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SEPARATION OF ESTROGEN GLUCURONIDES, SULPHATES AND PHOSPHATES ON ION-EXCHANGE CELLULOSE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

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SUMMARY

In continuation of previous work, the selectivity of anion-exchange cellulose systems with respect to thirty estrogen conjugates was studied. The influence of the type and particle size of cellulose anion exchangers, the type and concentration of the counter anion, pH and temperature was investigated. On the basis of the results the optimal choice of the ion-exchange material, the eluent and the operating conditions is demonstrated.

The results of the optimization are exemplified by a number of chromatograms showing rapid separations, including the determination of the estrogen conjugate profile of human pregnancy urine.

INTRODUCTION

Estrogen conjugates are major components of the urinary steroid hormones found during pregnancy and enable us to assess the physical condition of the foetus[1]. An increase in the excretion of estrogens during pregnancy has been known for some time, but their routine measurement has been achieved only after hydrolysis followed by extraction and chromatographic separation, a procedure which destroys the identity of the individual conjugates and is time consuming and subject to interference from other materials[2]. Among other chromatographic techniques, high-pressure liquid chromatography has been applied[3].

The alternative approach is the direct assay of the conjugates themselves after extraction from the urine. As estrogen conjugates are not sufficiently volatile to allow their direct gas chromatographic separation, high-performance anion-exchange chromatography was chosen for their final separation.

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Polystyrene–divinylbenzene resins have been found to be unsuitable for the separation of steroid conjugates because of the strong interaction of their matrix with the steroid skeleton[4]. Pellicular (superficially porous) anion exchangers combine an inferior selectivity with a moderate efficiency for estrogen glucuronides[4] compared with results obtained with cellulose anion exchangers. The efficiency of such cellulose columns has been dealt with previously[5]. In this paper, the selectivity of these anion exchangers for estrogen conjugates will be discussed.

EXPERIMENTAL

Apparatus

A high-pressure liquid chromatograph (Hewlett-Packard Model 1010 A) equipped with stainless-steel columns (250 × 3 mm I.D.) was used for the experiments with test mixtures. The detector was a variable-wavelength UV spectrophotometer (Hewlett-Packard 1030 B). The chromatograms were recorded with a linear potentiometric flat-bed recorder (Servogor RE 514.9).

For the analysis of extracts from pregnancy urine, a syringe-type pump (Perkin-Elmer 087-0201), a high-pressure sampling valve (Valco CV-6-UHPa-C20), thermostatted stainless-steel columns (250 × 3 mm) and a gas-segmented-flow reaction detector (Fig. 1), assembled from commercial parts, were used. Air, water and acetic acid were pumped by a 24-channel tubing pump (Cenco, Breda, The Netherlands), the oil-bath (Cenco, 34811) was equipped with a contact thermometer (0–250°) and the fluorocolorimeter (Aminco 4-7440) had a 535-nm narrow bandpass excitation filter and a 561-nm emission filter (Balzer B40). Both displacement bottles contained a 3-cm layer of 1,2-dichloroethane between the water and the acidic solution of higher density in order to prevent highly exothermic mixing, which might cause an explosion in the displacement bottle.

The columns were packed using a pressurized slurry technique, except for Cellex GE, which was dry-packed with small portions of material[6].

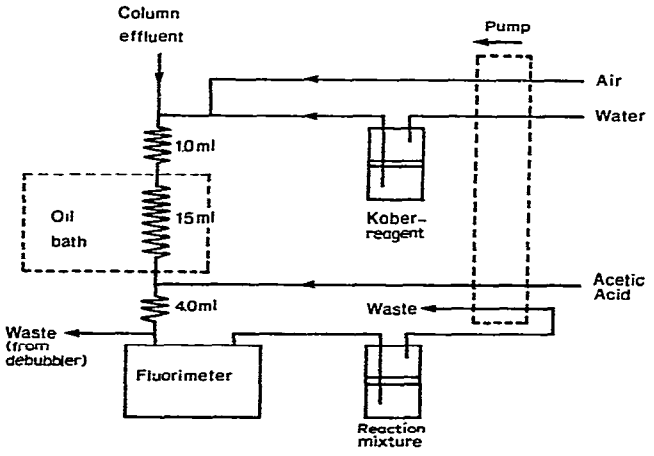


Fig. 1. Schematic diagram of the gas-segmented-flow reaction detector.

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), 17β-estradiol 17-β-D-glucuronide (E_2-17G), 17β-estradiol 3-sulphate-17-β-D-glucuronide (E_2-3S17G), 17β-estradiol 3-β-D-glucuronide-17-sulphate (E_3-3G17S) and estriol 3,16α,17β-trisulphate (E_3-triS), 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), 17β-estradiol 3,17-β-D-diglucuronide (E_2-diG), 17β-estradiol 3,17-diphosphate (E_2-diP), estriol 3,17-disulphate (E_3-3,17diS), estriol 16,17-disulphate (E_3-16,17diS) and 17β-estradiol 3,17-disulphate (E_2-3,17diS), all obtained from Steraloids (Pawling, N.Y., 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).

Some characteristic spectra, made with a scanning UV spectrophotometer (Beckman Acta M VI), are shown in Fig. 2. Ring D conjugates gave very similar spectra irrespective of the steroid aglycone or conjugating group; only the relative absorptivities differed. The same holds for ring A conjugates. The molar absorptivity

Fig. 2. UV spectra of estrogen conjugates in deionized water. –V–V–V–, 17α Eqe-3S; — — —, E_2-3G; —·—·—, E_2-3S; ·····, E_2-17G; ———, E_2-3P.

at 275 nm is at least 10^3. T-G and T-S show a maximum absorption at 248 nm, while Eqe-3S and 17αEqe-3S have a complex spectrum (see Fig. 2).

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

The methanolic XAD-2 extract of human pregnancy urine was kindly supplied by F. J. Gerhartl (Gynaecological Laboratory, University of Nijmegen, The Netherlands).

Column materials

All chemicals were of pro analysi quality (Merck, Darmstadt, G.F.R.). Eluent solutions were prepared with deionized water. The specified pH was adjusted with either orthophosphoric acid or sodium hydroxide.

Five types of anion-exchange celluloses were used as column packings:

(i) polyethyleneiminecellulose: Cellex PEI (Bio-Rad Labs., Richmond, Calif., U.S.A.), ion-exchange capacity (i.e.c.) 0.2 mequiv./g;

(ii) aminoethylcellulose: Cellex AE (Bio-Rad), i.e.c. 0.37 mequiv./g;

(iii) triethylaminoethylcellulose: Cellex T (Bio-Rad), i.e.c. 0.5 mequiv./g;

(iv) guanidoethylcellulose: Cellex GE (Bio-Rad), i.e.c. 0.9 mequiv./g; and

(v) ECTEOLA-cellulose: Cellex E (Bio-Rad), i.e.c. 0.3 mequiv./g; Whatman ET 41 (Whatman Ltd., Maidstone, Great Britain), i.e.c. not specified; and B 300 (Baker, Deventer, The Netherlands), i.e.c. 0.35 mequiv./g.

