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# A METHOD FOR THE DETERMINATION OF URINARY 15α-HYDROXYESTRIOL AND ESTRIOL

## PRELIMINARY RESULTS OF 15α-HYDROXYESTRIOL DETERMINATIONS IN PREGNANCY URINE

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SUMMARY

A quantitative gas chromatographic method for the simultaneous determination of 15α-hydroxyestriol (1,3,5(10)-estratriene-3,15α,16α,17β-tetrol = estetrol) and estriol in pregnancy urine is described. Its specificity was investigated by various chromatographic methods and by comparing the mass-spectra of the steroids determined with those of authentic reference standards. The accuracy and precision of the method were studied. Preliminary results obtained with 85 normal urine samples at various stages of pregnancy are presented.

## INTRODUCTION

THE FIRST reports on a new polar estrogen in urine were presented by Hagen *et al.*[1] in a study of malformed newborns receiving radioactive estradiol-17β and in normal pregnancy urine by Adlercreutz and Luukkainen[2] and Gurpide *et al.*[3]. In 1967 Zucconi *et al.*[4] isolated this polar estrogen and identified it as 1,3,5(10)-estratriene-3,15α,16α,17β-tetrol(estetrol). Hydroxylation of estradiol-17β to 15α-hydroxyestradiol-17β was demonstrated by Breuer *et al*[5] *in vitro* by incubating estradiol-17β with normal human liver slices. The maternal organism seems unable to synthesize estetrol from estrone to any high degree, because after an estrone sulphate load only very small amounts of estetrol could be identified in the urine of (1) a woman with a dead foetus in utero and of (2) another woman after delivery and these amounts did not differ from that found in the urine of (3) a postmenopausal woman[6, 7]. Hence practically all the estetrol found in pregnancy urine must be derived from the foetoplacental unit[3, 4, 8]. Thus, because of the foetal origin of estetrol, the amount of this estrogen excreted in maternal urine may better reflect the well-being of the foetus than the urinary level of estriol, especially at lower estrogen concentrations in pathological pregnancies. The daily variation in estriol excretion may be so great that a slow gradual reduction in excretion is not easy to detect and short-term collections are not usable[9]. Collections of more than 24 hr give better results but are clinically too time-consuming.

A quantitative method for determination of estetrol in late pregnancy urine was published in 1967 by Zucconi[10]. However, this method includes time-consuming paper and thin-layer chromatographic steps and the final quantitation of the estetrol is based on the Folin–Ciocalteau reaction, which is not sufficiently specific for the purpose. A shorter gas liquid chromatographic (GLC) method for the determination of urinary estetrol was presented from this laboratory in 1968 [11]. This method was only semiquantitative and in further studies it was observed

that the thin-layer chromatographic step could be replaced by silica gel column chromatography, thus increasing both the accuracy and the practicability of the method. The following is a report of this improved method, which can also be used for clinical purposes, with some results obtained in normal pregnancies.

## EXPERIMENTAL

### *Reference standards*

Estrone, estradiol and estriol were obtained from Mann Research Laboratories (New York 6, N.Y., U.S.A.). Estetrol (1,3,5(10)-estratriene-3,15$\alpha$,16$\alpha$,17$\beta$-tetrol) reference standards were obtained through generous gifts from Dr. J. Fishman (New York, N.Y., U.S.A.) and Dr. S. Solomon (Montreal, Canada). As internal standard for gas liquid chromatography (GLC), epicoprostanol (3$\alpha$-hydroxy-5$\beta$-cholestane) from Steraloids Inc. (Pawling, N.Y., U.S.A.) was used.

### *Solvents*

All solvents were analytical grade. The following solvents were used without further distillation: methanol (Merck, Darmstadt, Germany), absolute ethanol, AaS (Alko, Helsinki, Finland), cyclohexane (Merck). The following solvents were redistilled once in an all-glass fractionating distillation system: ethyl acetate (Merck), ethyl ether (Orion OY, Helsinki, Finland), benzene (Merck), chloroform (Merck), n-hexane (Merck). Acetone (Merck) was distilled twice. Pyridine (Fluka AG, Buchs, Switzerland) was distilled two or three times (purity controlled by GLC).

### *Reagents*

Sodium chloride, sodium acetate, acetic acid, sodium hydroxide, ferric chloride, potassium ferricyanide, Kieselgel G nach Stahl, alumina (acid), quartz (Merck); hexamethyldisilazane, trimethylchlorsilane and dimethylsulphate (Fluka), Regisil RC-1 (Regis Chemical, U.S.A.), Sephadex G 25 medium (Pharmacia, Uppsala, Sweden), Adsorbosil CAB mesh 140/200 (Applied Science Laboratories, U.S.A.), Suc d'*Helix pomatia* (stabilisé, standardisé) (Industrie Biologique Francaise, Gennevilliers, France).

