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A METHOD FOR THE QUANTITATIVE DETERMINATION OF URINARY ESTRIOL CONJUGATES IN HUMAN PREGNANCY

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SUMMARY

A new gas–liquid chromatographic method for the quantitative determination of estriol-3-glucuronide, estriol-3-sulfate,16-glucuronide, estriol-16-glucuronide and estriol-3-sulfate in human pregnancy urine is described. The accuracy of the method was improved by the use of both labeled and nonlabeled internal standards in every determination. Data on the accuracy, specificity, precision and sensitivity of the new method indicate that it fulfills the reliability criteria and is suitable for quantitative analysis of urinary estriol conjugates in human pregnancy.

INTRODUCTION

IT HAS BEEN suggested that in liver disease involving cholestasis most of the changes in estrogen metabolism can be related to impairment of their biliary excretion with resultant impairment of the enterohepatic circulation of these steroids[1]. Estriol, in conjugated form, is the main estrogen excreted in the bile, and the metabolism of estriol is mostly confined to interconversions of its conjugates[2–4]. Hence any changes in estrogen metabolism due to impairment of the enterohepatic circulation of estrogens will be especially well seen in the metabolism of estriol and its conjugates. We assumed that changes in the enterohepatic circulation of estriol are reflected in the rates of urinary excretion of the various conjugates of this steroid. Four estriol conjugates are known to be present in normal human pregnancy urine: estriol-3-glucuronide†, estriol-3-sulfate, 16-glucuronide, estriol-16-glucuronide and estriol-3-sulfate. Our aim was to devise a specific method for the determination of these four conjugates that would be sufficiently simple to allow adequate numbers of analyses of urine samples for comparisons between normal subjects and patients with liver disease.

EXPERIMENTAL

Storage of samples

The 24-hr urine samples were frozen immediately (− 18°C) unless the analysis was started the same day. For longer periods of storage the samples were mixed with methanol (70 parts methanol to 30 parts urine) and stored at − 18°C.

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†The following trivial names and abbreviations have been used in this text. Estriol-3-glucuronide (E_3-3Gl) = 16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronic acid; estriol-3-sulfate,16-glucuronide (E_3-3S,16Gl) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronic acid; estriol-16-glucuronide (E_3-16Gl) = 3,17β-dihydroxyestra-1,3,5(10)-trien-16α-yl-β-D-glucopyranosiduronic acid; estriol-3-sulfate (E_3-3S) = 16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-sulfate; estriol-17-glucuronide (E_3-17Gl) = 3,16α-dihydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronic acid; estriol-3,17-disulfate (E_3-3,17-diS) = 16α-hydroxyestra-1,3,5(10)-trien-3,17β-yl-disulfate.

Solvents and reagents

All reagents were of analytic grade. Distillations were made through all-glass fractionating columns. The following solvents were redistilled: ethyl acetate, benzene, n-hexane (Merck AG, Darmstadt, Germany); pyridine (Merck) was distilled twice and stored over potassium hydroxide pellets. The following solvents were used without further distillation: dioxan, methanol, chloroform, n-butanol, sec-butanol, tert-butanol (Merck), absolute ethanol (Oy Alkoholiliike Ab, Helsinki, Finland), ethyl ether, peroxide-free (Orion-Yhtymä, Helsinki, Finland, light petroleum ether (J. T. Baker, N.V., Holland). Other reagents used: hexamethyldisilazane, trimethylchlorosilane, dimethyl sulfate, uric acid (Fluka AG, Buchs, Switzerland), uridine 5'-diphosphoglucuronic acid, D-saccharic acid-1,4-lactone (Sigma Chemical Company, St. Louis, Mo., U.S.A.), adenosine 5'-triphosphate, crystalline sodium salt (C. F. Boehringer & Soehne GmbH, Mannheim, Germany), sodium chloride, sodium acetate, acetic acid, sodium hydroxide, sodium bicarbonate, sulfuric acid, boric acid, hydrochloric acid, ammonium hydroxide (Merch), Sephadex G-25 medium, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), alumina, neutral, grade I (Merck), extract of *Helix pomatia* (Suc d'Helix pomatia, Industrie Biologique Francaise, Genevilliers, France), Ketodase (Warner-Chilcott, Morris Plains, N.J., U.S.A.), Mylase P (Wallerstein Laboratories, New York, N.Y., U.S.A.).

