CHROM 4407

SEPARATION OF CONJUGATED URINARY ESTROGENS ON COLUMNS OF SEPHADEX®

I. OPTIMIZATION OF CONDITIONS*

W. PAUL, C. STITT, W. J. DIGNAM AND S. KUSHINSKY**

Department of Obstetrics and Gynecology, Harbor General Hospital, Torrance, Calif. 90509 and the UCLA School of Medicine, Los Angeles, Calif. 90024 (U.S.A.)

(Received September 29th, 1969)

SUMMARY***

Columns of Sephadex G-25, G-15 and G-10 were evaluated under a variety of conditions to determine which provided the most efficient separation of estrogens conjugated with glucuronic acid or sulfuric acid. Elution with 0.01 *M* ammonium formate of a column of Sephadex G-15 to which had been applied a complex mixture of conjugated ^{14}C-labeled estrogens in urine provided the separation of at least nine generally distinct radioactive fractions. Similar separations have been achieved with solutions of sodium chloride as eluant but ammonium formate is preferred because of its volatility.

INTRODUCTION

Several years ago BELING[1] used gel filtration on Sephadex G-25 with water as the eluant to separate into two major fractions (Peaks I and II) the conjugated estrogens present in late pregnancy urine. Subsequently, KUSHINSKY AND OTTERNESS[2] found that additional resolution could be achieved by using longer columns, slower flow rates and concentrated urine instead of intact urine. Primarily, the techniques provided a separation of conjugated estrogens from many non-steroidal constituents of urine but very little separation between individual or types of conjugated estrogens. More recently, BRETTHAUER AND GOLICHOWSKI[3] reported the reduction or elimination by judicious choice of eluant of the sorptive properties of Sephadex in the separation

* Supported by United States Public Health Service Grants AM-09908 and RCDA 5K3-AM-31,321 (S.K.), and a grant from the Ford Foundation.

** To whom requests for reprints should be sent (Harbor General Hospital).

*** The following abbreviations are used: E_1 = estrone = 3-hydroxyestra-1,3,5(10)-trien-17-one; E_2 = estradiol = 3,17β-dihydroxyestra-1,3,5(10)-triene; E_3 = estriol = 3,16α,17β-trihydroxyestra-1,3,5(10)-triene; E_3-3-GA = estriol-3-glucuronoside = 16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; E_3-16-GA = estriol-16α-yl-β-D-glucopyranosiduronate; E_2-17-GA = estradiol-17β-glucuronoside = 3-hydroxyestra-1,3,5(10)triene-17β-D-glucopyranosiduronate; E_1-3-SO_4 = estrone-3-sulfate = estra-1,3,5(10)trien-17-on-3-yl sulfate.

of phenylalanine peptides. This report and the availability of Sephadex G-15 and G-10 with the potential for separating compounds with molecular weights in the range of those of conjugated estrogens prompted us to reinvestigate the possibility of separating individual conjugated estrogens on columns of Sephadex.

A major objective of the study described here was to develop a convenient method for separating conjugated estrogens present in urine. Some effort was made to obtain separations based on molecular weight but most of the effort was directed toward developing conditions which provided the maximum number of peaks of radioactivity on plotting the elution pattern of urine containing conjugated metabolites of intravenously administered [4-^{14}C]estradiol.

EXPERIMENTAL

Materials and methods

Reagents and solvents, of analytical grade, were used without additional purification. Sephadex® (G-10, G-15 and G-25; Pharmacia, Piscataway, N.J.) was washed repeatedly with deionized glass-distilled water and decanted free of fine particles. Vials for liquid scintillation counting (18 × 53 mm) were purchased on special order from Acme Vial (Los Angeles, Calif.) or Owens-Illinois (Vineland, N.J.) at a cost of approximately one fifth that of standard, low-potassium vials. The same vials were used as receiving vessels in fraction collectors. Polyethylene vials (No. 6001075; Packard Instruments, Downers Grove, Ill.) were modified by cutting off the tops just above the shoulder (by means of a band saw) and enlarging the opening with a 13/16 in. drill. The polyethylene vials served as re-usable holders or carriers in the liquid scintillation counter[4].

A liquid scintillation counter (Model No. 6860, Nuclear Chicago, Des Plaines, Ill.) was used with settings optimized for the Balance Point Counting Procedure. Quenching was detected by the Channels Ratio Method and correction was made as necessary with internal standards. The scintillation fluid employed is a modification[5] of one described by BRUNO AND CHRISTIAN[6] and is particularly well suited for this type of work because of favorable characteristics with respect to both quenching and solubility.

Methanol (0.5 ml) was added to each vial before the scintillation fluid in order to facilitate solubility of the fractions to be analyzed. Under these conditions the counting efficiency for ^{14}C was approximately 85 % with a background count of approximately 40 c.p.m. Accuracy to within 5 % was achieved with samples containing radioactivity at least four times that of the background. Background counts have not been subtracted from data shown in the Figures.

