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SEPARATION OF OESTROGENS IN BIOLOGICAL FLUIDS AND SYNTHETIC MIXTURES ON SEPHADEX G-TYPE GELS

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CONTENTS

1 INTRODUCTION

The assay of oestrogens in biological fluids has many useful applications, the chief one being the determination of the total oestrogen levels in maternal urine (or blood) in order to aid the clinical assessment of the viability of the feto-placental unit[1]. The assay may also be applied to assist clinical studies of non-pregnant women, e.g., as a biochemical monitor of gonadal function[2].

However, there are a number of practical problems inherent in the assay of the three classical oestrogens oestrone, oestradiol and oestriol. First, the actual total concentration levels are very low (nanograms to milligrams per millilitre in urine; picograms to nanograms per millilitre in blood). Second, the oestrogens occur in different molecular forms; a mixture of both free and conjugated oestrogens exists in urine and blood. Third, in biological fluids the steroid mixture is present in an already exceedingly complex matrix of other compounds[3], some of which are structurally very similar to oestrogens. As a result, convenient spectrophotometric assay methods cannot be directly employed to quantitate the oestrogens because absorption of UV light by the aromatic A ring of the steroid nucleus also occurs with the other aromatic components of the biological fluid. Further, as these steroids are colourless, coloured chemical derivatives must first be formed if colorimetric methods are to be used. A number of the present assay methods are thus primarily dependent on the production of coloured derivatives.

As a consequence of the above difficulties in quantitating oestrogen levels, a considerable number of distinct assay methods have been devised and published. The

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considerable literature on this subject has recently been collated by members of the authors' research group to form a comprehensive bibliography[4].

The most favoured colorimetric assay method involves the formation of a red species (of unknown structure) by a reaction involving concentrated sulphuric acid, the Kober reaction[5]. This particular reaction is probably commonly utilized because it is believed to be specific for both free oestrogens and their conjugates and hence it can be applied directly to the biological fluid being investigated. Unfortunately, primarily because of the lack of fundamental understanding of the chemistry of this reaction, assays involving its use are difficult to reproduce within and between laboratories[6], probably owing to variations in the quality of the acid employed[7]. Because of this we decided to study in detail those methods which could possibly effect the separation of the oestrogens from the bulk of the other components of urine so that their quantitation could be more readily achieved, *e.g.*, by a UV method avoiding coloured derivative formation or via a colour-forming reaction of known chemistry[8]. Accordingly, we studied the papers listed in the chromatographic section of the above-mentioned bibliography[4] and as a result decided that methods using columns of Sephadex gels were the most promising for this purpose. These methods also have the potential to be used as a pre-purification/concentration step prior to further separation of the oestrogen mixture by high-performance liquid chromatography (HPLC).

2 SEPHADEX GEL METHOD OF BELING

Beling[9] in 1961 was the first to use a Sephadex gel for the purification of urinary (conjugated) oestrogens. He applied 1 ml of untreated late pregnancy urine to a column of Sephadex G-25, eluted it with distilled water and collected the total eluate as serial fractions. The presence of oestrogens in the fractions was ascertained by a method involving the use of the Kober (colorimetric) reaction. The elution curve forming Fig. 2 of Beling's paper showed that the oestrogen conjugates were eluted in two distinct Kober-positive fractions, which he later called peaks I and II. In his second paper, published in 1963, Beling[10] identified the major components of these peaks as probably oestrone-3-β-D-glucuronide, oestriol-3-β-D-glucuronide, oestriol-3,17-β-D-diglucuronide (peak I) and oestriol-16(17)-β-D-glucuronide (peak II). Further, two minor components tentatively identified as oestrone-3-sulphate and oestriol-3-sulphate were assigned to peak II. Smith and Kellie[11] in 1967, in a detailed combined ion-exchange–paper chromatographic study following Sephadex column procedures, confirmed Beling's positive assignments and also identified some additional conjugates in each peak. A complete listing of both positively and tentatively identified oestrogen constituents of the two peaks is given in Table 1. A study of Table 1 leads to the conclusion that the elution position of any oestrogen conjugate does not depend on the oestrogen moeity but rather on the position and nature of the conjugating group(s).

