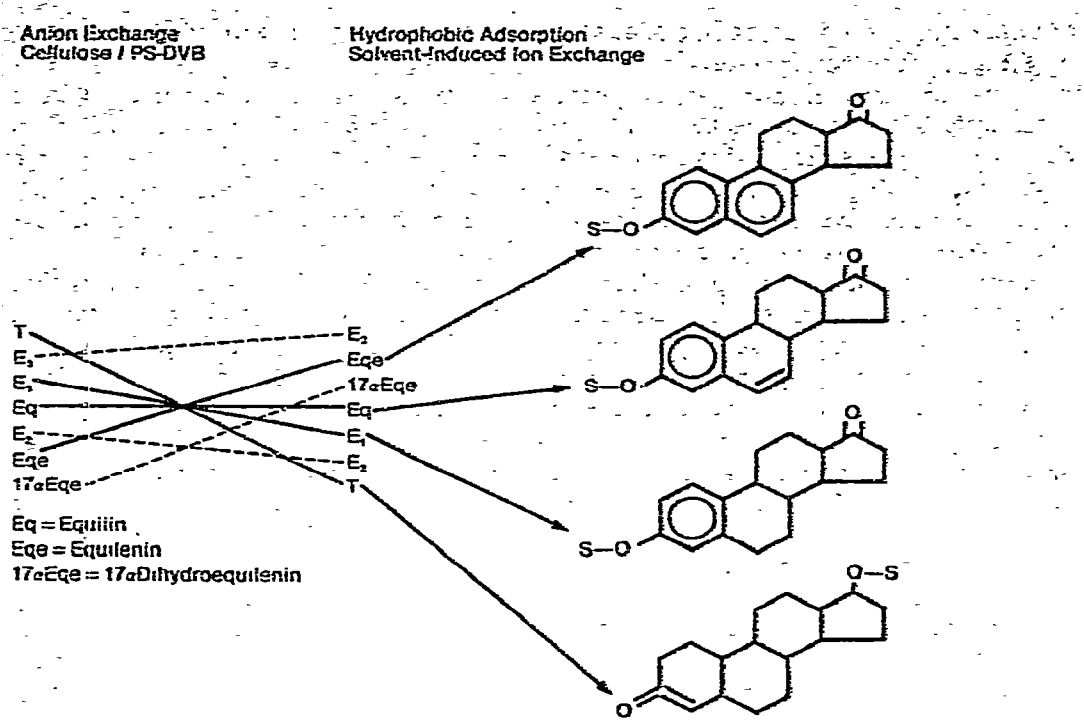


Fig. 5. Comparison of the order of elution of estrogen conjugates in different phase systems[12,36,37].

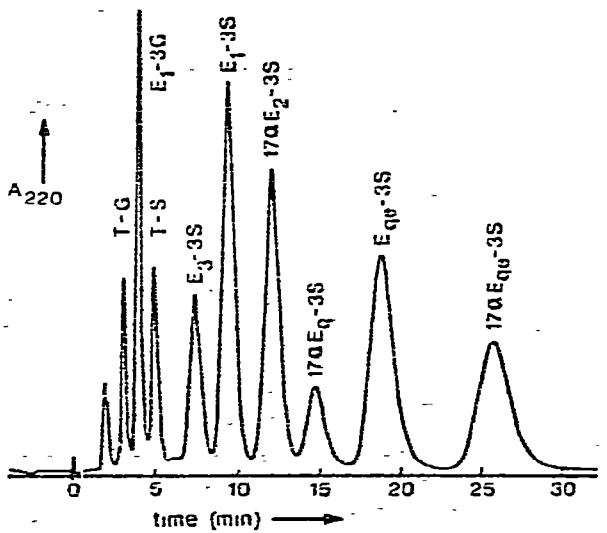


Fig. 6. Chromatogram of a test mixture of steroid conjugates. Column: 100 × 4 mm I.D. ECTEOLA-cellulose: B 300–7 μm diatomite (5:1); 0.025 *M* perchlorate + 0.01 *M* phosphate (pH 6.8). Temperature, 70°C; pressure, 36 bar. From ref. 38.

the capacity factor on non-polar stationary phases (*cf.*, T < E_t < Eq < Eqe). A representative chromatogram is shown in Fig. 6.

Most estrogen conjugates, having an aromatic A-ring, possess a UV absorption maximum at 273–287 nm of $\varepsilon_{M} = 500–3000$ in addition to a much more intense absorbance at 200–220 nm (see Fig. 7).

Electrochemical detection for this type of component is at least as sensitive as and more selective than UV detection at 200 nm[21]. The stability of electrochemical detectors, necessary for routine use, was not investigated.

