Separation of Urinary Oestrogen Sulphates from Oestrogen Glucosiduronates on Diethylaminoethyl-Sephadex Columns

By ROLAND HÄHNEL AND M. GHAZALI BIN ABDUL RAHMAN

*Queen Elizabeth II Coronation Gift Fund and University Department of Obstetrics and Gynaecology,
King Edward Memorial Hospital for Women, Subiaco, and Department of Biochemistry,
University of Western Australia, Nedlands, Western Australia*

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1. Gradient elution (sodium chloride concentration gradient) on DEAE-Sephadex columns is used to separate urinary oestrogen conjugates of pregnancy urine. Changes in the shape of the gradient alter the chromatograms in a predictable manner so that the dispersion of peaks can be modified at will. 2. Suitable choice of the gradient results in the separation from each other of oestrogen sulphates, oestrogen 16(or 17)-glucosiduronates, oestrogen 3-glucosiduronates and free oestrogens. 3. Evidence for the presence of oestriol 3-sulphate, oestrone 3-sulphate, 17 β -oestradiol 3-sulphate, 16-oxo-17 β -oestradiol 3-sulphate and oestriol 16(or 17)-sulphate in the sulphate fraction of DEAE-Sephadex chromatograms of pregnancy urine is provided.

In a previous paper (Hähnel, 1965) on the separation of conjugated urinary oestrogens on columns of the anion-exchanger DEAE-Sephadex it was reported that oestrogen sulphates partly overlap with the oestriol 16-glucosiduronate peak. Preliminary experiments at that time had demonstrated that a satisfactory separation can in principle be achieved by suitable adjustment of the sodium chloride gradient used for the elution. Altering the shape of the gradient to fit the requirements indicated by previous chromatograms has already proved successful for separating the overlapping peaks of pregnanediol glucosiduronate and oestrogen 3-glucosiduronates (Hähnel & Ghazali bin Abdul Rahman, 1966). The same principle (cf. Peterson & Sober, 1959) has now been employed again and a separation of oestrogen sulphates from oestriol 16-glucosiduronates has been achieved. Several oestrogen sulphates in fractions of DEAE-Sephadex chromatograms of pregnancy urine have been identified.

METHODS AND MATERIALS

Chromatography on DEAE-Sephadex. This was performed as described previously (Hähnel, 1965; Hähnel & Ghazali bin Abdul Rahman, 1966). A 10–30 ml. sample of diluted pregnancy urine (urine-water, 2:1, v/v) was put on top of a DEAE-Sephadex column (50 ml. burette, 11 mm. internal diam., height of DEAE-Sephadex filling approx. 500 mm. at the start of the experiment) prepared in water. When the urine had percolated into the bed material gradient elution was started. The new gradient for elution was obtained by filling into each of the nine identical chambers of a Buchler

Varigrad 95 ml. of the following aqueous solutions: 2 M-NaCl in chambers 8 and 9, 0.3 M-NaCl in chamber 3 and 0.05 M-NaCl in chambers 1, 2, 4, 5, 6 and 7. After completion of this gradient the DEAE-Sephadex column was further eluted with a gradient resulting from 50 ml. of 2 M-NaCl in chambers 1 and 2 and 50 ml. of 4 M-NaCl in chamber 3 (alternatively, the column was further eluted with 150 ml. of 2 M-NaCl). The column effluent was collected in fractions of 100 drops each. Samples of the effluent fractions were used for quantitative estimation of individual oestrogens by the method of Brown (1960) or of total oestrogens (Ittrich, 1958; Bradshaw, 1961).