Further, it was found that the Sephadex method also achieves a substantial separation of the oestrogens from other substances in human urine[9]; these include D-glucaric acid (MW = 210)[13], which is of interest since at the acidic pH values employed with β-glucuronidase preparations this saccharic acid is partially converted to its (1 $\rightarrow$ 4)-lactone, a potent inhibitor of β-glucuronidase activity[14]. Because of

TABLE 1

MOLECULAR IDENTITY OF CONSTITUENTS OF PEAKS ELUTED FROM APPLICATION OF URINE SAMPLES CONTAINING OESTROGEN CONJUGATES TO COLUMNS OF SEPHADEX G-25 GEL

Oestrogen moiety E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol Conjugate G = glucuronoside; S = sulphate, P = phosphate Specification of nature of conjugate(s) is preceded by number(s) signifying position(s) of conjugation(s) with steroid nucleus and symbols for their orientations are given if they were specified in the original papers Identities of conjugates given in square brackets are tentative only

Ref No	*Peak**	*Oestrogen conjugate(s) identified in peaks*
(A) Pregnancy urine applied to the columns		
10	I	E_3-3-G, [E_1-3-G], [E_3-3,17-di-G]
	II	E_3-16(17)-G; [E_1-3-S]; [E_3-3-S]
11	I	E_1-3-G, 16α-OHE_1-3S-16αG, E_3-3-G, E_3-3-S-16α-G
	II	16α-OHE_1-16α-G, E_2-17β-G, E_3-16α-G, 16-epiE_3-16β-G
12	I	E_1-3-G, 16α-OHE_1-3-G, E_3-3-G, 16α-OHE_1-3-S-16α-G, E_3-3-S-16α-G
	II	16α-OHE_1-16α-G, E_2-17β-G, E_3-16α-G, 16-epiE_3-16β-G
(B) Synthetic oestrogen conjugates added to urine applied to the columns		
9	I	None
	II	E_2-17-G; E_3-G, E_1-3-S, E_2-3-S, E_2-17-S, E_3-3-S, E_2-3-P, E_2-17-P
10	I	E_1-3-G, E_2-3,17-di-G, E_3-3-G
	II	E_2-17-G, E_3-16(17)-G, E_1-3-S, E_2-3-S, E_2-17-S, E_2-3,17-di-S, E_3-3-S, E_2-3-P, E_2-17-P, E_2-3,17-di-P

* I, Peak I of Beling, II, peak II of Beling

the similarities of the molecular weights of the oestrogen conjugates in Table 1 to each other (molecular weight range = 350–641) and to D-glucaric acid, the usual chromatographic mechanism of separation by Sephadex, which is by the process of molecular sieving, cannot be operating. Interestingly, whereas Beling in his first paper[9] ascribed the separation to the process of gel filtration, in his second paper[10] he ruled this out on the basis of the marked effect of salt on the retention volume and consequently stated that the urinary conjugated oestrogens were more or less adsorbed to the gel.

3 SEPARATION OF MIXTURES OF OESTROGEN CONJUGATES ON SEPHADEX

In order to understand the basis for the separation of oestrogen by the use of Sephadex gels, so as to assess the limitations of and suggest improvements in the methodology, it was decided to study and collate all of the publications listed under this heading in the previously mentioned bibliography[4]. As a direct result of this study, Tables 2, 3 and 4 were constructed, so as to present the reported separations in a unified, concise manner which would aid discussion of the various methods and of the results obtained.

Before proceeding to introduce Table 2, it should be noted that it is split into two parts on account of Beling's findings mentioned above; section A summarizes those separations obtained when the sample is eluted directly after its application; section B summarizes those separations achieved when the sample is applied and an

TABLE 2

SEPARATION OF CONJUGATED OESTROGENS ON SEPHADEX COLUMNS

Summary of experimental procedures and results. Abbreviations used general. I and II represent Beling's two peaks, NG = not given Sample: (NP) = non-pregnancy, (P) = pregnancy Pre-treatment of sample: Cen = centrifuged, Conc = concentrated, Dil = diluted; Fil = filtered, NaCl = sodium chloride saturated, *OE Conjs = radiolabelled oestrogen conjugates. Dilution factor = volume after pre-treatment/volume before pre-treatment N.B., when not stated the 24 h urinary excretion volume was taken to be 750 ml Vol of sample = volume of sample pre-treated Analyte ΣOE Conjs = total oestrogen conjugates. Assay/detection method Kober (C) = Kober colorimetric, Kober (F) = Kober fluorimetric, direct and indirect = Kober reaction applied directly and indirectly, respectively, to eluate. Elution position elution volumes refer to the volumes of eluate collected immediately following application of the eluent to the column, in order to distinguish between the liquids used to wash a column following sample application and to elute the sample off the column, only the latter liquid is referred to as the eluent.

