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IMPROVEMENT OF THE EFFICIENCY OF ION-EXCHANGE CELLULOSE COLUMNS AND THEIR APPLICATION

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SUMMARY

Packing procedures and operating conditions for microparticulate ion-exchange cellulose columns were investigated. The efficiencies of ion-exchange cellulose and mixed-bed ion-exchange cellulose columns were compared. The mixed-bed columns contained ion-exchange cellulose and diatomite, and those with a 5:1 volume ratio of ion-exchange cellulose (average particle size 7 μm) and diatomite were found to be superior in almost every respect.

Medical, pharmaceutical, biochemical and environmental applications of this type of column are shown.

INTRODUCTION

Ion-exchange high-pressure liquid chromatography, taking advantage of the ionic character of hydrophilic compounds, is widely used. The high-efficiency column packings that are commonly used in ion-exchange chromatography are polystyrene–divinylbenzene resins with sulphonic acid or quaternary ammonium groups attached. Recently, microparticulate ion exchangers with a silica matrix have been introduced[1,2]. Neither type of ion exchanger, however, could be applied successfully to the separation of certain types of hydrophilic compounds such as drugs and steroid conjugates, owing to strong adsorption at the matrix of the ion exchanger. This adsorption problem has been solved in the separation of estrogen conjugates[3] by using ion-exchange cellulose packings of small particle size, which combine a fairly high efficiency with excellent selectivity[4].

It therefore seems worthwhile to attempt to improve further the efficiency of cellulose ion-exchange columns, especially at high flow velocity. This paper describes the result of such an investigation, in which columns containing ion-exchange cellulose of small particle size were compared with mixed-bed columns containing ion-exchange cellulose and diatomite.

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EXPERIMENTAL

Apparatus

A commercial liquid chromatograph (Hewlett-Packard Model 1010A) and stainless-steel columns (250 × 3 mm) were used. The detector was a variable-wavelength UV spectrophotometer (Hewlett-Packard 1030B). The chromatograms were recorded with a linear potentiometric flat-bed recorder (Servogor RE 514.9). Estrogen conjugates were measured at 220 nm. Kanamycins were detected with an electrochemical detector developed in this laboratory[5].

Chemicals

Cytosine (E. Merck, Darmstadt, G.F.R.) was used as an unretarded tracer. The estrogen conjugates used as test compounds 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), 17β-estradiol 17-β-D-glucuronide (E_2-17G), all obtained from Sigma (St. Louis, Mo., U.S.A.); testosterone sulphate (T-S), estriol 3-sulphate (E_3-3S), estrone 3-sulphate (E_1-3S), 17β-estradiol 3-sulphate (E_2-3S), 17β-estradiol 17-sulphate (E_2-17S), estriol 3,17-disulphate (E_3-3,17diS), all obtained from Steraloids (Pawling, N.Y., U.S.A.); and 17α-estradiol 3-sulphate (17αE_2-3S), 17α-dihydro-equilin 3-sulphate (17αEq-3S), equilenin 3-sulphate (Eqe-3S) and 17α-dihydroequilenin 3-sulphate (17αEqe-3S), kindly given by Diosynth (Oss, The Netherlands).

Leucovorine (Ledervorin, 3 mg/ml, Lederle) and methotrexate (Ledertrexate, 5 mg per ampoule, Lederle) were a gift from E. van der Kleijn (University of Nijmegen, Nijmegen, The Netherlands).

The samples of fulvic acids obtained as fractions from a Sephadex G-25 column were kindly supplied by C. Beyer (Limnologic Institute, Nieuwersluis, The Netherlands).

Eluents were prepared with deionized water. The specified pH was adjusted with either orthophosphoric acid or sodium hydroxide.

ECTEOLA-cellulose (B-300, Baker, Deventer, The Netherlands, and ET 41, Whatman, Maidstone, Great Britain) and cellulose phosphate (P-300, Machery, Nagel & Co., Düren, G.F.R.), were used together with and without diatomite (Kieselgur, Merck) as column packings. ECTEOLA-cellulose, cellulose phosphate and diato-

TABLE I

PARTICLE SIZES OF ANION-EXCHANGE CELLULOSE FRACTIONS AND DIATOMITE

Type of material	*Particle diameter range 10–90% (μm)*	*Mean particle diameter (μm)*
B-300/13 μm	10–18	13
B-300/11 μm	8–16	11
B-300/ 7 μm	–11	7
B-300/ 4 μm	–9	4
ET41/11 μm	6–16	11
P 300/ 7 μm	–11	7
Diatomite	–7	4

mite were fractionated with an air classifier (Alpine Model 100 MZR) and the particle size distributions of the cellulose fractions were determined with a Coulter Counter (Model D) (see Table I). The cellulose anion exchangers were swollen by pre-cycling with 0.5 *N* sodium hydroxide solution, 0.5 *N* hydrochloric acid and 0.5 *N* sodium hydroxide solution and rinsed with deionized water. The ion-exchange capacity of 0.15 mequiv./g for B-300 was determined as the buffer capacity between pH 10 and 4 in 0.5 *N* sodium chloride solution using an automatic titrator (Radiometer TTT1). Settled bed volumes were calculated after storing a given amount of packing material suspended in 50% (v/v) aqueous ethanol overnight in a measuring cylinder.

The ortho phosphoric acid, hydrochloric acid, sodium hydroxide and sodium perchlorate used were of p.a. quality (Merck).

RESULTS AND DISCUSSION

Optimization of the chromatographic separation of two compounds *i* and *j* can be achieved on the basis of the three-term equation for the resolution, R_{ji}:

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

where

r_{ji} = selectivity coefficient = ratio of the capacity ratios of the components *j* and *i* in the phase system;
$\kappa_i = t_{Ri} - t_{R0}/t_{R0}$ = capacity ratio of component *i*;
t_{Ri} = retention time of component *i*;
t_{R0} = retention time of an inert tracer;
L = length of the column;
H_i = theoretical plate height for component *i*.