Chromatography of oestrogen sulphates. The fractions of DEAE-Sephadex chromatograms containing oestrogen sulphates were extracted with diethyl ether-ethanol (3:1, v/v) (Edwards, Kellie & Wade, 1953). The extracts were evaporated to dryness under reduced pressure and the residue was dissolved in methanol. Samples of this solution were used for thin-layer or paper chromatography in the following solvent systems (all by vol.): (1) butan-1-ol-acetic acid-water (10:1:9) (Menini & Diczfalussy, 1960); (2) 10% (v/v) formic acid-butan-1-ol-*n*-butyl acetate (5:1:4) (Lewbart & Schneider, 1955); (3) toluene-butan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (5:15:2:18) (Menini & Diczfalussy, 1961); (4) 0.4 M-NaHCO₃ (Payne & Mason, 1965); (5) diisopropylether-2-methylpropan-2-ol-aq. NH₃ (sp.gr. 0.88)-water (5:5:1:9) (Menini & Diczfalussy, 1960).

For the thin layers silica gel G (E. Merck A.-G., Darmstadt, Germany) was used. Two types of chromatograms were run, ascending on plates 20 cm. $\times$ 20 cm., and descending on plates 20 cm. $\times$ 45 cm. if the R_F values were rather similar and separations could not be achieved on the smaller plates.

Whatman no. 1 paper was used for paper chromatography.

For the detection and characterization of the oestrogen sulphates on the chromatograms Folin-Ciocalteu reagent (Barton, Evans & Gardner, 1952), anisaldehyde (Lisboa &

Diczfalussy, 1963) and rhodizonic acid combined with hydrolysis *in situ* were used (Schneider & Lewbart, 1956). The following authentic steroid sulphates were available as standards: oestrone sulphate (Schering A.-G., Berlin, Germany), oestriol 3-sulphate, oestradiol 3-sulphate and oestradiol 17-sulphate (Dipl. Eng. H. Fex, Leo A.B., Hälsingborg, Sweden).

Enzymic hydrolysis of oestrogen sulphates. Portions of the methanolic solution of the oestrogen sulphates were evaporated to dryness and redissolved in 0.05 M-tris-HCl buffer, pH 7.40. The solution was incubated with sulphatase (ox liver steroid sulphatase; Schering A.-G.) for 16 hr. at 37°. During the incubation the mixture was stirred or shaken continuously. After extraction of the liberated steroids with ether, the residue was adjusted to pH 4.8 and the mixture incubated with β -glucuronidase (Ketodase; Warner-Chilcott Laboratories, Morris Plain, N.J., U.S.A.) at 37° for 16 hr. After the extraction with ether the residue was finally hydrolysed for 1 hr. with HCl (3 ml. of conc. HCl/20 ml. of solution).

Characterization of free oestrogens. The free oestrogens in the hydrolysates were characterized by thin-layer chromatography in the following systems: (6) ethyl acetate-water-saturated *n*-hexane-ethanol (16:3:1, by vol.) (Lisboa & Diczfalussy, 1962); (7) cyclohexane-ethyl acetate (1:1, v/v) (Menini & Diczfalussy, 1961).

Oestriol, oestradiol-17 β and oestrone were also characterized by Brown's (1960) method.

RESULTS

Separation of oestrogen sulphates from oestrogen 16(or 17)-glucosiduronates

The results of the oestrogen estimations (total oestrogens) in the fractions obtained after chromatography of pregnancy urine on DEAE-Sephadex, together with the gradient used, are shown on Fig. 1. The peaks represent (from left to right): free oestrogens (if present, peak 1), oestrogen 3-glucosiduronates (peak 2) and oestrogen 16(and/or 17)-glucosiduronates (peak 3). These peaks correspond to those described previously (Hähnel, 1965), the position being displaced slightly because of the different gradient used. The last peak (4, fractions 68–78), however, did not appear on the previous chromatograms, owing to overlapping with the oestrogen 16-glucosiduronates. The new gradient employed for elution brought about the separation of this peak from the oestrogen 16-glucosiduronates.

Authentic oestrone sulphate, oestriol 3-sulphate and oestradiol 3-sulphate added to male urine and subjected to DEAE-Sephadex chromatography were eluted in the same fractions as the oestrogen sulphate peak of the chromatogram of pregnancy urine.