	(A) Direct elution							
	Ref							
	9	*10 (i)*	*10 (ii)*	*15*	*16*	*17*	*18*	*19*
Sephadex column								
G type	25	25M	25M	25M	15	15	25	25M
Length (cm)	50?	~48	35	145	40	100?	96	~409
I.D (cm)	1	~1	6 5	6 8	0 8	0 9?	~1	101 6
Total bed vol (ml)	39 3	37	1162	5268	20 1	63 6	73 9	33,172
Separation								
Sample	(P)urine	(P)urine	(P)urine	(NP)urine	(P)urine	(NP)urine	(P)urine	(P)urine
Pre-treatment	None	Fil	Fil	Conc,Fil	Dil,Cen	Conc (NG)	Four *OE	Steps to Purify + Conc
(dilution factor)	–	(1)	(1)	(~0 2) added	(~2.7)		Conjs added	OE Conjs (1/208)
Wash	–	–	–	–	–	–	–	–
Vol of wash (ml)	–	–	–	–	–	–	–	–
Flow-rate (ml/min)	–	–	–	–	–	–	–	–
Eluent	H_2O	H_2O	H_2O	H_2O	H_2O	0 01 *M* HCO_3NH_4	H_2O	H_2O
Flow-rate (ml/min)	0 21	0 2	5	0 3	0 15	NG	0 2	~2 1
Vol of sample (ml)	1	2-6	400	~2000	~9 3	1500	10	104,000
Vol applied (ml)	1	2 6	400	100	10	0 5	10	500
Analyte	Conjs of E_1, E_2, E_3	E_3 Conjs in peak II	ΣE_3 Conjs	*OE Conjs	OE Conjs in peak II	*OE Conjs	*OE Conjs	ΣOE Conjs
Assay/detection method	Kober (C), indirect	Kober (C), indirect	Kober (C), indirect	Radio-chemical	Kober (C), direct	Radio-chemical	Radio-chemical	Kober (C), direct
Elution position of oestrogens (ml)	Two peaks. (I) 33-36, (II) 40-43	Two peaks (I) ~33-38 5, (II) ~38 5 44	Two peaks (I) ~1000 –1200, (II) ~1200 1400	Six peaks ~3820 4890 Peaks 1 5 ≡ peak I, peak 6 ≡ peak II	Two peaks (I) ~15-21, (II) ~21 31	Nine peaks ~45–765	Two/four? peaks, elution volume NG	Six peaks· 20,000 90,000 Peak 3 ≡ peak I 23,700 26 600 Peak 4 ≡ peak II = 29,400 32,800
Separation mechanism	GFC	Adsorption	Adsorption	GFC	GFC	Adsorption	GFC	Adsorption

	(B) Elution after column wash						
	Ref						
	20	21	22	23	24 (i)	24 (ii)	24 (iii)
Sephadex column	10	15	15	25M	15	15	15
G type	~6 4?	30	30	25	50	50	50
Length (cm)	1	0 9	0 9	1	1	1	1
I D (cm)	5	19 1	19 1	19 6	39 3	39 3	39 3
Total bed vol (ml)							
Separation	(P)urine	(P)urine	(P)urine	(P)urine	(P)urine	(P)urine	(P)urine
Sample	0 3 *N* HCl	NaCl, Fil	NaCl, Fil	*OE Conjs	Dil, NaCl,	Dil, NaCl,	Dil, NaCl,
Pre-treatment (dilution factor)	added (41)	(1)	(1)	added, Fil, NaCl (1)	Cen (2 7)	Cen (2 7)	Cen (2 7)
Wash	0 3*N* HCl + H_2O	1 5% (w/v) NaCl	1 5% (w/v) NaCl	4*M* NaCl	1 5% (w/v) NaCl	1 5% (w/v) NaCl	1 5% (w/v) NaCl
Vol of wash (ml)	10+5	15	20	20	15	75	15
Flow-rate (ml/min)	NG	NG	0 4	0 2	0 45	0 45	0 45
Eluent	0 5 *N* NH_4OH	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
Flow-rate (ml/min)	NG	NG	0 4	0 2	0 45	0 45	0 45
Vol of sample (ml)	0 25	2	4	10	20	20	20
Vol applied (ml)	10 25	2	4	10	2	10	2
Analyte	ΣOE Conjs	OE Conjs in peak II	OE Conjs in peak II	*OE Conjs	OE Conjs in peak II	OE Conjs in peak II	OE Conjs in peak II
Assay/detection method	Kober (F), direct	Kober (C), direct	Kober (C), direct	Radio-chemical	Kober (C), direct	Kober (C), direct	UV monitor + Kober (C), direct
Elution position of oestrogens (ml)	ΣOE Conjs in fraction 0–20	One peak (II) 18 33	One peak (II) 6 31	*OE Conjs in fraction 18 36	One peak (II) 37 55	One peak (II) 33 51	Four peaks 6 73 One peak contains OE (peak 4)
Separation mechanism	Adsorption	Adsorption	GFC	GFC	Adsorption	Adsorption	Adsorption

intermediate wash stage, generally using a salt solution, is included before elution Within each section the analytical details have been abstracted from the papers arranged in chronological order.