### *Gel filtration*

The columns are prepared according to Beling[12], the column type used being the same as that used by him, its diameter being 2 cm and its length 30 cm; the length of the gel is 27 cm. The urine sample is eluted with distilled water. The first 80 ml is not collected and the following 15 ml (fraction I) and 45 ml (fraction II) are collected separately[13]. The flow rate is about 1 ml per minute. After gel filtration the columns are washed with large amounts of distilled water and after 10 gel filtrations they are cleaned by running 100 ml of 2·5% ammonia through, followed by distilled water until neutrality before re-use.

### *Hydrolysis of estrogen conjugates*

The gel filtration fractions I and II are hydrolysed separately with *Helix pomatia* extract according to Adlercreutz[13]. In experiments requiring acid hydrolysis, 15 vol.% of conc. HCl[14] is added to the gel-filtered urine sample and the mixture heated under a reflux condenser and kept at boiling point for 60 min.

*Silica gel chromatography*

3·0 g silica gel (Adsorbosil) was washed with 1 × 5 ml methanol, 2 × 5 ml ethyl acetate, and 2 × 5 ml cyclohexane. Washed silica gel suspended in cyclohexane is poured slowly into a glass column with a diameter of 10 mm. The column has a glass sinter on which the silica gel slowly sediments. A thin layer of quartz is placed on the silica gel. The following eluants are used:

(1) 2 × 10ml ethyl acetate : cyclohexane 1 : 9 (v/v)
(2) 2 × 10 ml ethyl acetate : cyclohexane 1 : 1 (v/v)
(3) 2 × 10 ml pyridine : methanol 1 : 40 (v/v).

The dry extract is first dissolved in the first of these solvents and the tube is then rinsed with the other solvents in turn before they are poured on the column.

*Chromatography on partially deactivated alumina*

In a glass tube with a diameter of 10 mm ending in a capillary with a diameter of 0·4 mm and length of 20 mm is placed a carefully washed piece of cotton wool, which is covered with a thin layer of quartz. The acid alumina was deactivated with 10% water. The column is composed of 2 g of deactivated alumina in benzene which is covered with a thin layer of quartz. The specimen is chromatographed, using the following eluants:

(1) 1 × 10 ml benzene : absolute ethanol : water 100 : 2·5 : 1 (v/v)
(2) 1 × 5 ml benzene : absolute ethanol : water 100 : 5 : 1 (v/v)
(3) 2 × 5 ml benzene : absolute ethanol : water 100 : 7·5 : 1 (v/v)
(4) 3 × 5 ml benzene : absolute ethanol : water 100 : 20 : 1 (v/v).

The specimen tube is rinsed with each of these solvent solutions before they are poured on the column.

*Thin layer chromatography (TLC)*

TLC is carried out with silica gel G as described by Stahl[15]. The silica gel plates are washed before use by eluting the impurities with methanol and then reactivated for 2 hr at 105°C. They may be stored in a desiccator. The walls of the glass chambers used for TLC are partially lined with filter paper. The following eluants (Lisboa and Diczfalusy[16]) are used: system A : cyclohexane : ethyl acetate : absolute ethanol 45 : 45 : 10 (v/v) and system D : chloroform : ethanol 90/10 (v/v). Standard estrogens are also chromatographed on these plates. The chromatograms are developed by spraying them with mixture of ferric chloride–potassium ferricyanide[17].

*Gas–liquid chromatography (GLC)*

F & M model 400 and 402 gas chromatographs are used. The preparation of the columns is done according to Vanden Heuvel *et al.*[18]. The following stationary phases are used: 1·7% SE-30 (gift from Dr. H. Fales) on 100/120 mesh Gas Chrom P in a 2 m glass U-tube, the oven temperature being kept at 240°C, and flash heater and detector temperatures both at 280°C; 1·5% QF-1 and 1% XE-60 (Applied Science Laboratories), both on 90/100 mesh Gas Chrom Q (Applied Science Laboratories), in a 2 m glass U-tube, the oven temperature being kept at 212°C, and flash heater and detector temperatures at 280°C. Nitrogen is used as carrier gas in all experiments and the instruments are equipped with hydrogen flame ionization detectors.