A specific and sensitive reaction of estrogenic conjugates is the Kober reaction[39]. Although known experimentally for almost 50 years[40], its mechanism is still a mystery[41,42]. The Kober reaction has been applied for the determination of estrogen conjugate profiles in human pregnancy urine in the segmented-flow type of post-column reactor represented schematically in Fig. 8. The influence of reaction coil material and geometry, air to liquid ratio, eluent flow-rate and reaction time on peak

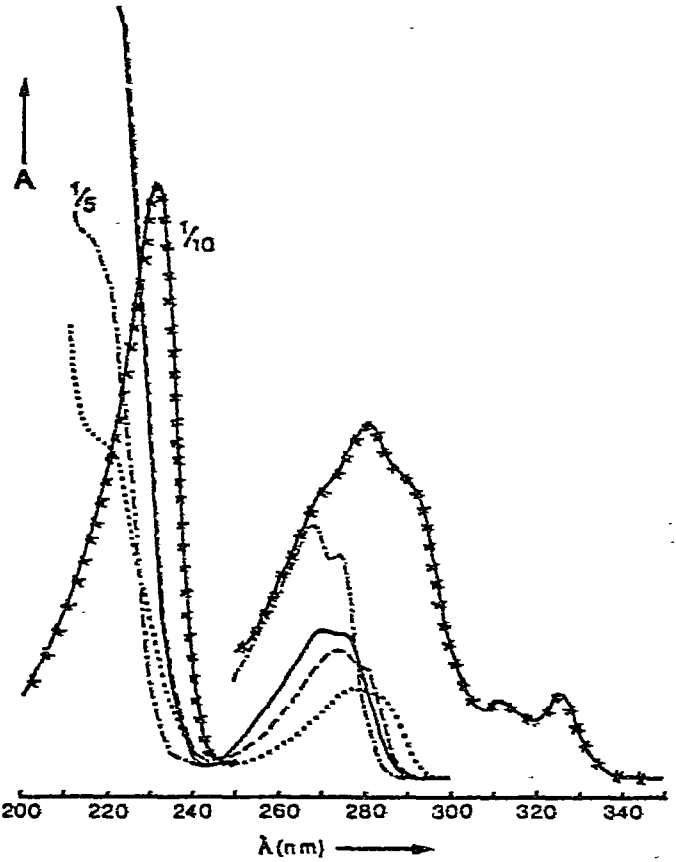


Fig. 7. UV spectra of estrogen conjugates in deionized water. —×—×—×—, 17α-Eqe-3S; ——, E_2-3G; - - -, E_2-3S; ....., E_2-17G; ———, E_2-3P. From ref. 37.

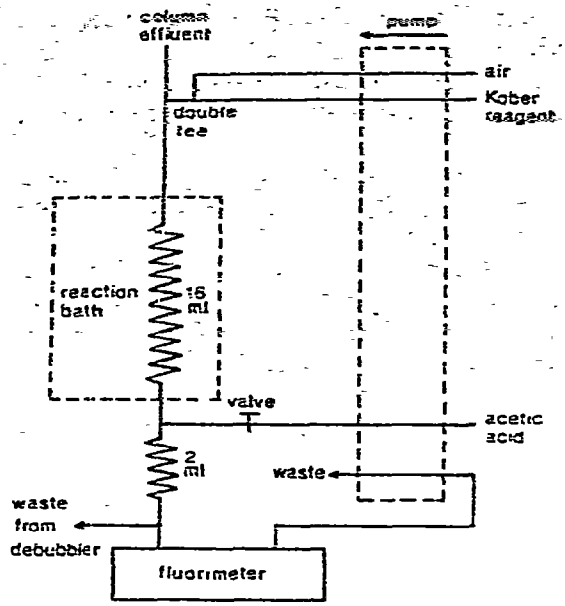


Fig. 8. Schematic diagram of gas-segmented flow reaction detector. From ref. 43.