The cellulose ion exchangers were swelled by pre-cycling with 0.5 *N* sodium hydroxide solution, followed by 0.5 *N* hydrochloric acid and 0.5 *N* sodium hydroxide solution and finally rinsed with deionized water. Except for Cellex GE, the packing materials were fractionated with an air classifier (Alpine Model 100 MZR) and the particle size distributions were determined with a Coulter Counter (Model D) (see Table I).

TABLE I

PARTICLE SIZES AND ION-EXCHANGE CAPACITIES OF ION-EXCHANGE CELLULOSES USED AS COLUMN PACKINGS

Anion exchanger	*Particle diameter range, 10–90% (μm)*	*Mean particle diameter (μm)*	*Ion-exchange capacity (mequiv./g)*
Cellex PEI	7–17	11	0.21
Cellex AE	10–19	14	0.29
Cellex T	11–19	14	0.15
Cellex GE	—	—	—
B 300	12–27	19	0.27
	9–19	13	
	8–16	11	0.15
ET 41	6–16	11	0.15
Cellex E	7–16	11	0.17

The actual ion-exchange capacities were determined as the buffer capacity between pH 10 and 4 in 0.5 *N* sodium chloride solution (Table I) by means of an automatic titrator (Radiometer TTT1).

RESULTS AND DISCUSSION

The choice of a phase system for a chromatographic separation is discussed on the basis of the equation for the resolution, R_{ji}, of two successive components, j and i:

$$R_{ji} = (r_{ji} - 1) \cdot \frac{\kappa_i}{1 + \kappa_i} \cdot (N_i)^{\frac{1}{2}} \tag{1}$$

where

r_{ji} = selectivity coefficient = ratio of the capacity ratios of the components j and i in the phase system;
κ_i = capacity ratio of component i;
$N_i = L/H_i$ = number of theoretical plates for component i;
L = length of the column;
H_i = theoretical plate height for component i.

In a first approach, phase systems are pre-selected for which the values of the factor $(r_{ji} - 1) \cdot [\kappa_i/(1 + \kappa_i)]$ for each pair of successively eluted components of the sample are large, with the restriction that extremely high capacity ratios and therefore separation times should be avoided. The final choice of the appropriate phase system is made by taking into account the column efficiency attainable.

Column selectivity

The selectivity coefficients were calculated from the capacity ratios, which were determined from the retention time, t_{Ri}, of component i and the hold-up time, t_{R0}, of the eluent:

$$\kappa_i = \frac{t_{Ri} - t_{R0}}{t_{R0}} \tag{2}$$

The hold-up time was measured by means of an inert tracer (cytosine). Capacity ratios were determined in triplicate, the standard deviation being about 1%. The capacity ratio of a component i is given by its distribution coefficient, K_i, and the ratio, q, of the amounts of the stationary and the mobile phases:

$$\kappa_i = K_i q \tag{3}$$

Regarding the ion-exchange equilibrium of the anion X^- and the dissociation equilibrium of the protonated form HX, an expression for the overall distribution coefficient, K_X, of component X can be derived:

$$K_X = \frac{[X^-]_s}{[X^-]_m + [HX]_m} = \frac{K_1}{[A^-]_m (1 + K_2 [H^+]_m)} \tag{4}$$

where

$[X^-]_s$ = anion concentration of the sample component in the anion exchanger;
$[X^-]_m$ = anion concentration of the sample component in the mobile phase;

$[HX]_m$ = concentration of the undissociated sample component in the mobile phase;
$[A^-]_m$ = anion concentration of the counter ion of the ion 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 HX.

From eqns. 3 and 4, it can be seen that, for a given sample anion, the capacity ratio depends on the nature of the fixed ionogenic group, the ion-exchange capacity, the nature of the matrix of the ion exchanger, the type and concentration of the eluent anion, the pH of the eluent and the temperature. The dependence of the capacity ratio on most of the parameters of eqn. 4 has been verified experimentally.

It was found for estrogen glucuronides that the elution order is determined primarily by the intrinsic nature of the sample components[5]. The regularities in elution behaviour appear to be applicable also to other estrogen conjugates. The site of conjugation in the estrogen conjugates is more important than the type of steroid. Ring

TABLE II

INFLUENCE OF THE TYPE OF CELLULOSE ANION EXCHANGER ON THE CAPACITY RATIO

Eluent: perchlorate + 0.01 *M* phosphate, pH 6.8.

