Journal of Chromatography, 102 (1974) 353–374
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CHROM. 7904

HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH ION-EXCHANGE CELLULOSES AND ITS APPLICATION TO THE SEPARATION OF ESTROGEN GLUCURONIDES

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SUMMARY

The selectivity of a number of different types of anion exchangers for estrogen glucuronides was investigated. The effects of the nature of the exchanger anion, its concentration in the eluent, the pH of the eluent and temperature on the chromatographic resolution parameters for estrogen glucuronides on ECTEOLA-cellulose are discussed in detail.

The preparation of high-efficiency columns from ion-exchange celluloses was investigated and the influence of the particle size and temperature on the efficiency is discussed.

Several chromatograms of test mixtures are presented in order to demonstrate the performance of ion-exchange cellulose columns. Significant variations in efficiency and selectivity were found for different batches of the same type of ion exchanger.

INTRODUCTION

Steroids in urine are excreted as conjugates. Fig. 1 shows an example of the structure of a steroid conjugate, an estrogen glucuronide. Estrogen glucuronides constitute a major part of the urinary steroid hormones during pregnancy[1–3] and give information on the physical condition of the foetus[4].

Since the discovery that steroids are produced, transported and metabolized as conjugates[5], the view has grown that physiological information is lost by hydrolysis of the conjugates. The formation of artifacts[6] and loss of time[7] during hydrolysis make it worthwhile to develop a method for the direct determination of these compounds.

Because of their low concentration and diversity, the separation of steroid conjugates must be performed in more than one step[8]:

(1) separation from other urine constituents;

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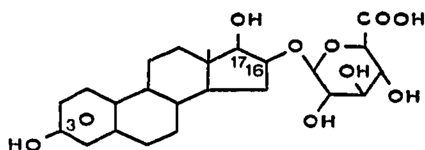


Fig. 1. Structure of estriol-16α-glucuronide (E_3-16G).

(2) separation into groups;
(3) separation of the individual compounds.

So far, most attention has been paid to the first two steps. The present work is concerned with the separation of individual components.

Steroid conjugates are not sufficiently volatile to allow their direct gas chromatographic separation: a derivatization step has to be carried out prior to the separation by gas chromatography. Liquid chromatography, especially ion-exchange chromatography, taking advantage of the ionic character of the estrogen glucuronides, makes it possible to separate the steroid conjugates themselves, and it should therefore be the method of choice.

Disappointing results in the chromatographic separation of steroid conjugates have been reported[9] for anion-exchange resins with hydrophobic polystyrene–divinylbenzene matrixes, probably because of interactions with the steroid skeleton.

Cellulose ion exchangers are promising with respect to their selectivity, but have a drawback in high-pressure liquid chromatography owing to their non-rigid structure. Few publications have been devoted to the packing of columns with these materials[10,11]. Encouraged by the progress made in the packing of columns with rigid materials, an attempt was made to prepare highly efficient columns from ion-exchange cellulose materials.

EXPERIMENTAL

Apparatus

Two types of liquid chromatograph were used. One liquid chromatograph was assembled from commercial and custom-made parts and consists of a thermostated eluent reservoir, a high-pressure pump, a manometer, a flow resistance, a septum injection port, a separation column, a UV detector with an amplifier and a flat-bed potentiometric recorder. The pumping device is a reciprocating piston pump (Lewa FL 1). The flow pulses are eliminated by means of a flow-through Bourdon-tube manometer and a stainless-steel capillary flow resistance. The injecton port, the separation column, its connections and the detector cell have been described in detail in previous publications[12–14]. The detector is a variable-wavelength spectrophotometer (Unicam SP 500), the output signal of which is amplified by an amplifier (Knick 72 W) and recorded by a linear potentiometric recorder (Servogor RE 511).

The other liquid chromatograph was a commercial apparatus (Hewlett-Packard 1010 A). This instrument was equipped with standard injection ports and two pumps. Stainless-steel columns (25 × 0.3 cm) were used. The detector was a variable-wavelength UV spectrophotometer (HP 1030 B). The chromatograms were recorded by a linear potentiometric flat-bed recorder (Servogor RE 511).

The columns were packed using a pressurized slurry technique.

Chemicals

Samples. The glucuronides used were the sodium salts of testosterone-β-D-glucuronide (T-G), estrone-β-D-glucuronide (E_1-G), β-estradiol-3β-D-glucuronide (E_2-3G), β-estradiol-17β-D-glucuronide (E_2-17G), estriol-3β-D-glucuronide (E_3-3G), estriol-16α-(β-D-glucuronide) (E_3-16G) and estriol-17β-(β-D-glucuronide) (E_3-17G) (all obtained from Sigma, St. Louis, Mo., U.S.A.). The molar absorptivity of estrogen glucuronides is about 10^3 at 275 nm and five times as high at 220 nm. Histamine was used as an unretarded tracer.

Column materials. Solutions of sodium chloride, sodium acetate, ammonium formate and citric acid in deionized water were used as eluents. The specified pH was adjusted by means of sodium hydroxide or acetic acid. All chemicals were of p.a. quality (Merck, Darmstadt, G.F.R.).

The following six types of anion-exchange materials were used as column packings: (*i*) Polydextran gel Sephadex G-15, Superfine (Pharmacia, Uppsala, Sweden), pre-swollen in deionized water and washed with pyridine[15]; (*ii*) dextran-based anion exchanger DEAE-Sephadex A-25 (Pharmacia), ion-exchange capacity (i.e.c.) 3.5 mequiv./g, left to swell for 1 week; (*iii*) polyalkylene amine-based anion-exchanger, Bio-Rex 5 (Bio-Rad Labs., Richmond, Calif., U.S.A.), i.e.c. 8.8 mequiv./g, swollen for 1 day; (*iv*) aminoethylcellulose Cellex AE (Bio-Rad), i.e.c. 0.37 mequiv./g; (*v*) diethylaminoethylcellulose Cellex D (Bio-Rad), i.e.c. 0.61 mequiv./g; (*vi*) ECTEOLA-cellulose Cellex E (Bio-Rad), i.e.c. 0.44 mequiv./g; MN 300 (Macherey, Nagel & Co., Düren, G.F.R.), i.e.c. 0.35 mequiv./g; Baker 300 (Baker, Deventer, The Netherlands), i.e.c. 0.35 mequiv./g; Servacel TLC p.a. (Serva, Heidelberg, G.F.R.), i.e.c. not specified; and Whatman ET 41 (W. & R. Balston, Maidstone, Great Britain), i.e.c. not specified. The cellulose ion exchangers were swollen by pre-cycling with 0.5 *N*

TABLE I

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

Anion exchanger	*Particle diameter range 10–90% (μm)*	*Mean particle diameter (μm)*	*Ion-exchange capacity (mequiv./g)*
Cellex E	18–26	20	0.31
	15.5–24	18	
	7–17.5	12	
	5.5–12.5	9	
MN 300, batch A	11–19	14	
MN 300, batch B	5–10	7	0.04
Baker 300	12–27	19	0.27
	9–19	13	
	<14	8	
ET 41	16–26	20	0.23
	<17	10	
Servacel TLC	9–24	17	0.10
	7–18	11	0.05
	<12	7	0.03
Cellex AE	10.5–19	14	0.29
	5–13	9	

TABLE II

CAPACITY RATIOS AND SELECTIVITY FACTORS OF SUCCESSIVE COMPONENTS FOR ESTROGEN GLUCURONIDES ON SOME ANION EXCHANGERS

Number of repetitive measurements, 3; relative standard deviation of measurement, 2%.