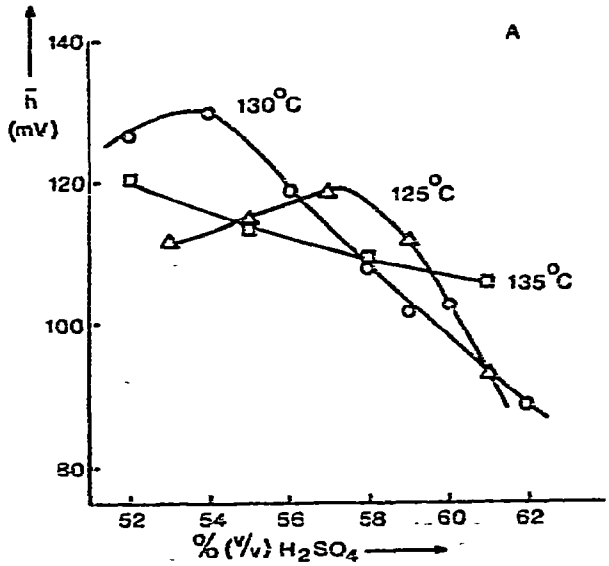


Fig. 9. Optimization of the peak height, $\bar{h}$, as a function of the sulfuric acid concentration and temperature. Reaction tubing, 16 ml; I.D. = 1.4 mm; reaction time = 500 sec. Sample: late pregnancy urine Amberlite XAD-2 extract. From ref. 43.

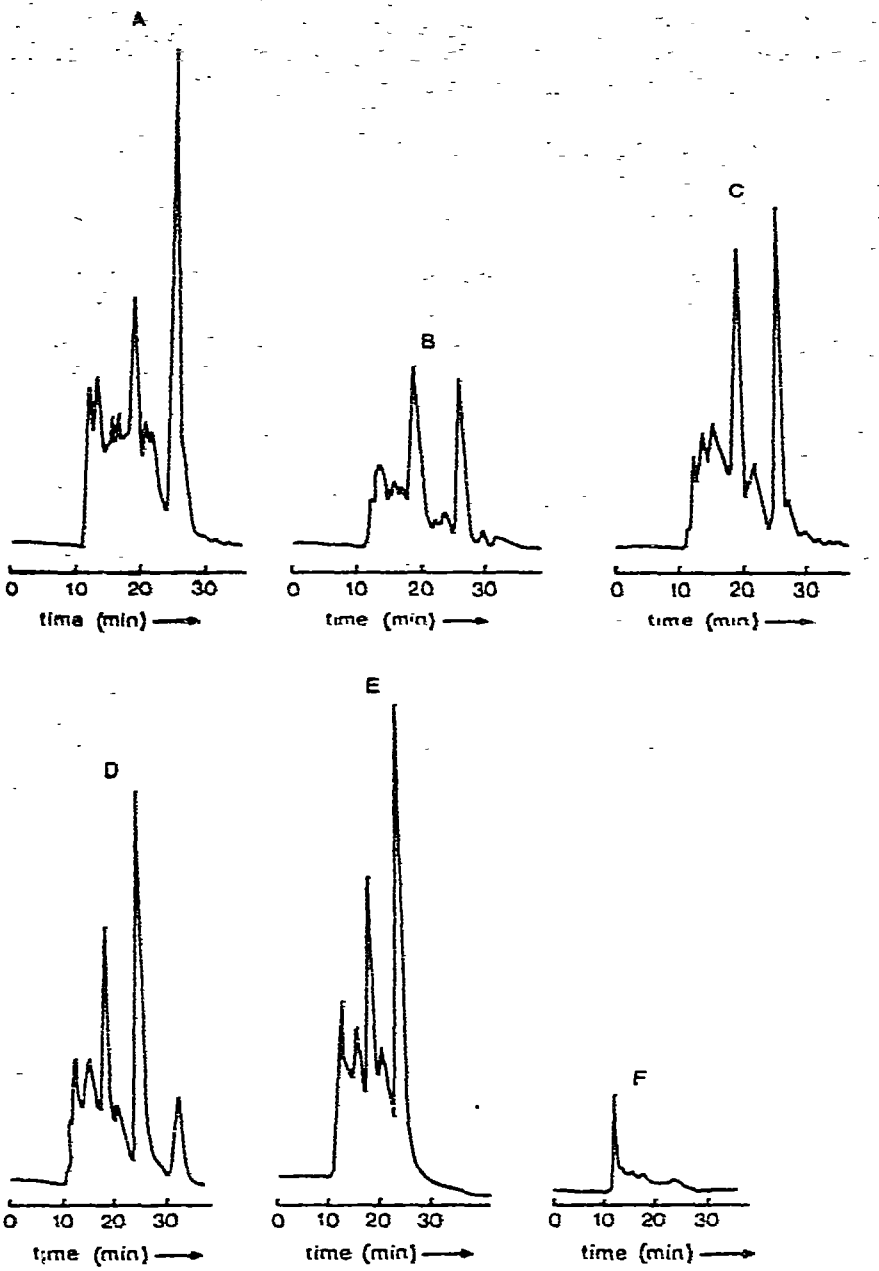


Fig. 10. Chromatograms of estrogen conjugate profiles of different urine specimens. Samples: Amberlite XAD-2 extracts of (A) aliquot of a pool of ten normal pregnancy urines; (B) late pregnancy urine of a patient with a urinary tract infection treated with Clamoxyl; (C) and (D) late pregnancy urine of patients with various disorders; (E) twin pregnancy urine; (F) male urine after a heavy meal. Column: 250 × 3 mm I.D.; packing as in Fig. 6. From ref. 43.

broadening in the detector was investigated and the optimum percentage of sulfuric acid and temperature were determined (Fig. 9). The Kober-positive chromophores in different urine samples give clearly distinctive patterns (see Fig. 10)[43].