Reference steroids

Estrone, estriol (Mann Research Laboratories, New York, N.Y., U.S.A.), estriol-3-methyl ether (Sigma), estriol-3-sulfate, estriol-3,17-disulfate (Leo Ab, Hälsingborg, Sweden), estriol-16-glucuronide (Steroid Reference Collection, London, England), (6,7-^{3}H)estriol, S.A. 14·7 Ci/mmol (New England Nuclear Corp., Boston, Mass., U.S.A.).

Measurement of radioactivity

Radioactivity was measured in a Wallac DECEM-NTL^{314} automatic liquid scintillation counter (Wallac Oy, Turku, Finland). Bray's liquid scintillator[5] was used. If necessary, the counting efficiencies for various samples were determined by the internal standard method.

Use of Sephadex gels

Gel filtration on Sephadex G-25.* Preparation of the G-25 gel, packing and testing of the columns (1 × 100 cm) and gel filtration were carried out as described by Beling[6]. Fractions (1–2 ml) were collected with an automatic fraction collector (Gilson Medical Electronics, Villiers-le-Bel, France). The solvent flow was maintained at the desired rate (1 ml/5 min in routine procedures) by connecting the column outlet with an LKB 4912 peristaltic pump (LKB Produkter AB, Stockholm-Bromma, Sweden).

Gel chromatography on Sephadex G-25. Two solvent systems were used: n-butanol–tert. butanol–water–conc. ammonia (30:10:34:6, by vol.) (system A), ethyl acetate–n-butanol–0·2 mol/l ammonium hydroxide (1:1:2, by vol.) (system B). The columns were prepared in the aqueous phase and eluted with the organic

*The terms *gel filtration* and *Peak I* and *Peak II* (indicating the two fractions of urinary estrogen conjugates obtained by this procedure) are used in this paper in the sense used by Beling[6].

phase[6]. In system B the elution of E_3-3S,16Gl and E_3-3Gl can be facilitated by changing the eluent to butanol.

For *chromatography on Sephadex LH-20* 4 g and 16 g columns (1 × 30 cm and 1·5 × 50 cm, respectively) were used. The columns were prepared in chloroform–methanol (1 : 1) containing 0·01 mol/l sodium chloride, according to the method of Vihko[7]. Elution was carried out with different chloroform–methanol mixtures containing sodium chloride[8]. When diconjugates were chromatographed their elution was facilitated by changing the eluent to methanol. The elution patterns of labeled conjugates were studied by transferring small portions of each fraction to vials for counting in a liquid scintillation counter.

Gas–liquid chromatography (GLC)

The gas chromatographs were F & M models 400 and 402 (F & M Scientific Corporation, Avondale, Penn., U.S.A.) with hydrogen flame ionization detectors and glass U columns (2–4 m by 2·5–3·0 mm i.d.). As stationary phases for GLC 2·5% QF-1, 1% SE-30 and 3% XE-60 on 80/100 mesh Gas Chrom Q (Applied Science Laboratories, Inc. P.O. Box 440, State College, Penn. 16801) were used. The columns were packed as described by others[9]. The 2·5% QF-1 coated support was prepared as described by Haahti[10]; the other supports were commercially available. The hydrogen flame was supported by air or oxygen and nitrogen was used as carrier gas. The column temperatures were: SE-30: 235°C, QF-1: 235°C and XE-60: 210°C. The flash heater and detector temperatures were kept at 280°C. The samples were injected into the columns with Hamilton micro-syringes.