A study of the elution profile data in Table 2A shows that when a urine sample is applied to a column of Sephadex gel and eluted, either two, four, six or nine separate oestrogen or oestrogen metabolite fractions are obtained. This separation of up to nine fractions from urine is feasible, as the presence of at least nine oestrogen conjugates in this fluid has been documented earlier (Table 1). A comparative study of the elution volumes of the oestrogen conjugates with the other information in Table 2A clearly shows that the exact number of the distinct fractions obtained is dependent on the experimental conditions, and this finding will now be discussed in detail

Four of the eight separations detailed in Table 2A gave only two distinct oestrogen-containing fractions. The first of these separations[9] is the original one performed by Beling discussed earlier and two of the other separations (ref. 10, i and ii) are modifications of this method, performed again by Beling himself. The remaining two peak separation profile[16] was achieved using a 40-cm column of Sephadex G-15 (bead form) rather than Sephadex G-25 because the latter was no longer commercially available in the original block form. A study of the relevant sections in Table 2A shows that for each of the three procedures reported by Beling, the elution volume at which the separation was completed (V_{elu}) was approximately equal to the total bed volume (V_t). The actual ratios of V_{elu}/V_t range between 1.1 and 1.2 and the closeness of these values, over the large range of elution volumes employed, quantitates the good reproducibility of the two peak separation. This ratio for the remaining two peak separations[16] has the greater value of 1 5. It is suggested, for reasons that will be discussed later, that the origin of this significant increase lies in the fact that this separation was performed at 5°C rather than at the ambient temperature adopted throughout by Beling, or in the use of Sephadex G-15.

Unfortunately, it was not clear from a careful study of ref. 18 (Table 2A) if complete separation of the four labelled oestrogen conjugates was obtained when these were added to urine or whether they co-eluted off the Sephadex column in pairs to correspond to the two peaks of Beling. For this reason, this paper will not be discussed further

Now let us consider two of the separations summarized in Table 2A[15,19]. The elution profile section indicates that six peaks containing oestrogen were obtained. A study of the relevant sections of Table 2A showed that the general methodology of each of these two separations had certain similarities to those eluting only two oestrogen fractions and discussed above, namely, that all of the procedures involved the separation of the oestrogens from human urine which was eluted from the columns with water directly following application. However, in these two methods achieving the higher degree of fractionation, the urine samples were pre-concentrated before application and much longer columns of Sephadex gel were employed, the smallest column being 145 cm long[15]. Thus these column lengths are approximately three times or more greater than those used to resolve two oestrogen fractions. Interestingly, the two separations under discussion produced values of 0.9 and 1.0 for the ratio V_{elu}/V_t, which are very similar to the corresponding values calculated for the ratio from the papers by Beling. Because of this experimental finding, it is sug-

gested that the increase in the number of oestrogen fractions obtained in the separations is due primarily to the increased lengths of the Sephadex columns employed. The fact that as many as six separated peaks could be detected in these separations probably follows from the use of very sensitive radiochemical detection for the slightly concentrated non-pregnancy urine studied in ref. 15, and from the use of very concentrated urine (208 ×) when the Kober colorimetric detection procedure was used[19].

The best chromatographic separation of urinary oestrogen conjugates is that reported in ref. 17 (Table 2A) A study of the chromatographic data given in Table 2A shows that for this separation the calculated ratio of V_{elu}/V_t is 12. When this is compared with the range of values of 0.9–1.5 for all of the other separations summarized in Table 2A, it is logical to conclude that in this separation a higher degree of adsorption must be occurring. The basis of this difference must lie in the use of dilute aqueous ammonium formate instead of water as the eluent. A study of ref. 17 showed that the use of dilute aqueous sodium chloride solutions (0.1–0.01 *M*) also gave similar separation of the oestrogen components of urine, but ammonium formate was recommended by the original authors on account of its volatility.

Of the seven separations summarized in Table 2B, five involve the process of saturation of the urine sample with sodium chloride followed by filtration or centrifugation prior to application to the Sephadex columns where they are then washed with sodium chloride solution before final elution with water. Only one oestrogen-containing peak is eluted by this procedure; this corresponds to peak II of Beling, as the salt saturation process removes the peak I conjugates from the urine[25]. The column salt-wash step is included, as it achieves the removal of other saturated sodium chloride-soluble (interfering) Kober chromogens from the subsequently eluted fraction of eluate containing oestrogens. All of these separations have good reproducibility, and the V_{elu}/V_t ratio is 1.6–1.8 for columns of length 25–30 cm and 1.3–1.4 for columns of length 50 cm. These ratio values are generally larger than those in Table 2A, but this finding is not unexpected in view of the large increase (in elution volume) found when salts such as ammonium formate or sodium chloride are used[19] (Table 2A).

The remaining separation procedure summarized in Table 2B[20] involves the addition of dilute hydrochloric acid to the sample before application to the column, followed by a wash with this dilute acid before elution with dilute ammonia solution. Unfortunately, it is not possible to calculate the V_{elu}/V_t ratio from the experimental information provided by the authors and so it cannot be discussed further.