3. 17-KETOSTEROID AND CORTICOSTEROID CONJUGATES

Mixtures of several 17-ketosteroid glucuronides and sulfates were analysed with moderate success[19,20].

Korpi *et al.*[44] demonstrated the anion-exchange effect of solvent-induced ion-exchange systems differentially, keeping the retention of hydrocortisone constant at $\kappa = 2.2$ while increasing the capacity factor for hydrocortisone phosphate, by adding a series ammonium salts of increasing hydrophobic chain length to the mobile phase, from $\kappa = 0$ to 19.7.

No accounts of separations of 17-ketosteroid or corticosteroid conjugates in body fluids by HPLC were found in the literature.

Conjugates of 3-hydroxy-Δ4-steroids have a UV adsorption maximum at 232–244 nm of $\varepsilon_M = 1 \cdot 10^4$–$2 \cdot 10^4$.

The Zimmerman reaction (Z) on 3-, 6-, 17- or 20-ketosteroids is used on-line but batchwise; the column eluate is collected in tubes that are stored to allow an extended reaction time[45]. However, this reaction can be accelerated after modification[46].

Specific reactions used for detection are the isonicotinic acid hydrazide (INH) reaction on Δ4-3-ketosteroids[47] and the Porter–Silber (PS) reaction[48] and blue tetrazolium reaction (BT) on 17,21-dihydroxy-20-ketosteroids[49] (see Fig. 11). These reactions employ strongly basic or acidic conditions which hydrolyse the conjugates prior to the reaction proper, and this may slow the reaction[50].

4. CONJUGATED BILE ACIDS

Shaw and Elliott[51] separated bile acid conjugates by HPLC on silica columns in basic systems that limited the lifespan of the columns and in acidic systems that showed tailing for the taurine conjugates because of their strong interaction with the silanol groups on the silica surface.

Bloch and Watkins[17] demonstrated that the concept of relating a solute structural change to a change in selectivity coefficient is valid for conjugated bile acids. An additional hydroxy group in the equatorial position decreases the retention more (see

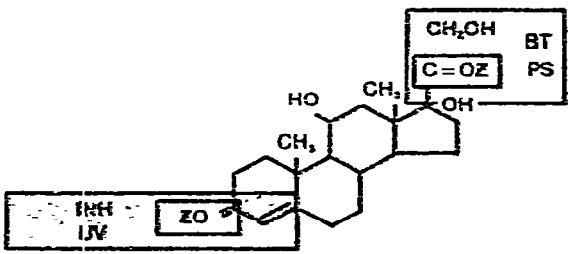
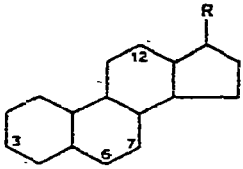


Fig. 11. Different detection methods possible for corticosteroids.

TABLE 2
RELATIONSHIP BETWEEN STRUCTURE AND CHROMATOGRAPHIC RETENTION FOR CONJUGATED BILE ACIDS



Position of hydroxyl group	*Cholate*					
	Oursodeoxy	*Hyodeoxy*	*Litho*	*Deoxy*	*Chenodeoxy*	—
3	α	α	α	α	α	α
6(e)	—	α	—	—	—	—
7	β(e)	—	—	—	α(a)	α(a)
12(a)	—	—	—	α	—	α

Compound	$r^{*}_{j/i}\ \frac{j = glyco}{i = tauro}$		$r_{j/i}$ *(tauro)*	$r_{j/i}$ *(glyco)*
Hyodeoxycholate (HDC)	$\frac{3.5}{1.27} = 2.7$	$\frac{HDC}{ODC} = 1.0$		1.0
Oursodeoxycholate (ODC)	$\frac{3.6}{1.27} = 2.8$	$\frac{C}{ODC} = 1.3$		1.1
Cholate (C)	$\frac{4.1}{1.66} = 2.5$	$\frac{CDC}{C} = 1.6$		1.7
Chenodeoxycholate (CDC)	$\frac{7.1}{2.7} = 2.6$	$\frac{DC}{CDC} = 1.2$		1.2
Deoxycholate (DC)	$\frac{8.7}{3.3} = 2.6$	$\frac{LC}{DC} = 1.8$		1.5
Lithocholate (LC)	$\frac{15.6}{5.04} = 3.1$			

* Values from Shaw *et al.*[52] with the assumption of $\varepsilon_p = 0.65$. Especially for the small r_s the values are not accurate. The data of Sian and Harding Rains[57] confirm this.

