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Studies on Steroid Conjugates. XII. Occurrence of 16-Epiestriol 16-Glucuronide in Human Pregnancy Urine[1]

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A new estrogen conjugate, 16-epiestriol 16-glucuronide, was separated from human late-pregnancy urine. The structure was definitely characterized by transforming into the ^{3}H-acetate-methyl ester with use of ^{3}H-acetic anhydride, followed by reverse isotope dilution analysis.

It is well known that a large amount of estrogen is excreted in human pregnancy urine mainly as sulfate and/or glucuronide and in a certain case as N-acetylglucosaminide.[3] Of the estrogen conjugates estriol glucuronide is the most principal one and plays an important role in the feto-placental unit. The complete structure of estriol glucuronide had remained unclear until Hashimoto and Neeman characterized it to be the 16-glucuronide by the degradative means.[4] In the subsequent study the second estriol monoglucuronide was isolated from pregnancy urine with success and was identified as the 3-glucuronide.[5] Besides the classic estrogens the considerable attentions have recently been drawn to 16-epiestriol in respect with the physiological significance. Several workers have already reported the urinary excretion of a small amount of 16-epiestriol glucuronide in pregnant women.[6,7] However, the attached position of the glucuronyl moiety to the steroid nucleus has not yet definitely been determined. As a series of our studies on the steroid conjugates three possible 16-epiestriol monoglucuronides were synthesized by the unequivocal route from the necessity of the authentic specimens.[8] The availability of these synthetic samples prompted us to explore the specificity of hepatic 16-epiestriol uridine diphosphate (UDP)-glucuronyltransferase in man.[9] In this paper we wish to report the separation and characterization of 16-epiestriol 16-glucuronide excreted in human pregnancy urine.

An initial effort was directed to the efficient separation of the 16-epiestriol conjugate from pregnancy urine. It has been demonstrated that Amberlite XAD-2 resin, a neutral cross-linked styrene polymer, adsorbs the steroid conjugates quantitatively from urine, which

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1) This paper constitutes Part LXXII of the series entitled "Analytical Chemical Studies on Steroids"; Part LXXI: T. Nambara, M. Takahashi, and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), 22, 1167 (1974). In this paper the following trivial names were used: 16-epiestriol, estra-1,3,5(10)-triene-3,16β,17β-triol; estriol, estra-1,3,5(10)-triene-3,16α,17β-triol.

2) Location: *Aobayama, Sendai.*

3) H. Jirku and M. Levitz, *J. Clin. Endocrinol. Metab.*, **29**, 615 (1969).

4) M. Neeman and Y. Hashimoto, *J. Am. Chem. Soc.*, **84**, 2972 (1962); Y. Hashimoto and M. Neeman, *J. Biol. Chem.*, **238**, 1273 (1963).

5) S. Ladany, *Steroids*, **12**, 717 (1968).

6) E.R. Smith and A.E. Kellie, *Biochem. J.*, **104**, 83 (1967); J. Ahmed and A.E. Kellie, *J. Steroid Biochem.*, **3**, 31 (1972).

7) R. Hähnel, *Clin. Chim. Acta*, **7**, 768 (1962); *idem*, *Anal. Biochem.*, **10**, 184 (1965); *idem*, *Biochem. J.*, **105**, 1047 (1967); M. Ghazali bin Abdul Rahman and R. Hähnel, *Clin. Chim. Acta*, **17**, 59 (1967).

8) *a*) T. Nambara, Y. Matsuki, and T. Chiba, *Chem. Pharm. Bull.* (Tokyo), **17**, 1636 (1969); *b*) T. Nambara, Y. Matsuki, and Y. Kawarada, *ibid.*, **19**, 844 (1971).

9) T. Nambara, Y. Matsuki, and M. Kurata, *Chem. Pharm. Bull.* (Tokyo), **20**, 2607 (1972).