4 SEPARATION OF FREE OESTROGENS ON SEPHADEX

In a number of clinical chemistry methods for analysing the total oestrogen content of biological fluids, the fluid is first subjected to a hydrolysis procedure so as to convert the oestrogen conjugates into free oestrogens. The problem of separating the resulting mixture can be achieved in a number of ways. First, the free oestrogens may be recovered from the aqueous biological matrix by the process of solvent extraction as they are soluble in a number of organic solvents which are immiscible with water Second, the free oestrogens may be separated from other components of the hydrolysed biological fluid using columns of various adsorbents,

TABLE 3

SEPARATION OF FREE OESTROGENS ON SEPHADEX COLUMNS

Summary of experimental procedures and results Abbreviations used General NG = not given Sample (NP) = non-pregnancy, (P) = pregnancy, (*E_1, *E_2, *E_3) syn = synthetic mixture of radiolabelled oestrone, oestradiol and oestriol Pre-treatment of sample ACD = acid added, ADH = acid hydrolysed, Alk = alkali added, Dil = diluted, EZH = enzyme hydrolysed, Fil = filtered, > = several treatment steps (see original paper for details) Dilution factor = volume after pre-treatment/volume before pre-treatment Vol of sample = volume of sample pre-treated. Analyte *E_1, *E_2, *E_3 = radiolabelled oestrone, oestradiol and oestriol, respectively, Σ Free OE = total free oestrogens. Assay detection method· Kober (C) = Kober colorimetric; Kober (F) = Kober fluorimetric, Direct and Indirect = Kober reaction applied directly and indirectly, respectively, to eluate. Elution position· as in Table 2

	(A) Direct elution				
	Ref				
	26 (i)	*26 (ii)*	*27*	*28 (i)*	*28 (ii)*
Sephadex column					
G type	25M	25M	25F	25F	15
Length (cm)	15	15	23 7	7.0	4.2
I D (cm)	1	1	1 27	0 85	0 85
Total bed vol (ml)	11 8	11 8	30	4	2 4
Separation.					
Sample	(NP) urine	(P) urine	(*E_1,*E_2,*E_3) syn	(*E_1,*E_2,*E_3) syn	(*E_1,*E_2,*E_3) syn
Pre-treatment (dilution factor)	Dil, Fil, ACD, EZH. Alk (~2 1)	Dil, Fil, ACD, EZH, Alk (~2 1)	None	None	None
Wash	–	–	–	–	–
Vol of wash (ml)	–	–	–	–	–
Flow-rate (ml/min)	–	–	–	–	–
Eluent	H_2O	H_2O	H_2O	H_2O	H_2O
Flow-rate (ml/min)	NG	NG	~0 46	NG	NG
Vol of sample (ml)	~2.5	~2.5	2	2	2
Vol. applied (ml)	3	3	2	2	2
Analyte	Σ Free OE	Σ Free OE	*E_1,*E_2,*E_3	*E_1,*E_2,*E_3	*E_1,*E_2,*E_3
Assay/detection method	Kober (F), Indirect	Kober (F), Direct	Radio-chemical	Radio-chemical	Radio-chemical
Elution position of oestrogens (ml)	Σ Free OE in fraction 32 57	Σ Free OE in fraction 32–57	Two peaks ~69–154 Peak 1 =*E_1+*E_3, peak 2 =*E_2	One peak then shoulder ~4–16. Peak 1 =*E_1+*E_3, shoulder =*E_2	Two peaks: ~11–58 Peak 1 =*E_1+*E_3, peak 2 =*E_2
Separation mechanism	GFC	GFC	Adsorption	Adsorption	Adsorption

	(B) Elution after column wash				
	Ref				
29	*28 (in)*	*30*	*31*	*32*	*33*
25F	15	15	15	15	15
45	4 2	4 2	3.5	~3 1	~3 5
2 5?	0 85	0 85	2 1	1	~1
22 1	2 4	2 4	12 1	2 4	3
(NP) urine Fil, ACD, EZH (NG)	(NP) urine Dil, Fil, ACD, EZH, Fil (~2.1)	(NP) urine ADH ···> E_3 in aqueous phase (4)	(P) urine Dil, ADH (5 8)	(NP) serum Dil, ACD (8)	(P) urine Dil, Fil, ACD, EZH, Fil (~2 3)
–	H_2O	H_2O	H_2O	H_2O	H_2O
–	9 5	9 5	38	7 5	9.5
–	NG	NG	NG	NG	NG
H_2O	EtOAc	EtOAc	EtOAc	C_6H_6	EtOAc
NG	NG	~0.67	NG	NG	NG
~40	~2.5	20	10	1 1	~1
42,25	2	2	57 5	4	2
E_1,E_2,E_3	Σ Free OE	E_3	Σ Free OE	$E_1 + E_2$	Σ Free OE
Kober (C), indirect	Kober (F), direct	Kober (F), direct	Kober (F), direct	RIA	Kober (C), direct
Two peaks ~440–880	Σ Free OE in	E_3 in fraction	Σ Free OE in	$E_1 + E_2$ in fraction	Σ Free OE in
Peak 1 = $E_1 + E_3$, peak 2 = E_2	fraction 0 10	0–10	fraction 0–50	0 10	fraction 0 10
GFC	Adsorption	Adsorption	Adsorption	Adsorption	Adsorption

including those of Sephadex gel. Third, the above two methods may be combined so that the free oestrogens are first extracted into a suitable organic solvent, the solvent is evaporated off and the solid residue containing the oestrogens is reconstituted in the aqueous phase for application to columns of Sephadex gel[30] (Table 3).