*Formation of trimethylsilyl ethers (TMSi)*

These derivatives are made according to Luukkainen *et al.*[19] or by adding 0·1 ml Regisil RC-1 to the sample dissolved in 1 ml of pyridine. After the sample has stood overnight at room temperature the pyridine is evaporated to dryness and the TMSi ethers of estrogens are dissolved in 3 × 1 ml n-hexane and transferred to small tubes for GLC.

*Quantification*

The peak areas are calculated by multiplying the peak height by the peak width at half-height and comparing the values for the estrogen peaks with that of the internal standard (epicoprostanol). The calibration curves made with the liquid phases used are linear in the range from 0·01–1·0 μg estrogen.

*Formation of acetonide of estetrol* is done essentially according to Adlercreutz and Luukkainen[20] as follows: the dry sample is dissolved in 25 ml 1 *N* NaOH and extracted twice with 15 ml of chloroform, the residue is acidified with 3 *N* HCl, saturated with sodium chloride and extracted with 3 × 25 ml of ethyl acetate. The ethyl acetate extract is evaporated to dryness under a nitrogen stream, the residue is dissolved in 10 ml of freshly distilled acetone, and 0·6 ml of trimethylchlorsilane is added. After shaking for 30 min 25 ml of 1 *N* NaOH is added and the acetone is evaporated. The acetone-free residue is extracted with 2 × 15 ml of distilled chloroform. The aqueous residue is acidified with 3 *N* HCl and extracted with 3 × 25 ml of ethyl ether or ethyl acetate. This extract contains the estetrol acetonide (and estriol, if the sample is contaminated with this steroid).

*Methylation* of estrogens is carried out according to Brown[21].

*Mass spectrometry*

The mass spectra are recorded with a LKB 9000 gas chromatograph-mass spectrometer combination instrument (GLC-MS instrument), using a 3% SE-30 column, length 2 m, oven temperature 250°C. The molecular separator is maintained at 260°C and the ion source at 290°C. The ionizing energy is 70 or 16 eV.

## METHOD FOR THE DETERMINATION OF ESTRIOL AND ESTETROL IN PREGNANCY URINE

The method is presented in flow-sheet 1.

If the urine cannot be processed immediately, it is kept in a deepfreeze at −20°C until analysed.

The chromatographic fraction 2 from the silica gel column is usually not collected, but by using this fraction it is possible to carry out an analysis of estrone, estradiol-17β and 11-dehydroestradiol-17α according to Adlercreutz and Luukkainen[22].

When the urinary fractions are chromatographed on partially deactivated alumina, most of the estriol is eluted in fraction 3, but small amounts may be eluted with fraction 4(0–3%) containing all the estetrol. For the final GLC determination of estetrol the whole of the fraction 4 is used, but for the determination of estriol in samples collected during 20–28 weeks of pregnancy a 1/10 aliquot and in samples collected during 29–40 weeks of pregnancy only a 1/20 aliquot of fraction 3 is used for GLC.

*Flow-sheet 1. Method for the determination of estriol and estetrol in pregnancy urine*

50 ml of filtered pregnancy urine is gel-filtered through a 2 × 27 cm Sephadex G 25 medium column. Peak I and peak II of conjugated estrogens are collected separately.

Peak I and peak II are hydrolysed separately with *Helix pomatia* extract, according to Adlercreutz[13].

Hydrolysates of peak I and peak II are combined, saturated with NaCl and extracted with 3 × 1/1 vol. of ethyl acetate. The extracts are combined and evaporated to dryness.

The dry residue is chromatographed on a 1 × 4 cm silica gel column, with the following eluants:

(1) 2 × 10 ml ethyl acetate : cyclohexane 1 : 9 (v/v)
(2) 2 × 10 ml ethyl acetate : cyclohexane 1 : 1 (v/v)
(3) 2 × 10 ml pyridine : methanol 1 : 40 (v/v).

Fraction 2: see text

From fraction 3 1/10 is measured and evaporated to dryness (contains estriol and estetrol). The dry residue is chromatographed on partially deactivated acid alumina (10% water) with the following eluants:

(1) 1 × 10 ml benzene : absolute ethanol : water 100 : 2·5 : 1 (v/v)
(2) 1 × 5 ml benzene : absolute ethanol : water 100 : 5 : 1 (v/v)
(3) 2 × 5 ml benzene : absolute ethanol : water 100 : 7·5 : 1 (v/v)
(4) 3 × 5 ml benzene : absolute ethanol : water 100 : 20 : 1 (v/v).