Table 2) than a hydroxy group in an axial position. The amino acid moiety-induced selectivity is shown to be independent of the selectivity associated with the number and/or orientation of hydroxy groups of the parent steroid and the selectivity coefficient for two bile acid conjugates which differ in the number or position of hydroxy groups is independent of the taurine or glycine group (see Table 2)[52].

The decreasing dissociation of the glyco-conjugates ($pK_a \approx 5$) with decreasing pH can be used to increase the retention times of these conjugates relative to the tauro-conjugates in order to overcome their interference, while the hydroxy group selectivity stays relatively constant. Parris[53] demonstrated this feature with solvent-induced ion exchange using a UV-absorbing quaternary ammonium compound. At 7.5 bile acids form ion pairs and are retained and detected, at pH 4.7 their tauro- and glyco-conjugates form ion pairs and at pH 3.3 only the tauro-conjugates are formed. induced ion exchange using a UV-absorbing quaternary ammonium compound. At pH 7.5 bile acids form ion pairs and are retained and detected, at pH 4.7 their tauro- and glyco-conjugates form ion pairs and at pH 3.3 only the tauro-conjugate ion pairs are formed.

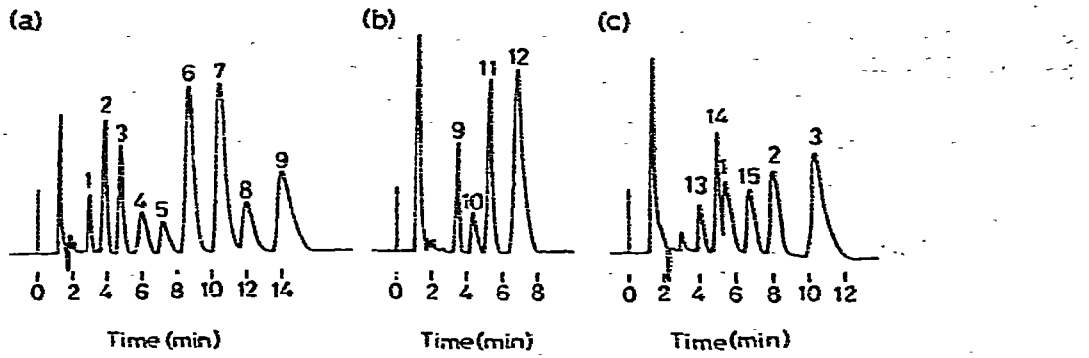


Fig. 12. Separation of a mixture of 3-sulfated bile acids. 1 = Cholate; 2 = glycocholate; 3 = taurocholate; 4 = chenodeoxycholate; 5 = deoxycholate; 6 = glycochenodeoxycholate; 7 = glycodeoxycholate; 8 = taurochenodeoxycholate; 9 = taurodeoxycholate; 10 = lithocholate; 11 = glycolithocholate; 12 = taurolithocholate; 13 = oursodeoxycholate; 14 = glycoursodeoxycholate; 15 = tauroursodeoxycholate. Mobile phase: (a) 0.5% ammonium carbonate–acetonitrile (26:8). 2.1 ml/min; (b) 0.5% ammonium carbonate– acetonitrile (20:8), 2.0 ml/min; (c) 0.04% ammonium carbonate–acetonitrile (36:8), 1.6 ml/min. Column: ODS SC-02 (250 × 4.6 mm I.D.); detection, 205 nm. From ref. 56.

Shimada *et al.*[16] used a mobile phase pH that was too high to separate the tauro-, glyco- and unconjugated bile acids and utilized thin-layer chromatography to separate these groups. They studied the effect of different organic modifiers on the hydroxy group selectivity. Their data are scanty and not completely consistent (*e.g.*, the litho/tauro selectivity ratio for 9:4 buffer–acetonitrile is 0.8, for a 10:4 proportions 0.5 and for 11:4 proportions 1.0), but indicate that increased pH leads to larger selectivity coefficients for a given difference in number of hydroxy groups.

The order of elution in the separation of the glucuronides[54] and sulfates[55] of (conjugated) bile acids on non-polar adsorbents with a low concentration of organic modifier in the mobile phase could be predicted and was recently confirmed experimentally[56] (see Fig. 12).