[4-^{14}C]Estrone, [4-^{14}C]estradiol and [4-^{14}C]estriol were purchased from Amersham/Searle (Des Plaines, Ill.). [6,7-^{3}H]Estradiol-17β-glucuronic acid, [6,7-^{3}H]-estrone, [6,7-^{3}H]estradiol, [6,7-^{3}H]estriol and [6,7-^{3}H]estrone-3-sulfate were purchased from New England Nuclear (Boston, Mass.). Estriol-3-glucuronide was biosynthesized using a preparation of guinea-pig liver[7] and estriol-16α-glucuronide was biosynthesized using a preparation of human liver[8].

Gel filtration. A dilute slurry of gel is poured into a chromatographic column (Fischer and Porter, Warminster, Pa.) fitted with an extension tube to accommodate the excess liquid used in packing the column. When the bed has settled the excess

eluant is percolated through the column and the extension is removed. Eluant from a reservoir is percolated through the column before adding the charge. The void volume and the inner volume are determined by passing a mixture of Blue Dextran® (Pharmacia) and sodium chloride through the column and determining the elution volumes. If the Blue Dextran does not migrate down the column uniformly the column is backwashed[2] with the aid of a polyethylene or teflon tube of appropriate diameter inserted down through the column to help loosen the bed.

Preliminary treatment of specimens of urine. Urine was collected for 48 h from subjects who had been given 10 μCi of [4-^{14}C]estradiol by i.v. injection. The urine was distilled to dryness *in vacuo* and redissolved in a volume of 0.01 *M* ammonium formate, which resulted in a solution containing at least 10,000 c.p.m./ml.

Comparison of separation of conjugated estrogens in urine with columns of Sephadex G-25, G-15 and G-10, using water and ammonium formate as eluants

A 1.0 ml specimen of concentrated urine containing conjugated ^{14}C-labeled estrogens was applied to columns of Sephadex G-25, G-15 and G-10 and eluted with 0.1 *M* ammonium formate, pH 6.8. In each case the bed volume was 2 × 100 cm. The volume of eluant required to elute the radioactivity from the column of G-15 was greater than that for the column containing G-25. Although the elution volume

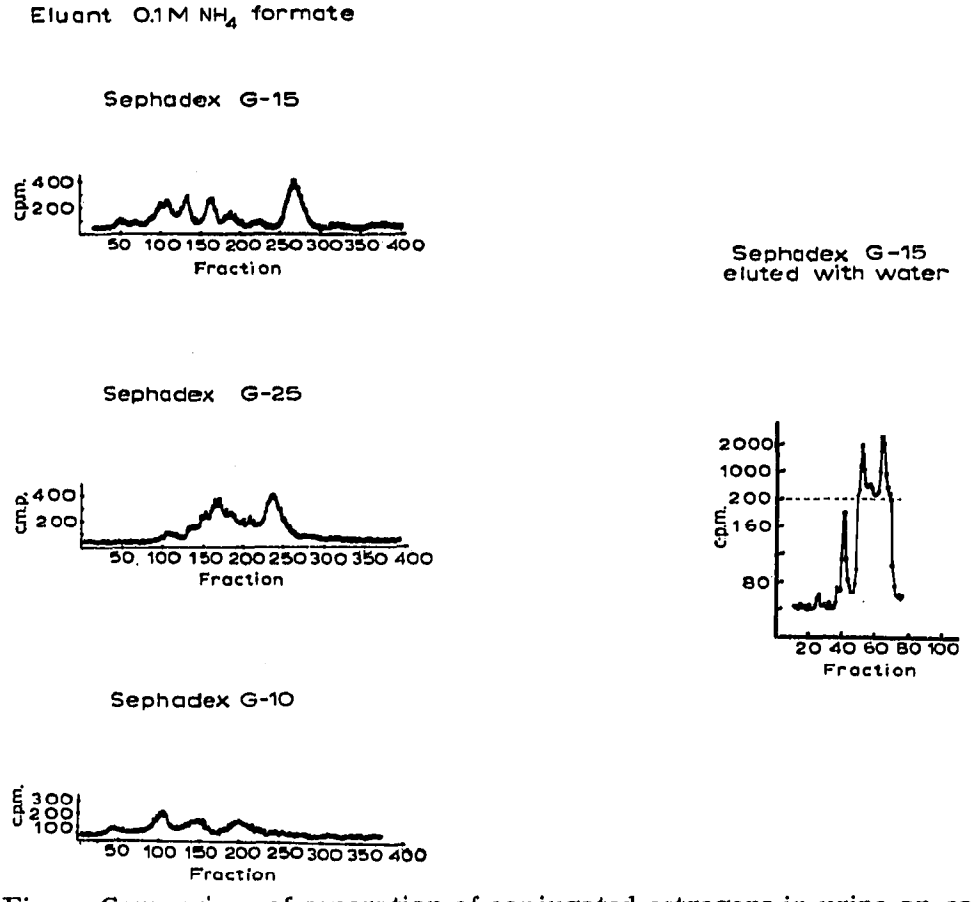

Fig. 1. Comparison of separation of conjugated estrogens in urine on columns of Sephadex G-25, G-15 and G-10, using water and ammonium formate as eluants. Column dimensions: 2 × 90 cm; charge: E.F. urine, day 1 (1.0 ml); volume per fraction: 5.0 ml.

for the column of G-10 was greatest, and in fact represents an incomplete elution because the column was stopped prematurely, the separation clearly was better with G-15 than with G-10 or G-25. The results of these studies are shown in Fig. 1, along with another in which a column of G-25 was prepared and eluted with water instead of with ammonium formate.