Other methods

Acid hydrolysis was performed according to the method of Brown[11] and *solvolysis* as described by Vihko[7]. *Hydrolysis in situ* of estriol sulfate on paper chromatograms was carried out as described by Schneider and Lewbart[12]. *Enzymatic hydrolysis* of estriol conjugates with extract of *Helix pomatia* was carried out in 0·15 mol/l acetate buffer, pH 4·1, using an enzyme concentration of 1000 units/ml (16–18 h at 37°C). Estriol glucuronides were hydrolyzed with 1000 units/ml of *Ketodase* (hereafter called β-glucuronidase) by incubating in 0·1 mol/l acetate buffer, pH 5·0, at 37°C for 16 h[13]. Aryl sulfates were hydrolyzed with 2–4 mg/ml of *Mylase P* (hereafter called arylsulfatase) in 0·1 mol/l acetate buffer, pH 6·0, at 37°C for 16 h[13]. *Methylation* was carried out as described by Brown [11] but with only one-third of each reagent. If estriol conjugates were methylated, the subsequent extraction was carried out with n-butanol from a slightly acid (pH 4–5) solution[14]. *Chromatography* of the methylated estriol fraction was carried out on partially deactivated alumina (for details of deactivation, preparation and standardization of columns see Ref. 15) according to the method of Brown[11]. *Colorimetric* determination of estrogens was carried out with the Kober[16] reaction according to the method of Nocke[17]. The Allen[18] correction of the absorbancies measured after the Kober reaction was applied as described by Brown[11]. For *paper chromatography* Bush-type systems were used; the samples were applied to dry Whatman No. 2 filter paper strips, which were then equilibrated for 16 h in chromatography tanks before the run with the mobile phase was started. Two solvent systems were used: isopropyl ether–tert. butanol–conc. ammonia–water (75 : 125 : 20 : 80) (system C by vol.) and sec.

butanol–water (1 : 1) (system D). Estrogen was detected on paper with the ferricyanide–ferric chloride reagent[19]. Radioactivity on paper chromatograms was determined by the immersion method[cf. 20].

Preparation of labeled estriol conjugates

In the purification procedures separation of estriol conjugates on Sephadex G-25 was achieved by dissolving them in 10 ml of 0·1 mol/l phosphate buffer. pH 6·5, containing 8 mg uric acid[8, 21]. For chromatography on Sephadex LH-20 16 g columns were used and elution was performed with chloroform–methanol (1 : 1), containing 0·01 mol/l sodium chloride. The biosynthetically prepared estriol conjugates were stored in methanol at − 18°C.

Tritiated *estriol-16-glucuronide* was prepared from (6,7-^{3}H)estriol as described by Slaunwhite *et al.*[22]. The E_3-16Gl-like material was purified by passing it through Sephadex G-25 and Sephadex LH-20 columns several times. Treatment of the purified material with β-glucuronidase rendered it 96 per cent ether-extractable, but this hydrolysis could be completely inhibited with 1 mg/ml of saccharic acid lactone. Solvolysis did not release any radioactivity into the ether phase. One portion of the purified material was methylated and the methylated product extracted with butanol from a slightly acid (pH 4–5) solution and submitted to acid hydrolysis. The mixture was made strongly alkaline by addition of 5 mol/l sodium hydroxide and extracted once with benzene. Chromatography on alumina led to recovery of 85 per cent of the original radioactivity in the second (methylated estriol) fraction; 15 per cent of the estriol is known to be lost during these procedures[14]. Authentic nonradioactive E_3-16Gl was mixed with the purified labeled material and chromatographed on paper in system C and on Sephadex LH-20. After the E_3 zone had been demonstrated with the ferricyanide–ferric chloride reagent the paper strip was assayed for radioactivity. This indicated identical mobility (R_f 0·51) of the labeled material and authentic E_3-16Gl. After chromatography on Sephadex LH-20 the fractions were assayed for radioactivity and Kober chromogens. The elution curves obtained coincided. The tritium-labeled E_3-16Gl was converted to *estriol-3-sulfate,16-glucuronide* as described by Levitz *et al.*[13]. The E_3-3S,16Gl-like material was submitted to successive gel filtrations on Sephadex G-25 and chromatographies on Sephadex LH-20. The labeled material was strongly retarded in the Sephadex LH-20 column, but emerged after the eluent had been changed to methanol. When partitioned on Sephadex G-25 in systems A and B and on paper in system D the labeled material gave single symmetrical peaks of radioactivity. Portions of the purified material were treated with (a) β-glucuronidase, (b) arylsulfatase (2 mg/ml) and (c) extract of *Helix pomatia*. After hydrolysis the labeled materials from incubations (a) and (b) were gel-filtered on Sephadex G-25 and the respective peak II fractions were characterized further as described for E_3-3S (see below) and for E_3-16Gl. Treatment with the *Helix pomatia* extract rendered 99 per cent of the radioactivity ether-extractable. Characterization of the partially hydrolyzed conjugates from incubations (a) and (b) showed that the material obtained by β-glucuronidase hydrolysis was identical with E_3-3S and the material obtained by arylsulfatase hydrolysis was identical with E_3-16Gl. Tritium-labeled *estriol-3-sulfate* was prepared by treating E_3-3S,16Gl with β-glucuronidase. After hydrolysis the labeled material was gel-filtered on Sephadex G-25 and more than 90 per cent of the radioactivity was recovered in the peak II fraction. This material was