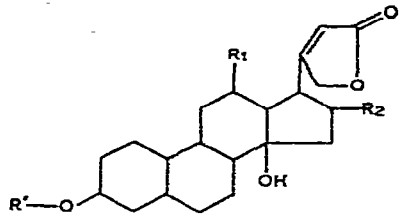
Okuyama *et al.*[28] applied a post-column packed-bed immobilized enzyme reactor for the detection of bile acid conjugates and claimed a detection limit of 10 ng, relative to a limit of detection for UV absorption of 100 ng at 193 nm[57].

5. CARDENOLIDE GLYCOSIDES

Cardenolide glycosides are not steroid conjugates in the narrow sense but extensive HPLC investigations with digitalis glycosides can give some insight in the chromatography of structurally analogous conjugates. The following elution order with a non-polar adsorbent of the digitalis aglycones is expected on the basis of the number and position of hydroxy groups: diginatigenin, digoxigenin, gitoxigenin, gitaloxigenin and digitoxigenin[13] (see Table 3). The same elution order is seen for the respective glycosides and lanatosides[13], with selectivities that are characteristic of the number and site of the hydroxy groups; a selectivity coefficient due to the tridigitoxose group or the lanatoside side-chain is hard to attribute when acetonitrile is used as a modifier, but is clear when dioxan is employed (see Table 3). From Table 3 it can also be concluded that it is unlikely that the gitoxin capacity factor is correct in the THF-containing phase system. As discussed by the authors, lanatoside E is converted

TABLE 3

CORRELATION OF CHROMATOGRAPHIC SELECTIVITY WITH STRUCTURE OF DIGITALIS GLYCOSIDES[12]



R_1	R_2	R'*					Series
		H	DDD	G Ac DDD	G DDD	Ac DDD	
H	H	Digitoxigenin	Digitoxin	Lanatoside A	Purpurea glucoside A	Acetyl-digitoxin	A
H	OH	Gitoxigenin	Gitoxin	Lanatoside B			B
OH	H	Digoxigenin	Digoxin	Lanatoside C	Desacetyl-lanatoside C	Acetyl-digoxin	C
OH	OH	Diginatigenin	Diginatin	Lanatoside D			D
H		Gitaloxigenin	Gitaloxin	Lanatoside E	Gluco-gitaloxin		E

* G = Glucose; D = digitoxose; Ac = acetyl.

Series	37% acetonitrile		45% dioxan		67% THF–dioxan (2:1)	
	Y**	Z**	Y**	Z**	Y**	Z**
D	1.3	0.9	4.7	4.1	1.8	1.8
C	1.5	1.0	4.7	3.9	1.5	1.7
B	1.8	1.2	4.1	3.5	1.9	2.2
E	2.1	0.8	4.2	2.2	3.0	1.7
A	1.9	1.2	4.3	3.5	2.1	2.5

r_{ij}	Aglycone	Glycoside	Lanatoside	Agly	Glyc	Lan	Agly	Glyc	Lan
C/D	1.5	1.8	1.6	1.8	1.8	1.7	1.8	1.5	1.7
B/C	2.0	2.5	2.4	3.7	3.2	3.1	1.9	2.5	2.5
E/B	1.5	1.7	1.8	1.6	1.6	1.8	1.2	1.9	0.9
A/E	1.8	1.6	2.6	1.5	1.5	2.4	1.4	1.8	2.1
A/B	2.7	2.8	2.8	2.3	2.4	2.3	1.7	1.8	1.9

** $Y = \frac{\kappa \text{ glycoside}}{\kappa \text{ aglycone}}$; $Z = \frac{\kappa \text{ lanatoside}}{\kappa \text{ aglycone}}$