Influence of salt, molarity, and pH on the pattern of elution of conjugated urinary estrogens from columns of Sephadex G-15

A charge of conjugated urinary estrogens was chromatographed on columns of Sephadex G-15, prepared and eluted with 0.01 *M* and 0.1 *M* solutions of ammonium formate (pH 6.8), sodium chloride and ammonium hydroxide. The elution profile was similar with ammonium formate and sodium chloride as eluant. Ammonium hydroxide as eluant resulted in a smaller elution volume and a generally different pattern of elution compared with those of the other eluants. The results are summarized in Fig. 2. With 10^{-3} *M* eluant the resolution was considerably worse than with 0.01 *M* eluant and the recovery of radioactivity from the columns was poor at concentrations below 10^{-3} *M*.

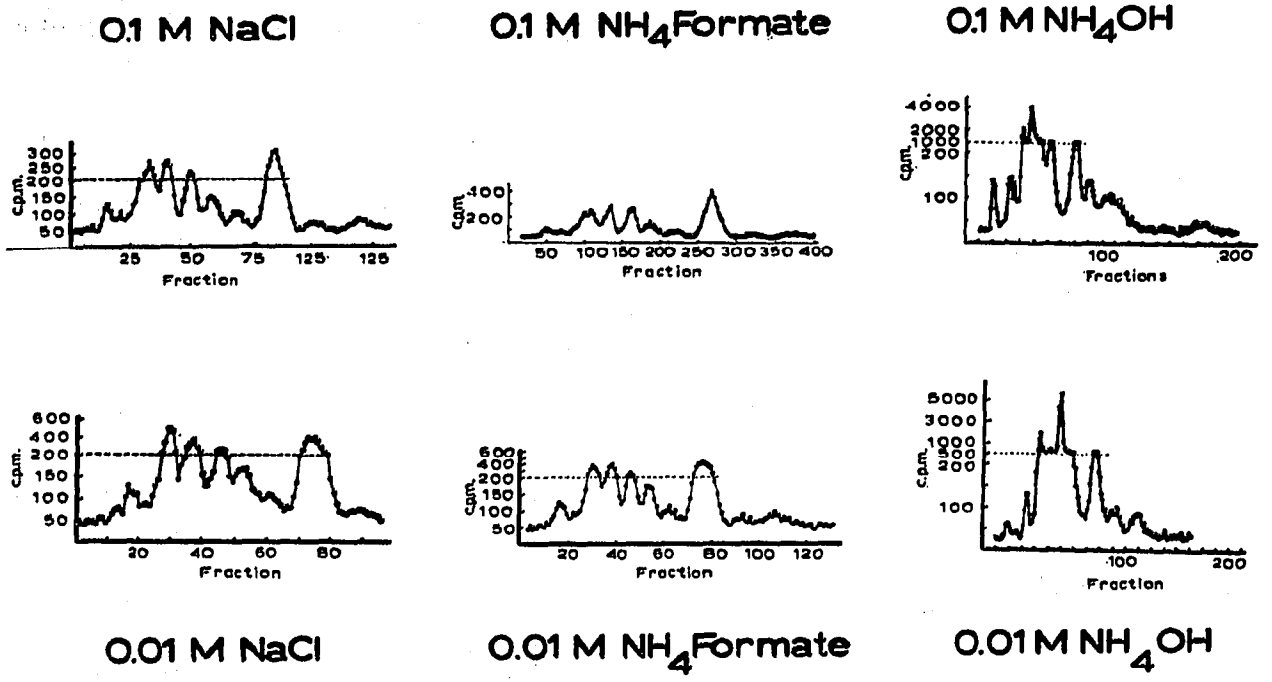

Fig. 2. Influence of salt and molarity on the pattern of elution of conjugated urinary estrogens from columns of Sephadex G-15. Column dimensions: 2 × 90 cm; charge: E.F. urine, day 1 (1.0 ml); volume per fraction: 5.0 ml (0.1 *M* ammonium formate, 0.01 *M* and 0.1 *M* ammonia) or 15 ml (sodium chloride and 0.01 *M* ammonium formate).

Other columns were prepared and eluted with 0.01 *M* ammonium formate, pH 8.7, 6.8 and 3.4. The elution volume and resolution on the columns decreased with an increase in pH. The data are summarized in Fig. 3.

Attempt to decrease the sorptive effects of Sephadex G-15

Three columns were packed and eluted with (a) methanol–water (80:20),

(b) pyridine (1.0 *M*), and (c) acetic acid–phenol–water (1:1:1), respectively. In each case the resolution was poor (Fig. 4) and the relatively large elution volumes preclude separations based exclusively on molecular weights. While there may be some separation based on molecular weights superimposed on the separation due to sorptive properties these columns were not pursued further because of the superior separations obtained with ammonium formate as the eluant.