Conditions	*Ion exchanger*													
	Sephadex G-15		*DEAE-Sephadex*		*Cellex D*		*Cellex E*		*Cellex E*		*Cellex AE*		*Bio-Rex 5*	
Eluent	0.01 *M* formate		0.5 *M* chloride + 0.05 *M* acetate		1.0 *M* acetate		0.5 *M* chloride + 0.05 *M* acetate		0.25 *M* citrate		0.05 *M* formate		0.2 *M* citrate	
pH	6.7		5.0		5.0		5.0		5.0		6.7		5.0	
Temperature (°C)	25		25		24		30		30		30		50	
Component	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	0.87	1.42	1.52	2.55	0.57	1.77	0.57	2.10	1.15	1.85	1.86	1.32	0.21	5.4
E_3-3G	1.24	1.33	3.78	1.00	1.01	1.11	1.20	1.16	2.13	1.21	2.46	1.02	1.69	1.27
E_1-G	1.65	1.70	3.78	1.38	1.12	1.39	1.39	1.59	2.57	1.54	2.52	1.30	1.13	1.50
E_2-3G	2.81	1.42	5.23	1.06	1.56	1.14	2.21	1.57	3.96	1.50	3.28	1.65	2.14	1.03
E_3-17G	4.22	1.68	5.53	1.03	1.78	1.13	3.46	1.08	5.94	1.10	5.40	1.04	2.20	1.05
E_3-16G	3.98	1.06	5.72	1.23	2.01	1.16	3.73	1.25	6.53	1.16	6.13	—	2.32	1.34
E_2-17G	7.04	—	7.05	—	2.34	—	4.68	—	7.58	—	5.59	1.10	3.12	—

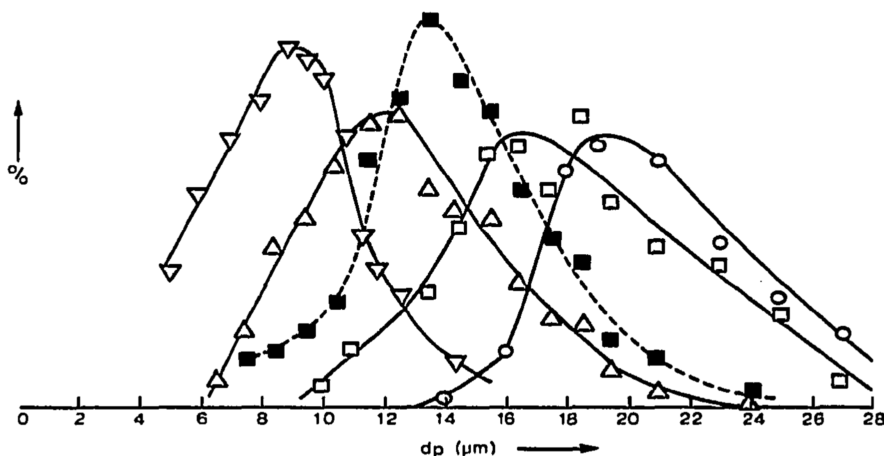


Fig. 2. Particle size distribution of air-classified fractions. ———, Cellex E; – – –, MN 300 (batch A). Mean particle diameter: ○, 20 µm; □, 18 µm; △, 12 µm; ▽, 9 µm; ■, 14 µm.

sodium hydroxide solution, 0.5 *N* hydrochloric acid and 0.5 *N* sodium hydroxide solution and rinsed with deionized water.

The ECTEOLA- and AE-celluloses 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 and Fig. 2).

The actual ion-exchange capacities were determined as the buffer capacity between pH 10 and pH 4 in 0.5 *N* sodium chloride solution (Table II). An automatic titrator (Radiometer TTT 1) was used.

RESULTS AND DISCUSSION

The choice of the phase system for a chromatographic separation should be based on the equation for the resolution, R_{ji}, of two components, j and i:

$$R_{ji} = (r_{ji} - 1)\cdot\frac{\kappa_i}{\kappa_i + 1}\cdot(N_i)^{\frac{1}{4}} \quad (1)$$

where

r_{ji} = selectivity factor = 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, those phase systems should be selected for which the values of the factor $(r_{ji} - 1)\cdot[\kappa_i/(\kappa_i + 1)]$ for each pair of successively eluting components of the sample are largest, with the restriction that extremely high capacity ratios should be avoided, because otherwise the separation time becomes too long. The final choice of the appropriate phase system is made by taking into account the

column efficiency attainable with those phase systems which had been pre-selected because of their selectivity.

The capacity ratio was determined from the retention time, t_{Ri}, of the 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 determined by means of an inert tracer (histamine).

Column selectivity

According to eqn. 1, the resolution of two components depends on the factor $(r_{ji} - 1) \cdot [\kappa_i/(\kappa_i + 1)]$, which is determined mainly by the distribution coefficients of the components, as the capacity ratio and the distribution coefficient, K, are proportional:

$$\kappa = q \cdot K \tag{3}$$

where the proportionality factor q is the phase ratio of the ion exchanger and the mobile phase.

From the equations describing 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.

Influence of the type of anion exchanger. The ion-exchange equilibrium constant in eqn. 4 depends on, in addition to the nature of the sample anion, the nature of the matrix, the fixed ionogenic group and the mobile counter ion of the ion exchanger. A number of anion-exchange systems were screened for their selectivity with respect to estrogen glucuronides. The results are shown in Table II. The separation of a pair of compounds becomes difficult when the factor $(r_{ji} - 1) \cdot [\kappa_i/(\kappa_i + 1)]$ is less than 0.1.

It can be seen that the nature of the matrix, the fixed cationic group and the mobile counter anion of the ion exchanger have little influence on the elution order, which is determined primarily by the nature of the sample. The site of conjugation

in the estrogen conjugates was found to be more important than the type of steroid. Estrogen-3-glucuronides are eluted first, followed by 17- and then 16-glucuronides. The elution order according to the type of steroid is estriol, estrone and estradiol, except on Bio-Rex 5, where the order is estrone, estriol and estradiol. The separation of the pair E_3-17G and E_3-16G is difficult on all ion-exchange systems shown in Table II, whereas the separations of E_1-G and E_3-3G as well as of E_3-17G and E_2-3G are difficult only on certain systems. The separations of the other pairs of estrogen conjugates in Table II give no serious problems. Resolutions involving E_3-17G are of minor interest from the clinical point of view, as this conjugate is reported not to occur in human urine in significant concentrations[2]. Therefore, the discussion of the choice of an optimal phase system will be focused on the resolution of E_1-G and E_3-3G and occasionally E_3-16G and E_2-3G.

The separation of estrone and estradiol conjugates on DEAE-Sephadex has been reported earlier[16,17]. From Table II, it can be concluded that the selectivity of this type of anion exchanger is insufficient when estriol conjugates are present.