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can be readily eluted from the resin with an organic solvent.[10] The late-pregnancy urine collected from two women was percolated through a column packed with the resin. After thorough washing with distilled water, the conjugate fraction was obtained by elution with methanol. The eluate was then extracted with butanol at pH 4 and the extract in turn was submitted to gel filtration on Sephadex G-25. The nature and amount of estrogen conjugate in the effluent were determined by carrying out enzymatic hydrolysis and Kober reaction on a small portion of each fraction. A model experiment with use of the synthetic samples showed that the 16-glucuronide of 16-epiestriol was eluted more easily than that of estriol. As illustrated in Fig. 1, the 16-epiestriol glucuronide could be separated from the much more abundant estriol glucuronide. Further purification by gel filtration on Sephadex G-25 followed by thin-layer chromatography (TLC) on silica gel provided the desired 16-epiestriol monoglucuronide as a single compound.

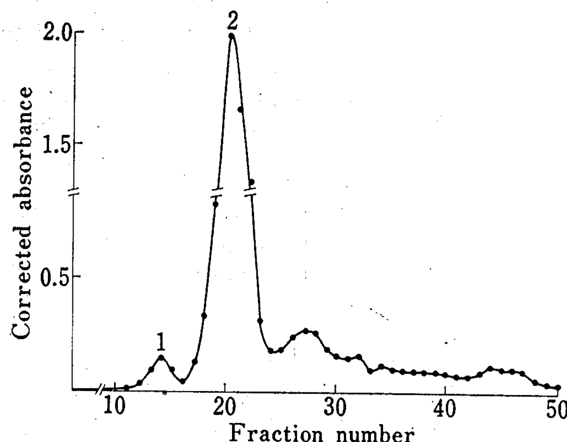

Fig. 1. Separation of 16-Epiestriol Glucuronide from Late-Pregnancy Urine by Gel Filtration on Sephadex G-25

1: 16-epiestriol glucuronide, 2: estriol glucuronide

The thin-layer and paper chromatographic separation of three possible 16-epiestriol monoglucuronides has previously been established employing the synthetic specimens.[9] The 3-glucuronide is most polar of the three and easily distinguishable from the others. The chromatographic behaviors of the 16- and 17-glucuronides are quite similar, but the former is slightly less polar than the latter. Being developed for a prolonged time, these two can be differentiated with each other. According to these criteria the urinary conjugate seemed very likely to be the 16-glucuronide. In order to establish the complete structure the glucuronide was converted into the ^{3}H-acetate-methyl ester by treatment with diazomethane and then with ^{3}H-acetic anhydride-pyridine as shown in Chart 1. The homogeneity of this derivative was confirmed by the reverse dilution method employing the authentic methyl (3,17β-diacetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate as a carrier (see Table I).

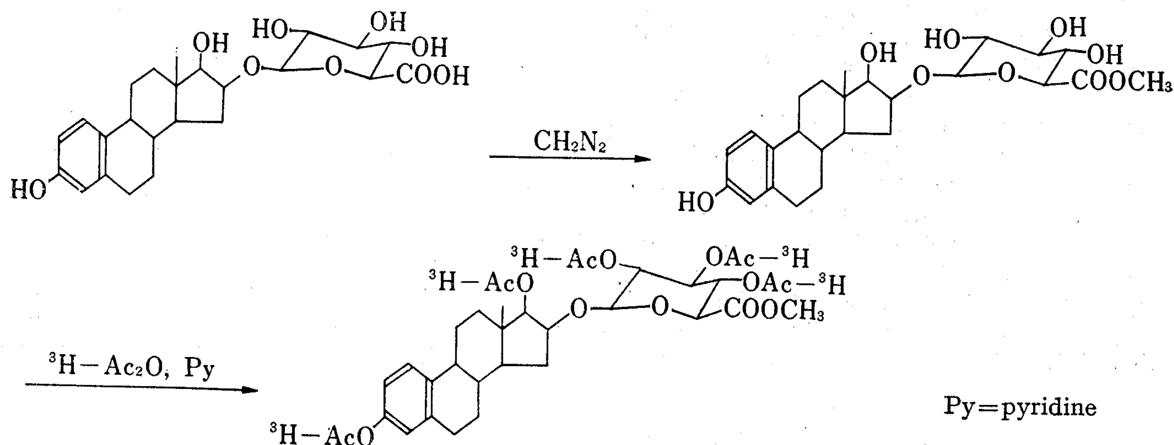

Chart 1

10) H.L. Bradlow, *Steroids*, **11**, 265 (1968); T. Nambara, Y.H. Bae, T. Anjyo, and S. Goya, *J. Chromatog.*, **62**, 369 (1971).