No influence of the pH of the urine on the chromatographic pattern of the oestrogen glucosiduronates was observed between pH 5.5 and 7.0 and the recovery (as judged by measuring the oestrogens in the urine and in the combined effluent

Fig. 1. Separation of oestrogen conjugates of pregnancy urine on DEAE-Sephadex columns. Column: 50 ml. burette, 11 mm. internal diam., height of DEAE-Sephadex filling approx. 500 mm. at the start of the experiment. The column was loaded with 20 ml. of late-pregnancy urine diluted with 10 ml. of water. Gradient elution was as described in the text. Effluent fractions (100 drops each) were collected. The ordinate is the corrected extinction in the Kober reaction. Peak 1, free oestrogens; peak 2, oestrogen 3-glucosiduronates; peak 3, oestrogen 16(or 17)-glucosiduronates; peak 4, oestrogen sulphates. The NaCl gradient shown is calculated from Peterson & Sober (1959).

fractions) was quantitative (Hähnel, 1965). Subsequently, however, it was noticed that extreme pH values influence the pattern. Taking the elution of oestriol 16-glucosiduronate (in fractions 40–50) from a pregnancy urine with physiological pH as reference it is found that with decreasing pH of the applied urine the position of the peaks shift to lower fractions (oestriol 16-glucosiduronate in fractions 36–44 at pH 2.0). Increased pH values retard the elution of the peaks (oestriol 16-glucosiduronate in fractions 57–64 at pH 12.0). The positions of the other oestrogen conjugates on the DEAE-Sephadex chromatograms change similarly.

Preliminary evidence suggests that the recovery of oestrogen sulphates decreases with increasing pH.

Characterization of oestrogen sulphates and identification of individual oestrogen sulphates in peak 4 of DEAE-Sephadex chromatograms of pregnancy urine

The fractions of several DEAE-Sephadex chromatograms forming the new peak (fractions 68–78) were combined and extracted with ether-ethanol, and the extract was evaporated to dryness under reduced pressure. The residue was redissolved in methanol and samples were used for the characterization of the oestrogen conjugates. Steroid sulphatase quantitatively split all Kober-positive material present; very little further Kober-positive material was released by subsequent β -glucuron-

Table 1. *Fractionated hydrolysis of the oestrogen sulphates of peak 4 of DEAE-Sephadex chromatograms of pregnancy urine*

The combined fractions forming peak 4 were extracted with ether: (1) before hydrolysis, (2) after sulphatase hydrolysis, (3) after subsequent β -glucuronidase hydrolysis, (4) after subsequent acid hydrolysis. The amount of oestrogens in the ether extracts was estimated by the method of Bradshaw (1961). The spectrophotometer readings of the final colour extracts are given in the Table. Corrected extinctions were calculated by using the formula given by Brown (1960), namely:

$$\text{Corrected extinction} = 2E_{532} - (E_{502} + E_{562})$$

Ether extraction	E_{502}	E_{532}	E_{562}	Corrected extinction
(1) Before hydrolysis	0.067	0.053	0.041	—
(2) After sulphatase hydrolysis	0.201	0.267	0.171	0.162
(3) After β -glucuronidase hydrolysis of sulphatase hydrolysate	0.075	0.070	0.051	0.014
(4) After acid hydrolysis of enzyme hydrolysate	0.091	0.087	0.069	0.014