further purified by chromatography on Sephadex LH-20. A mixture of the labeled E_3-3S-like material and authentic nonradioactive E_3-3S was gel-filtered on Sephadex G-25 and chromatographed on Sephadex LH-20. Suitable fractions were collected and assayed for radioactivity and Kober chromogens. No dissociation of radioactivity from the Kober color was shown by the elution curves in either system. A mixture of the carrier and labeled material was chromatographed on paper in system C. After hydrolysis *in situ* the chromatogram was treated with the ferricyanide–ferric chloride reagent. The color zone coincided with the radioactivity (R_f 0·71). Treatment with arylsulfatase (4 mg/ml) rendered 96 per cent of the radioactivity ether-extractable and solvolysis rendered 97 per cent of the activity ether-extractable. When partitioned in a solvent system devised to separate E_3-3S from other estriol conjugates[14, p. 130], 98 per cent of the activity was found in the sulfate fraction. Tritium-labeled *estriol-3-glucuronide* was prepared by the method of Goebelsmann *et al.*[23]. The labeled material was purified by chromatography on Sephadex LH-20 and during this process behaved in exactly the same way as E_3-16Gl. The E_3-3Gl-like material was further gel-filtered on Sephadex G-25. One portion of the labeled material was submitted to methylation followed by acid hydrolysis and chromatography on alumina, as described for E_3-16Gl. No radioactivity was recovered in the methylated estriol fraction after alumina chromatography, indicating that methylation had not taken place at position C-3. Single zones of radioactivity were obtained when portions were chromatographed on paper in system C (R_f 0·27) and partitioned on Sephadex G-25 in systems A and B. Treatment with β-glucuronidase rendered 99·5 per cent of the E_3-3Gl-like material ether-extractable. In the presence of 1 mg/ml of saccharic acid lactone only 15·5 per cent of the material was liberated by β-glucuronidase; in the presence of 5 mg/ml of this inhibitor 4 per cent became ether-extractable. The fact that saccharic acid lactone is a more potent inhibitor of E_3-16Gl hydrolysis than of E_3-3Gl hydrolysis has been reported by others[2]. Solvolysis did not render any radioactivity ether-extractable.