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Table I. Determination of Purity of 16-Epiestriol 16-Glucuronide ^{3}H-Acetate-Methyl Ester by Reverse Isotope Dilution

No.	Crystallization from	Weight (μg)	Radioactivity (dpm)	Specific activity (dpm/mg)
1	EtOH	886	183×10^3	206×10^3
2	Acetone–hexane	695	128×10^3	184×10^3
3	Acetone–hexane	1083	208×10^3	193×10^3
4	Acetone–hexane	368	70×10^3	191×10^3

These evidences lent a support to assign the structure 16-epiestriol 16-monoglucuronide to the urinary conjugate. Kellie, *et al.* reported the excretion of this conjugate in human late-pregnancy urine, although the definite evidence was unavailable.[11] On the other hand, Diczfalusy and his co-worker recently demonstrated that the quantitatively most important urinary conjugate of 16-epiestriol was the 17-glucuronide followed by the 16-glucuronide, when labeled 16-epiestriol was administered to pregnant women.[12] In our case, however, no evidence for the occurrence of any other conjugates than the 16-glucuronide has so far been obtained.

It is to be noted that *in vivo* glucuronidation of 16-epiestriol occurs exclusively at C-16. This finding is fairly consistent with the result of *in vitro* experiment with the human liver microsomes.[9] It is also of interest that glucuronic acid conjugates preferentially with the C-16 hydroxyl group in 16-epiestriol as well as in estriol. Recently Breuer, *et al.* clarified that the soluble UDP-glucuronyltransferase which catalyzes the formation of estriol 17-glucuronide is localized in the ground plasma ($150000\times \boldsymbol{g}$ supernatant) of human intestine.[13] Although any evidence for the urinary excretion of 16-epiestriol 3- and 17-glucuronides has so far been obtained, it is necessary to examine whether or not the intestinal UDP-glucuronyltransferase is capable of catalyzing the transfer of glucuronic acid to the remaining positions in 16-epiestriol.

Experimental

Syntheses of 16-Epiestriol Glucuronide Acetate-Methyl Esters[14]

Methyl (3,17β-Diacetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate——Treatment of methyl (3-hydroxy-17β-acetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate[8a] (25 mg) with Ac_2O (0.1 ml) and pyridine (0.2 ml) in the usual manner followed by recrystallization from EtOH gave methyl (3,17β-diacetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (20 mg) as colorless needles. mp 204—205°. $[\alpha]_D^{27}$ −26.3° (c=0.11, $CHCl_3$). *Anal.* Calcd. for $C_{35}H_{44}O_{14}$: C, 61.03; H, 6.44. Found: C, 61.17; H, 6.65.

Methyl (3,16β-Diacetoxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate——Treatment of methyl (3-hydroxy-16β-acetoxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate[8a] with Ac_2O and pyridine in the manner as described above followed by recrystallization from EtOH gave methyl (3,16β-diacetoxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate as colorless needles. mp 253—255°. $[\alpha]_D^{20}$ +6.9° (c=0.15, $CHCl_3$). *Anal.* Calcd. for $C_{35}H_{44}O_{14}\cdot 1/2H_2O$: C, 60.25; H, 6.50. Found: C, 60.10; H, 6.32.

Separation of 16-Epiestriol 16-Glucuronide from Human Late-Pregnancy Urine——The late-pregnancy (35—38 weeks) urine (37 liters) collected from two volunteers was divided into five portions. Each portion of urine was layered gently onto a column packed with Amberlite XAD-2 resin (Roam & Haas Co., Philadelphia) (1000 ml) and allowed to percolate through the column at a rate of 1000 ml/hr, and the effluent was discarded. When all the urine sample had entered the column, distilled water (3 liters) was added for

11) E.R. Smith and A.E. Kellie, *Biochem. J.*, **104**, 83 (1967); J. Ahmed and A.E. Kellie, *J. Steroid Biochem.*, **3**, 31 (1972).
12) B. de la Torre and E. Diczfalusy, *Acta Endocrinol., Suppl.* **155**, 124 (1971).
13) K. Dahm and H. Breuer, *Biochim. Biophys. Acta*, **113**, 404 (1966); *idem, ibid.*, **128**, 306 (1966); G.S. Rao, M.L. Rao, and H. Breuer, *Biochem. J.*, **118**, 625 (1970).
14) All melting points were taken on a micro hot-stage apparatus and are uncorrected.