idase hydrolysis or acid hydrolysis (Table 1). The Kober-positive material could be quantitatively released also by solvolysis by the method of Burstein & Lieberman (1958). The remainder of the solution of oestrogen conjugates in methanol was subjected in several runs to thin-layer chromatography in butan-1-ol-acetic acid-water (10:1:9, by vol.). Part of the developed chromatograms was stained with anisaldehyde reagent. Two bands with R_F 0.46 and R_F 0.40 respectively were obtained. The band with R_F 0.46 on acid hydrolysis, ether extraction and thin-layer chromatography yielded three oestrogens that behaved like oestrone, oestriol and 17β -oestradiol in two solvent systems (nos. 6 and 7; see the Methods and Materials section). The band with R_F 0.40 contained two oestrogens. After acid hydrolysis of the band, ether extraction and thin-layer chromatography of the free oestrogens, these were identified as oestriol and 16α -oxo- 17β -oestradiol on account of identical R_F values in the same two solvent systems (6 and 7). The remaining portions with R_F 0.46 and 0.40 of the chromatograms were scraped off and collected separately in test tubes. The oestrogen conjugates were eluted from the scrapings with methanol (2 hr. at 37°), and portions of the methanolic solution, containing the oestrogen conjugates of bands with R_F 0.46 and R_F 0.40 respectively, were used for the following experiments.

Band with R_F 0.46. Rechromatography in toluene-butan-1-ol-aq. ammonia (sp.gr. 0.88)-water (5:15:2:18, by vol.) resolved this fraction into two subfractions with R_F 0.86 and 0.91. The spot with R_F 0.91 gave a positive Folin-Ciocalteu reaction; the spot with R_F 0.86 did not react with this reagent but did so after solvolysis *in situ*. Both spots gave a positive sulphate reaction with rhodizonate after solvolysis *in situ*. Staining with anisaldehyde reagent and heating for 10 min. at 80° produced a lilac colour in the region with R_F 0.91,

whereas the area with R_F 0.86 turned olive-green. If the compound with R_F 0.91 was hydrolysed and subjected to Brown's (1960) method it behaved like authentic oestriol in every respect (solvent partition, methylation, alumina chromatography, Kober reaction). In addition, thin-layer chromatography of the ether extracts of the hydrolysed compound of R_F 0.91 yielded a phenolic oestrogen with the R_F of authentic oestriol in two solvent systems (nos. 6 and 7). Likewise, it was found that the spot with R_F 0.86 contained two oestrogens that behaved like oestrone and 17β -oestradiol in Brown's (1960) method and had R_F values identical with those of authentic oestrone and 17β -oestradiol in two solvent systems (nos. 6 and 7).

Separation of the two conjugates with identical R_F 0.86 in system (3) could be achieved by rechromatography in system (5). Two spots were observed after staining with anisaldehyde reagent. One had R_F 0.54, as authentic 17β -oestradiol 3-sulphate, and showed the characteristic colour changes from lilac to emerald green; the other one had R_F 0.48 and a lilac colour, identical in both respects with authentic oestrone 3-sulphate. It is concluded from these results that 17β -oestradiol 3-sulphate and oestrone 3-sulphate are present in the oestrogen sulphate peak of DEAE-Sephadex chromatograms of pregnancy urine.

The properties of the conjugate with R_F 0.91 (in system 3) are suggestive of an oestriol sulphate in which the sulphate group is attached to one of the alcoholic hydroxyl groups.

Band with R_F 0.40. Rechromatography of this fraction in toluene-butan-1-ol-aq. ammonia (sp.gr. 0.88)-water (5:15:2:18, by vol.) gave two compounds with R_F 0.50 and 0.52 respectively that gave negative phenol and sulphate tests on the chromatograms. After solvolysis *in situ* both tests became positive. Elution of the areas corresponding to R_F 0.50 followed by acid hydrolysis of the

Table 2. Comparison of R_F values of reference standards with oestrogen sulphates isolated from peak 4 of DEAE-Sephadex chromatograms of pregnancy urine