ECTEOLA- and AE-cellulose ion exchangers were found to be more rigid and therefore more suited for high-pressure liquid chromatography than the other materials. They were chosen for further investigations, although polydextran gel would be more favourable in terms of overall selectivity. The variation of the properties of different batches of the same type of anion exchanger was investigated for six batches of ECTEOLA-cellulose, the particle sizes and ion-exchange capacities of which are given in Table I. Their selectivity data are given in Table III. It can be seen that the ion-exchange capacity and also the selectivity factor change significantly from batch to batch. An exact interdependence between the two parameters does not exist, however. The selectivity for E_1-G–E_3-3G, for instance, is smallest on ET 41, although its ion-exchange capacity is intermediate. The materials Baker 300 and MN 300/A appear to be identical, while MN 300/B resembles Servacel TLC. The ion-exchange capacity of Servacel TLC was found to be strongly dependent on the particle size, whereas that of Baker 300 was found to be constant, as reflected in the capacity ratios.

The effect of the nature of the mobile counter anion of the anion exchanger was studied in more detail. The results are presented in Table IV, from which it can be concluded that the nature of the counter anion has a significant influence on the absolute value of the capacity ratio but has a less distinct influence on the relative value, the selectivity factor.

Effect of eluent composition. According to eqn. 4, the distribution coefficient and with it the capacity ratio depend on the concentrations of the ion-exchanger counter anion and the hydrogen ion in the eluent.

The influence of the anion concentration was studied for chloride anions (Table V). In agreement with eqn. 4, it can be seen that the anion concentration has an insignificant influence on the selectivity factor. The reciprocal inter-relationship between the capacity ratio and the anion concentration is confirmed by the plots shown in Fig. 3. Extrapolation to infinitely large anion concentration gives significant residue values, which make it likely that the distribution process involved is a mixed mechanism including ion exchange and adsorption.

The influence of the pH of the eluent in an ion-exchange process is also described by eqn. 4, which predicts that the distribution coefficient is independent of

TABLE III

CAPACITY RATIOS AND SELECTIVITY FACTORS OF SUCCESSIVE COMPONENTS FOR ESTROGEN GLUCURONIDES ON DIFFERENT BATCHES OF ECTEOLA-CELLULOSE AT 70°

Number of repetitive measurements, 3; relative standard deviation of measurement, 2%.

Conditions	*Ion exchanger*																			
	Cellex E		*MN 300*		*MN 300*		*ET 41*		*Baker 300*		*Baker 300*		*Baker 300*		*Servacel TLC*		*Servacel TLC*		*Servacel TLC*	
Mean particle size (μm)	18		14		7		20		19		13		8		17		11		7	
Eluent	500 m*M* chloride + 50 m*M* acetate		250 m*M* chloride + 50 m*M* acetate		50 m*M* acetate		250 m*M* chloride + 50 m*M* acetate		125 m*M* chloride + 50 m*M* acetate		125 m*M* chloride + 50 m*M* acetate		125 m*M* chloride + 50 m*M* acetate		125 m*M* chloride + 50 m*M* acetate		125 m*M* chloride + 50 m*M* acetate		50 m*M* acetate	
pH	5.0		5.0		5.0		5.0		5.0		5.0		5.0		5.0		5.0		5.0	
Component	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	0.66	1.57	0.62	1.37	0.48	1.40	0.89	1.59	0.96	1.35	0.94	1.33	0.88	1.30	0.88	1.39	0.24	1.62	0.35	1.61
E_3-3G	1.04	1.15	0.84	1.11	0.67	1.31	1.42	1.06	1.29	1.12	1.25	1.13	1.11	1.15	1.22	1.21	0.39	1.29	0.56	1.31
E_1-G	1.19	1.67	0.94	1.52	0.88	1.68	1.50	1.52	1.45	1.47	1.41	1.47	1.28	1.51	1.48	1.61	0.50	1.65	0.74	1.69
E_2-3G	1.98	1.30	1.42	1.14	1.48	1.25	2.29	1.16	2.13	1.08	2.08	1.07	1.93	1.06	2.38	1.26	0.83	1.31	1.25	1.31
E_3-17G	2.56	1.04	–	–	–	–	2.65	1.04	2.30	1.02	2.21	1.02	2.04	1.02	3.00	1.01	1.09	1.01	1.64	1.08
E_3-16G	2.70	1.26	1.62	1.30	1.85	1.19	2.76	1.19	2.34	1.29	2.28	1.30	2.07	1.33	3.03	1.27	1.10	1.29	1.78	1.28
E_2-17G	3.36	–	2.17	–	2.20	–	3.28	–	3.03	–	2.96	–	2.76	–	3.84	–	1.42	–	2.28	–

TABLE IV

DEPENDENCE OF THE CAPACITY RATIO AND THE SELECTIVITY FACTOR OF SUCCESSIVE COMPONENTS ON THE FORM OF THE ANION EXCHANGER

Number of repetitive measurements, 3; relative standard deviation of measurement, 2%.

Conditions	*Ion exchanger*													
	Cellex E								*Cellex AE*					
	Formate		*Acetate*		*Acetate*		*Chloride*		*Formate*		*Acetate*		*Chloride*	
Eluent	1.0 *M* formate		1.0 *M* acetate		0.5 *M* acetate		0.5 *M* chloride + 0.05 *M* acetate		0.05 *M* formate		0.05 *M* acetate		0.125 *M* chloride + 0.05 *M* acetate	
pH	5.0		5.0		5.0		5.0		6.7		5.0		5.0	
Temperature (°C)	25		25		25		30		30		25		25	
Component	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	0.85	2.31	1.26	1.82	1.79	1.87	0.57	2.10	1.86	1.32	3.81	1.34	0.82	1.24
E_3-3G	1.82	1.18	2.29	1.23	3.35	1.18	1.20	1.16	2.46	1.02	5.12	1.02	1.01	1.09
E_1-G	2.15	1.48	2.82	1.54	3.95	1.53	1.39	1.59	2.52	1.30	5.22	1.33	1.10	1.26
E_2-3G	3.29	1.53	4.35	1.45	6.05	1.50	2.21	1.57	3.28	1.65	6.94	1.67	1.39	1.68
E_3-17G	5.25	1.04	6.32	1.13	9.10	1.09	3.46	1.08	5.40	1.03	11.56	1.03	2.34	1.08
E_3-16G	5.77	1.26	7.15	1.10	9.95	1.17	3.73	1.25	6.13	–	12.7	–	2.70	–
E_2-17G	6.61	–	7.88	–	11.60	–	4.68	–	5.59	1.10	11.89	1.07	2.52	1.07

TABLE V

DEPENDENCE OF THE CAPACITY RATIO AND SELECTIVITY FACTOR OF SUCCESSIVE COMPONENTS ON THE ANION CONCENTRATION OF THE ELUENT

Number of repetitive measurements, 2; relative standard deviation of measurement, 3%.

