Separation of Estriol Conjugates on Sephadex

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Beling's method used gel filtration on Sephadex G-25 to separate estrogen conjugates in late pregnancy urine into two major fractions, referred to as peaks I and II. This method eliminates most of the urinary constituents that would otherwise interfere with enzymatic hydrolysis, and separation of estrogen 3-glucuronides from estrogen 16-glucuronides is achieved. However, sulfates and sulfoglucuronides cannot be separated from glucuronides by this procedure. Methods to subfractionate peaks I and II by reapplication on various hydrophilic Sephadex columns have been described. A gel chromatographic system in which the Sephadex G-25 column was eluted with an organic solvent has been used in the separation of various peak I and II conjugates.

The advantages of a lipophilic gel, Sephadex LH-20, for the purification and separation of neutral steroid conjugates have been demonstrated but its possible merits in the separation of conjugated estrogens have seemingly been overlooked. Accordingly, we set out to explore the potentialities of Sephadex LH-20 for the separation of peak I and II conjugates. This communication describes preliminary studies on a method to separate four urinary estriol conjugates by sequential gel filtration on Sephadex G-25 and chromatography on Sephadex LH-20. A similar scheme can probably be used for the fractionation of a number of other urinary estrogen conjugates as well.

Methods. Sephadex G-25 and Sephadex LH-20 were purchased from Pharmacia AB, Uppsala, Sweden. Gel filtration of urine specimens on Sephadex G-25 columns was performed according to Beling. When reference compounds were gel-filtered in the absence of urine, the samples were dissolved in 10 ml of 0.1 M phosphate buffer, pH 6.5, containing 8 mg of uric acid, which led to elution of peak I and II conjugates in the correct position. Flow rate was maintained at 0.2 ml/min with a peristaltic pump, and fractions were collected automatically. The gel chromatographic procedures were modified from a method used in the separation of urinary neutral steroid conjugates. Columns of 4 g of Sephadex LH-20 (300 × 10 mm) were prepared in chloroform/methanol 1:1 containing 0.01 mol/l sodium chloride giving a bed height of approximately 25 cm. The samples were applied in 2–3 ml of the eluting solvent, which consisted of chloroform/methanol mixtures containing 0.01 mol/l sodium chloride, or, when higher percentages of chloroform were used, the eluent was saturated with sodium chloride (Ref. 9). Solvent flow rate was about 0.5 ml/min and fractions were collected automatically. Radioactivity was measured in a Wallac NTL 314 liquid scintillation spectrometer.

Labeled reference compounds. Four labeled estriol conjugates, estriol-3-glucuronide (E3-3G), estriol-3-sulfate, 16-glucuronide (E3-3S, 16G), estriol-16-glucuronide (E3-16G) and estriol-3-sulfate (E3-3S), were prepared biosynthetically from estriol-6,7,16H and estriol-4,14C (New England Nuclear Corporation, Boston, Mass., U.S.A.). Gel filtration on Sephadex G-25 in the presence of uric acid (see above) proved to be a convenient method for the initial separation of estriol conjugates after the biosynthetic procedure. Labeled E3-16G, synthesized according to Slauwhite et al., was converted to E3-3S, 16G, by incubating in a 100 000 g supernatant of homogenized guinea-pig liver in the presence of ATP and MgSO4 (Levitz et al.13). Gel filtration on Sephadex G-25 in urate-phosphate buffer gave the synthesized E3-3S, 16G (90–90% of the radioactivity) in the frac-

tions corresponding to peak I and the unchanged E₂₃G in the fractions corresponding to peak II. After evaporation, the peak I fractions were incubated with 1000 U/ml of β-glucuronidase (Ketodase, Warner-Chilcott Laboratories, Morris Plains, New Jersey, U.S.A.) in 0.1 M acetate buffer, pH 4.5, for 16 h and refiltered through Sephadex G-25 in urea-phosphate buffer. In the peak II fractions, 97% of the radioactivity was eluted and only traces were found in the peak I fractions, indicating almost quantitative cleavage of E₂₃G to E₂₃.

Labeled E₂₃G was prepared according to Goebelsman et al. 14

Results and discussion. Gel filtration studies. When in separate experiments labeled reference compounds were added to 10 ml specimens of pregnancy urine and gel-filtered on Sephadex G-25 columns (500 × 10 mm), sharp symmetrical peaks were obtained for E₂₃G (peak I) and for E₂₃ and E₁₆G (peak II). E₂₃G (peak I), however, was invariably eluted somewhat later than E₂₃G and gave a broader peak with incomplete separation from peak II conjugates. By increasing the column lengths with twice the amount of gel, an acceptable separation of peak I and II fractions was achieved.