The first two terms depend strongly on the type of the sample components *j* and *i*, while the last term depends mainly on the column geometry and only to a minor extent on the type of sample component *i*. The dependence of the theoretical plate height on the process parameters can be approximated by the equation[6]

$$H_i = \frac{a_d}{u} + \frac{a_c' \bar{d}_p}{1 + a_c''(u \cdot \bar{d}_p)^{-\frac{1}{3}}} + a_f u^{\frac{1}{3}} \bar{d}_p^{3/2} \left(\frac{\kappa_i}{\kappa_i + 1}\right)^2 + a_b u \bar{d}_p^{\,2} \cdot \frac{\kappa_i}{(\kappa_i + 1)^2} \tag{2}$$

where

$a_d, a_c', a_c'', a_f, a_b$ = factors dependent on the diffusion coefficients of component *i* in the fluid stream and in the fixed bed, respectively, and describing the effect of the packing geometry with respect to diffusion, convective mixing, mass transfer in the flowing fluid and mass transfer in the fixed bed;
u = linear flow velocity;
$\bar{d}_p$ = mean particle size of the column packing material.

The first term is the contribution to the theoretical plate height due to longitudinal diffusion and is negligible at the linear velocities used. The second term arises

from the convective mixing and approaches a constant value at higher flow velocities. The third and fourth terms are the contributions to the theoretical plate height related to the speed of mass transfer in the fluid stream and in the fixed bed. They both depend on the flow velocity and the capacity ratio of the component and decrease strongly with increasing particle size. The fourth term is most dependent on particle size.

TABLE II

INFLUENCE OF PARTICLE SIZE, PACKING SOLVENT AND PACKING PRESSURE ON THE EFFICIENCY OF COLUMNS OF ECTEOLA-CELLULOSE

0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

(A) B-300/13 μm:

Packing pressure 35 bar, $\frac{\Delta p}{u L} = 5$ *bar·sec/cm², packed in eluent*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.55	0.38	0.39	0.40	0.42
0.85	0.45	0.46	0.47	0.46
1.08	0.48	0.48	0.52	0.52
1.39	0.53	0.61	0.60	0.57
1.94	0.62	0.71	0.70	0.73
κ_i	1.0	2.0	5.2	11.8

Packing pressure 35 bar, $\frac{\Delta p}{u L} = 6$ *bar·sec/cm², packed in 50% aqueous ethanol*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.52	0.28	0.27	0.22	0.22
0.67	0.26	0.26	0.24	0.25
0.83	0.27	0.28	0.27	0.29
1.00	0.30	0.32	0.32	—
1.23	0.33	0.35	0.34	0.36
1.46	0.37	0.40	0.37	0.40
1.74	0.445	0.46	0.44	0.455
κ_i	0.85	1.7	4.4	10.3

(B) B-300/11 μm:

Packing pressure 20 bar, $\frac{\Delta p}{u L} = 7$ *bar·sec/cm², packed in eluent, eluent pH 7.0*

u (mm/sec)	$H_{T\text{-}G}$ (mm)	$H_{E_2\text{-}17G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{E_2\text{-}17S}$ (mm)	$H_{E_3\text{-}3,17dlS}$ (mm)
0.96	0.26	0.29	0.29	0.33	0.44
1.02	0.32	0.33	0.33	0.35	0.42
1.14	0.33	0.43	0.40	0.37	0.47
1.24	0.32	0.35	0.38	0.37	0.46
1.39	0.41	0.42	0.39	0.43	0.53
1.67	0.38	0.47	0.42	0.43	0.61
1.81	0.43	—	0.44	0.46	0.60
κ_i	0.45	2.48	3.33	5.80	7.58

TABLE II (*continued*)

(B) B-300/11 μm:

Packing pressure 40 bar, $\frac{\Delta p}{u L} = 12$ *bar·sec/cm², packed in 50% aqueous ethanol*

u (*mm/sec*)	$H_{E_3\text{-}3G}$ (*mm*)	$H_{E_2\text{-}3G}$ (*mm*)	$H_{E_1\text{-}3S}$ (*mm*)	$H_{Eqe\text{-}3S}$ (*mm*)
0.27	0.16	0.16	0.14	0.14
0.46	0.16	—	0.15	0.16
0.60	0.19	0.17	0.17	0.19
0.76	0.22	0.22	0.22	0.22
0.90	0.23	0.23	0.22	0.24
1.12	0.24	0.25	0.24	0.26
1.21	0.26	0.29	0.27	0.31
κ_t	0.8	1.6	4.1	9.4

(C) B-300/7 μm:

Packing pressure 40 bar, $\frac{\Delta p}{u L} = 13$ *bar·sec/cm², packed in 50% aqueous ethanol*

u (*mm/sec*)	$H_{E_3\text{-}3G}$ (*mm*)	$H_{E_2\text{-}3G}$ (*mm*)	$H_{E_1\text{-}3S}$ (*mm*)	$H_{Eqe\text{-}3S}$ (*mm*)
0.32	0.15	0.125	0.115	0.12
0.42	0.15	0.14	—	0.13
0.55	0.16	0.15	0.14	0.14
0.68	0.17	0.16	0.145	0.145
0.76	0.22	0.20	0.165	0.175
κ_t	0.9	1.8	4.7	10.8

Ion-exchange cellulose columns

Columns of ECTEOLA-cellulose of different particle size were packed and their chromatographic characteristics measured (see Table II).

The conventional slurry packing method gives good results with glass tubes, provided that the particle diameter range is narrow[3]. This slurry technique, in which a suspension of cellulose anion exchanger in the eluent to be used for the separation is pumped from a pre-column into the separation column, has been found to be susceptible of improvement when using commercial stainless-steel tubes. The methods tried to pack these tubes were dry packing, packing in aqueous caesium chloride solutions of various concentrations and packing with a slurry in ethanol, 50% aqueous ethanol, ethylene glycol, 50% aqueous glycerol and 40% sucrose in water.

Dry packing[7] gave irreproducible and less efficient columns, except for microcrystalline Servacel CM 32, a carboxymethylcellulose cation exchanger, which is a free-flowing powder that can be poured into the tube. These microcrystalline cellulose columns have good efficiencies but can be employed only at low linear flow velocities owing to their low permeability.