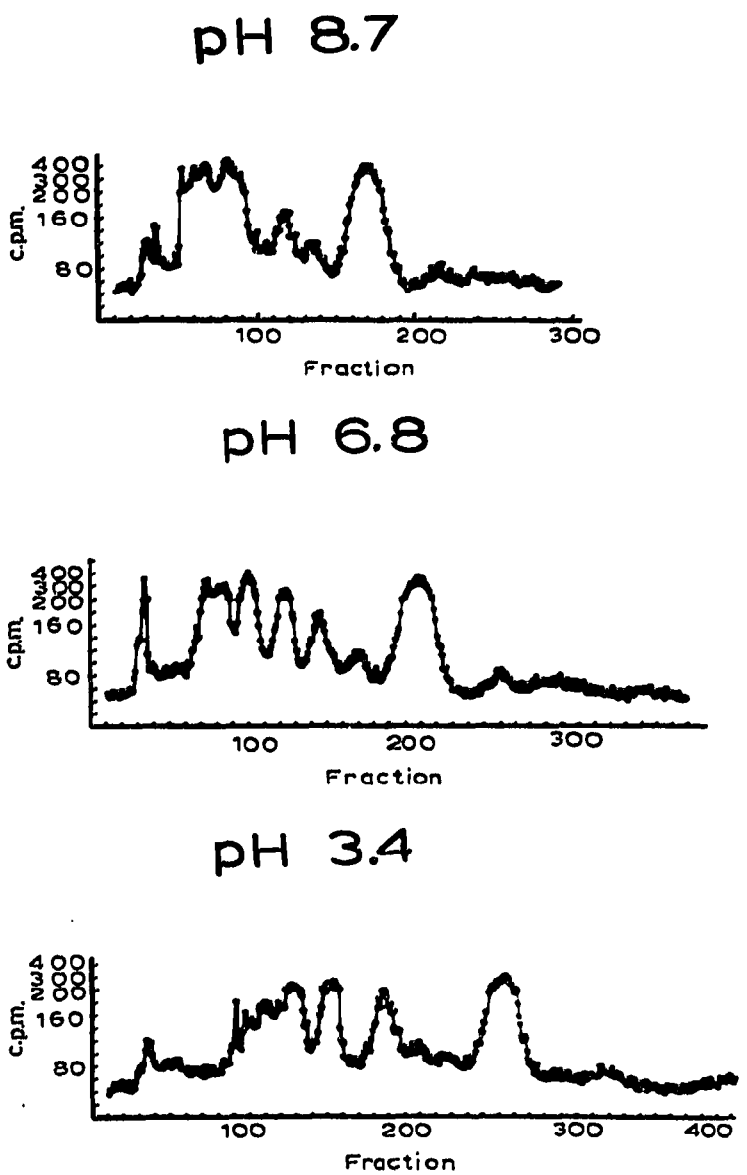

Fig. 3. Influence of pH on the pattern of elution of conjugated urinary estrogens from columns of Sephadex G-15 with ammonium formate. Column dimensions: 0.9 × 100 cm; charge: E.F. urine, day 1 (1.0 ml); eluant: 0.01 *M* ammonium formate (pH as indicated); volume per fraction: 1.7 ml.

Capacity of columns of Sephadex G-15

A charge of 0.2, 1.0, 5.0 and 20 ml of concentrated urine was applied to a 0.9 cm × 100 cm column and eluted with 0.01 *M* ammonium formate, pH 6.8. In