The next part of this discussion is concerned with the Sephadex gel procedures involved in the last two methods above. The discussion will start from a consideration of the maximum number of free oestrogens to be expected from a separation of a hydrolysate of various biological fluids. For example, if the fluid contained only the conjugates of the classical oestrogens then the maximum number of free oestrogens resulting from the hydrolysis procedure is three. In terms of the complexity of the mixture this number may be favourably compared and contrasted with the much larger number of oestrogen conjugates originally present in the biological fluid; for details of those in pregnancy urine, see Table 1.

As before, all of the relevant publications listed under the heading of Sephadex gels in the bibliography[4] were collected together, summarized and collated to form Table 3. Table 3 is divided into two sections according to whether the free oestrogens were eluted off the column directly with water (section A) or with an organic solvent immediately after a column wash with water (section B).

Table 3A summarizes details of six reported separations of free oestrogens either from hydrolysed human urine or from synthetic mixtures. Although this type of procedure, involving direct elution of the sample of non-pregnancy hydrolysate off the column with water, was first reported by Eechaute[34] in 1964, this particular work is not included in the table as the required comprehensive experimental details were not given until the following year (ref. 26,i; Table 3A). None of the methods summarized in Table 3A achieves complete separation of the three oestrogens, probably because the columns used were relatively short (maximum length 45 cm). In those methods for which the elution profile was characterized[27–29] two oestrogen-containing peaks were reproducibly obtained, the first being composed of oestriol and oestrone. It appears likely from one study[29] that oestriol would be eluted before oestrone from a mixture if longer columns were employed. The observed elution volumes for these partial separations of the free oestrogens are much larger than those found for the conjugates on columns of comparable size Thus the calculated ratio V_{elu}/V_t for these separations using Sephadex G-25 where the elution profile is given lies within the range 4.0–5.1 for the free oestrogens, whereas the corresponding range for the conjugates found previously is 0.9–1.2. This finding indicates that it should be possible to separate free oestrogens from the conjugates by direct elution with water of an applied unhydrolysed urine sample off the Sephadex column. Interestingly, this experimental possibility has been partially confirmed by the work of Eechaute and Demeester[26] To conclude this discussion of Table 3A, it should perhaps be noted that the elution volume for the free oestrogens increases markedly when Sephadex G-15 is employed (ref. 28, ii) rather than Sephadex G-25. Further, Van Baelen *et al.*[28] presented some data which indicates that this elution volume increases even more when columns of Sephadex G-10 are used.

Table 3B summarizes the experimental conditions and results for five separations of free oestrogens present in either serum or hydrolysed urine, achieved when columns of Sephadex (G-15 only) were employed All of the separations involve a water wash–suction removal procedure immediately following the application of the

biological sample and the ratio V_{wash}/V_t varies from 3.1 to 4.0. The ratio for the volume of water required to begin to elute the free oestrogens off a column of Sephadex G-15 gel to the volume of the gel was calculated (from ref. 28, ii, Table 3A) to be 4 6. The interpretation of these two ratios is that whereas weakly adsorbed substances may be removed by the water wash procedure, all of the free oestrogens remain on the Sephadex column for subsequent elution by the organic solvent. Experimental confirmation of this interpretation is afforded by the high percentage recovery figures for added free oestrogens quoted by the authors of refs. 28, 32 and 33. The actual separation of the free oestrogens is then effected by the solvent used as the eluent. Thus, when the hydrophobic solvent benzene is employed oestrone and oestradiol are eluted but the oestriol is retained on the Sephadex column[32], in direct contrast to oestriol being the first eluted with water as discussed above. When ethyl acetate, a hydrophilic solvent, is employed all three oestrogens are eluted.