Solvent system	Oestrone 3-sulphate		Oestriol 3-sulphate		Oestradiol 3-sulphate		16-Oxo-oestradiol 3-sulphate	Oestriol 16 (or 17)-sulphate
	Isolated	Authentic	Isolated	Authentic	Isolated	Authentic	Isolated	Isolated
(1)	0.46	0.43	0.40	0.40	0.46	0.46	0.40	0.46
(2)	0.24	0.24	0.21		0.24	0.24		0.24
(3)	0.86	0.88	0.50	0.49	0.86	0.88	0.52	0.91
(4)	0.50	0.51	0.36		0.50	0.50		0.50
(5)	0.48	0.48	0.17	0.19	0.54	0.54		

methanol extract and thin-layer chromatography in systems (6) and (7) together with authentic oestriol demonstrated that oestriol (R_F 0.37 and 0.05 respectively) was present in the compound with R_F 0.50. If the free oestrogen was subjected to Brown's (1960) method it behaved in every respect like authentic oestriol. Rechromatography of the oestriol conjugate with R_F 0.50 in system (5) together with authentic oestriol 3-sulphate gave R_F values in agreement with that of the reference sample. Staining with anisaldehyde reagent for 10 min. at 80° produced a lilac colour. It is concluded from these results that oestriol 3-sulphate is one of the components of the band with R_F 0.40, and that it is present in the oestrogen sulphate peak of DEAE-Sephadex chromatograms of pregnancy urine. Hydrolysis of the other compound of the R_F 0.40 band (with R_F 0.52 in system 3) with acid and thin-layer chromatography of the ether extract of the hydrolysate in two solvent systems (nos. 6 and 7) gave R_F values of 0.65 and 0.30 respectively, which were in agreement with those of authentic 16-oxo-17 β -oestradiol in these solvent systems. The results suggest that 16-oxo-17 β -oestradiol is present in the sulphate fraction of DEAE-Sephadex chromatograms of pregnancy urine in the form of the 3-sulphate.

The separation and identification of the various oestrogen sulphates is shown schematically on the flow sheet (Scheme 1); the R_F values of the isolated oestrogen conjugates are compared with those of authentic reference compounds in Table 2.

DISCUSSION

Several methods are available for the separation of oestrogen sulphates from oestrogen glucosiduronates. Both Crépy, Jayle & Meslin (1957) and Barlow & Kellie (1959) used alumina chromatography of butan-1-ol or ether-ethanol extracts respectively of urine for this purpose. Baulieu (1961) employed Florisil chromatography. In these methods separation of the oestrogen sulphates from the glucosiduronates is achieved by eluting the

two classes of compounds that have been absorbed on the aluminium oxide with solvent mixtures of different polarity.

Good separation can also be achieved by counter-current distribution, paper and thin-layer chromatography and electrophoresis (see e.g. Schneider & Lewbart, 1959; Menin & Diczfalusy, 1961; Oertel, Tornero & Groot, 1964), and these methods have found numerous applications for particular problems. They are less well suited for group separations; very limited amounts can be processed on paper and thin-layer chromatograms only and countercurrent distribution requires rather specialized and bulky apparatus.

The separation of oestrogen glucosiduronates from oestrogen sulphates by anion-exchange chromatography on DEAE-Sephadex columns as described in this paper has the advantage of being applicable to urine rather than urine extracts. Thus the first step in all the other methods mentioned above, namely the extraction of the oestrogen conjugates from urine by organic solvents, is eliminated. Owing to the formation of emulsions, the use of high-boiling solvents etc., the extractions and the subsequent evaporation of the solvents require considerable time before the actual group separations can be commenced.