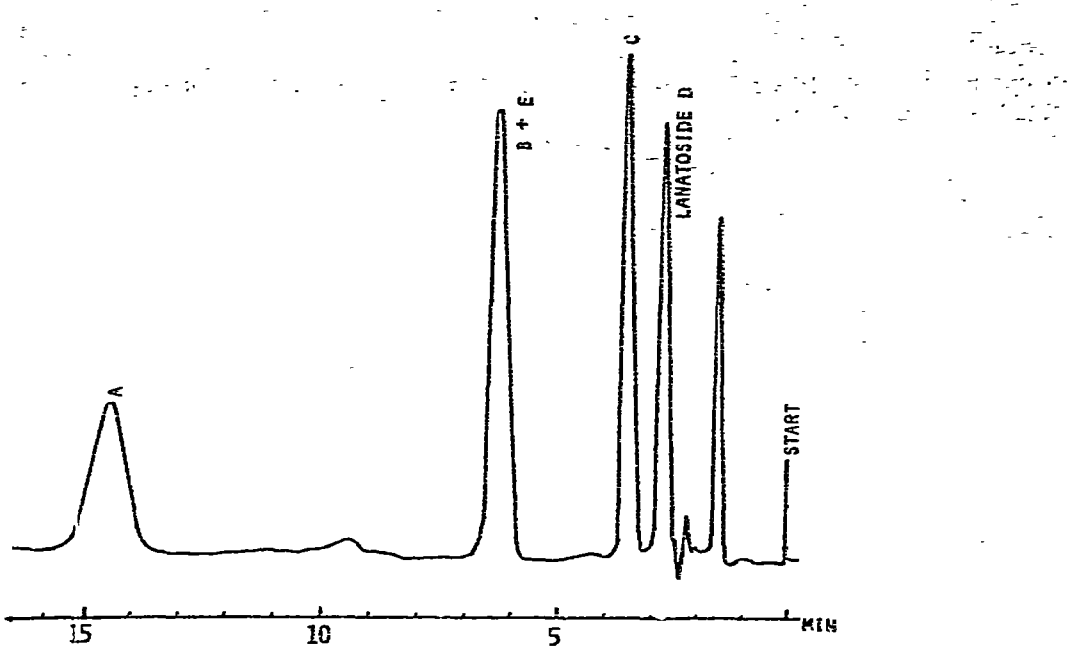


Fig. 13. Separation of lanatosides. Column, Nucleosil C18 (300 × 3.5 mm I.D.); mobile phase, 37% acetonitrile in water at 1.4 ml/min (pressure 100 bar); injection, 25 μl loop; detection wavelength, 220 nm. From ref. 13.

to lanatoside B and there is some doubt about the identity of the sample. When we consider the analogous aglycones and glycosides in Table 3, it becomes clear that the capacity factor of lanatoside E should be 4.6–5.3 with the 37% acetonitrile-containing mobile phase, corresponding to a retention time of approximately 9 min in Fig. 13, at which small peak is observed.

Silica was also used as a solid support in the liquid–liquid chromatographic separation of digitalis glycosides[58]. Chromatography with a non-polar mobile phase and a polar stationary phase with polar digitalis glycosides shows no major advantages over chromatography with a polar mobile and a non-polar stationary phase[13]. It is the method used, however, after the polarity of these compounds has been lowered by pre-column derivatization to 4-nitrobenzoates[39].

6. BUFOGENIN CONJUGATES

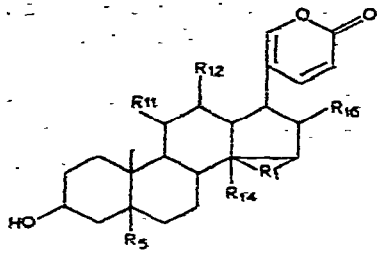
The order of elution of bufotoxins on an octadecylsilica column eluted with a methanol-containing mobile phase is as shown in Table 4 and is in full agreement with the decreasing number and polarity of the groups on the steroid moiety: –H > =O > –OAc[16]. An example is shown in Fig. 14. The 3-sulfates behave analogously to the bufotoxins (3-suberoylarginines).

7. CHOICE OF TYPE OF COLUMN

The choice of a chromatographic phase system is strongly dependent on the composition of the sample because the order of elution of the components varies in

TABLE 4

STRUCTURE OF BUFOGENINES



Compound	R_1	R_{14}	R_{11}	R_{16}	R_{12}	R_5
Gamabufotalin	–	OH	OH	H	H	H
Cinobufotalin	O	–	H	–O Ac	H	OH
Arenobufagin	–	OH	H	H	=O	H
Bufotalin	–	OH	H	–O Ac	H	H
Bufalin	–	OH	H	H	H	H
Cinobufagin	O	–	H	–O Ac	H	H
Resibufogenin	O	–	H	H	H	H

the different phase systems (*cf.*, Fig. 5). The final choice of a phase system for the chromatographic separation of steroid conjugates depends not only on selectivity and column efficiency but also on load capacity, stability, convenience and commercial availability of reproducible column packings.

Polar chemically bonded stationary phases on microparticulate silica lack, depending on the mobile phase, either stability or selectivity. A simple mixture containing no trace components might be separable on a pellicular or superficially porous anion exchanger.