ECTEOLA-cellulose (0.025 M ClO_4^-, 70°)		*Polyethyleneimine-cellulose (0.100 M ClO_4^-, 50°)*		*Aminoethylcellulose (0.025 M ClO_4^-, 70°)*		*Triethylaminoethyl-cellulose (0.100 M ClO_4^-, 70°)*		*Guanidoethyl-cellulose (0.250 M ClO_4^-, 75°)*	
n	κ_n	*n*	κ_n	*n*	κ_n	*n*	κ_n	*n*	κ_n
T-G	0.54	E_3-3G	0.42	T-G	0.55	T-G	0.82	E_3-3G	1.57
E_3-3G	0.76	T-G	0.46	E_3-3G	0.73	E_3-3G	1.01	T-G	1.59
E_1-3G	1.03	E_3-17G	1.03	E_1-3G	0.75	E_1-3G	1.49	E_1-3G	2.09
E_1-3P	1.24	E_3-16G	1.16	T-S	0.90	E_2-3G	1.76	E_3-17G	2.40
T-S	1.57	E_1-3G	1.18	E_2-3G	1.05	E_1-3P	1.94	T-S	2.48
E_2-3G	1.59	E_2-3G	1.34	E_3-3S	1.45	E_3-17G	2.05	E_2-3G	2.60
E_2-3P	1.86	E_3-3S	1.42	E_1-3S	1.49	E_2-diG	2.16	E_3-16G	2.69
E_3-17G	1.98	T-S	1.69	E_3-17G	1.63	E_3-16G	2.17	E_3-3S	3.09
E_3-16G	2.14	E_2-17G	1.75	E_2-17G	1.72	E_2-diP	2.31	E_2-17G	3.43
E_2-diG	2.39	E_2-diG	1.85	17αE_2-3S	1.80	E_2-3P	2.32	E_1-3S	3.92
E_2-17G	2.68	E_1-3S	4.65	E_3-16G	1.82	T-S	2.37	17αE_2-3S	4.20
E_3-3S	3.13	Eq-3S	4.85	Eq-3S	1.90	E_2-17G	2.56	Eq-3S	4.50
E_2-17P	3.27	17αE_2-3S	4.97	E_2-3S	2.08	E_2-17P	3.07	E_2-3S	4.94
E_1-3S	3.99	E_2-3S17G	4.97	17αEq-3S	2.49	E_3-3S	3.84	E_2-diG	5.01
Eq-3S	5.13	E_2-3S	5.26	E_2-diG	2.74	E_1-3S	5.64	17αEq-3S	5.32
E_2-diP	5.20	17αEq-3S	5.65	Eqe-3S	2.94	17αE_2-3S	6.02	E_2-17S	5.70
17αE_2-3S	5.53	E_2-17S	6.04	E_2-17S	3.04	E_2-3S	6.77	Eqe-3S	6.91
E_2-3S	6.06	E_3-3,17diS	6.70	17αEqe-3S	3.56	Eq-3S	6.82	17αEqe-3S	7.88
17αEq-3S	6.98	E_2-17P	6.74	E_2-3S17G	4.97	E_2-17S	7.38	E_2-3S17G	9.17
E_2-17S	7.04	E_1-3P	7.95	E_2-17P	5.25	E_2-3S17G	7.47	E_3-3,17diS	9.86
E_2-3S17G	7.09	E_2-diP	9.04	E_1-3P	6.46	17αEq-3S	7.78	E_2-diP	13.5
Eqe-3S	9.04	E_2-3P	9.17	E_3-3,17diS	6.97	Eqe-3S	12.4	E_3-16,17diS	14.1
E_3-3,17diS	10.9	Eqe-3S	9.23	E_2-diP	8.51	E_3-3,17diS	13.75	E_2-3,17diS	14.35
17αEqe-3S	13.25	17αEqe-3S	10.75	E_2-3,17diS	8.51	17αEqe-3S	13.9	E_1-3P	16.0
E_3-16,17diS	16.3	E_2-3,17diS	12.9	E_2-3P	8.71	E_3-16.17diS	18.1	E_2-diP	19.2
E_2-3,17diS	16.8	E_3-16,17diS	16.15	E_3-16,17diS	13.0	E_2-3,17diS	20.65	E_2-3P	19.6

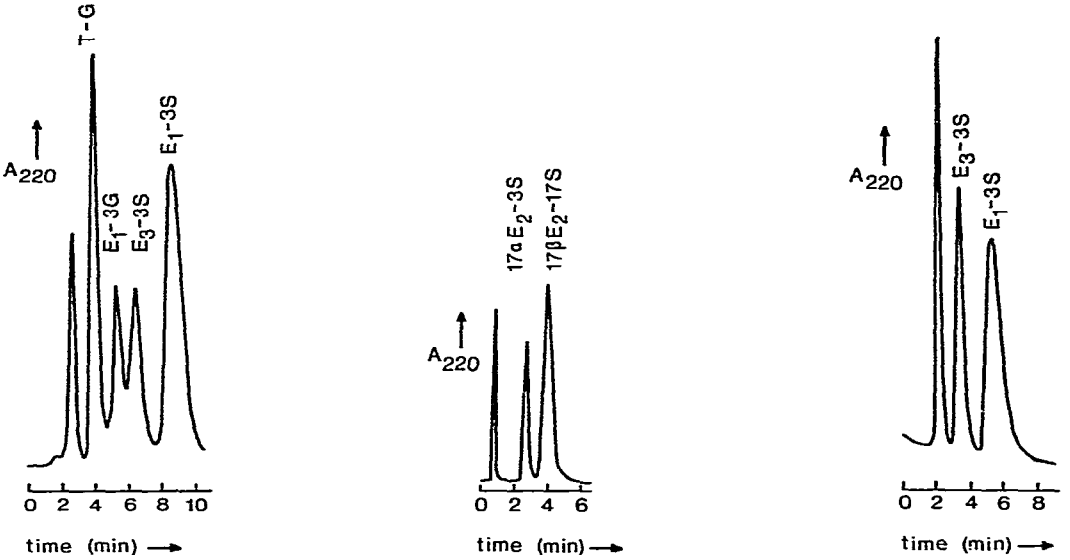


Fig. 3. Separation of four steroid conjugates on Cellex T, 14 μm. Eluent: 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 6.8. Temperature: 75°.

Fig. 4. Separation of two estrogen sulphates on Cellex AE, 14 μm. Eluent: 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8. Temperature: 70°.

Fig. 5. Separation of estriol 3-sulphate and estrone 3-sulphate on Cellex PEI, 11 μm. Eluent: 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5. Temperature: 50°.

A conjugates are eluted first, than ring D conjugates; the elution order according to the type of steroid is estriol, estrone, equilin, estradiol, 17α-dihydroequilin, equilenin and 17α-dihydroequilenin. Of corresponding compounds, the glucuronides are usually eluted in front of the phosphates, while the sulphates have the largest capacity ratios.

Influence of the nature of the stationary phase. Only a few reversals in elution sequence are caused by the type of cellulose anion exchanger (Table II). Estrogen phosphates are relatively more retarded on aminoethyl- and polyethyleneiminecellulose and less on ECTEOLA- and triethylaminoethylcellulose. Equilin and equilenin sulphates have relatively high capacity ratios on triethylaminoethylcellulose.

Chromatograms of some test mixtures are shown in Figs. 3 and 4. The large selectivity of polyethyleneiminecellulose in the separation of estriol 3-conjugates and estrone 3-conjugates is illustrated in Fig. 5.

Variation in the mean particle size ($\bar{d}_p$) of the anion-exchange cellulose fractions has only a slight effect on the capacity ratio, and variation in brand has a more pronounced effect (see Table III).

From Table IV, it can be seen that under the same experimental circumstances with the same material it is possible to make columns that are highly reproducible with respect to their retention behaviour. An increase in the pressure used during the packing procedure beyond a certain limit results in an increase in the capacity ratio due to an increased phase ratio, the selectivity coefficient remaining constant.