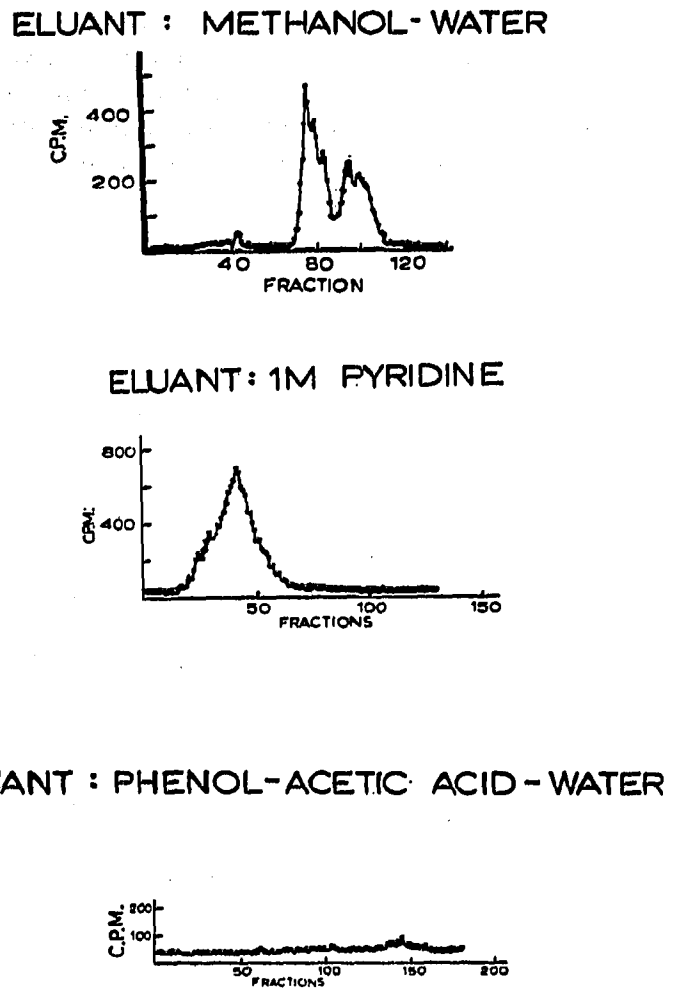

Fig. 4. Attempt to decrease sorptive effects of Sephadex G-15. Phenol-acetic acid–water (1:1:1): charge, 0.3 ml R.B. urine (2–4 h); column dimensions, 0.9 × 97 cm; volume per fraction, 5.0 ml. 1.0 *M* pyridine: charge, 0.3 ml R.B. urine (2–4 h); column dimensions, 0.9 × 45 cm; volume per fraction, 1.0 ml. Methanol–water (80:20): charge, peak I (5.0 ml) from M.S. urine; column dimensions, 2 × 88 cm; volume per fraction, 13.5 ml.

addition, a 1.0 ml charge was diluted to 20 ml with water before application to the column. The results of this study are summarized in Fig. 5. When the volume of the charge was 5 ml or less the resolution was generally constant. With a charge of 20 ml the resolution was considerably worse. The charge of 1.0 ml diluted to 20 ml yielded a similarly poor resolution, suggesting that when the volume of the charge approaches that of the void volume (27 ml) a significant deterioration of the separation occurs.

Partial separation of conjugated urinary estrogens from other substances on the basis of weights and radioactivity

A specimen of concentrated urine containing ^{14}C-labeled conjugated estrogens was applied to a column of Sephadex G-15 (0.9 × 100 cm) and eluted with 0.01 *M* ammonium formate. An aliquot (one fifth) was removed for measurement of radioactivity and for determination of the weight of the residue on evaporation to dryness.

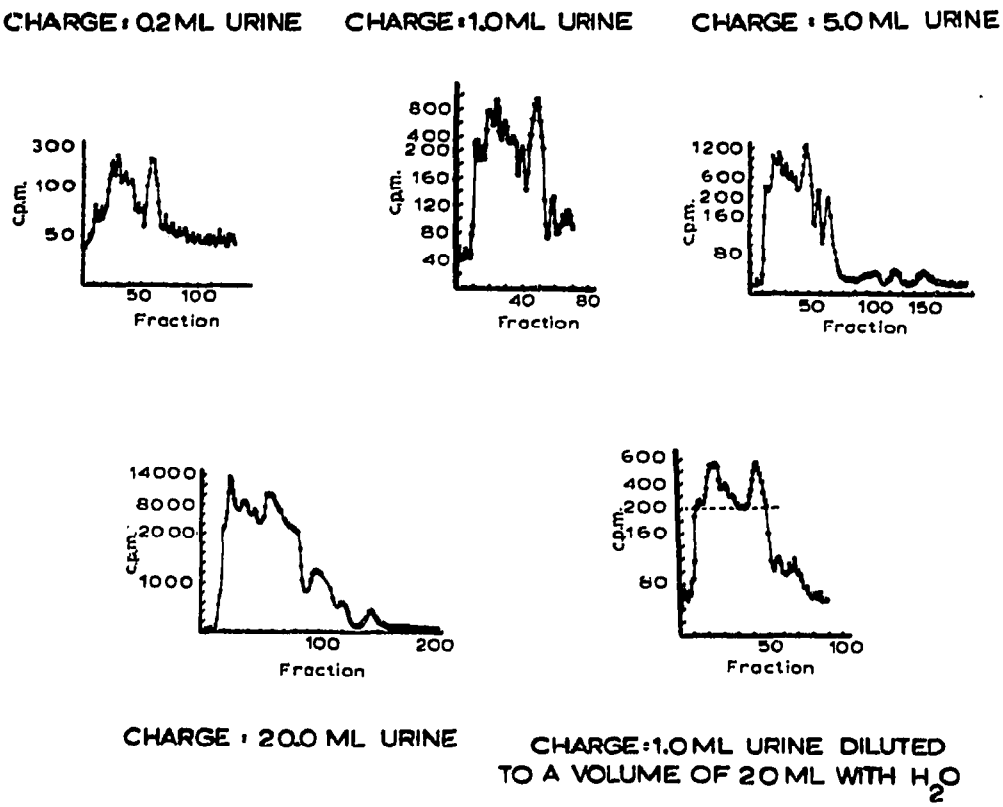

Fig. 5. Capacity of columns of Sephadex G-15. Column dimensions: 0.9 × 100 cm; charge: E.F. urine, day 1; eluant: 0.01 *M* ammonium formate; volume per fraction: 5.0 ml.