Component	*Phase system: Cellex E; chloride + 0.05 M acetate; 70°*																			
	pH 4.7										*pH 3.5*									
	Chloride concentration (M)										*Chloride concentration (M)*									
	1.0		*0.4*		*0.2*		*0.1*		*0.04*		*2.0*		*1.0*		*0.5*		*0.2*		*0.1*	
	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	–	–	0.92	1.53	1.48	1.49	2.13	1.39	4.45	–	–	–	–	–	0.89	1.50	1.75	1.50	2.6	1.58
E_3-3G	1.15	1.18	1.41	1.09	2.21	1.13	2.05	1.07	–	–	1.59	1.07	1.30	1.19	1.34	1.15	2.62	1.12	4.1	1.14
E_1-G	1.36	1.54	1.54	1.58	2.51	1.46	3.14	1.74	7.60	–	1.70	1.69	1.55	1.55	1.52	1.58	2.93	1.47	4.7	1.51
E_2-3G	2.10	1.36	2.43	1.28	3.63	1.29	5.46	1.15	–	–	2.87	1.16	2.40	1.20	2.40	1.17	4.31	1.18	7.1	1.21
E_3-17G	2.85	1.05	3.10	1.02	4.67	1.03	6.27	1.07	–	–	3.32	1.05	2.88	1.04	2.80	1.035	4.73	1.035	8.6	–
E_3-16G	3.00	1.24	3.17	1.27	4.81	1.24	6.71	1.23	–	–	3.50	1.52	3.00	1.40	2.90	1.29	4.90	1.36	–	–
E_2-17G	3.73	–	4.04	–	5.97	–	8.25	–	–	–	5.33	–	4.20	–	3.75	–	6.67	–	10.7	–

Component	*Phase system: Cellex AE; chloride + 0.05 M acetate; pH 5.0; 25°*							
	Chloride concentration (M)							
	0.125		*0.050*		*0.025*		*0.000*	
	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	0.82	1.24	1.67	1.28	2.72	1.34	3.81	1.34
E_3-3G	1.01	1.09	2.14	1.08	3.66	1.07	5.12	1.02
E_1-G	1.10	1.26	2.30	1.26	3.92	1.23	5.22	1.33
E_2-3G	1.39	1.68	2.91	1.72	4.82	1.70	6.94	1.67
E_3-17G	2.34	1.08	4.99	1.03	8.17	1.03	11.56	1.03
E_3-16G	2.70	–	5.43	–	9.17	–	12.7	–
E_2-17G	2.52	1.07	5.13	1.06	8.38	1.08	11.89	1.07

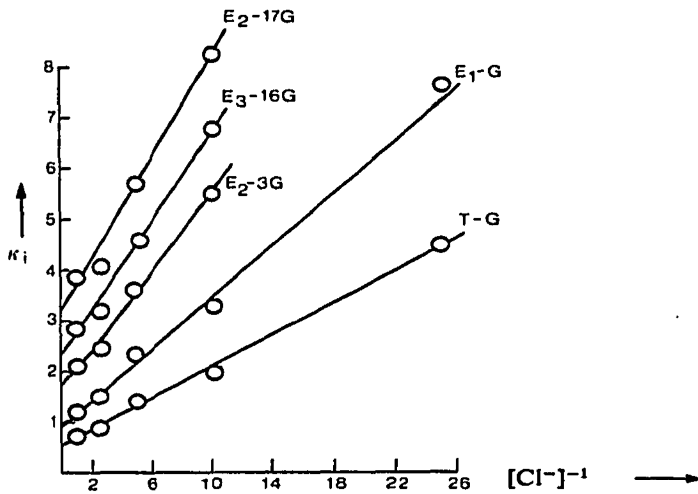


Fig. 3. Dependence of the capacity ratio on the anion concentration in the eluent. Phase system: Cellex E; chloride + 0.05 M acetate, pH 4.7; 70°.

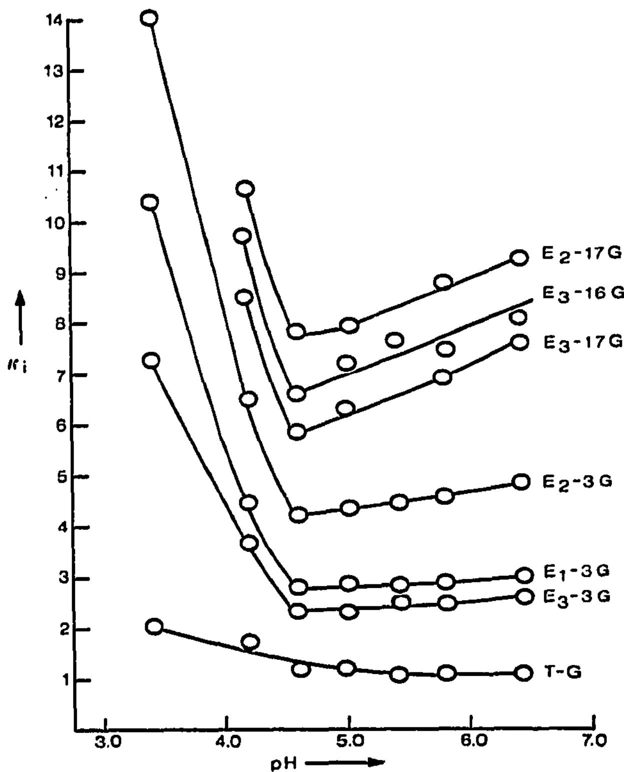


Fig. 4. Dependence of the capacity ratio on the pH of the eluent. Phase system: Cellex E; 1.0 M acetate; 25°.

TABLE VI

DEPENDENCE OF THE CAPACITY RATIO AND THE SELECTIVITY FACTOR OF SUCCESSIVE COMPONENTS ON THE PH OF THE ELUENT

Number of repetitive measurements, 3; relative standard deviation of measurement, 3%.

Component	*Phase system: Cellex E; 0.5 M chloride + 0.05 M acetate; 70°*															
	pH 3.0		*pH 4.0*		*pH 4.5*		*pH 5.0*		*pH 5.5*		*pH 6.0*		*pH 6.5*		*pH 7.0*	
	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$
T-G	0.83	1.40	0.64	1.61	0.64	1.56	0.66	1.58	0.67	1.57	0.66	1.52	0.65	1.51	0.62	1.48
E_3-3G	1.16	1.10	1.03	1.16	1.00	1.16	1.04	1.15	1.05	1.14	1.00	1.15	0.98	1.12	0.92	1.13
E_1-G	1.27	1.69	1.20	1.63	1.16	1.67	1.19	1.66	1.20	1.67	1.15	1.67	1.10	1.67	1.04	1.71
E_2-3G	2.14	1.08	1.96	1.29	1.94	1.30	1.98	1.30	2.00	1.31	1.92	1.34	1.84	1.37	1.78	1.37
E_3-17G	2.30	1.05	2.52	1.07	2.52	1.04	2.56	1.05	2.61	1.04	2.57	1.04	2.52	1.03	2.44	1.04
E_3-16G	2.42	1.45	2.69	1.24	2.62	1.27	2.70	1.25	2.71	1.25	2.67	1.25	2.58	1.30	2.53	1.24
E_2-17G	3.52	–	3.34	–	3.33	–	3.36	–	3.38	–	3.35	–	3.36	–	3.13	–

Component	*Phase system: Cellex E; 1.0 M acetate; 25°*													
	pH 3.4		*pH 4.2*		*pH 4.6*		*pH 5.0*		*pH 5.4*		*pH 5.8*		*pH 6.4*	
	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$
T-G	2.05	3.6	1.67	2.20	1.18	1.97	1.26	1.82	1.07	2.34	1.16	2.07	1.11	2.30
E_3-3G	7.25	1.42	3.67	1.20	2.33	1.18	2.29	1.23	2.50	1.13	2.39	1.20	2.55	1.14
E_1-G	10.4	1.31	4.4	1.48	2.75	1.54	2.82	1.54	2.83	1.57	2.86	1.60	2.92	1.63
E_2-3G	13.65	–	6.52	1.30	4.23	1.38	4.35	1.45	4.46	–	4.58	1.51	4.77	1.59
E_3-17G	–	–	8.45	1.15	5.83	1.13	6.32	1.13	–	–	6.90	1.09	7.60	1.08
E_3-16G	–	–	9.7	1.09	6.57	1.19	7.15	1.10	7.63	–	7.51	1.16	8.17	1.13
E_2-17G	–	–	10.6	–	7.8	–	7.88	–	–	–	8.75	–	9.25	–