Effect of eluent composition. For ECTEOLA-cellulose, the type of eluent anion and cation has been varied, resulting in minor selectivity changes (see Table V). A

TABLE III

DEPENDENCE OF THE CAPACITY RATIO (κ_n) ON THE BATCH AND PARTICLE SIZE OF ECTEOLA-CELLULOSE

(B 300, 19 μm and 13 μm, are two fractions from the same batch, separately regenerated). Eluent: 0.025 *M* perchlorate + 0.0125 *M* phosphate, pH 7.0; 70°.

n	*ET 41* ($\bar{d}_p$ = 11 μm)	*Cellex E* ($\bar{d}_p$ = 11 μm)	*B 300* ($\bar{d}_p$ = 11 μm)	*B 300* ($\bar{d}_p$ = 13 μm)	*B 300* ($\bar{d}_p$ = 19 μm)
T-G	1.58	1.09	0.47	0.41	0.45
E_3-G	2.46	1.71	0.67	0.51	0.67
E_1-3G	3.20	2.43	0.92	0.65	0.84
E_1-3P	3.90	4.44	1.00	0.85	0.88
E_2-diG	10.0	4.15	1.33	1.13	1.24
E_2-3G	4.38	3.71	1.42	1.05	1.30
T-S	4.80	4.50	1.49	1.04	1.27
E_3-17G	5.07	4.65	1.74	1.28	1.68
E_2-3P	4.76	6.44	1.47	1.27	1.32
E_3-16G	5.30	4.86	1.86	1.38	1.80
E_2-17G	6.49	6.29	2.44	1.74	2.24
E_3-3S	9.9	8.62	2.89	1.77	2.56
E_2-diP	11.7	10.0	2.40	2.15	2.32
E_2-17P	13.95	14.9	2.86	2.23	2.77
E_1-3S	12.6	12.15	3.81	2.14	3.11
E_2-3S	17.1	18.45	5.87	3.48	4.91
E_2-17S	20.8	22.65	6.81	3.99	5.75
E_3-3,17diS	66.5	42.5	9.55	5.55	7.91
E_7-3,17diS	—	66.6	15.8	8.65	12.7

significant abnormality, however, is the small capacity ratios of estrogen phosphates when using magnesium as eluent cation, which indicates complex formation.

The concentration of eluent anion and the pH have been varied using ECTEOLA-cellulose with perchlorate as eluent anion (see Tables VI and VII).

Fig. 6A shows the dependence of the capacity ratios on the inverse of the anion concentration for different types of conjugates. The glucuronides are eluted first, then the phosphates and finally the sulphates. Note, however, the great change in selectivity and even reversals of elution order. At low ionic strength, mono-conjugates

TABLE IV

REPRODUCIBILITY OF THE CAPACITY RATIO (κ_n) AND INFLUENCE OF THE PACKING PRESSURE

Phase system: B 300, 19 μm; 0.025 *M* perchlorate + 0.0125 *M* phosphate, pH 7.0; 70°.

n	*Packing pressure (bar)*	
	15–23	*30*
E_2-3G	1.30	1.51
E_2-17P	2.73	3.16
E_2-3S	5.09	6.02
E_3-3,17diS	8.18	9.8
Standard deviation (%)	1.5	
Number of columns	4	1

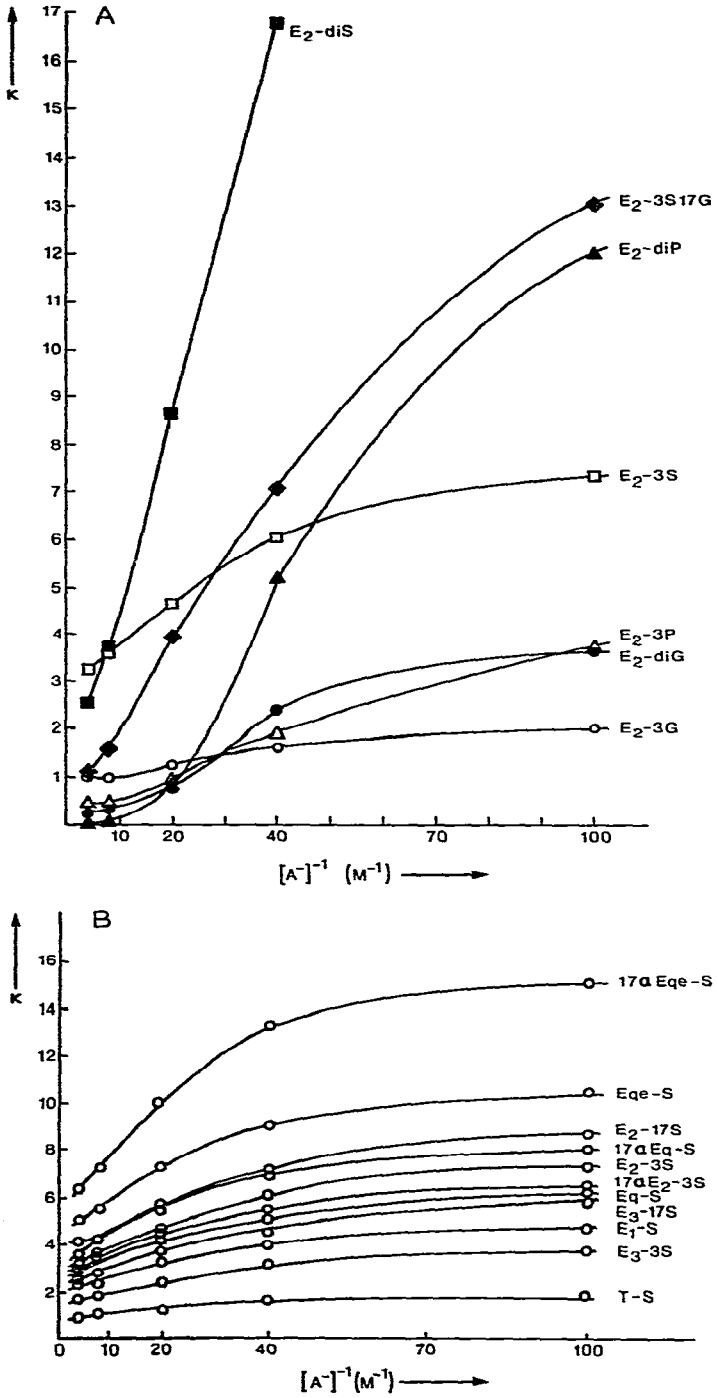


Fig. 6. Dependence of the capacity ratio, κ_i, on the inverse of the eluent anion concentration, $[A^-]^{-1}$. A, 17β-Estradiol conjugates. Phase system: B 300, 19 μm; perchlorate + 0.01 *M* phosphate, pH 6.8; 70°. B, Estrogen sulphates. Phase system: B 300, 19 μm, perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

TABLE V

INFLUENCE OF THE TYPE OF ELUENT ANION AND CATION ON THE CAPACITY RATIO (κ_n)

Phase system: B 300, 19 μm; 0.01 *M* phosphate, pH 6.8; 70°.