The main advantage of the DEAE-Sephadex chromatography, however, is that the separation is not confined to oestrogen sulphates from oestrogen glucosiduronates, but that a separation of four groups from each other is obtained at the same time: free oestrogens, oestrogen 3-glucosiduronates, oestrogen 16(or 17)-glucosiduronates and oestrogen sulphates. In late-pregnancy urine the main constituents of the oestrogen 3-glucosiduronate peak have been identified as oestriol 3-glucosiduronate and oestrone 3-glucosiduronate (Hähnel & Ghazali bin Abdul Rahman, 1966). Smaller amounts of 16-epioestriol 3-glucosiduronate have also been found (Ghazali bin Abdul Rahman & Hähnel, 1967). The oestrogen 16(or 17)-glucosiduronate peak represents mainly oestriol 16 α -glucosiduronate (Hähnel, 1965), with smaller

amounts of 16-epioestrinol 16(or 17)-glucosiduronate also present (Ghazali bin Abdul Rahman & Hähnel, 1967). The dispersion of the oestrogen conjugate peaks on the DEAE-Sephadex chromatograms can be modified at will by changes in the shape of the eluting salt gradient. This makes the method very adaptable to particular problems, as shown by the separation of interfering pregnanediol glucosiduronate from oestrinol 3-glucosiduronate and oestrone 3-glucosiduronate (Hähnel & Ghazali bin Abdul Rahman, 1966) and by the separation of oestrinol 16-glucosiduronate from oestrogen sulphates in this paper.

In some ways anion-exchange chromatography of urinary oestrogen conjugates on DEAE-Sephadex columns is similar to gel filtration (Beling, 1963), in which separation into oestrogen 3-glucosiduronates and oestrogen 16(or 17)-glucosiduronates is achieved. However, no separation of oestrogen sulphates from oestrogen 16(or 17)-glucosiduronates by gel filtration has been reported. Further, the gel-filtration technique cannot be used for free oestrogens. Though the behaviour of the oestrogen conjugates during gel filtration is poorly understood (molecular-sieve action cannot explain the separation of oestrinol 3-glucosiduronate from oestrinol 16-glucosiduronate, for instance), it seems reasonable to regard anion-exchange of the oestrogen conjugates as the predominant cause for the separation of the different types of conjugates on DEAE-Sephadex columns, as suggested previously (Hähnel, 1962). The influence of salt concentration on the elution of the conjugates, and the order of elution of the different types of conjugates, among others, lend support to this view.

Some of the oestrogen sulphates that were found to be present in the oestrogen sulphate peak of DEAE-Sephadex chromatograms of pregnancy urine have been identified in urine previously. The presence of oestrone 3-sulphate in pregnancy urine was demonstrated by Butenandt & Hofstetter (1939) and numerous others.

Indirect evidence for the occurrence of oestrinol 3-sulphate in pregnancy urine was first presented by Cohen & Bates (1949).

17 β -Oestradiol 3-sulphate to the best of our knowledge has not been found in pregnancy urine so far. Analytical data for the characterization of this compound, however, have been reported in the literature (Payne & Mason, 1963, 1965; Diezfalussy, Cassmer, Alonso & de Miquel, 1961), and an authentic sample was available to us. One of the compounds in the oestrogen sulphate peak of DEAE-Sephadex chromatograms of pregnancy urine behaved like authentic 17 β -oestradiol 3-sulphate.

16-Oxo-17 β -oestradiol is known to occur in pregnancy urine (Layne & Marrian, 1958), but no

information is available on its mode of conjugation. The urine from which 16-oxo-17 β -oestradiol was isolated by Layne & Marrian (1958) had been hydrolysed with a crude enzyme preparation from *Patella vulgata*, which contained β -glucuronidase but the sulphatase activity of which had not been determined (Marrian, Watson & Panattoni, 1957). The results obtained by us suggest that 16-oxo-oestradiol is present in pregnancy urine as 16-oxo-17 β -oestradiol 3-sulphate. The limited amount of material available and the lack of the authentic conjugate have not permitted further studies so far. The evidence for oestrinol 16(or 17)-sulphate in the oestrogen sulphate peak of DEAE-Sephadex chromatograms should be regarded as preliminary only, as sufficient material for detailed investigation was not available. From the experiments it cannot be decided if conjugation occurs at C-16 or C-17 or at both sites. A disulphate, however, seems unlikely, as one would expect R_F values distinctly different from those of the monosulphates.

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