THE METHOD (FIG. 1)

Separation of urinary estriol conjugates

The development of the separation procedure for the four known estriol conjugates has been described previously[8]. It involves gel filtration on Sephadex G-25 followed by chromatography on Sephadex LH-20. *Gel filtration* is performed as follows: Suitable amounts (representing 15,000 c.p.m.) of the four tritiated estriol conjugates are added to a 10 ml urine specimen, which is gel-filtered on a 1 × 100 cm Sephadex G-25 column according to the method of Beling[6]. Alternatively, if the urine has been stored in 70% methanol, the following procedure is used: After addition of the labeled standards the urine-methanol mixture is centrifuged and the supernatant evaporated to dryness. The dry residue is dissolved in urate-phosphate buffer (see *Preparation of labeled estriol conjugates*) and gel-filtered as usual. Urines with unusually high or low pH values can be processed similarly[24]. Peaks I and II are collected separately and evaporated to dryness under reduced pressure. *Chromatography on Sephadex LH-20* is carried out as follows: 4-g Sephadex LH-20 columns are prepared in chloroform–methanol 1 : 1, containing 0·01 mol/l sodium chloride. For elution two solvents are prepared: *Solvent I* consists of chloroform–methanol (2 : 3 v/v), containing 0·01 mol/l sodium chloride and *solvent II* consists of chloroform–

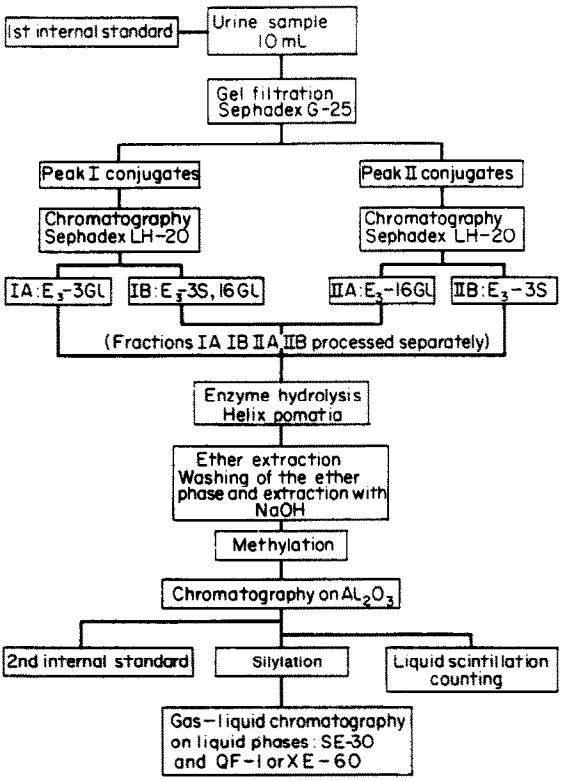

Fig. 1. Outline of the GLC method.

methanol (2:1 v/v), saturated with sodium chloride. The dry residues of peaks I and II are taken up in 3 ml of solvents I and II, respectively, applied on the Sephadex LH-20 columns, and eluted with the same solvents. *Peak I:* E_3-3Gl emerges in the effluent between 20 and 40 ml (fraction IA); after elution of the column with 70 ml of solvent I elution is continued with methanol which results in elution of E_3-3S,16Gl in the effluent between 80 and 105 ml (fraction IB). *Peak II:* E_3-16Gl emerges in the effluent between 40 and 80 ml (fraction IIA) and E_3-3S between 95 and 135 ml (fraction IIB). These elution volumes should be checked with radioactive standards for new batches of Sephadex LH-20.

Hydrolysis of estrogen conjugates

After evaporation, each fraction (IA, IB, IIA and IIB) is dissolved in 0·15 mol/l acetate buffer, pH 4·1, and incubated with extract of *Helix pomatia* containing to 1000 units/ml of β-glucuronidase for 16–18 h at 37°C. The incubation mixture is extracted three times with equal volumes of ethyl ether. The combined ether extract is washed successively with 1/5 Vol. of Brown's carbonate buffer, pH 10·5, 1/20 Vol. of 8% sodium bicarbonate (w/v), and 1/40 Vol. of distilled water, and the volume of the ether phase is reduced to 4 ml under a stream of nitrogen. The ether phase is extracted twice with 3 ml of 1 mol/l sodium hydroxide and the organic phase is discarded.