Columns packed with a slurry in pure ethanol were comparable in efficiency with those packed with a slurry in eluent; aqueous caesium chloride solutions caused aggregation of the cellulose anion exchanger particles and gave poorer columns.

More efficient columns can be packed when the cellulose anion exchanger is

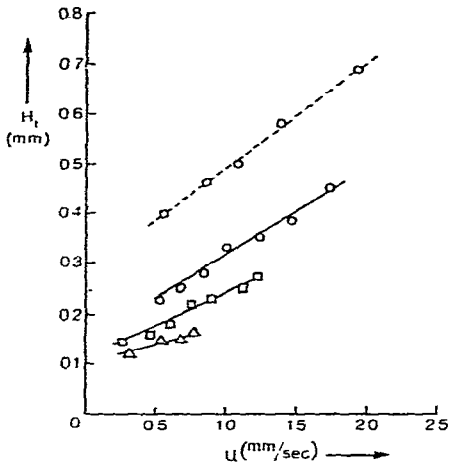
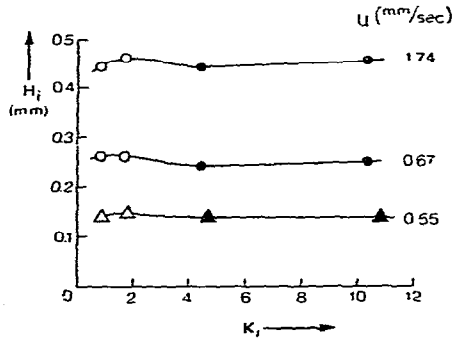


Fig. 1. H_t *versus* u curves for anion-exchange cellulose columns with different mean particle size, packed in eluent (– – –) and 50% (v/v) aqueous ethanol (———). Test compound, E_1-3S. ○—○, $\kappa = 5.2$; ○—○, $\kappa = 4.4$; □—□, $\kappa = 4.1$; △—△, $\kappa = 4.7$. Columns: ○, B-300/13 μm; □, B-300/11 μm; △, B-300/7 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

suspended in 50% aqueous ethanol, a viscous ($\eta = 2.9$ cP) liquid from which the ethanol is absorbed by the exchanger causing it to alter and shrink. The flow resistance of columns packed in 50% aqueous ethanol is higher than that of columns packed in eluent at the same pressure; the capacity ratios are up to 20% lower than those in the absence of ethanol. No noticeable selectivity changes due to this packing technique have been observed. The way in which the column efficiency is affected by this novel packing method can be seen in Fig. 1. The broken line for the eluent-packed column is about parallel with the line for the ethanol-packed column, which indicates that the contribution of convective mixing to the theoretical plate height has been minimized



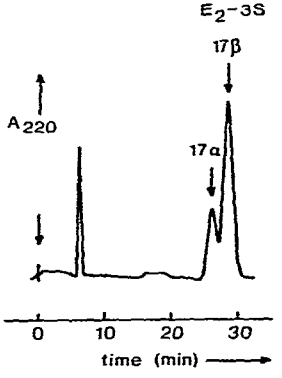


Fig. 2. H_i *versus* κ_i curves for anion-exchange cellulose columns. Values are corrected for extra-column contribution. Test compounds: ○, △, E_3-3G and E_2-3G; ●, ▲, E_1-3S and Eqe-3S. Columns: ○, ●, B-300/13 μm; △, ▲, B-300/7 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

Fig. 3. Separation of the isomers of 17α-estradiol 3-sulphate and 17β-estradiol 3-sulphate. Column: ET-41/11 μm; 0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5; 70°; $\Delta p = 30$ bar.

TABLE III

INFLUENCE OF THE SETTLED-BED VOLUME RATIO OF ECTEOLA-CELLULOSE–DIATOMITE MIXED-BED COLUMNS ON THEIR EFFICIENCY

B-300/13 μm–diatomite; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°. Columns packed in 50% aqueous ethanol at 35 bar.

B-300–diatomite 5:5 (settled-bed volume ratio), $\frac{\Delta p}{u L} = 5$ *bar·sec/cm²*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{E_{qe}\text{-}3S}$ (mm)
0.66	0.36	0.40	0.62	0.64
0.99	0.38	0.42	0.66	0.75
1.32	0.39	0.48	0.75	0.89
1.63	0.42	0.51	0.78	0.93
1.93	—	0.59	0.81	1.04
2.25	—	0.71	0.96	1.14
κ_t	0.23	0.46	1.09	2.55

B-300–diatomite 5:3, $\frac{\Delta p}{u L} = 6$ *bar·sec/cm²*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{E_{qe}\text{-}3S}$ (mm)
0.88	0.25	0.31	0.47	0.49
1.12	0.29	0.36	0.54	0.58
1.41	0.35	0.42	0.59	0.61
1.74	0.40	0.47	0.63	0.73
2.06	0.42	0.51	0.67	0.80
2.32	0.44	0.56	0.73	0.89
2.52	0.48	0.62	0.82	0.94
2.78	0.57	0.69	0.82	1.02
κ_t	0.35	0.70	1.7	3.9

B-300–diatomite 5:1, $\frac{\Delta p}{u L} = 9$ *bar·sec/cm²*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{E_{qe}\text{-}3S}$ (mm)
0.60	0.17	0.20	0.225	0.225
0.76	0.215	0.225	0.235	0.235
0.97	0.22	0.25	0.265	0.265
1.39	0.28	0.34	0.37	(0.44)
κ_t	0.65	1.3	3.0	6.9

B-300, $\frac{\Delta p}{u L} = 11$ *bar·sec/cm²*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{E_{qe}\text{-}3S}$ (mm)
0.61	0.25	0.255	0.24	0.25
0.78	0.26	0.29	0.25	0.285
0.84	0.28	0.29	0.295	—
1.24	0.34	0.345	0.35	0.37
1.39	0.36	0.37	0.37	0.38
κ_t	0.9	1.85	4.6	10.5

TABLE IV

INFLUENCE OF PARTICLE SIZE AND PACKING SOLVENT ON THE EFFICIENCY OF COLUMNS OF ECTEOLA-CELLULOSE MIXED WITH DIATOMITE

B-300–diatomite 5:1 (settled-bed volume ratio); 0.025 *M* perchlorate + 0.01 *M* phosphate pH 6.8; 70°.