The results of this study, summarized in Fig. 6, show that most of the components which contribute significant weight are eluted before the conjugated estrogens.

Elution volume of standards and reproducibility of columns

In preliminary studies the elution volume of several conjugated estrogens applied to columns of Sephadex G-15 was found to vary considerably in successive

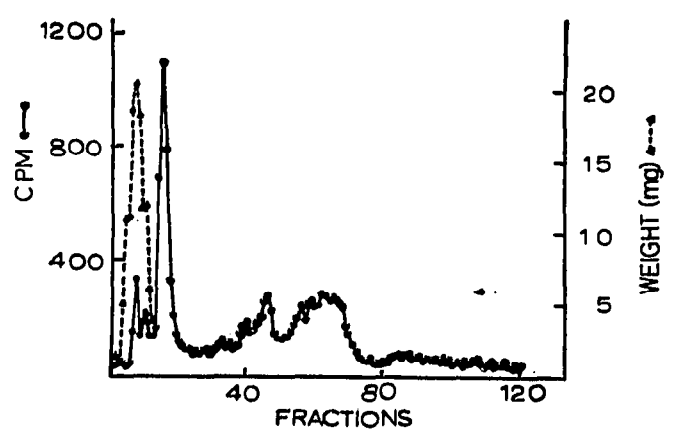

Fig. 6. Partial separation of conjugated urinary estrogens from other substances on the basis of weights and radioactivity. Column dimensions: 0.9 × 100 cm; charge: R.B. urine (4.0 ml); eluant: 0.01 *M* ammonium formate; volume per fraction: 4.3 ml.

experiments, with an apparent increase in the elution volume each time a charge was applied to the same column. To characterize the phenomenon more thoroughly two columns (0.9 × 95 cm) were prepared with fresh Sephadex G-15. A charge containing [4-^{14}C]estriol-16-glucuronide and [6,7-^{3}H]estradiol-17-glucuronide was applied to each column and eluted with 0.01 *M* ammonium formate. Using the same columns and more of the same charge the study was repeated three times. The results are summarized in Table I. Although the elution volumes increased in successive columns the ratio of the elution volumes remained constant.

TABLE I

CHANGE IN ELUTION VOLUME (ml) ON REPEATED USE OF COLUMNS OF SEPHADEX G-15 FOR SEPARATION OF PURE COMPOUNDS

Column dimensions: 0.9 × 100 cm; eluant: 0.01 *M* ammonium formate, pH 6.8; volume per fraction: 5 ml.

Run	*Column 1*			*Column 2*		
	E_3-16-GA	*E_2-17-GA*	*E_3-16-GA/ E_2-17-GA*	*E_3-16-GA*	*E_2-17-GA*	*E_3-16-GA/ E_2-17-GA*
1	375	510	0.74	345	480	0.72
2	400	535	0.75	400	530	0.75
3	480	660	0.73	425	560	0.76
4	> 1000	> 1000		> 1000	> 1000	

A large batch of used Sephadex G-15 was washed with ten bed volumes each of 1.0 *M* pyridine, 0.2 *M* formic acid, water and 0.01 *M* ammonium formate. Reference standards of [6,7-^{3}H]estrone-3-sulfate, [6,7-^{3}H]estradiol-17-glucuronide, [4-^{14}C]-estriol-16-glucuronide and [4-^{14}C]estriol-3-glucuronide were added in various combinations to different specimens of urine or to 0.01 *M* ammonium formate and the elution volumes were determined with columns containing pyridine-washed Sephadex. The results of this study, summarized in Table II, show no clear trend for a change

TABLE II

ELUTION VOLUME OF CONJUGATED ESTROGENS WITH COLUMNS OF PYRIDINE-WASHED, "USED" SEPHADEX G-15

Column dimensions: 0.9 × 47 cm; eluant: 0.01 *M* ammonium formate, pH 6.8; volume of urine, if used: 1.0 ml; volume per fraction: 3 ml.

Condition of Sephadex[a]	*Type of urine added to charge*	*Elution volume (ml)*			
		E_3-3-GA (^{14}C)	*E_3-16-GA* (^{14}C)	*E_2-17-GA* (^{3}H)	*E_1-3-SO_4* (^{3}H)
Used	Female (SH)	66			248
Used	Female (RB)	64	168	226	226
Used	None	82			246
Used	None		162		242
Used	None	61	143	214	214
Re-used	Pregnancy (MH)		161		240
Used	Pregnancy (FA)		163		
Re-used	None		152		222
Re-used	None		163		

[a] See text for definition of "Used".

in elution volume in the presence or absence of urine or on re-use of the same column. It appears that pre-treatment with pyridine stabilized the system but the extent of stabilization, if real, is not known. Of the four standards tested only estrone-3-sulfate and estradiol-17-glucuronide did not separate on the columns. Two of the peaks in the elution profile of ^{14}C-labeled conjugated estrogens in urine correspond in elution volume with those of estradiol-17-glucuronide and estriol-16-glucuronide (Fig. 7). A mixture of ^{3}H-labeled estrone, ^{3}H-labeled estradiol, ^{3}H-labeled estriol and concentrated urine containing conjugated metabolites of ^{14}C-labeled estradiol was applied to a column of Sephadex G-15 (0.9 × 100 cm) and eluted with 0.01 *M* ammonium formate. The K_{av} value[9] for the free estrogens was 22.4 or greater while the K_{av} value for the conjugated estrogens was between 0.97 and 10.1, thereby affording complete separation of these classes of compounds.