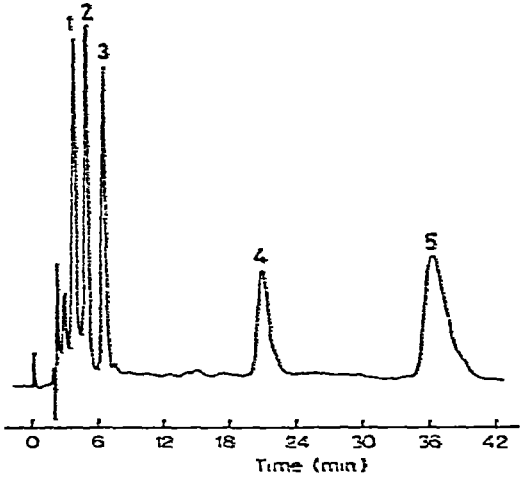


Fig. 14. Separation of bufogenin 3-sulfates. 1, Sarmentogenin 3-sulfate; 2, gamabufotalin 3-sulfate; 3, arenobufagin 3-sulfate; 4, bufotalin 3-sulfate; 5, bufalin 3-sulfate. Column, μBondapak C_{18}; eluent, methanol–0.03 *M* $NH_4H_2PO_4$ (2:3); flow-rate, 1.5 ml/min; detection, 254 nm. From ref. 16.

Non-polar adsorption chromatographic systems with an aqueous mobile phase offer advantages also with respect to sample preparation and often detection over adsorption or partition chromatography on silica with a mobile phase of which the composition of the mobile phase as well as the temperature of the phase system have to be carefully monitored.

The commercial non-availability of microparticulate ECTEOLA-cellulose and the skill required to pack and maintain mixed-bed cellulose–diatomite columns make this phase system an unlikely choice, although the speed of separation is generally equivalent to that of the best hydrophobic adsorption systems. This is demonstrated with a comparison of the speed of separation of five estrogen sulphates present in pregnant mare's urine (Table 5). Because of its selectivity, this type of column could be interesting for use in multi-stage chromatography.

Solvent-induced anion-exchange systems offer retention that can be predictably varied by means of the concentration of the anion exchanger and organic modifier, a selectivity that can be widely changed through the type of organic modifier used and a column efficiency, load capacity and stability that are equal to or higher than those in hydrophobic adsorption systems.

8. CHOICE OF DETECTOR

It can be concluded that at low wavelength (<210 nm), UV detection of steroid conjugates is sensitive but non-selective. Some classes of steroid conjugates

TABLE 5

COMPARISON OF THE SPEED OF SEPARATION OF A MIXTURE OF ESTROGEN SULPHATES ON DIFFERENT CHROMATOGRAPHIC PHASE SYSTEMS[12]

Phase systems:

A LiChrosorb RP-8, 5 μm; 40% methanol–60% 0.07 *M* phosphate (pH 4.5) + 0.1 *M* trimethylamine; 25°C.

B LiChrosorb RP-2, 5 μm; 2% dichloromethane–28% methanol–70% 0.05 *M* phosphate (pH 8.0); 25°C.

C LiChrosorb RP-2, 5 μm; 2% chloroform–35% methanol–63% 0.05 *M* phosphate (pH 8.0); 70°C.

D LiChrosorb RP-18, 5 μm; 20% acetonitrile–80% 0.05 *M* phosphate (pH 8.0); 70°C.

E ECTEOLA-cellulose B 300, 19 μm; 0.10 *M* $NaHSO_4$–0.01 *M* phosphate (pH 6.8); 70°C.

F ECTEOLA-cellulose B 300, 19 μm; 0.025 *M* perchlorate–0.01 *M* phosphate (pH 8.4); 70°C.

	k_1'					
	A	*B*	*C*	*D*	*E*	*F*
Eqe-S	19.2	5.1	7.5	14.1	6.58	2.67
17α-Eqe-S	12.0	2.4	8.4	10.5	9.97	3.92
Eq-S	20.6	5.5	9.3	15.3	3.67	1.53
E_1-S	38	7.2	10.3	19.2	2.84	1.12
E_2-3S	16.1	3.3	12.3	12.0	4.72	1.99
$(r_{\beta})_{min}$	1.19	1.08	1.11	1.09	1.29	1.30
$(H/u)_{min}$ (sec)	0.03	0.02	0.04	0.03	0.2	0.2
$t_{R_n}^{(4)}$* (sec)	580	590	880	1370	760	480

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

can be detected at higher wavelengths with lower sensitivity. For estrogen conjugates electrochemical detection is the method of choice. Direct fluorimetric detection is limited to a few steroid conjugates. Adding a detectable ion-pairing or complex-forming agent to the mobile phase before or after the separation can improve detection. In general, on-line post-column reaction detection of steroid conjugates should be selective and sensitive, but is not yet optimized. The use of immobilized enzymes in detection is expected to increase dramatically.

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