n	*Eluent*											
	$NaClO_4$	*$NaHSO_4$*	*$NaNO_3$*	*NaCl*	*KCl*	*NH_4Cl*	*$MgCl_2$*	*NH_4 phosphate*	*Na citrate*	*Na formate*	*Na acetate*	*Na propionate*
$[ClO_4^-]$	*0.025 M*	*0.100 M*	*0.100 M*	*0.100 M*	*0.100 M*	*0.100 M*	*0.050 M*	*0.500 M*	*0.005 M*	*0.100 M*	*0.100 M*	*0.125 M*
T-G	0.54	0.41	0.29	0.35	0.36	0.36	0.37	1.12	0.42	0.37	0.42	0.36
E_3-3G	0.76	0.63	0.50	0.58	0.62	0.60	0.63	1.27	0.68	0.61	0.68	0.58
E_1-3G	1.03	0.74	0.59	0.67	0.71	0.69	0.72	1.72	0.74	0.68	0.77	0.67
E_1-3P	1.24	0.67	0.64	0.75	0.84	0.79	0.49	0.93	1.34	0.87	1.04	0.77
T-S	1.57	1.17	0.94	1.03	1.08	1.07	1.25	3.01	1.07	1.07	1.14	1.01
E_2-3G	1.59	1.22	0.98	1.09	1.16	1.11	1.19	2.56	1.17	1.14	1.20	1.09
E_2-diG	2.39	1.04	0.88	1.19	1.28	1.30	1.53	1.47	1.81	1.36	1.62	1.18
E_2-3P	1.86	1.08	1.02	1.19	1.31	1.21	0.78	1.33	1.97	1.35	1.61	1.21
E_2-diP	5.20	1.28	1.55	1.18	1.31	1.21	0.76	0.59	1.97	2.76	3.62	2.14
E_3-17G	1.98	1.69	1.30	1.43	1.50	1.47	1.52	3.15	1.60	1.54	1.60	1.52
E_3-16G	2.14	1.78	1.42	1.55	1.61	1.51	1.54	3.38	1.72	1.59	1.71	1.56
E_2-17G	2.68	2.18	1.75	1.92	2.05	1.95	2.03	4.05	2.11	2.02	2.17	1.89
E_3-3S	3.13	2.53	2.04	2.26	2.31	2.29	2.74	5.03	2.34	2.25	2.53	2.27
E_2-17P	3.27	2.52	2.06	2.34	2.50	2.26	2.04	3.35	2.82	2.47	2.68	2.30
E_1-3S	3.99	2.84	2.37	2.56	2.73	2.62	3.15	6.45	2.50	2.52	2.73	2.43
Eq-3S	5.13	3.67	3.05	3.32	3.51	3.34	3.97	8.43	3.24	3.23	3.52	3.12
17$\alpha$$E_2$-3S	5.53	4.32	3.45	3.83	4.04	3.83	4.54	9.8	3.54	3.75	3.92	3.48
E_2-3S	6.06	4.72	3.76	4.20	4.47	4.20	5.00	10.6	3.94	4.16	4.42	3.94
17αEq-3S	6.98	5.40	4.50	5.03	5.15	4.95	5.83	12.0	4.69	4.74	5.23	4.54
E_2-3S17G	7.09	4.08	3.50	4.80	5.34	4.96	6.76	6.29	5.57	4.95	5.98	4.42
E_2-17S	7.04	5.72	4.53	5.07	5.34	4.91	5.72	11.95	4.95	4.93	5.36	4.68
Eqe-3S	9.04	6.58	5.41	5.86	6.22	5.91	7.07	13.8	5.54	5.86	6.17	5.53
E_3-3,17diS	10.9	7.32	6.02	8.34	9.22	8.94	13.5	10.9	9.17	8.10	9.88	7.31
17αEqe-3S	13.25	9.87	8.22	9.10	9.58	9.05	10.85	20.5	8.25	8.86	9.30	8.09
E_3-16,17diS	16.3	10.1	9.17	11.7	12.6	12.7	18.0	11.2	14.6	12.3	14.3	10.4
E_2-3,17diS	16.8	11.6	9.44	13.0	14.8	14.1	20.4	19.6	13.6	12.7	15.7	10.6

TABLE VI

DEPENDENCE OF THE CAPACITY RATIO (κ_n) ON ELUENT ANION CONCENTRATION, pH AND TEMPERATURE FOR PHASE SYSTEM A

Phase system A: B 300, 19 μm; perchlorate + 0.01 *M* phosphate.

n	*0.025 M, pH 5.0, 25°*	*0.025 M, pH 6.8, 25°*	*0.025 M, pH 4.2, 70°*	*0.025 M, pH 5.0, 70°*	*0.025 M, pH 6.0, 70°*	*0.025 M, pH 6.8, 70°*	*0.025 M, pH 7.9, 70°*	*0.025 M, pH 8.4, 70°*	*0.010 M, pH 6.8, 70°*	*0.050 M, pH 6.8, 70°*	*0.125 M, pH 6.8, 70°*	*0.250 M, pH 6.8, 70°*
T-G	1.33	0.51	2.36	1.76	0.93	0.54	0.14	0.01	0.68	0.42	0.38	0.36
E_3-3G	2.04	0.87	2.84	2.26	1.30	0.76	0.26	0.12	1.01	0.61	0.45	0.46
E_1-3G	2.98	1.21	3.99	3.08	1.70	1.03	0.30	0.17	1.25	0.84	0.68	0.70
E_3-3P	3.09	2.11	2.91	2.92	2.81	1.25	0.10	–	2.24	0.80	–	0.30
E_1-3P	4.29	2.73	4.61	4.12	3.01	1.24	0.10	–	2.39	0.65	0.32	0.24
E_2-3G	3.75	1.65	5.03	3.82	2.38	1.59	0.63	0.42	2.00	1.23	0.99	1.02
T-S	4.60	2.48	5.72	4.16	2.46	1.57	0.63	0.34	1.89	1.24	1.04	0.98
E_2-3P	5.01	3.44	5.84	5.32	4.03	1.86	0.37	0.06	3.72	0.96	0.45	0.42
E_3-17G	4.08	2.18	5.02	4.20	2.72	1.98	0.94	0.67	2.55	1.60	1.28	1.29
E_3-16G	4.32	2.41	5.22	4.35	2.88	2.14	1.02	0.83	2.63	1.85	1.44	1.49
E_2-17G	5.75	2.98	7.20	5.52	3.68	2.68	1.26	0.99	3.51	2.12	1.56	1.74
E_2-diG	10.3	3.42	18.1	11.3	5.15	2.39	0.70	0.41	3.63	0.76	0.35	0.25
E_3-3S	8.65	5.48	8.74	6.29	4.58	3.13	1.30	0.91	3.84	2.39	1.86	1.60
E_2-17P	7.14	5.58	–	5.80	4.57	3.27	1.28	0.64	4.48	2.23	1.69	1.45
E_2-diP	13.6	8.52	25.5	19.7	12.5	5.20	0.76	0.34	12.0	0.77	0.05	0.02
E_2-3G17S	32.4	10.8	–	–	12.3	5.27	1.48	1.02	9.92	2.86	1.22	0.67
E_1-3S	14.6	7.27	12.55	–	6.07	3.99	1.59	1.12	4.82	3.23	2.45	2.36
E_3-17S	11.3	7.44	10.6	8.84	6.25	4.52	2.23	1.62	5.89	3.77	2.87	2.44
Eq-3S	19.9	10.8	15.8	12.65	7.42	5.13	2.16	1.53	6.24	4.24	3.31	2.82
17αE_2-3S	14.5	9.05	14.55	11.9	7.65	5.53	2.36	1.73	6.54	4.41	3.46	2.85
E_2-3S	17.3	9.87	16.4	12.95	8.55	6.06	2.62	1.99	7.32	4.68	3.55	3.23
17αEq-3S	21.4	12.9	18.6	14.9	9.88	6.98	3.04	2.17	8.16	5.59	4.24	3.62
E_2-3S17G	40.7	14.0	45.6	37.8	17.2	7.09	2.08	1.44	13.0	3.96	1.58	1.08
E_2-17S	20.0	12.1	17.7	14.1	9.80	7.04	3.20	2.45	8.79	5.60	4.21	4.06
Eqe-3S	40.8	22.0	28.0	21.9	13.2	9.04	3.76	2.67	10.5	7.30	5.51	5.02
E_3-3,17diS	–	26.4	–	–	25.9	10.9	3.12	2.08	–	5.28	2.22	1.53
17αEqe-3S	47.1	27.3	34.6	27.9	17.75	13.25	5.26	3.92	15.05	10.1	7.26	6.35
E_3-16,17diS	–	44.0	109	93.1	40.5	16.3	4.42	3.00	49.4	8.58	3.39	2.12
E_2-3,17diS	–	43.9	–	–	41.9	16.8	4.72	3.39	–	8.64	3.66	2.53
E_3-triS	–	–	–	–	–	62.2	7.85	4.38	–	15.0	3.17	1.46