Fraction 3 (contains estriol) and fraction 4 (contains estetrol) are collected. The epicoprostanol internal standard is added and the solvent is evaporated to dryness under a stream of nitrogen.

Formation of TMSi derivatives of fractions 3 and 4. GLC is carried out on a 1·7% SE-30 and/or 1·5% QF-1.

Owing to the high polarity of estetrol, which renders it rather insoluble in organic solvents, the chromatographic solvents are divided in several portions, with which the dry residue is washed every time before the eluant is poured on the column.

## RESULTS

### *Specificity of the method*

The specificity of the method was first studied by determination of the relative retention time (to cholestane) of the TMSi derivative of the estriol-like material obtained by the method from pregnancy urine on 3 different liquid phases (SE-30, QF-1 and XE-60) and comparing them to those of the corresponding derivative of authentic estriol (Table 1). Simultaneously a quantitative determination of estriol was carried out. It was found that the quantity of the estriol-like compound

Table 1. Relative retention times of the TMSi derivatives of estriol, estetrol and epicoprostanol and of the TMSi derivative of the estetrol acetonide

| | SE-30 | XE-60 | QF-1 |
|---|---|---|---|
| Estetrol-TMSi | 2·3 | 3·4 | 2·7 |
| Estetrol-acetonide-TMSi | 1·0 | 1·5 | 1·2 |
| Estriol-TMSi | 1·3 | 1·7 | 1·4 |
| Epicoprostanol-TMSi | 1·9 | 2·2 | 1·8 |

was independent of the column used, and the retention times were the same as those of the reference compound. The retention behaviour of the TMSi derivative and of the TMSi derivative of the acetonide of the estetrol-like material isolated from urine by this method was studied on the same three liquid phases. The relative retention times of the derivatives of the estetrol-like material and of the corresponding derivatives of authentic estetrol were identical. Two gas chromatograms of the estriol and estetrol fractions obtained from pregnancy urine are presented in Fig. 1. Further evidence of the identity of the compounds was obtained by investigating extracts containing these estrogens, obtained from several low and high-titre urine samples, by combined gas chromatography-mass spectrometry and recording multiple mass-spectra when the compound was emerging from the gas chromatographic column. It was shown that not only the mass-spectrum recorded at the correct retention time, but also the mass-spectra recorded from other parts of the peak emerging from the column were identical with the mass-spectra of the reference standard. Two mass-spectra of the derivatives of estetrol are shown in Figs. 2. and 3. The mass-spectrum of the TMSi

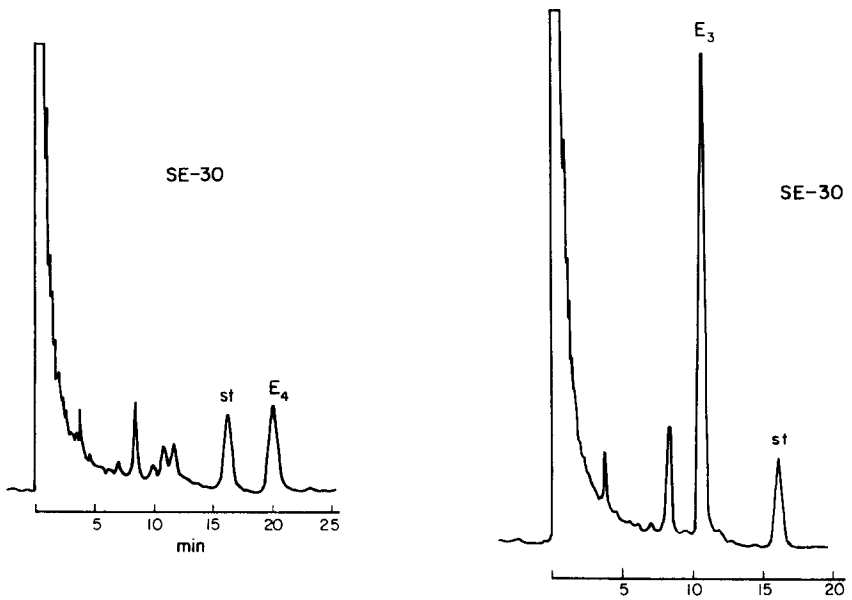


Fig. 1. Gas chromatograms of estriol and estetrol fractions obtained from late pregnancy urine, in which the concentration of estriol is 25·2 μg/ml urine and of estetrol 2·24 μg/ml urine. The stationary phase is 1·7% SE-30.