(A) B-300/13 μm:

Packing pressure 35 bar, $\frac{\Delta p}{u L} = 5$ *bar·sec/cm², packed in eluent*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_2\text{-}17G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{E_2\text{-}3S}$ (mm)
0.72	0.195	0.205	0.20	0.20	0.23
1.14	0.215	0.255	0.27	0.295	0.30
1.63	0.26	0.31	0.32	0.35	0.36
2.03	0.30	0.33	—	0.38	0.41
2.32	—	—	0.41	—	0.46
2.72	0.34	0.45	—	0.50	0.53
3.08	0.42	0.50	0.57	0.58	0.60
3.68	0.58	0.66	0.64	0.68	0.76
3.80	0.60	0.64	0.72	0.74	0.83
κ_i	0.6	1.2	1.9	2.9	4.4

Packing pressure 40 bar, $\frac{\Delta p}{u L} = 9$ *bar·sec/cm², packed in 40% aqueous sucrose*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.58	0.15	0.145	0.15	0.16
0.79	0.17	0.17	0.18	0.185
0.98	0.19	0.19	0.18	0.21
1.24	0.21	0.23	0.215	0.22
1.41	0.205	0.25	0.225	0.255
1.57	0.225	0.25	0.255	0.295
1.81	0.25	0.265	0.265	0.32
1.98	0.27	0.31	0.305	0.38
κ_i	0.5	1.1	2.9	6.9

(B) B-300/11 μm:

Packing pressure 35 bar, $\frac{\Delta p}{u L} = 5.5$ *bar·sec/cm², packed in eluent*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.74	0.16	0.18	0.18	0.19
1.10	0.20	0.20	0.23	0.26
1.44	0.21	0.26	0.28	0.32
1.74	0.22	0.32	0.42	0.40
2.08	0.26	0.35	0.43	0.39
2.32	0.33	0.50	0.43	0.45
2.58	0.45	0.50	0.53	0.52
κ_i	0.65	1.35	3.6	8.1

TABLE IV (*continued*)

(B) B-300/11 μm:

Packing pressure 35 bar, $\frac{\Delta p}{u L} = 8$ *bar·sec/cm², packed in 50% aqueous ethanol*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.52	0.135	0.14	0.145	0.15
0.76	0.14	0.17	0.195	0.195
0.97	0.17	0.23	0.20	0.21
1.23	0.18	0.23	0.245	0.24
1.39	0.20	0.25	0.245	0.26
1.60	0.26	0.30	0.28	0.30
1.82	0.28	0.29	0.28	0.35
1.98	0.295	0.325	0.32	0.35
κ_t	0.5	1.1	3.0	6.9

(C) B-300/7 μm:

Packing pressure 35 bar, $\frac{\Delta p}{u L} = 9$ *bar·sec/cm², packed in eluent*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.62	0.13	0.115	0.11	0.11
0.82	0.135	0.145	0.13	0.14
1.04	0.145	0.17	0.155	0.165
1.23	0.18	0.18	0.17	0.17
1.39	0.22	0.24	0.21	0.22
1.84	0.29	0.24	0.26	0.29
κ_t	0.7	1.5	3.9	8.9

(D) B-300/4 μm:

Packing pressure 40 bar, $\frac{\Delta p}{u L} = 11$ *bar·sec/cm², packed in eluent*

u (mm/sec)	$H_{E_3\text{-}3G}$ (mm)	$H_{E_2\text{-}3G}$ (mm)	$H_{E_1\text{-}3S}$ (mm)	$H_{Eqe\text{-}3S}$ (mm)
0.44	0.12	0.115	0.105	—
0.63	0.15	0.145	0.13	0.14
0.91	0.185	0.16	0.16	0.16
1.09	0.17	0.18	0.185	0.17
1.25	0.20	0.195	0.20	0.195
1.41	0.225	0.225	0.23	0.23
κ_t	0.6	1.25	3.3	7.6

owing to an improvement in the bed geometry. Ethylene glycol, 50% aqueous glycerol and 40% aqueous sucrose as suspension media gave about the same results as 50% aqueous ethanol.

The dependence of the efficiency of columns packed in 50% aqueous ethanol on the mean particle size is also shown in Fig. 1. A considerable decrease in the theo-

retical plate height can be achieved by using smaller particles although the maximum attainable flow velocity also decreases.

Fig. 2. demonstrates that the theoretical plate height is fairly independent of the capacity ratio, so neither of the mass transfer terms in eqn. 2 is dominating.

An illustration of the performance of such a microparticulate column packed in 50% aqueous ethanol is shown in Fig. 3.

Mixed-bed ion-exchange cellulose columns

In column chromatography with ion exchangers that have a cellulose matrix, the flow velocity cannot be increased to the limit given by the maximum pressure of a modern high-pressure liquid chromatograph. This is a drawback of this type of ion exchanger with respect to the maximum attainable speed of separation.

In order to decrease the flow resistance, mixed-bed anion-exchange cellulose columns containing a fraction of diatomite were prepared and tested (see Tables III and IV). As expected, the capacity ratio decreases owing to the decrease in the amount of anion-exchange material in the column, which is related to the fraction of anion-exchange cellulose present in the slurry, as demonstrated in Fig. 4.

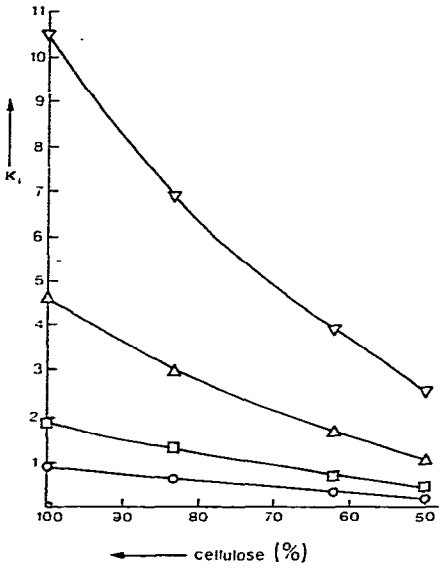


Fig. 4. Dependence of the capacity ratio on the volume percentage of anion-exchange cellulose in the packing material. Test compounds: ○, E_3-3G; □, E_2-3G; △, E_1-3S; ▽, Eqe-3S. Column: B-300/13 μm–diatomite; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°; packing pressure 35 bar.