TABLE VII

DEPENDENCE OF THE CAPACITY RATIO (κ_n) ON ELUENT ANION CONCENTRATION, pH AND TEMPERATURE FOR PHASE SYSTEM B

Phase system B: 0.25 *M* perchlorate + 0.01 *M* phosphate.

n	*B 300, 19 μm*					*ET 41, 11 μm*			
	pH 4.2, 70°	*pH 4.9, 70°*	*pH 5.8, 70°*	*pH 6.8, 70°*	*pH 7.7, 70°*	*pH 8.5, 25°*	*pH 8.5, 40°*	*pH 8.5, 55°*	*pH 8.5, 70°*
E_2-3G17S	0.92	0.89	0.73	0.50	0.37	0.72	0.82	0.78	0.83
T-S	0.90	0.89	0.80	0.64	0.57	0.77	0.82	0.85	0.88
E_2-3S17G	1.21	1.14	0.99	0.72	0.55	1.10	1.17	1.12	1.16
E_1-3S	1.42	1.46	1.35	1.15	1.03	1.91	1.86	1.78	1.72
E_3-3,17diS	1.72	1.74	1.52	1.07	0.80	2.18	2.20	2.06	2.06
E_1-3S	2.09	2.25	1.93	1.54	1.33	2.84	2.76	2.50	2.35
E_3-17S	2.05	2.07	1.97	1.75	1.65	3.08	3.01	2.74	2.57
E_3-16,17diS	2.29	2.40	2.07	1.50	1.19	—	—	—	—
E_3-triS	2.39	2.50	1.94	1.00	0.63	—	—	—	—
E_2-3,17diS	2.79	2.85	2.47	1.71	1.26	3.61	3.57	3.36	3.31
17αE_2-3S	2.55	2.58	2.34	1.96	1.71	3.33	3.19	3.10	3.02
Eq-3S	2.61	2.78	2.46	1.99	1.70	3.99	3.69	3.28	3.00
E_2-3S	2.79	2.86	2.60	2.18	1.93	3.83	3.67	3.56	3.41
17αEq-3S	3.16	3.27	3.05	2.54	2.25	5.02	4.67	4.27	3.99
E_2-17S	3.32	3.43	3.13	2.73	2.50	5.12	4.90	4.50	4.01
Eqe-3S	4.64	4.83	4.33	3.46	2.98	8.12	7.22	6.16	5.46
17αEqe-3S	5.67	5.97	5.47	5.54	3.99	10.0	9.05	8.10	7.26

move faster than di-conjugates. At high anion concentration, the less polar mono-conjugates obscure this true ion-exchange behaviour by stronger additional adsorption than the di-conjugates.

For different types of conjugates, we found that the counter anion concentration is a very important parameter. However, for a series, *e.g.* the sulphates (see Fig. 6B), it alters only the absolute value of the capacity ratios and not the elution sequence.

The influence of the pH of the eluent on the capacity ratio is shown in Fig. 7A. Three observations can be made: (i) the glucuronides are eluted first, then the phosphates and finally the sulphates; (ii) there is stronger retardation at lower pH values and the weaker retardation at high pH is due to diminution of the ion-exchange capacity; at lower pH, adsorption processes probably occur and reinforce the retardation; (iii) the order of elution of equivalent conjugates is estriol, estrone, estradiol. The stronger retardation at lower pH values is also apparent at higher eluent anion concentrations, although for sulphates there seems to be a maximum at pH 5 (Fig. 7B).

Effect of temperature. The influence of temperature on the capacity ratio is shown in Tables VI and VII. Although in principle the capacity ratios decrease as the temperature increases, the decrease is much greater for some compounds than for others. For example, equilin 3-sulphate can be separated from 17α-estradiol 3-sulphate at 25°, but at 70° under otherwise identical conditions this separation is impossible. On the other hand, the opposite is true for the separation of equilin 3-sulphate from 17β-estradiol 3-sulphate (see Fig. 8).

Choice of an optimal phase system

Knowing the selectivity coefficients and capacity ratios of different types of anion-exchange columns for the estrogen conjugates, the number of theoretical plates required for the separation of each pair of successive compounds with a given resolution and the minimal time needed for separation can be calculated[5].

The theoretical plate number, N_R, required for a resolution of R_{ji} is given by the equation

$$N_R = R_{ji}^2 \left(\frac{1 + \kappa_i}{(r_{ji} - 1)\,\kappa_i}\right)^2 \tag{5}$$

The corresponding length of the column required is given by $L_{Ri} = N_{Ri}\,H$. In practice, the maximum length is limited by the pressure limit of the apparatus and the permeability of the column packing.

The time in which the separation of the total mixture can be performed is given by the retention time, t_{Rn}, of the last compound, n, eluted:

$$t_{Rn} = \frac{L_{Rm}}{u}(1 + \kappa_n) \tag{6}$$

where m refers to that component of the mixture which has the largest value of L_{Rm}. In order to increase the speed of separation, the value of the capacity ratio, κ_n, of the last component eluted as well as the largest value of L_{Rm}/u occurring in the mixture should be minimized.