Methylation

The combined alkaline fraction is diluted with water to a volume of 15 ml and methylation is carried out according to the method of Brown[11]. Boric acid, 0·3 g, is added to the alkaline fraction, which is prewarmed for 5 min at 37°C. Then 0·3 ml of dimethyl sulfate is added, and the mixture is shaken until homogeneous and incubated at 37°C for 30 min. After addition of 0·7 ml of 5 mol/l sodium hydroxide and 0·3 ml of dimethyl sulfate and shaking, the mixture is incubated for 30 min at 37°C. The methylation procedure is carried out in test tubes.

Chromatography on alumina

The methylation mixture is made strongly alkaline by adding 3·3 ml of 5 mol/l sodium hydroxide. The methylated estriol fraction is then extracted with 8 ml of water–saturated benzene, which is washed with 1 ml of water. The benzene phase is applied to the alumina column and chromatography is performed as described by Brown[11]; the columns are eluted successively with the following eluents:

I 12 ml of 1·4% ethanol in benzene (v/v)
II 16 ml of 6% ethanol in benzene (v/v)
III 10 ml of 10% ethanol in benzene (v/v).

All solvents applied on the columns are water-saturated. The methylated estriol is eluted in fraction II. A suitable amount (e.g. 10–25 μg) of estrone internal standard is added to the fraction, and 1/10 Vol. of the fraction is removed for liquid scintillation counting.

Silylation

Silylation is performed according to the method of Luukkainen *et al.*[25], but in dry pyridine, as follows: After evaporation of fraction II, 1 ml of freshly distilled pyridine is added to the dry residue, 100 μl of hexamethyldisilazane and 10 μl of trimethylchlorsilane are then added, and the silylation mixture is kept overnight at room temperature. Simultaneously, two reference standards are prepared by silylating two mixtures each containing a weighed amount (e.g. 20 μg) of estriol methyl ether and an amount of estrone equal to that added to the samples. The silylation mixtures are then carefully evaporated under nitrogen and the silylated materials are transferred with several portions of dry n-hexane to conical glass tubes in which the n-hexane is again evaporated and the residue redissolved in 100 μl of n-hexane.

Gas–liquid chromatography

Gas–liquid chromatography is carried out both on the SE-30 and on the XE-60 or QF-1 liquid phases. In addition to the samples, the two reference standards are injected into the gas chromatograph both before and after injection of the samples.

Calculation of results

The results are calculated in terms of the two internal standards. The calculation is based on a comparison between the peak area (peak height times peak width at half-height) produced by the estriol derivative in the sample and the mean peak area produced by a known amount of the estriol derivative in the reference

standards. The amount of estriol excreted in 24 h is calculated as follows:

$$x\,(\mu g/24\,h) = 0{\cdot}95\,\frac{V}{V_s}\cdot\frac{C}{10\cdot C_x}\cdot\frac{E_s}{I_s}\cdot\left[\frac{\sum\left(\frac{I}{E}\right)_n}{n}\right]\cdot M$$

where 0·95 is the factor that converts values expressed as methylated estriol to estriol, V is the volume of the 24-h urine and V_s the volume of the urine sample. C is the amount of radioactivity added at the start and C_x the amount of radioactivity found in 1/10 Vol. of the second fraction after alumina chromatography. E_s and I_s are the peak areas of the estriol derivative and the estrone internal standard in the sample, and E and I are the corresponding peak areas in a given reference standard chromatogram. M is the mass (usually 20 μg) of methylated estriol in the reference standards. The index n indicates the number of reference standards injected into the gas chromatograph.