For small values of the capacity ratio, the higher efficiency of a mixed-bed column in its effect on the resolution is compensated for by the decrease in capacity ratio compared with a pure anion-exchange cellulose column, as shown in Fig. 5A and 5B. The capacity ratio can be increased by lowering the temperature, but the decrease in efficiency keeps the resolution almost constant (Fig. 5C). The main improvement due to the use of mixed-bed columns is an increase in peak height, which is an advantage in trace analysis. The nucleotide bases are usually separated by chromato-

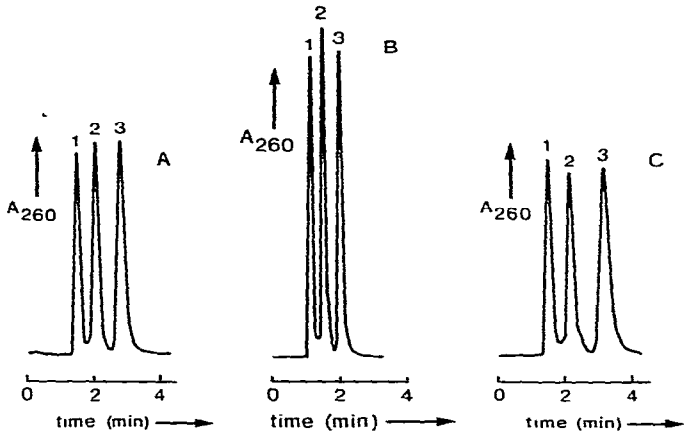


Fig. 5. Chromatograms of a mixture of nucleotide bases illustrating the improvement of mixed-bed compared with plain anion-exchange cellulose columns. Samples: 1 = xantosine; 2 = hypoxanthine, 3 = adenosine. Eluent: 0.01 *M* phosphate, pH 7.4. Columns: (A) P-300/7 μm, 70°, Δp = 27 bar; (B) P-300/7 μm–diatomite (5:1), 70°, Δp = 28 bar; (C) P-300/7 μm, 37°, Δp = 29 bar.

graphy on a polystyrene–divinylbenzene cation exchanger, which appears to involve a mixed mechanism of ion exchange and adsorption, the latter accounting for most of the high selectivity and strong retention on those resins.

Fig. 6 shows that the theoretical plate height decreases first when diatomite

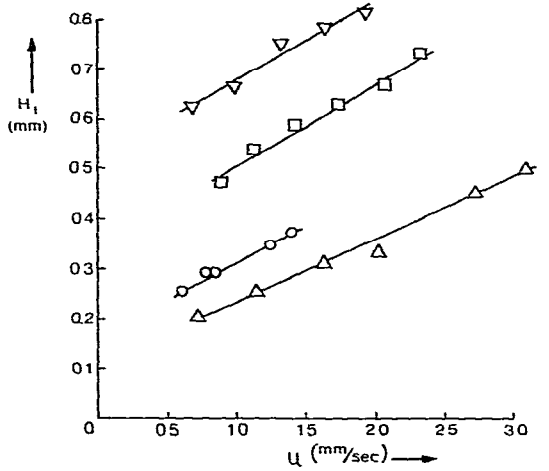


Fig. 6. H_t *versus* u curves for mixed-bed columns with varying ion exchange cellulose to diatomite ratios. Column: B-300/13 μm–diatomite; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°; packing pressure 35 bar.

Symbol	*Settled bed volume fraction of diatomite*	*Test compound*	κ
▽	1/2	E_1-3S	1.09
□	3/8	E_1-3S	1.7
△	1/6	E_2-3G	1.2
○	0	E_2-3G	1.85

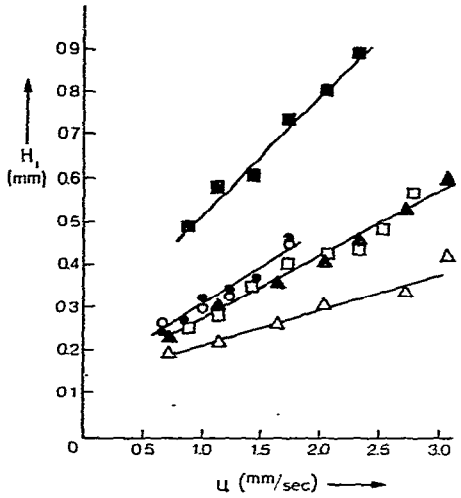


Fig. 7. H_t *versus* u curves for mixed-bed columns illustrating the influence of the capacity ratio on the efficiency. Column as in Fig. 6.

Symbol	*Settled bed volume fraction of diatomite*	*Test compound*	κ
○	0	E_3-3G	0.85
●	0	E_1-3S	4.4
△	1/6	E_3-3G	0.6
▲	1/6	E_2-3S	4.4
□	3/8	E_3-3G	0.35
■	3/8	Eqe-3S	3.9

is added to the cellulose anion exchanger and then increases on the further addition of diatomite. Fig. 7 shows the effect of the capacity ratio on the graph of theoretical plate height *versus* flow velocity for different ratios of anion-exchange cellulose and diatomite. It can be seen that the contribution of the mass transfer term to the theoretical plate height according to eqn. 2 increases with increasing fraction of diatomite to such an extent that it overrides the effect of the better packing geometry of the mixed-bed columns. For capacity ratios between 0 and 5, mixed-bed columns with a settled bed volume ratio of 5:1 are more efficient than plain cellulose anion-exchange columns.

At a constant ratio of cellulose anion exchanger to diatomite, the effect of the mean particle size of the ion-exchange cellulose on the graphs of theoretical plate height *versus* flow velocity and theoretical plate height *versus* capacity ratio were determined experimentally (see Figs. 8 and 9). As expected on the basis of eqn. 2, the contribution of the mass transfer term to the theoretical plate height also decreases with the particle size of the cellulose anion exchanger in mixed-bed columns.