Component	*Phase system: Cellex E; 1.0 M formate; 25°*							
	pH 3.4		*pH 4.0*		*pH 5.0*		*pH 5.6*	
	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$	K_n	$r_{(n+1)n}$
T-G	1.38	2.12	0.86	2.31	0.85	2.14	0.85	2.03
E_3-3G	2.93	1.17	1.99	1.18	1.82	1.18	1.73	1.21
E_1-G	3.43	1.48	2.35	1.48	2.15	1.53	2.10	1.53
E_2-3G	5.07	1.45	3.48	1.53	3.29	1.60	3.21	1.68
E_3-17G	7.37	1.31	5.34	1.04	5.25	1.10	5.38	1.10
E_3-16G	7.34	1.00	5.55	1.26	5.77	1.15	5.94	1.14
E_2-17G	9.65	–	7.00	–	6.61	–	6.78	–

pH when the formation constant is much smaller than the reciprocal of the hydrogen ion concentration. It is obvious that in this case the selectivity factor is constant. When the formation constant K_2 is of the order of or larger than the reciprocal of the hydrogen ion concentration, the distribution coefficient decreases with decreasing pH. In this pH range, a change in the selectivity factor with pH occurs if the formation constants differ. For the case when the protonated form HA is a weak acid, the A^- concentration will decrease with decreasing pH and consequently the distribution coefficient increases.

The results in Table VI and Fig. 4 can be explained by the prediction of eqn. 4, considering the proportionality of the capacity ratio and the distribution coefficient. For the chloride anion, the capacity ratio is about constant, while for the

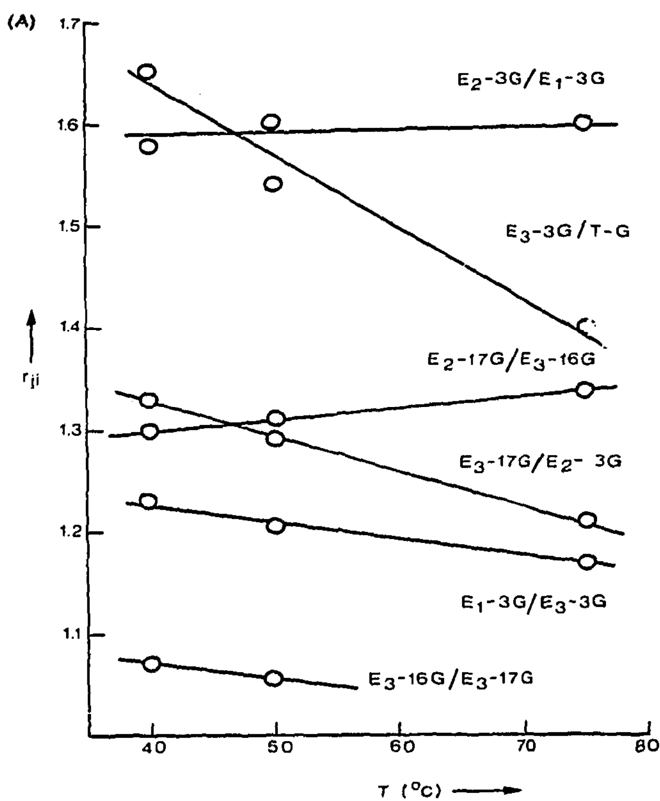
TABLE VII

EFFECT OF TEMPERATURE ON THE CAPACITY RATIO AND THE SELECTIVITY FACTOR OF SUCCESSIVE COMPONENTS

Component	*Phase system: Cellex E; 0.5 M chloride + 0.05 M acetate; pH 4.5*					
	40°		*50°*		*75°*	
	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	1.20	1.65	1.20	1.54	1.20	1.40
E_3-3G	1.98	1.23	1.85	1.21	1.68	1.17
E_1-G	2.44	1.58	2.24	1.60	1.96	1.60
E_2-3G	3.86	1.33	3.58	1.28	3.13	1.20
E_3-17G	5.14	1.07	4.58	1.06	—	—
E_3-16G	5.51	1.30	4.83	1.32	3.76	1.34
E_2-17G	7.17	—	6.37	—	5.04	—

Component	*Phase system: Baker 300; 0.125 M chloride + 0.05 M acetate; pH 5.0*							
	25°		*40°*		*55°*		*70°*	
	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	0.92	1.38	0.92	1.35	0.89	1.31	0.87	1.29
E_3-3G	1.26	1.25	1.24	1.21	1.16	1.18	1.11	1.15
E_1-G	1.58	1.40	1.50	1.44	1.38	1.45	1.28	1.51
E_2-3G	2.21	1.13	2.17	1.09	2.00	1.09	1.93	1.05
E_3-17G	2.49	1.07	2.38	1.07	2.18	1.03	2.03	1.02
E_3-16G	2.65	1.35	2.55	1.33	2.23	1.34	2.07	1.33
E_2-17G	3.59	—	3.39	—	2.99	—	2.76	—

Component	*Phase system: Cellex AE; 0.125 M chloride + 0.05 M acetate; pH 5.0*			
	25°		*70°*	
	κ_n	$r_{(n+1)n}$	κ_n	$r_{(n+1)n}$
T-G	0.82	1.24	0.73	1.15
E_3-3G	1.01	1.09	0.84	1.04
E_1-G	1.10	1.26	0.87	1.32
E_2-3G	1.39	1.68	1.15	1.37
E_3-17G	2.34	1.08	1.57	1.06
E_3-16G	2.70	—	1.65	1.00
E_2-17G	2.52	1.07	1.66	—



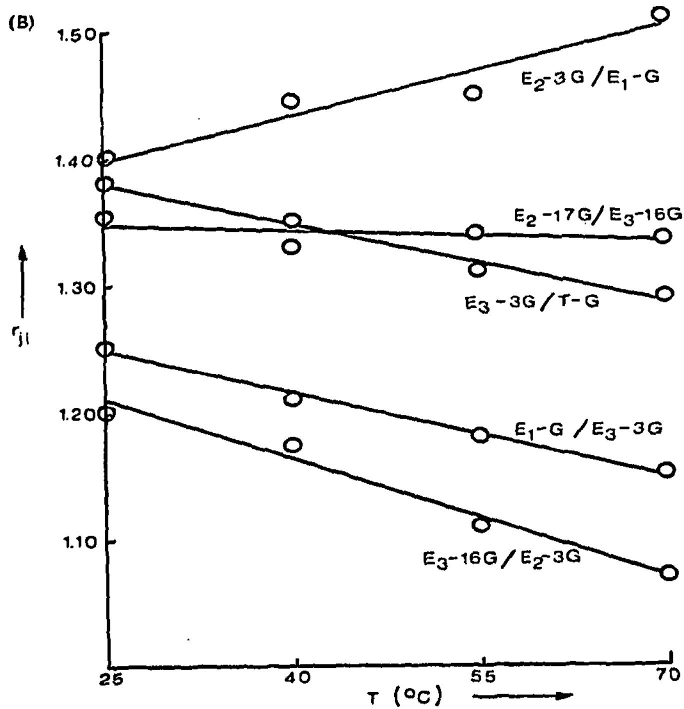


Fig. 5. The effect of temperature on the selectivity factor. Phase systems: A, Cellex E; 0.5 *M* chloride + 0.05 *M* acetate, pH 4.5; B, Baker 300; 0.125 *M* chloride + 0.05 *M* acetate, pH 5.0.

acetate anion the capacity ratio increases when the pH decreases beyond the pK_a of the acetic acid. The selectivity factor is only slightly influenced by the pH.