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removal of the polar substances. The effluent with 90% MeOH (3 liters) was combined and concentrated *in vacuo* below 45° to bring the whole volume to 2 liters. Being adjusted to pH 4 with 5% HCl, the resulting solution was extracted with *n*-BuOH (5 liters). The BuOH extract was concentrated *in vacuo* below 45° to give an oily residue, which in turn was redissolved in distilled water (30 ml) and divided into two portions. One of these was submitted to gel filtration on Sephadex G-25 (50 g) and each 10 ml was fractionally collected. Employing a 0.5 ml aliquot of each fraction the content of estrogen was colorimetrically determined by the Ittrich's method.[15] Another 0.5 ml aliquot was submitted to hydrolysis with beef-liver β-glucuronidase (Tokyo Zōkikagaku Co., Tokyo). Eluate obtained from fr. 31—53 was combined and re-chromatographed on Sephadex G-25 (20 g). The eluate of each fraction was similarly checked by the Ittrich's method and enzymatic hydrolysis. The presence of 16-epiestriol glucuronide was detected in fr. 11—16 as illustrated in Fig. 1. The eluate thus obtained was purified by preparative TLC on silica gel H (E. Merck AG, Darmstadt) using $CHCl_3$/iso-PrOH/HCOOH (15:5:4) as developing solvent. Staining with both Folin-Ciocalteu reagent and conc. H_2SO_4 showed a single spot on the chromatogram. The unstained part of this area was collected and eluted with hot *n*-BuOH. Evaporation of the solvent *in vacuo* gave an oily residue, which was divided into several portions and used for characterization, *i.e.* enzymatic hydrolysis, TLC, and reverse isotope dilution analysis. The t_R value (12.0 cm/14 hr) of the isolated 16-epiestriol 16-glucuronide was identical with that of the authentic material, when subjected to TLC under the same conditions as described above.

Hydrolysis of 16-Epiestriol 16-Glucuronide with β-Glucuronidase——A portion of the isolated 16-epiestriol 16-glucuronide was dissolved in 0.1M acetate buffer (2 ml) and incubated with beef-liver β-glucuronidase (13000 Fishman Unit/ml, 0.5 ml) at 37° for 48 hr. The incubation fluid was saturated with NaCl and extracted with AcOEt (20 ml × 3). The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue thus obtained was submitted to TLC on silica gel H employing benzene–ether (1:2) and hexane–AcOEt (1:1) as solvent and then stained with Folin-Ciocalteu reagent and conc. H_2SO_4 whereby the hydrolyzate exhibited a single spot with *Rf* values of 0.33 and 0.23, which proved to be identical with those of the authentic 16-epiestriol, respectively. No further estrogens were liberated by heating the enzymatic hydrolyzate with acid.

Transformation of 16-Epiestriol 16-Glucuronide into ^{3}H-Acetate-Methyl Ester——To a portion of the 16-epiestriol 16-glucuronide was added an ethereal solution of CH_2N_2 and allowed to stand at room temperature for 1 hr. After addition of a drop of AcOH the resulting solution was evaporated with an aid of N_2 gas stream. To a solution of this residue dissolved in pyridine (0.3 ml) was added ^{3}H-Ac_2O (25 mCi/0.25 ml, 0.13 ml) (Daiichi Chemicals Co., Tokyo), heated at 70° for 5 hr, and allowed to stand at room temperature overnight. After usual work-up the crude product obtained was subjected to preparative TLC on silica gel H. On multiple runs using hexane–AcOEt (2:1) as solvent the authentic sample was located by spraying the edges of the plate with Folin-Ciocalteu reagent and conc. H_2SO_4. The unstained part of this area (^2Rf 0.31) was eluted with AcOEt to give the radioactive acetate-methyl ester. To this eluate was added methyl (3,17β-diacetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (20.5 mg) as a carrier and crystallized repeatedly up to constant specific activity as listed in Table I.

Counting of Radioactivity——Samples containing ^{3}H were counted in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer. Toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (200 mg/liter) was used as a scintillant.

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15) G. Ittrich, *Z. Physiol. Chem.*, 312, 1 (1958).

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