The columns with a ratio of cellulose anion exchanger to diatomite of 5:1 were packed as a slurry in eluent, 50% aqueous ethanol or 40% aqueous sucrose. The latter two suspension media gave no significant improvement in column efficiency compared with eluent.

The relative efficiency of chromatographic systems can generally be indicated by two characteristic quantities: the minimum theoretical plate height, H_i^{min}, and the minimum value, $(H_i/u)_{min}$, determining the minimum time of separation. At the mini-

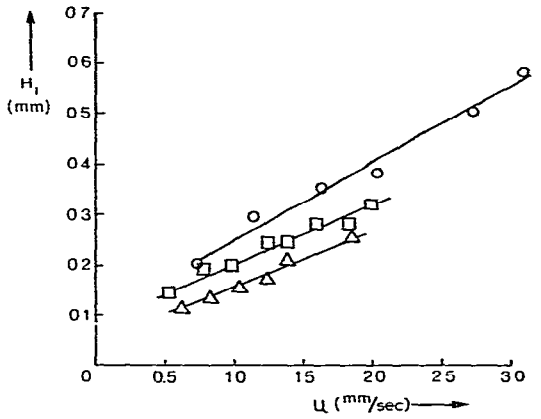


Fig. 8. Dependence of the H_t *versus* u curves for mixed-bed (5:1) columns on the mean particle size of the anion-exchange cellulose. Test compound: E_1-3S. ○, $\kappa = 2.9$; □, $\kappa = 3.0$; △, $\kappa = 3.9$. Columns: ○, B-300/13 μm; □, B-300/11 μm; △, B-300/7 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

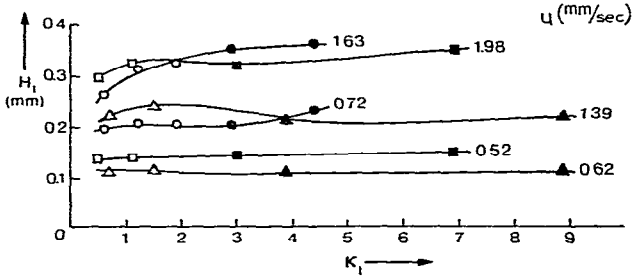


Fig. 9. H_t *versus* κ_t curves for mixed-bed (5:1) columns at different flow velocities and mean particle sizes. Values are corrected for extra-column contribution. Test compounds: ○, □, △, E_3-3G and E_2-3G; ●, ■, ▲, E_1-3S and Eqe-3S. Columns: ○, ●, B-300/13 μm; □, ■, B-300/11 μm; △, ▲, B-300/7 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

mum theoretical plate height, the maximum resolution on a given phase system is obtained, irrespective of time. In general, however, this value, H_t^{min}, is found at a very low flow velocity and the separation time becomes impractically long. The minimum time of separation required for a given resolution is described by the equation

$$t_{jt} = \left(\frac{R_{jt}}{r_{jt} - 1}\right)^2 \cdot \frac{(\kappa_t + 1)^3}{\kappa_t^2} \cdot \frac{H_t}{u} \tag{3}$$

The ratio H_t/u should be small when high-speed separation is required. It decreases with decreasing particle size and increasing flow velocity, as demonstrated in Fig. 10. Fig. 10 shows that mixed-bed columns of 7-μm anion-exchange cellulose are superior to other types of columns with respect to minimum theoretical plate height and speed of separation. An illustration of the performance of such a column is given in Fig. 11.