Effect of temperature. Capacity ratios and selectivity factors were measured at different temperatures on three ion-exchange systems with ECTEOLA- and AE-cellulose. The results are presented in Table VII and Fig. 5. It can be seen that the capacity ratio decreases with temperature, as usual. In some instances, the selectivity factor changes only slightly with temperature, while in others significant changes can be seen. In terms of resolution, the changes in the selectivity factors are mostly not very important, as they are either small or the values of the selectivity factors and those of the corresponding capacity ratios are relatively large, making the separation easy. An exception is the selectivity factor of E_3-16G and E_2-3G on Baker 300 ECTEOLA-cellulose, which increases from 1.07 at 70° to 1.20 at 25°.

Column efficiency

Theoretical plate height versus fluid velocity. In routine analysis, such as the determination of estrogens in urine, the speed of separation is of particular importance. An expression describing the retention time required for a given resolution is derived by substituting the theoretical plate number in eqn. 1 by means of the expression $NH_i = t_{Ri} \cdot v/(\kappa_i + 1)$:

$$t_{Ri} = \left(\frac{R_{ji}}{r_{ji} - 1}\right)^2 \cdot \frac{(\kappa_i + 1)^3}{\kappa_i^2} \cdot \frac{H_i}{v} \tag{5}$$

where v = average velocity of the eluent.

The ratio H/v should be made as small as possible when high-speed separation is required. It decreases with increasing fluid velocity and decreasing particle size. In

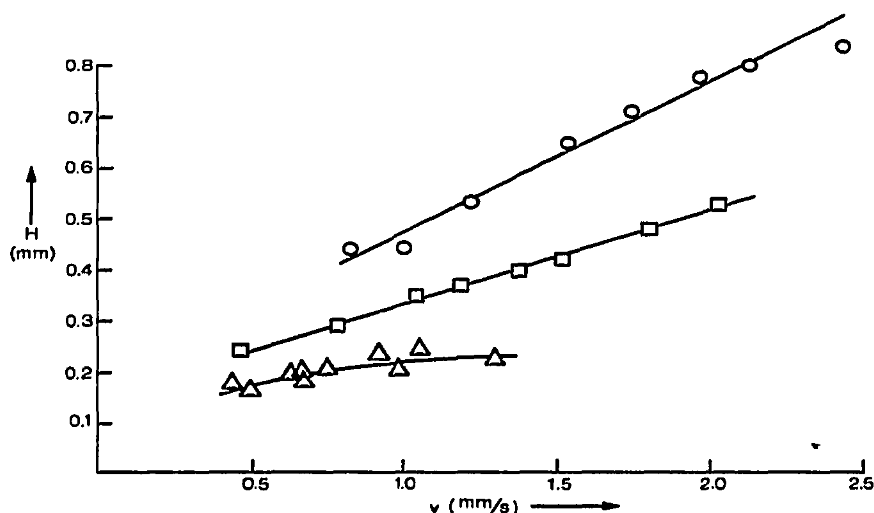


Fig. 6. H *versus* v curves for ECTEOLA-celluloses of different average particle size: ○, 20 μm; □, 18 μm; △, 12 μm. The test component is E_3-3G with κ = 0.99 (20 μm), 1.36 (18 μm) and 1.18 (12 μm). Phase system: Cellex E; 0.5 M chloride + 0.05 M acetate, pH 5.0; 70°.

TABLE VIII

COMPARISON OF THE EFFICIENCIES OF COLUMNS PACKED WITH DIFFERENT TYPES OF ANION EXCHANGERS

Property	*Anion exchanger*							
	Servacel TLC		*MN 300/A (Baker 300)*		*Cellex E*		*ET 41*	
Mean particle diameter (μm)	11		14		12		10	
Eluent	0.125 *M* chloride + 0.01 *M* acetate		0.5 *M* chloride + 0.05 *M* acetate		0.5 *M* chloride + 0.05 *M* acetate		0.25 *M* chloride + 0.025 *M* acetate	
pH	5.0		5.0		5.0		5.0	
Temperature (°C)	70		70		70		70	
κ (E_3-3G)	0.5		0.8		1.18		2.3	
v and H	v *(mm/sec)*	H *(mm)*	v *(mm/sec)*	H *(mm)*	v *(mm/sec)*	H *(mm)*	v *(mm/sec)*	H *(mm)*
	0.38	0.29	0.42	0.14	0.48	0.16	0.42	0.12
	0.82	0.48	0.87	0.18	0.82	0.19	0.88	0.16
	1.28	0.70	1.36	0.22	1.29	0.22	1.34	0.23
	1.64	0.76	1.74	0.25	–	–	–	–
	2.11	0.99	–	–	–	–	2.19	0.29
$(H/v)_{min.}$ (sec)	0.47		0.14		0.17		0.13	

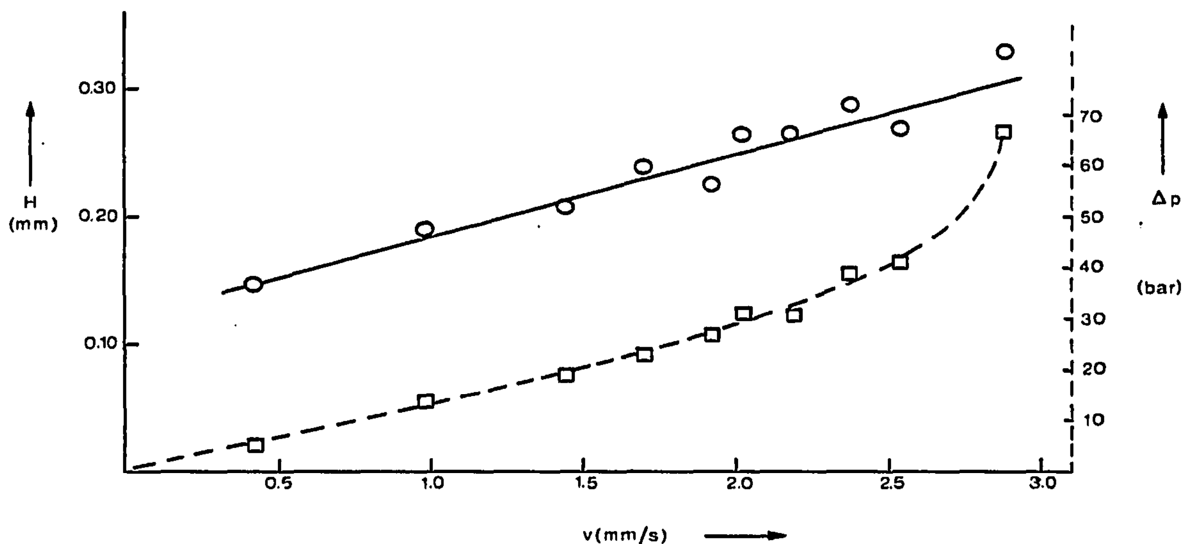


Fig. 7. *H versus v* (———) and *Δp versus v* (— — —) curves for aminoethylcellulose. Phase system: Cellex AE, 14 μm; 0.05 *M* acetate, pH 5.0; 70°.

practice, the lowest attainable value, $(H/v)_{min.}$, is determined by the maximum attainable fluid velocity, which is determined in the case of cellulose ion exchangers by the type of ion exchanger, its particle size and the pressure drop used during the packing of the column. Above the maximum fluid velocity, the column packing collapses as the pressure drop becomes too large.