RELIABILITY OF THE METHOD

Specificity

Elution curves obtained from gel filtration of urine samples containing labeled estriol conjugates showed that separation of E_3-3S,16Gl from peak II estriol conjugates was good. However, slight "tailing" to subsequent fractions was observed when E_3-3Gl emerged from the column, which necessitated further studies; (^{3}H)E_3-3Gl was added to several urine samples, which were then processed as usual, and fractions IA, IB, IIA and IIB were assayed for radioactivity. The radioactivity was found exclusively in the expected fraction (IA), indicating that in these experiments E_3-3Gl had not "tailed" into peak II. *The homogeneity of the four estriol conjugate fractions* obtained from pregnancy urine was studied by adding a suitable amount of the corresponding labeled conjugate to each fraction for correction of losses during the different procedures and submitting the fractions to analytic procedures as follows:

Fractions IA *and* IIA. Aliquots were either treated with (a) extract of *Helix pomatia*, or (b) β-glucuronidase, or (c) β-glucuronidase in the presence of saccharic acid lactone or (d) were methylated, the methylated fractions extracted with butanol from a slightly acidified (pH 4–5) methylation mixture; the butanol extracts were submitted to acid hydrolysis, and the methylated fraction was then extracted with benzene from an alkaline solution, chromatographed on alumina and processed according to the GLC method. After incubation (a)–(c) were extracted with ether and the extracts processed according to the GLC method. The results (corrected values expressed as μg/sample; ND = not detectable) may be summarized as follows:

		(a)	(b)	(c)	(d)
Fraction IA		13·0	12·9	1·9	ND
Fraction IIA	(1)	81·8	76·4	ND	81·9
	(2)	23·0	23·3	ND	23·8

Saccharic acid lactone (1 mg/ml) completely inhibited β-glucuronidase hydrolysis of the conjugate in IIA, whereas for IA the inhibition was 85 per cent (see results obtained with (^{3}H)E_3-3Gl). The other values corresponded, within the limits of precision of the GLC method. Experiment (d) indicated that a free phenolic

hydroxyl was available for methylation in the conjugate in fraction IIA but not in IA. These results indicate that fraction IA consists of E_3-3Gl and IIA of E_3-16Gl. The view that no significant amounts of E_3-17Gl are present in normal pregnancy urine[26] was adopted here.

Fraction IB. Aliquots were either treated with (a) the *Helix pomatia* extract, or (b) β-glucuronidase, or (c) arylsulfatase or (d) solvolyzed. After incubation, (a) was processed further according to the GLC method, and (b) and (c) were gel-filtered on Sephadex G-25 after impurities had been removed by precipitation in methanol at −18°C overnight. In both filtrations (b and c) the peak II material was collected and incubated, (b) with arylsulfatase and (c) with β-glucuronidase and the hydrolysates were processed according to the GLC method. After solvolysis, the ethyl acetate was evaporated, the residue dissolved in water, and the pH of the solution adjusted to neutral. The aqueous phase was extracted with ether and processed by the GLC method. The corrected values obtained (expressed as μg/sample) were as follows:

(a)	(b)	(c)	(d)
6·27	6·91	6·05	ND

Experiments with authentic E_3-3,17diS showed that this conjugate is eluted in peak I and on Sephadex LH-20 in fraction IB. Solvolysis of this fraction obtained from urine did not release any estriol into the ether phase, indicating the absence of estriol conjugated only with sulfuric acid from this fraction. The results from (a)–(c) are consistent with IB being E_3-3S,16Gl.

Fraction IIB. Aliquots were either treated with (a) the *Helix pomatia* extract or (b) arylsulfatase or (c) β-glucuronidase or (d) partitioned in a solvent system devised for separation of E_3-3S from other estriol conjugates[14, p. 130], or (e) solvolyzed. After incubation (a)–(c) were processed according to the GLC method. After partition (d) the sulfate fraction was hydrolyzed with extract of *Helix pomatia* and the products were processed by the GLC method. After solvolysis (e) the procedure described for the solvolyzed fraction of IB was followed. The corrected values obtained are given below (μg/sample):

(a)	(b)	(c)	(d)	(e)
16·9	17·4	ND	15·7	15·3

The results are in accordance with the view that fraction IIB consists of E_3-3S.