An example of such an optimized separation for six glucuronides is shown in Fig. 9. Similarly, the commercially available monophosphates can be separated within 15 min (Fig. 10). In this manner, some impurities in 17β-estradiol 3-phosphate were discovered. For the analysis of seven sulphates, half an hour was needed (Fig. 11). Here, too, some degradation products were found after storage for 2 months at 4°. However, they did not interfere with the separation.

Applications

An application of the separation of estrogen sulphates is the analysis of Premarin, an extract of pregnant mare's urine (see Fig. 12). The identity of the peaks was confirmed by varying the chromatographic conditions. Because the ethanolic extract was relatively clean and the retention of the sulphates was strong, we were able to use direct UV detection.

The main reason for the interest in the separation of estrogen conjugates is, of course, the possibility of obtaining physiological information from the changing estrogen profile of urine during pregnancy. The problem is to find an extraction that is selective for a whole group of compounds without significant selectivity for individual components. Adsorption of the estrogen conjugates on Amberlite XAD-2 and elution with methanol[7] appeared feasible. However, this extract was too impure for direct UV detection of the effluent. Fig. 13 shows a chromatogram of such an XAD-2 extract. Instead of, or in addition to, improving the specificity of the extraction, one can also try to improve the specificity of the detection. The dotted line in Fig. 13 represents an off-line Kober–Ittrich analysis of the chromatogram, obtained with a segmented-flow apparatus[8].

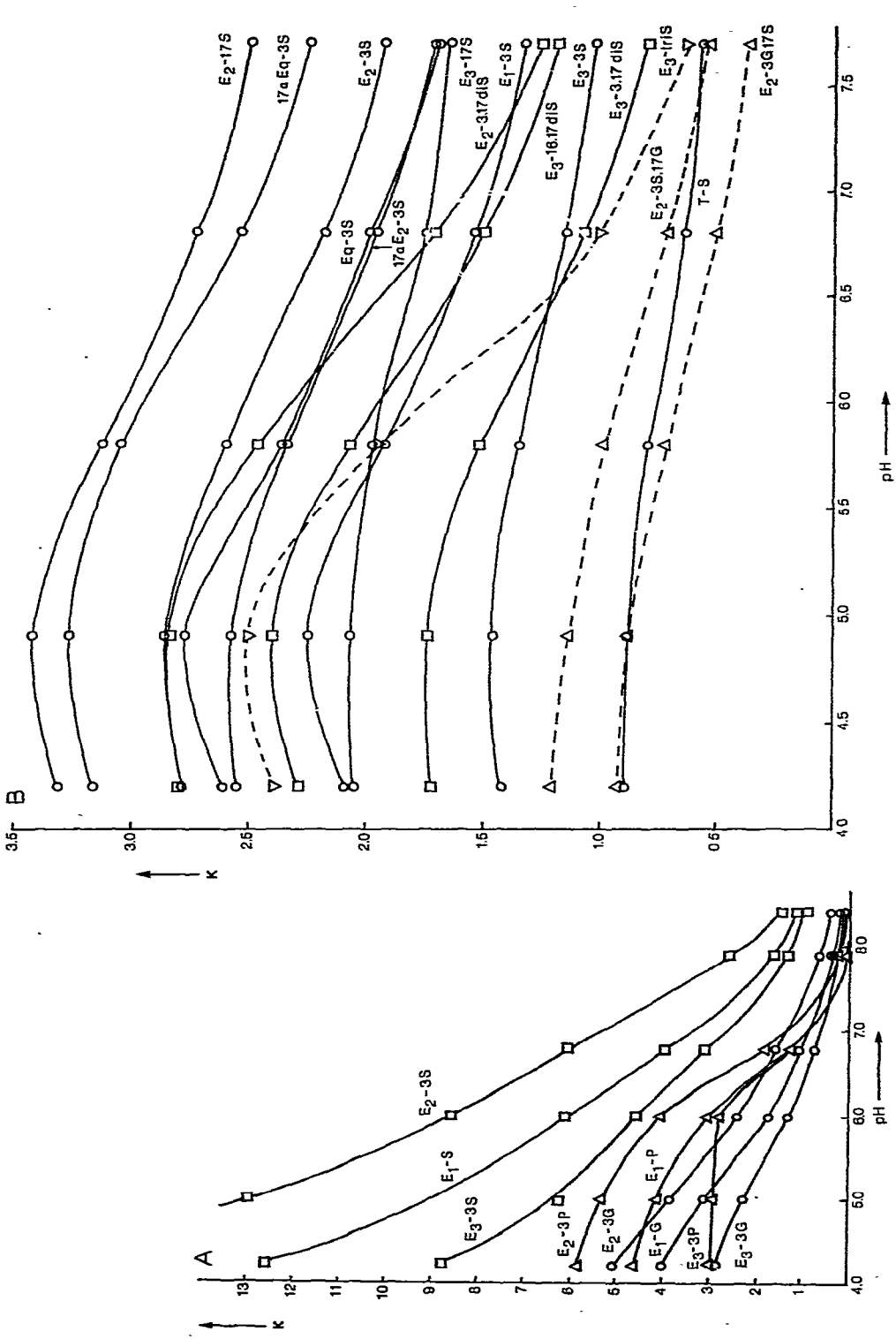


Fig. 7. Influence of the pH of the eluent on the capacity ratio. A, Estrogen conjugates. Phase system: B 300, 19 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate; 70°. B, Estrogen sulphates. Phase system: B 300, 19 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate; 70°.

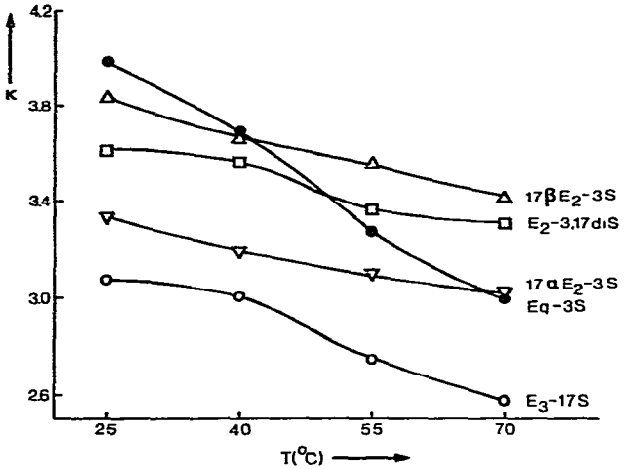
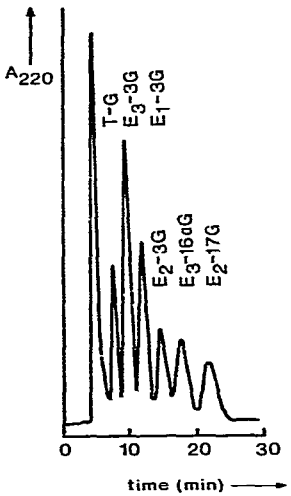


Fig. 8. Dependence of the capacity ratio on temperature for some estrogen sulphates. Phase system: ET 41, 11 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5.