Effect of particle size. The theoretical plate height was measured as a function of the fluid velocity at different average particle sizes for a given batch of ECTEOLA-cellulose, the columns being packed at 10 bar. The results are plotted in Fig. 6. Other batches of ECTEOLA-cellulose, with one exception, gave similar results, as can be seen from Table VIII. When an ECTEOLA-cellulose (Baker 300) with a mean

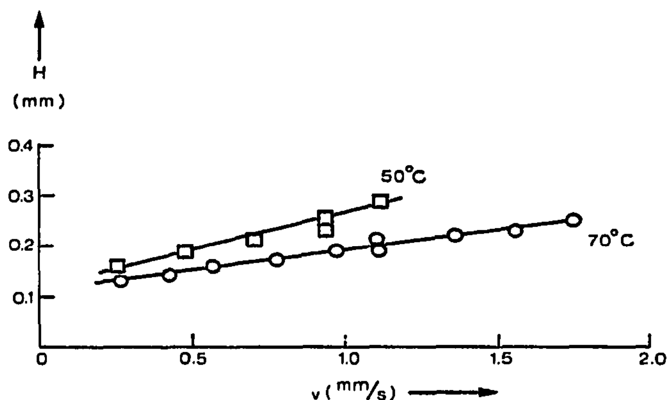


Fig. 8. *H versus v* curves at different temperatures. Test compound, E_3-3G; κ = 0.8 (70°) and 0.9 (50°). Phase system: MN 300, 14 μm; 0.5 *M* chloride + 0.05 *M* acetate, pH 5.0.

TABLE IX

RESOLUTION PARAMETERS OF THE MOST DIFFICULT TO SEPARATE ESTROGEN GLUCURONIDES FOR SELECTED PHASE SYSTEMS

Property	*Anion exchanger*									
	Baker 300			*Cellex E*			*Cellex AE*			
Mean particle size (μm)	13			12			14			
Eluent	0.125 *M* chloride + 0.05 *M* acetate			0.5 *M* chloride + 0.05 *M* acetate			0.125 *M* chloride + 0.05 *M* acetate			
pH	5.0			4.5			5.0			
Temperature (°C)	25			75			25		70	
Components: n	E_3-3G	E_2-3G	E_3-17G	E_3-3G	E_2-3G	E_3-17G	E_3-3G	E_3-17G	E_3-3G	E_3-17G
$n+1$	E_1-G	E_3-16G	E_3-16G	E_1-G	E_3-16G	E_3-16G	E_1-G	E_3-16G	E_1-G	E_3-16G
$(r_{(n+1)/n} - 1)\,\kappa_n/(\kappa_n + 1)$	0.14	0.14	0.05	0.10	0.15	<0.03	0.05	0.11	0.02	0.04
$N_{R=4}$	800	800	6000	1700	700	–	8000	1400	50 000	10 000
$v_{max.}$ (mm/sec)	1.5	–	–	1.3	–	–	1.3	–	2.5	–
$(H_n/v)_{min.}$ (sec)	0.27	–	–	0.17	–	–	0.28	–	0.11	–
t_{R_1} (min)	17	17	130	30	12	–	130	25	240	57
$(\Delta P/L)_{max.}$ (bar/cm)	0.65	–	–	0.60	–	–	2.5	–	1.6	–

particle size of 8 μm was used and the column was packed at a pressure of 30 bar, a theoretical plate height of 0.08 mm at a maximum allowable flow-rate of 0.4 mm/sec was achieved at a capacity ratio of 1.3. Another batch (MN 300/B) gave results similar to those with Servacel TLC.

An example of the results obtained with AE-cellulose is shown in Fig. 7, which demonstrates also the more than proportional increase of the pressure drop near to the maximum attainable fluid velocity.

Effect of temperature. An example of the influence of temperature on the theoretical plate height is shown in Fig. 8. The minimum attainable value of the ratio H/v increases in this case from 0.14 sec at 70° to 0.25 sec at 50°. In general, the minimum H/v ratio increases by a factor of about two when the temperature is decreased from 70° to 25°.

Choice of an optimum phase system for the separation of estrogen glucuronides

For a given selectivity factor and capacity ratio, a certain number of theoretical plates is required in order to achieve a given resolution. The theoretical plate number, N_R, required for the resolution R_{jl} can be calculated from eqn. 1:

$$N_R = \left(\frac{R_{jl}}{r_{jl} - 1}\right)^2 \left(\frac{\kappa_l + 1}{\kappa_l}\right)^2 \tag{6}$$

The corresponding length of the column is given by $L_R = N_R H$. In practice, the maximum length is limited by the pressure limit of the apparatus or the column packing.

The time in which the separation of the total mixture can be performed is given by the retention time of the last-eluting compound 1:

$$t_{R_1} = \frac{N_R H_n}{v} \cdot (1 + \kappa_1) \tag{7}$$

where n refers to that component of the mixture which has the largest value of $N_R H_n/v$. In order to increase the speed of separation, the value of the capacity ratio, κ_1, of the last-eluting component as well as the largest value of $N_R H_n/v$ occurring in the mixture should be minimized.

The required theoretical plate number, $N_{R=4}$, for a resolution of four was calculated for each successive pair of components from the data in Tables II–VII. The groups of values obtained under the same conditions were compared. Because E_3-17G is clinically unimportant, the comparison was made both inclusive and exclusive of this compound. The systems and temperatures which gave the lowest values for $N_{R=4}$ were further evaluated with regard to the practical minimum value of H/v and the minimum separation time was calculated according to eqn. 7. The results are summarized in Table IX, in which those components which are easily separated are not included. The following conclusions can be drawn from Table IX.

(1) The changes in the separation time are caused mainly by the changes in the factor $(r_{(n+1)n} - 1)\ \kappa_n/(\kappa_n + 1)$ and less by the changes in the value of $(H/v)_{min.}$.

(2) None of the phase systems is the best for the separation of each pair of estrogen glucuronides. E_1-G and E_3-3G are best separated in the first system, E_3-16G and E_2-3G in the second system and E_3-16G and E_3-17G at 25° in the third system.

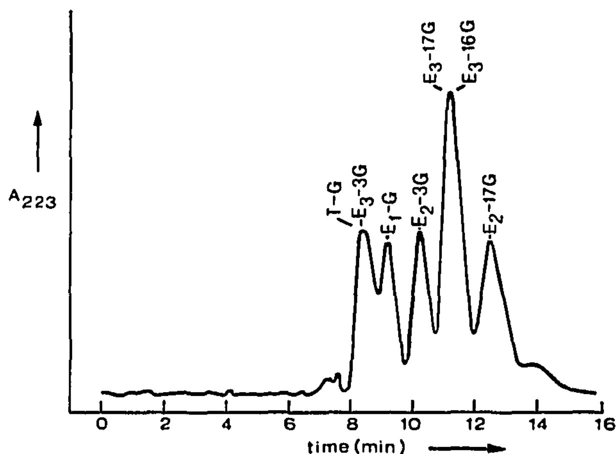


Fig. 9. Separation of a test mixture of estrogen glucuronides on an ECTEOLA-cellulose of low ion-exchange capacity. Phase system: MN 300, 7 μm; 0.25 *M* chloride + 0.05 *M* acetate, pH 5.0; 70°.