Gel chromatographic studies. When applied on a Sephadex LH-20 column and eluted with chloroform/methanol 1:1 containing sodium chloride, the two labeled estriol monoglucuronides (E₂₃G and E₂₃G) were recovered in the effluent between 20 and 45 ml. The estriol monosulfate (E₂₃S) was eluted in the fractions between 40 and 65 ml. This closely resembles the elution of neutral steroid glucuronides and monosulfates, respectively, under similar experimental conditions. 11, 14 The estriol double conjugate (E₂₃G) was strongly retarded in the column and was not eluted in the first 150 ml of effluent. In subsequent experiments the elution of E₂₃G was facilitated by changing the eluent to methanol after elution of the “glucuronide” fraction. In all chromatographic experiments with Sephadex LH-20 free estriol was eluted in the first 15 ml of effluent.

Separation of labeled peak I conjugates on Sephadex LH-20. In the system described above, complete separation of E₂₃G and E₂₃G could be obtained. To eliminate slight tailing of E₂₃G to subsequent fractions, chloroform/methanol 2:3 containing sodium chloride (0.01 mol/l) was used as eluent in the final method. Under these conditions E₂₃G was recovered in the fractions between 20 and 40 ml. After elution of the column with 70 ml, the eluent was changed to methanol, which led to elution of E₂₃G in the effluent between 80 and 105 ml (Fig. 1).

Fig. 1. Separation of labeled E₂₃G and E₂₃G on a 4 g Sephadex LH-20 column. Solvent: a, 0.01 M NaCl in CHCl₃:CH₃OH 2:3; b, CH₃OH.

Separation of labeled peak II conjugates on Sephadex LH-20. When chloroform/methanol 1:1 containing sodium chloride (0.01 mol/l) was used as eluent, overlapping of E₂₃G with E₂₃S occurred (see above). An increase in the proportion of chloroform caused retardation of both conjugates in the column, but E₂₃S was retained to a higher degree. With chloroform/methanol 2:1 (saturated with sodium chloride) as eluent, satisfactory separation of E₂₃S (40–80 ml), from E₂₃G (95–135 ml) was obtained (Fig. 2).

Separation of labeled estriol conjugates added to pregnancy urine. Labeled estriol

Fig. 2. Separation of labeled E₂₃G and E₂₃S on a 4 g Sephadex LH-20 column. Solvent: CHCl₃:CH₃OH 2:1, saturated with NaCl.

conjugates added to pregnancy urine were gel-filtered on Sephadex G-25, and peaks I and II were collected separately and evaporated to dryness in vacuo. The dry residues were taken up in 3 ml of the eluting solvent and chromatographed in the systems designed for peak I and II conjugates. The presence of urinary peak I and II contents in the samples did not alter the elution pattern of the labeled reference compounds.

**Application.** In chromatographic experiments on Sephadex LH-20 the elution pattern of labeled estradiol glucuronides and estradiol sulfate closely resembled that of the neutral steroid glucuronides and monosulfates. In addition, the estradiol double conjugate (E2-3S,16G1) was strongly retarded in the column, as is the case with the neutral steroid disulfates, which seems to confirm earlier studies, which suggested that it is the mode of conjugation, rather than the steroid nucleus, which determines the elution behaviour of conjugated steroids on lipophilic Sephadex. Experiments with labeled estradiol conjugates indicate that by chromatography on Sephadex LH-20 a convenient group separation of estrogen conjugates may be achieved, where at least glucuronides, sulfates, and sulfogluconides can be separated from each other. In addition, gel filtration on Sephadex G-25 can be used in the separation of estrogen 3-glucuronides from estrogen 16-glucuronides. Thus it can be concluded that gel chromatography on Sephadex LH-20 in combination with gel filtration on Sephadex G-25 provides a useful means for group separation of urinary estrogen conjugates.

**Acknowledgements.** We are indebted to Mrs. Diana Godenhjelm and Mr. Henrik Brunholm for skilful technical assistance.

The expenses of this investigation were defrayed by grants from the Population Council, New York (Nos. M69.7 and M70.10C) and from the Nordisk Insulinfond.

One of us (M.J.T.) is a research fellow of the National Research Council for Medical Sciences in Finland.


Received November 13, 1970.

**Studies on Orchidaceae Alkaloids**

XX.* The Constitution and Relative Configuration of Crepidine, an Alkaloid from *Dendrobium crepidatum* Lindl.

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Five closely related crystalline alkaloids have been isolated from *Dendrobium crepidatum* Lindl.* One of the bases (C14H24NO3), named crepidine (I), crystal-

* No. XIX of this series, see Ref. 1.