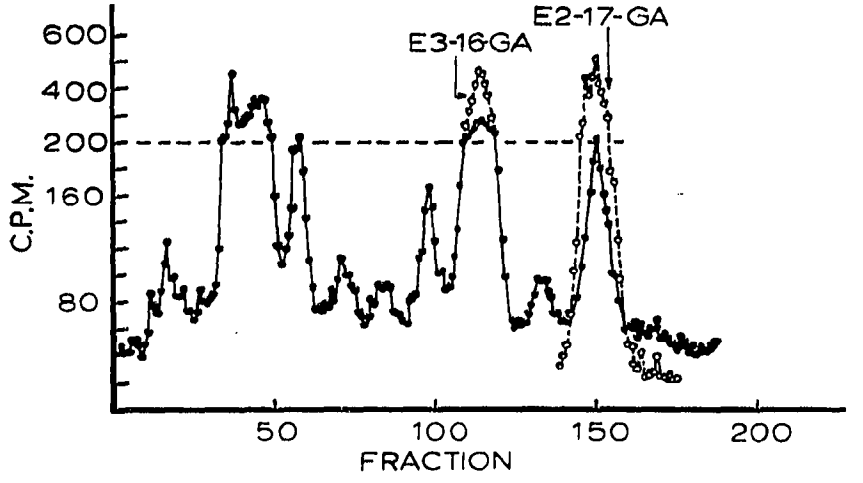

Fig. 7. Elution of synthetic radioactive standards in the presence of conjugated ^{14}C-labeled estrogens in urine. Column dimensions: 0.9 × 100 cm; charge: E.F. urine, day 2 (0.5 ml) with and without designated standards; eluant: 0.01 *M* ammonium formate; volume per fraction: 4.5 ml.

Effect of height of column on efficiency of separation

A comparison was made among the separations achieved on columns of 50, 100 and 200 cm length, of a mixture of conjugated ^{14}C-labeled estrogens in urine. The results, summarized in Fig. 8, show clearly that the best separation was obtained with the longest column.

DISCUSSION

As part of a continuing search for better methods to separate conjugated estrogens in urine, columns of Sephadex G-25, G-15 and G-10 were evaluated under a variety of conditions to determine which provided the most efficient resolution. Particular attention was paid to the selection of conditions not likely to cause structural changes in the conjugated estrogens during the process of separation. The use of some pH values away from that at neutrality was done primarily to help understand the system and for the sake of completeness.

Nominally, gel filtration implies a technique whereby separations based on differences in molecular weight are achieved. In practice separation based exclusively