The corresponding minimum separation times are 17, 12 and 25 min, respectively.

(3) The best system for the separation of all six estrogen glucuronides, representing a compromise, is the first system, although the separation of E_3-16G and E_3-17G is difficult and takes 130 min. Excluding E_3-17G, the separation can be performed in 17 min.

The choice of the best phase system and the best working conditions is illustrated by a number of chromatograms. Fig. 9 demonstrates an insufficient separation resulting from a too low capacity ratio due to the low ion-exchange capacity of the column packing. Fig. 10 shows the improvement in resolution that can be achieved with the same separation time with a better packing material. Fig. 11 demonstrates that the resolution can be improved further in a longer time. The most difficult separation, that of E_3-16G and E_3-17G, can be accelerated by using a system that is more selective for this pair of compounds, as shown in Fig. 12.

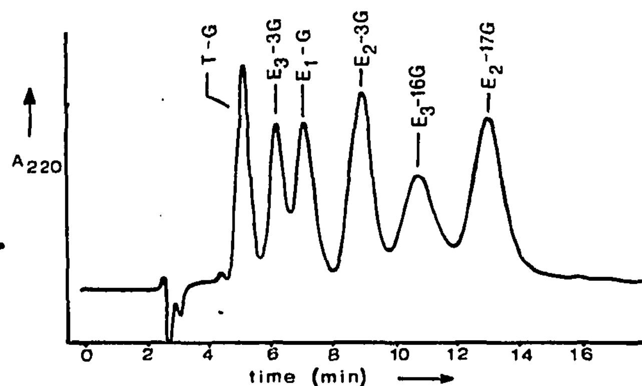


Fig. 10. Separation of a test mixture of estrogen glucuronides on an ECTEOLA-cellulose of high ion-exchange capacity. Phase system: Baker 300, 13 μm; 0.125 *M* chloride + 0.05 *M* acetate, pH 5.0; 25°.

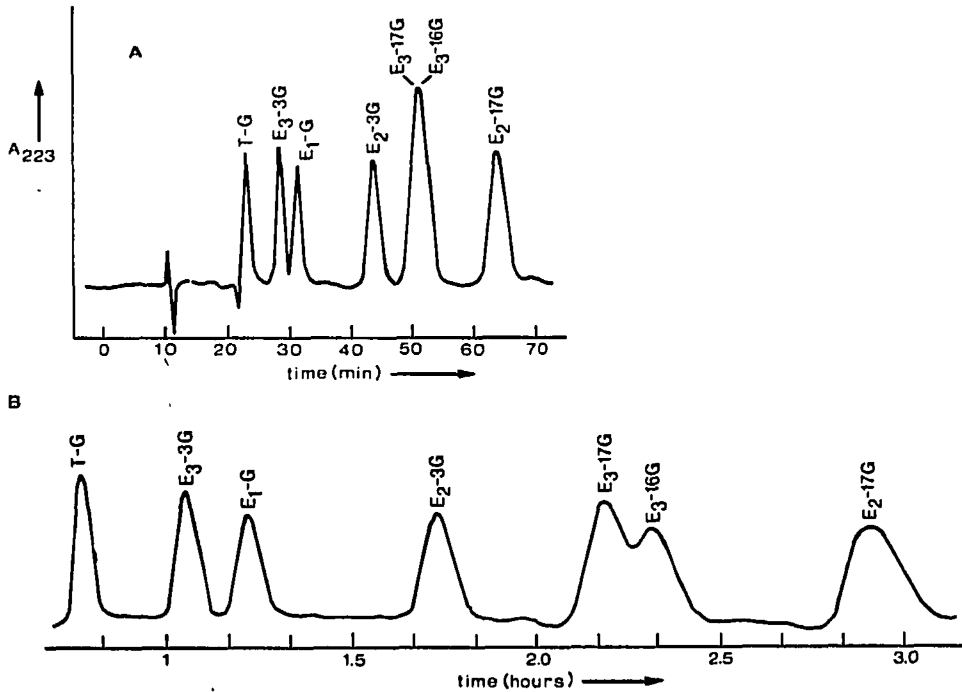


Fig. 11. Improvement in the resolution of a test mixture of estrogen glucuronides in a longer time. Phase system: Cellex E, 9 μm; 0.5 M chloride + 0.05 M acetate, pH 4.5; 75° (A) and 40° (B).

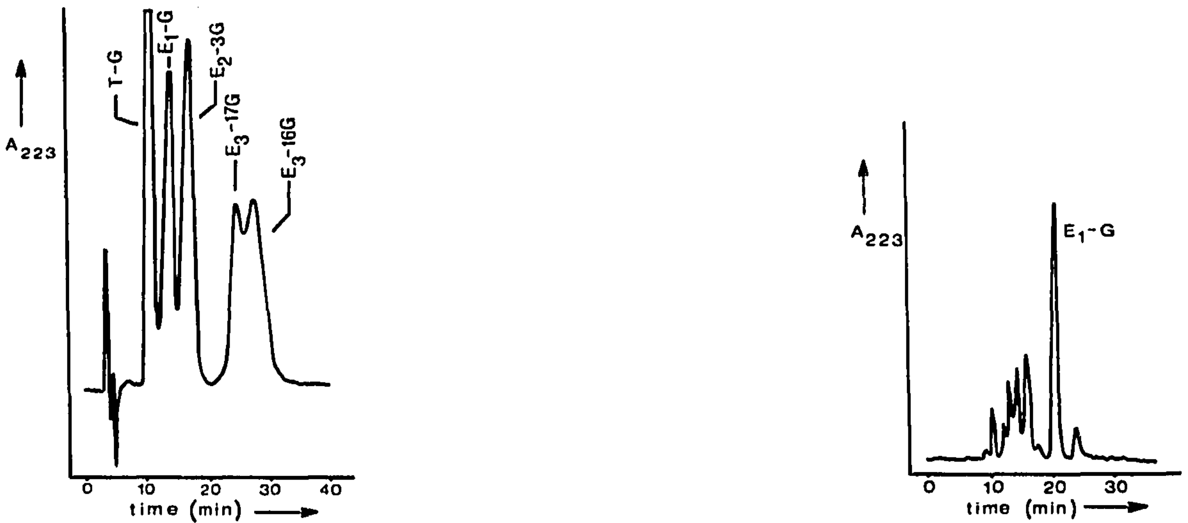


Fig. 12. Separation of a test mixture of estrogen glucuronides. Phase system: Cellex AE, 14 μm; 0.05 M chloride + 0.05 M acetate, pH 5.0; 25°.

Fig. 13. Purity control of a commercial product of E_1-G. Phase system: Baker 300, 8 μm; 0.125 M chloride + 0.05 M acetate, pH 5.0; 70°.

A first application is shown in Fig. 13. The chromatogram is the result of the purity control of a commercial product. This application illustrates the performance of the method in the analysis of steroid conjugates.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. K. Camstra, whose technical skill was indispensable, and Messrs. L. T. M. Schilder, E. Endert and M. P. Uitendaal for their technical assistance.

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