Overall specificity of the method. Apart from the initial fractionation of conjugates, the GLC method is based on extensively studied procedures that have been shown to result in specific estimates of estriol. Apart from chromatography on Sephadex LH-20 and alumina, the present method is basically similar to that of Adlercreutz and Luukkainen[27], the specificity of which has been established by gas chromatography-mass spectrometry[28, 29]. In order to enhance the specificity, two GLC columns (selective and nonselective) are used in every determination. In some cases the identity and purity of the GLC peaks obtained with the present method was confirmed by gas chromatography-mass spectrometry.

Accuracy and precision

A complete internal control has been incorporated into the method by the use of two internal standards. The labeled estriol conjugates (first internal standard) serve as partial internal standards until non-radioactive estrone (second internal

standard) is added. The mean recoveries of radioactivity in 76 determinations (expressed as mean of percentages ± SD) were:

E_3-3Gl	E_3-3S,16Gl	E_3-16Gl	E_3-3S
72·0 ± 13·2%	78·1 ± 10·2%	71·7 ± 10·9%	77·0 ± 9·6%

The precision of the method was calculated according to the method of Snedecor [30]. From 13 duplicate determinations the following values (precision expressed as coefficient of variation) were obtained:

E_3-3Gl	E_3-3S,16Gl	E_3-16Gl	E_3-3S
±9·0%	±7·5%	±7·7%	±9·6%

Sensitivity

Statistical assessment of the sensitivity of the GLC method was omitted, since the method was not intended for analysis of low-level urine samples. The practical sensitivity limit was found to be in the range of 20–25 μg of estriol conjugate (expressed as μg estriol) per 24-h urine sample. The sensitivity may be enhanced without difficulty by starting the analysis with larger urine samples, e.g. 30 ml. The initial separation of conjugates on Sephadex LH-20 results in a marked purification of fractions IB and IIB which are retarded in the columns and the conjugates present in these fractions can be estimated at lower concentrations than glucuronides.

DISCUSSION

To our knowledge there is only one previous report in which the urinary excretion of all four endogenous estriol conjugates was measured. In connection with an investigation designed to study labeled estriol conjugates in pregnancy urine after intravenous infusion of labeled precursors, Goebelsmann and Jaffe [31] estimated the amounts of endogenous estriol in conjugate fractions obtained from countercurrent distributions. Urines from two pregnant women were studied and the estrogen was determined with fluorometry or colorimetry. No data on the reliability of the procedure used were given. There do not seem to be methods sufficiently reliable and practicable to allow serial analyses during the course of pregnancy or comparisons between groups of normal and pathological urines. This is at least partly due to the methodological difficulties encountered in the separation of intact conjugates. We overcame this difficulty by extending the use of Sephadex LH-20, which had been successfully employed in the analysis of conjugated neutral steroids [7], to the separation of urinary estriol conjugates [8].

Another reason for the lack of methods is the minimal interest in the separate determination of individual conjugates, and the belief that only negligible amounts of sulfated conjugates of estriol are excreted in pregnancy urine. We have reported results obtained with the present method in three preliminary communications [32–34]. Our results indicate that in normal late pregnancy approximately 10 per cent of the urinary estriol is present as E_3-3S,16Gl and E_3-3S, and that in intrahepatic cholestasis the relative amount of these conjugates increases markedly [33]. An even more significant change can be seen in the excretion of E_3-3Gl in cholestasis. This conjugate is synthesized exclusively in the mucosal cells of the intestine [35–37] and rapidly eliminated in the urine without undergoing further

metabolism[38]. In liver disease involving cholestasis the biliary excretion of estrogens is impaired[39] and in consequence the synthesis of E_3-3Gl diminishes. Therefore, the amount of this conjugate probably reflects the degree of impairment of the enterohepatic circulation of estrogens, i.e. the degree of impairment of the excretory function of the liver for those steroids. Accordingly, we regard the determination of individual urinary estriol conjugates as a useful tool in the study of liver function and the role of the liver in estrogen metabolism. The method developed was shown to satisfy the reliability criteria and can be used for accurate quantitative assays of these urinary estriol conjugates in pregnancy.

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