5 IMMUNOCHEMICAL SEPARATION OF FREE OESTRIOL ON SEPHADEX

Table 4 summarizes two similar experimental procedures, using very small amounts of Sephadex G-10, which were devised for an indirect (radioimmunoassay) method of determining the free oestriol concentration in plasma and serum. Both methods involve four distinct steps, the first of which is that of the retention of the oestrogens, certain other components of the blood sample and of a known amount of tritiated oestriol by the Sephadex column. The second step is that of the removal of the oestrogen conjugates, serum proteins and some interfering factors from the column by elution with water It should perhaps be noted that the actual volumes of water employed in this step of the procedure varied considerably Thus the method reported in ref. 36 used twice the volume employed in the procedure reported in ref. 35, which evidently only partially eluted the oestrogen conjugates. The corresponding V_{elu}/V_t ratio for this improved method is 8, and this value should be compared with the ratio of approximately 1.0 obtained earlier for elution of oestrogen conjugates from Sephadex G-25. One plausible explanation of the large difference in these ratios required to elute the oestrogen conjugates is that it is due to the use of different G-types of Sephadex The third step in these methods, which is not summarized in Table 4, is that of the "equilibration" of the remaining adsorbed free oestriol with an added oestriol antibody. The fourth and final step is summarized in Table 4 and is that of the elution of antibody by a buffer solution, a process which also removes proportional amounts of sample and labelled oestriol for the subsequent counting procedure.

Consideration of this final stage of the separation process detailed in Table 4 shows that it is clearly different from all of the other processes discussed earlier in that it depends on the formation of an antibody–oestriol complex. For this reason it will not be included further in the general discussion of the separation of oestrogens achieved with Sephadex presented below.

6 DISCUSSION

Tables 2 and 3 contain experimental details of 26 separations of mixtures of either free or conjugated oestrogens using columns of Sephadex gels presented in 20

TABLE 4

OESTROGEN ASSAYS INVOLVING ELUTION OF ANTIBODY-BOUND OESTRIOL FROM SEPHADEX COLUMNS

Summary of experimental procedures and results Abbreviations used General NG = not given Sample (P) = pregnancy Pre-treatment of sample *E_3 = radiolabelled oestriol Dilution factor = volume after pre-treatment/volume before pre-treatment Vol of sample = volume of sample pre-treated Assay/detection method RIA = radioimmunoassay Elution position as in Table 2

	Ref	
	35	*36*
Sephadex column		
G type (cm)	10	10
Length (cm)	~1 1	1 5
I D (cm)	1 07	0 98
Total bed vol (ml)	1	1 1
Separation		
Sample	(P) plasma	(P) serum
Pre-treatment (dilution factor)	*E_3 in buffer added (11)	*E_3 in buffer added (11)
Wash	H_2O + buffer	H_2O + buffer
Vol of wash (ml)	4+1	8+2
Flow-rate (ml/min)	NG	NG
Eluent	Antiserum + buffer	Antiserum + buffer
Vol of eluent (ml)	0 25 + 1 75	0 25 + 1 5
Flow-rate (ml/min)	NG	NG
Vol of sample (ml)	0 2	0 1
Vol applied (ml)	1	1 1
Analyte	E_3	E_3
Assay/detection method	RIA	RIA
Elution position of oestrogens (ml)	E_3 in fraction 0–2	E_3 in fraction 0 1 75
*Separation mechanism**	Adsorption	Adsorption

papers during the period 1961–82. This total consists of 15 separations of oestrogen conjugates, all from urine, and 11 separations of free oestrogens from urine (7), serum (1) and synthetic mixtures (3). A study of the relevant sections of Tables 2 and 3 shows that detection of the separated oestrogens was most commonly achieved by use of the Kober reaction using either colorimetric (12) or fluorimetric (6) procedures. The remaining separations were monitored by radiochemical procedures. The majority (68%) of the separations listed were designed as an integral part of an assay procedure for oestrogens in biological fluids for use in clinical chemistry laboratories. Indeed, two of these[10,21] were reported only after they had been in routine clinical laboratory use for a considerable time.

This study of the experimental methods of separating oestrogens will be concluded by discussing its main functions. The first function is to correlate, understand and summarize the various types of separation methods published and the second is to identify any shortcomings of these methods. The third and most interesting function is the listing of detailed proposals for future improvements.

Various correlations between similar separations of the conjugated oestrogens and of the free oestrogens have been detailed in the accounts accompanying Tables 2 and 3, respectively, and so these need not be discussed further. However, before proceeding to make general recommendations for improvements in Sephadex gel procedures it is necessary to draw attention to certain important shortcomings of published experimental practices or in the reporting of these. The most common failing is that of the almost complete lack of information concerning the temperature at which the separations were performed, despite the fact that Kreutzer and Meulendijk[16] in 1968 reported that the separation of the oestrogen conjugates present in human urine deteriorated abruptly when the separation was performed at temperatures higher than 20°C. Only one other group of workers took this finding into account[22] and they adopted the practice of keeping the column of gel at a constant temperature within the range 4–20°C. The deliberate omission of temperature data in Tables 2 and 3 is due to this paucity of reports of the temperature at which the separations were achieved The probable reason for this finding is that the majority of authors did not realise the importance of this variable in the present application because they were using gel filtration chromatographic procedures and these do not normally depend critically on temperature.