In conclusion, it can be said that microparticulate ion-exchange cellulose–

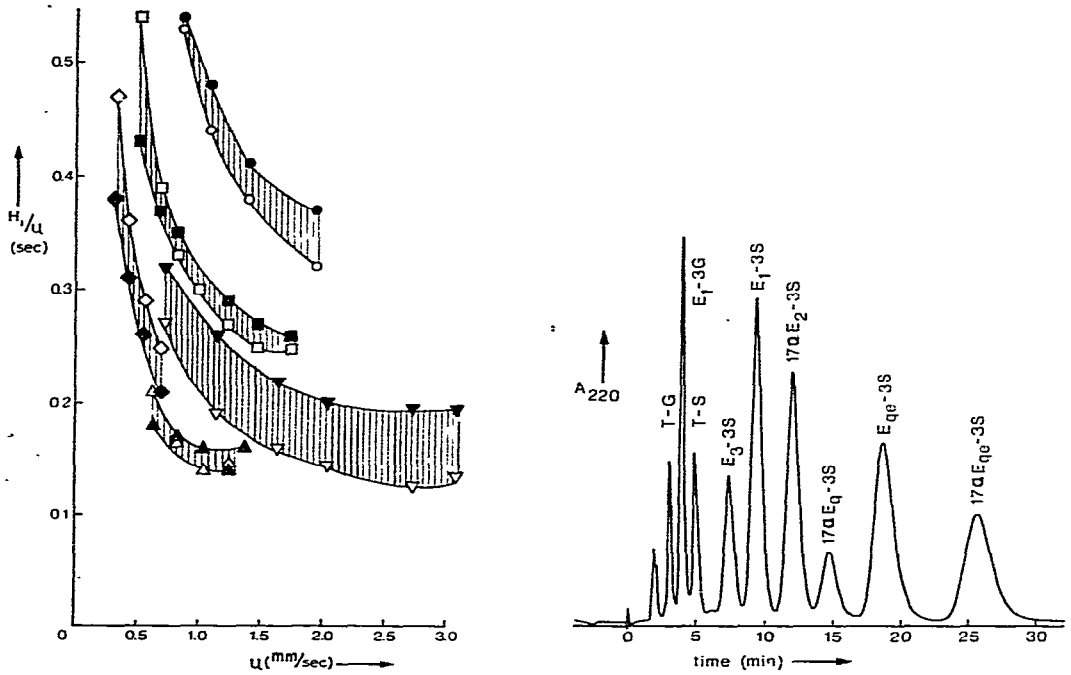


Fig. 10. Comparison of the speed of separation obtainable with different types of anion-exchange cellulose columns. Test compounds as follows. E_3-3G: ○, $\kappa = 1.0$; □, $\kappa = 0.85$; ◇, $\kappa = 0.9$; ▽, $\kappa = 0.6$; △, $\kappa = 0.7$. E_2-3S: ▼, $\kappa = 4.4$. Eqe-3S: ●, $\kappa = 11.8$; ■, $\kappa = 10.3$; ◆, $\kappa = 10.8$; ▲, $\kappa = 8.9$. Columns: ○, ●, B-300/13 μm, packed in eluent; □, ■, B-300/13 μm, packed in 50% aqueous ethanol; ◇, ◆, B-300/7 μm, packed in 50% aqueous ethanol; ▽, ▼, B-300/13 μm–diatomite (5:1), packed in eluent; △, ▲, B-300/7 μm–diatomite (5:1), packed in eluent; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

Fig. 11. Chromatogram of a test mixture of steroid conjugates. Column: 100 × 4 mm, B-300/7 μm–diatomite (5:1); 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°; $\Delta p = 36$ bar.

diatomite mixed-bed columns can be packed by means of a simple slurry technique to give columns that meet the requirements of high-performance liquid chromatography.

Applications

The potential of the analytical application of high-efficiency anion-exchange cellulose liquid chromatography columns can be demonstrated by a number of examples.

Chemotherapy is used to treat cancer, but is frequently limited by the toxicity of the drugs and the knowledge of the pharmacokinetics is very limited. The folic acid analogue methotrexate (MTX) has been used for more than 20 years as an anti-neoplastic agent, and non-acute MTX toxicity has been recognized in the brain, liver, kidney, lung and bone[8], which stresses the urgent need for a rapid and specific method for its analysis in blood. Fluorimetry suffers from interference by leucovorin (calcium folinate)[9], while enzymatic methods and radioimmunoassay demand much manual effort and time[10]. A feasible alternative might be the separation by liquid column

chromatography with detection at 290 nm after extraction from serum. Fig. 12A shows a chromatogram of serum to which 3.5 nmole of MTX had been added. Leucovorin at the level of 3 nmole showed no interference (Fig. 12B). Overlapping of the MTX signal by serum components can be prevented by variation of the chromatographic conditions owing to the selectivity of the phase system. An extraction step remains necessary in order to concentrate the MTX by about two orders of magnitude.

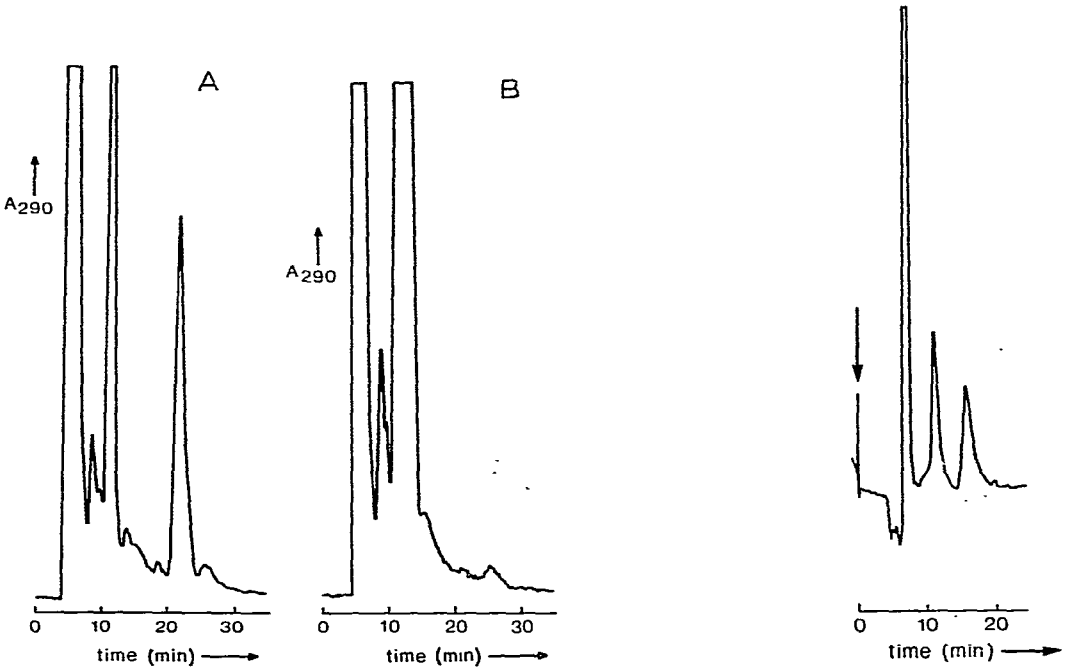


Fig. 12. Chromatograms of serum after addition of 3.5 nmole of methotrexate (A) and 3 nmole of leucovorin (B). Column: 100 × 4 mm, B-300/4 μm–diatomite (5:1); 0.02 *M* perchlorate + 0.008 *M* phosphate + 0.005 *M* chloride, pH 7.3; 70°; Δp = 65 bar.

Fig. 13. Chromatogram of a kanamycin sample, detected with an electrochemical detector[5]. Column as in Fig. 12.

Kanamycins are broad-spectrum antibiotics with different selectivities and toxicities, produced by *Streptomyces kanamyceticus*. They have been analysed by a chromatographic method after derivatization[11]. A more direct approach involves analysis by liquid column chromatography on a polystyrene–divinylbenzene anion exchanger[12]. A better separation of the three main components is obtained on a mixed-bed column, and a chromatogram of an old commercial kanamycin sample is shown in Fig. 13.

Very polar polymeric substances of biological origin, such as fulvic acids, are another field of application. Fulvic acids can be separated into several fractions, presumably according to molecular weight, on Sephadex G-25 and LH-20 and CPG-10-75 (ref. 13). Fractions of the Sephadex G-25 column eluate containing sedimentary fulvic acids were analysed by mixed anion-exchange cellulose–diatomite column chro-

matography (Fig. 14). A remarkable difference between the high-molecular-weight (*ca.* 10^4 dalton) sample and the partly hydrolysed lower-molecular-weight fraction was found.