As off-line batch determinations are tedious and time consuming, an on-line segmented-flow detector has been designed (Fig. 1). This modification is a compromise with respect to specificity, sensitivity and peak broadening. The first stage is a Kober reaction at 127° for approximately 10 min, followed by enhancement of fluorescence with acetic acid[9]. The system is still under development and a comparison with other



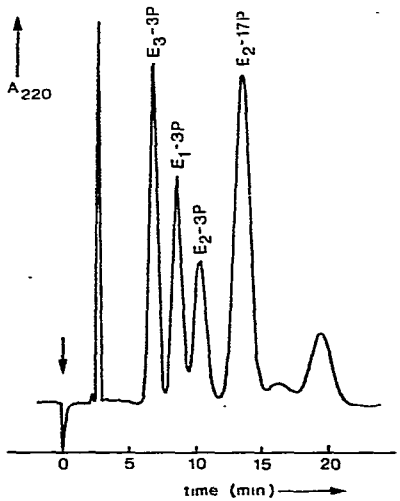


Fig. 9. Separation of an estrogen glucuronide mixture. Phase system: B 300, 13 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 7.0. Temperature, 25°; pressure, 16 bar.

Fig. 10. Separation of estrogen monophosphates. Phase system: ET 41, 11 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5. Temperature, 70°; pressure, 30 bar.

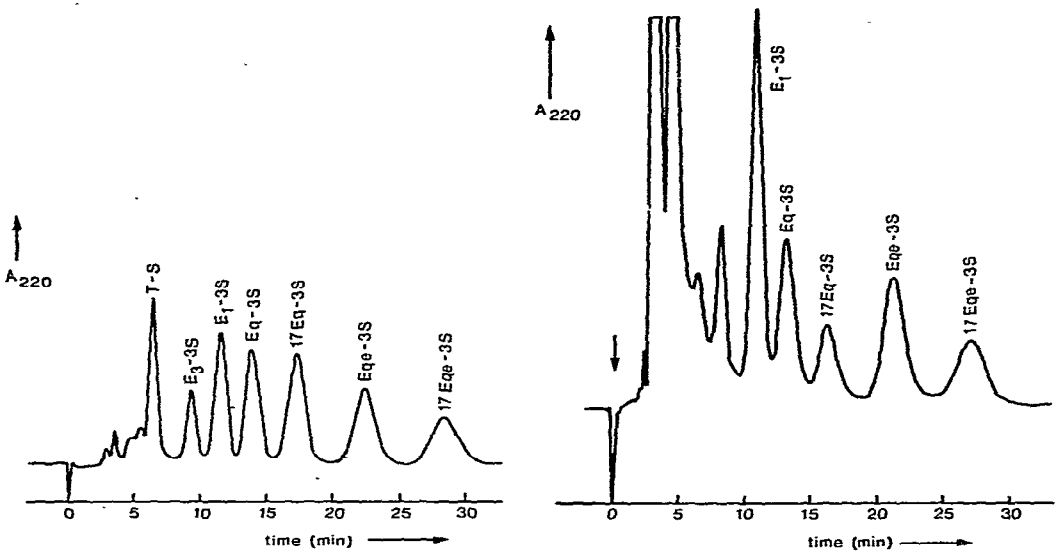


Fig. 11. Separation of estrogen sulphates. Phase system: ET 41, 11 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5. Temperature, 70°; pressure, 30 bar.

Fig. 12. Analysis of ethanolic Premarin extract. Phase system: ET 41, 11 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5. Temperature, 70°; pressure, 30 bar.

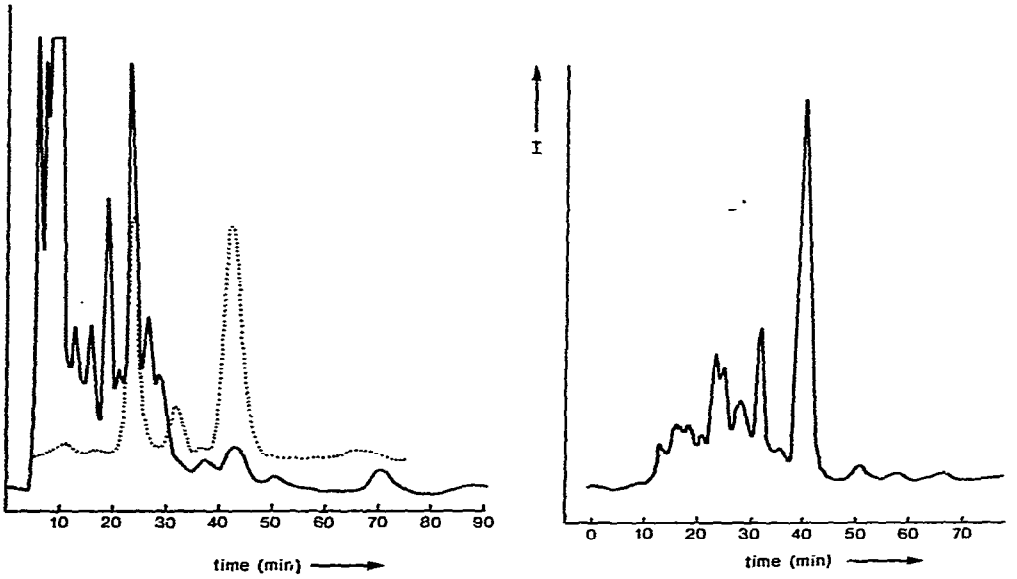


Fig. 13. Chromatogram of a methanolic XAD-2 extract of human pregnancy urine. Solid line, absorbance at 220 nm; dotted line, chromatogram obtained by off-line batch determination of estrogenic material. Phase system: B 300, 11 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8. Temperature, 70°; pressure, 20 bar.

Fig. 14. Chromatogram of an XAD-2 extract of late pregnancy urine detected with the on-line continuous gas-segmented-flow reaction detector. Phase system: ET 41, 11 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8. Temperature, 70°; pressure, 14 bar.

reaction systems will be given later[4]. Fig. 14 shows a chromatogram of an Amberlite XAD-2 extract of late pregnancy urine, analysed with this segmented-flow reaction detector. Such an estrogen pattern can be obtained within 2 h with little manual work; the detection limit is less than 20 nmoles of estriol 16-glucuronide.

In order to develop this procedure into a routine method for the determination of estrogen-conjugate urinary profiles during pregnancy, the extraction and detection procedures must be improved. Such work is in progress at present.

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