Another overlooked key experimental variable, and one which should always be reported in any column chromatographic separation, is the flow-rate of the eluent. In spite of its importance, a study of the eluent flow-rate section in Tables 2 and 3 shows that 12 out of the 26 separations reported do not have values for this variable because they were not reported by the original workers. The same consideration, with regard to importance, applies to the flow-rate of wash liquid for those procedures involving a washing step. Examination of the Tables 2B and 3B shows that it was not specified in 7 of the 12 reported separations. Failure to provide flow-rate information makes it difficult to reproduce a separation, and it is hoped that these comments will highlight the need to ensure that the requisite values are given in future publications.

7 CONCLUSIONS

Attention will now be focused on the principles underlying the separation. A study of the last sections of Tables 2 and 3 shows that for 17 of the 26 separations the authors give adsorption as the mechanism for the separation. The remaining separations listed the authors describe as gel filtration chromatographic (GFC) separations. Apart from Eechaute and Demeester[26], none of these authors commented on the principles by which a GFC separation of these low-molecular-weight compounds is supposedly achieved.

We are of the considered opinion that *all* of the separations listed in Tables 2 and 3 are due primarily to adsorption effects, for the following reasons:

(1) The range of gels employed would not be expected to separate the oestro-

gens by the process of molecular sieving, as the molecular weights included in their fractionation ranges[37] are much higher than those of the oestrogens.

(2) The separations are very dependent on the concentration of salt[21] and the pH of the oestrogen-containing mixture[10,20]. This is not the case for separations effected by molecular size alone. In particular, Smith and Kellie[11] reported that if uric acid was removed from a urine sample and Beling's Sephadex procedure adopted, then *no* separation of the oestrogen conjugates into two peaks could be achieved.

(3) As mentioned earlier, the separation of oestrogens (in urine) is critically dependent on small changes in temperature around room temperature, whereas in molecular sieving processes temperature does not markedly affect the separation.

The realization that the separations listed in the tables are due primarily to the operation of adsorption effects leads us to ask if it is appropriate for future workers to employ column gel chromatographic procedures at all! The reason for posing this question is a realization that GFC techniques are necessarily slow because of the inability of columns of Sephadex gels to be operated at high eluent flow-rates owing to their inability to withstand the requisite high pressures. Thus, for example, it took Beling[9] about 3.3 h to separate the oestrogen conjugates after the application of sample to a column of Sephadex G-25 gel It was therefore of great interest to us to find that recently a paper has been published[38] in which oestrogen conjugates have been extracted by adsorption from urine by a very rapid method involving the addition of a small amount of Sephadex G-15 powder to the specimen. This adsorption procedure was completed in 15 sec; the Sephadex G-15 powder was then rapidly filtered off using a sintered-glass filter funnel under a water-pump vacuum before the oestrogens were eluted off the Sephadex with 0.1 *N* sodium hydroxide solution. The total time taken for the transfer of the oestrogens from urine to the sodium hydroxide solution was estimated by us to be less than 10 min. Further, by controlling the volumes of the urine and the base used, this adsorption procedure opens up the possibility of the investigator controlling the final levels of the oestrogens. In particular, the possibility of increasing the concentration of the oestrogens in the eluting base over that of the original biological fluid in order to assist the detection/analytical problem is an appealing one.

Experimental studies based on these considerations are currently under active investigation in the authors' laboratory and it is hoped that these will form the basis of a subsequent publication in which the adsorption method is subsequently combined with an HPLC separation.

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9 SUMMARY

The separations achieved when mixtures of both free and conjugated oestrogens from a variety of sources are chromatographed on columns of Sephadex gel are

reviewed. The molecular identities of oestrogen conjugates which have been separated from human urine by these methods are listed in Table 1. Tables 2 and 3 contain the key experimental details for a total of 26 separations of oestrogen mixtures, abstracted from the total of 20 papers which were published during the period 1961-82. Table 4 details corresponding experimental data for the separation of free oestriol (in human blood) achieved by methods involving a combined Sephadex gel and immunochemical procedure abstracted from a further two papers.

A careful analysis of the separation data given in the tables leads to the initial conclusion that the elution profile depends on the expected chromatographic variables for gel filtration chromatography, namely, type of Sephadex gel, length of column, nature and amount of sample applied and the sensitivity of detection methods. However, the separation achieved by the Sephadex columns is also shown to be critically dependent on the column temperature and the pH and chemical composition of the eluent and wash solvents. These latter effects, together with the realization that the molecular weights of the oestrogens being separated are very similar, leads to the conclusion that the separations summarized in Tables 2 and 3 are all being achieved by an adsorption process

This being the case, it is suggested that the time-consuming methods of gel filtration chromatography need not be used. Confirmation of this proposal is afforded by a discussion of a recent paper in which the rapid separation of the oestrogens from other components in the biological matrix (urine) was achieved by an adsorption procedure.

It is suggested that in the future, separations of oestrogens in biological materials may be most rapidly achieved in combining this type of adsorption procedure with HPLC.

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