Although it has been shown that peak dispersion in columns with a mixed packing is less than in plain cellulose ion-exchanger columns, such columns remain indispensable in those cases where a large phase ratio is necessary in order to increase the resolution for components with little retention or when compounds that are not compatible with diatomaceous earth are to be separated.

Novobiocin, an antibiotic produced by *Streptomyces niveus*, contains isomers

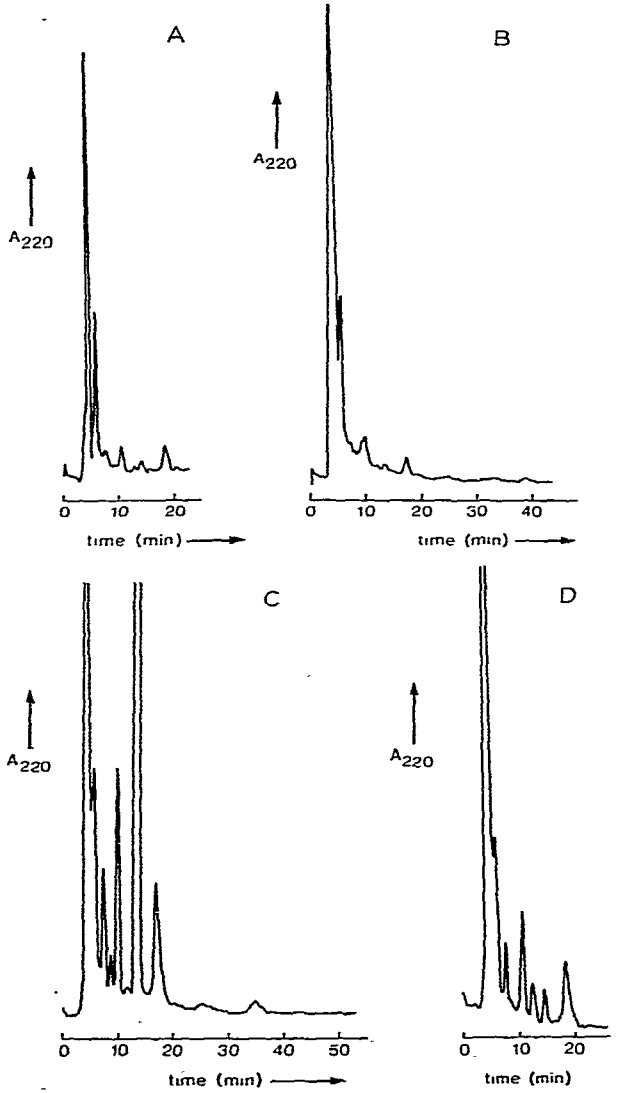


Fig. 14. Chromatograms of the four successively eluted fractions of fulvic acids from a Sephadex G-25 column. Column as in Fig. 11.

and degradation products. Six components could be separated by hydrophobic surface chromatography in half an hour[14]. An old sample, stored for 1 year in a refrigerator, shows at least 16 components with an analysis time of 3 h on an ECTEOLA-cellulose column (Fig. 15).

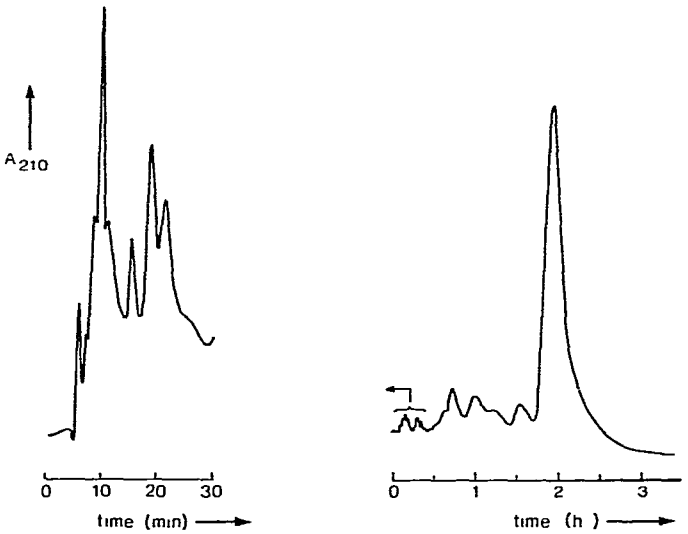


Fig. 15. Chromatogram of a novobiocin sample. Column: ET-41/11 μm; 0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8; 70°.

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REFERENCES

1 K. Unger and D. Nyamah, *Chromatographia*, 7 (1974) 63.
2 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
3 Sj. van der Wal and J. F. K. Huber, *J. Chromatogr.*, 102 (1974) 353.
4 Sj. van der Wal and J. F. K. Huber, *J. Chromatogr.*, 135 (1977) 305.
5 J. Lankelma and H. Poppe, *J. Chromatogr.*, 125 (1976) 375.
6 J. F. K. Huber, *Ber. Bunsenges. Phys. Chem.*, 77 (1973) 159.
7 J. F. K. Huber and J. A. R. J. Hulsman, *Anal. Chim. Acta*, 38 (1967) 305.
8 E. Arons, S. P. Rothenberg, M. da Costa, C. Fischer and M. Perwaiz Iqbal, *Cancer Res.*, 35 (1975) 2033.
9 B. Overdijk, W. van der Kroef, A. A. M. Visser and G. J. M. Hooghwinkel, *Clin. Chim. Acta*, 59 (1975) 177.
10 C. Bohuon, F. Duprey and C. Boudene, *Clin. Chim. Acta*, 57 (1975) 263.
11 K. Tsuji and J. H. Robertson, *Anal. Chem.*, 42 (1970) 1661.
12 T. Ottake and M. Yachuchi, *Liquid Chromatography at Work*, No. 5, Varian, Palo Alto, Calif., 1973.
13 H. de Haan, *Freshwater Biol.*, 2 (1972) 235.
14 K. Tsuji and J. H. Robertson, *J. Chromatogr.*, 94 (1974) 245.