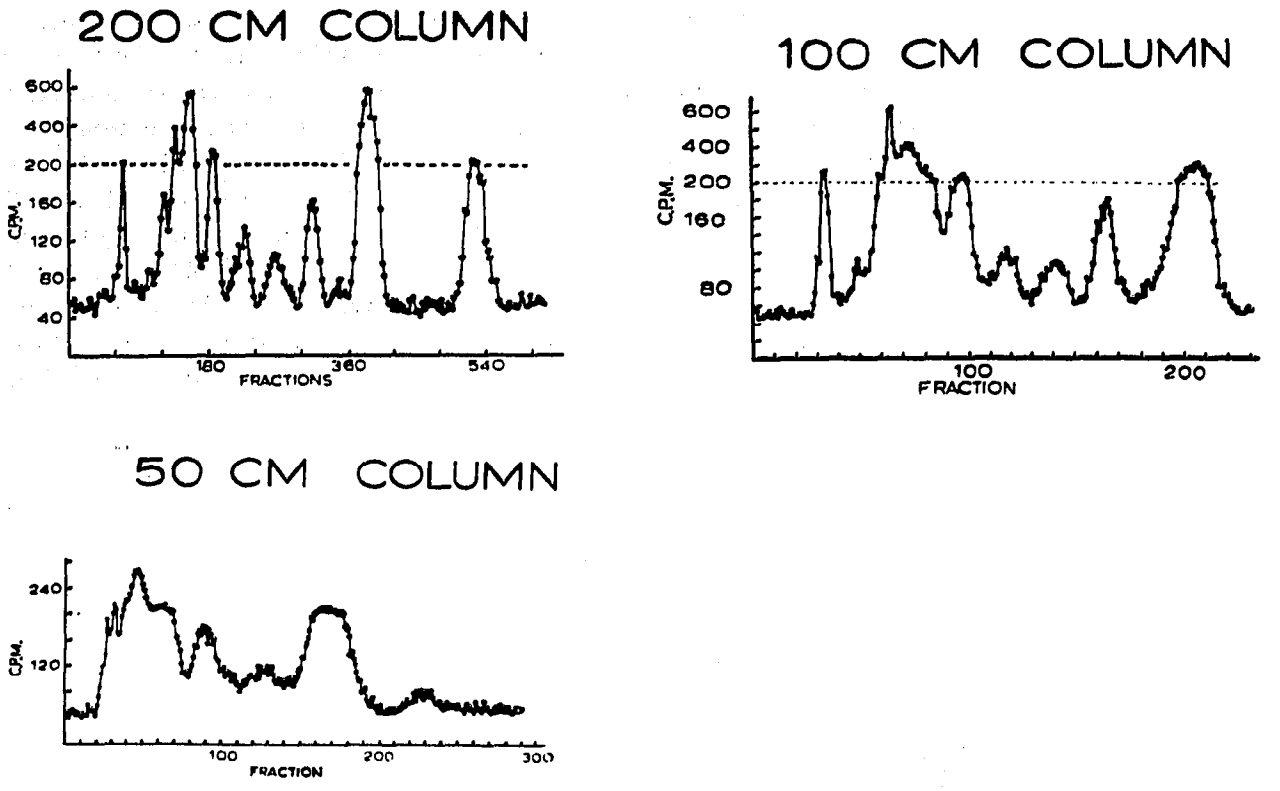

Fig. 8. Effect of height of column on efficiency of separation. Column diameter: 0.9 cm; charge: E.F. urine, day 2 (0.5 ml); eluant: 0.1 *M* ammonium formate; volume per fraction: 5.0 ml.

on molecular weight is not always achieved. Sorptive properties of the gel which are influenced by both pH and the ionic environment cause the retention of many types of relatively small molecules with potentially ionizable functional groups[3,10–17]. With some types of compounds which generally are sorbed in an unpredictable manner, careful choice of eluant has led to separations based primarily on molecular weights[3,18–20]. Other types of compounds have been resolved very effectively by empirically determining the solvent system which provides the best separation, regardless of the molecular weights. Primarily the latter approach was employed in the present investigation.

Clearly the use of some eluants such as dilute solutions of ammonium formate or sodium chloride improved the separations compared with that obtained with water as the eluant. The precise reason for the improvement is not known. From the K_{av} values it is apparent that under all conditions used in this paper the separations were a result of factors other than differences in molecular weight. BELING[1] reported that pure conjugated estrogens are eluted from columns of Sephadex G-25 at close to void volume with water as the eluant. Adding even a small quantity of salt or buffer to the charge but still using water as the eluant caused the conjugated estrogens to be eluted considerably beyond the void volume. A possible explanation is that the inner matrix of the gel acts as a statically charged cage which repels the conjugated estrogens and that the charge is dissipated in the presence of inorganic ions. The changes in the sorptive properties of Sephadex with changes in the pH, in the ionic environment

or in the dielectric constant are not readily explained by classical concepts of adsorption or ion exchange. It appears that the separations which are achieved are the result of the interaction of a number of factors.

REFERENCES

1 C. G. BELING, *Acta Endocrinol.*, 43, *Suppl.* 79 (1963) 1.
2 S. KUSHINSKY AND I. OTTERNESS, *Steroids*, 3 (1964) 311.
3 R. K. BRETTHAUER AND A. M. GOLICHOWSKI, *Biochim. Biophys. Acta*, 155 (1967) 549.
4 S. KUSHINSKY, W. PAUL AND M. FOOTE, to be published.
5 K. HONDA, D. OSTERGARD AND S. KUSHINSKY, *Am. J. Obstet. Gynecol.*, 104 (1969) 528.
6 G. A. BRUNO AND J. E. CHRISTIAN, *Anal. Chem.*, 33 (1961) 1216.
7 U. GOEBELSMANN, E. DICZFALUSY, J. KATZ AND M. LEVITZ, *Steroids*, 6 (1965) 859.
8 M. LEVITZ, J. KATZ AND G. TWOMBLY, *Steroids*, 6 (1965) 553.
9 T. C. LAURENT AND J. KILLANDER, *J. Chromatog*, 14 (1964) 317.
10 B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
11 D. EAKER AND J. PORATH, *Separation Sci.*, 2 (1967) 507.
12 J.-C. JANSON, *J. Chromatog.*, 28 (1967) 12.
13 I. LINDQVIST, *Acta Chem. Scand.*, 21 (1967) 2564.
14 A. J. W. BROOK AND S. HOUSLEY, *J. Chromatog.*, 41 (1969) 200.
15 J. DE BERSAQUES, *J. Chromatog.*, 31 (1967) 222.
16 C. G. NORDSTROM, *Acta Chem. Scand.*, 21 (1967) 2885.
17 L. SWEETMAN AND W. L. NYHAN, *J. Chromatog.*, 32 (1968) 662.
18 A. NILSONN, *Acta Chem. Scand.*, 16 (1962) 31.
19 T. C. SOMERS, *Nature*, 209 (1966) 368.
20 J. PORATH, *Biochim. Biophys. Acta*, 39 (1960) 193.