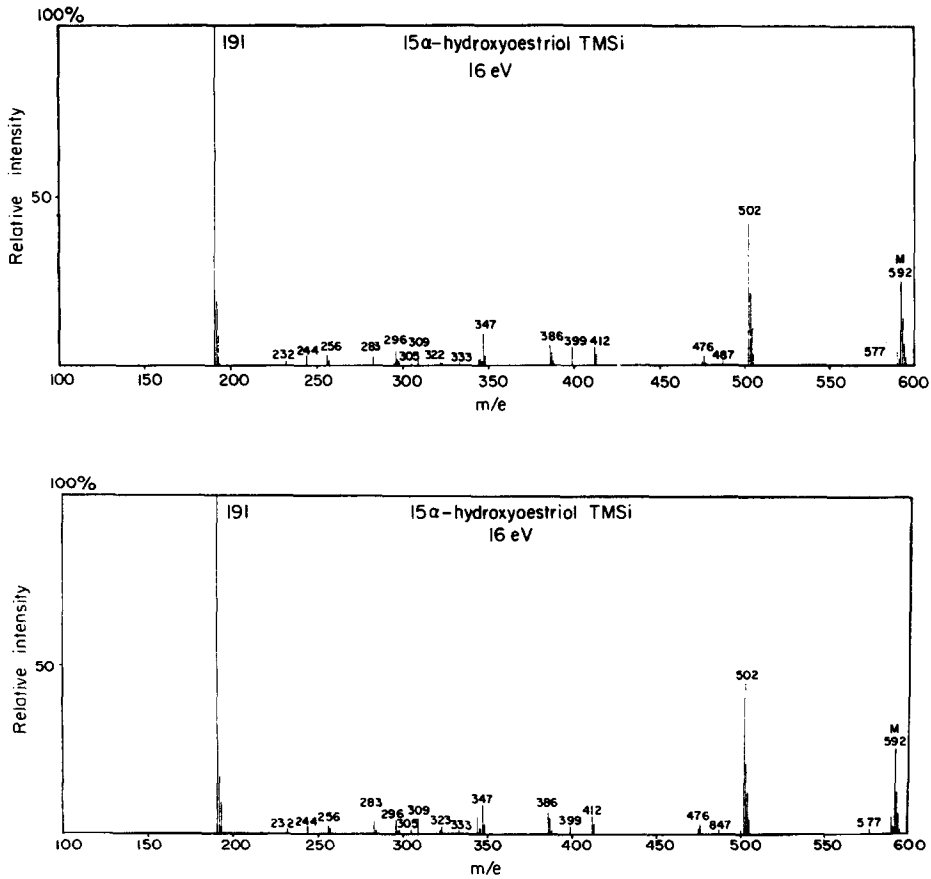


Fig. 2. Mass-spectra of urinary estetrol (as TMSi derivative) estimated by the method used (upper) and that of authentic standard estetrol (lower).

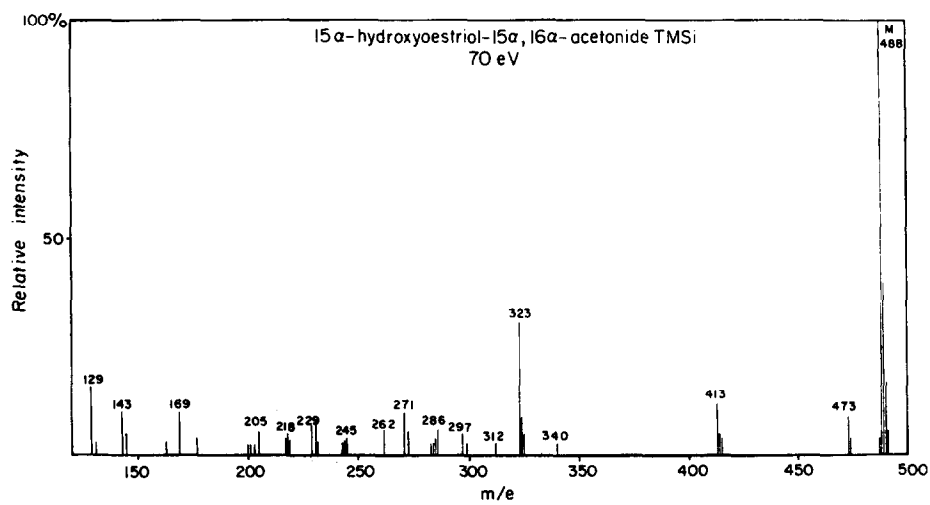


Fig. 3. Mass-spectrum of urinary estetrol acetonide (as TMSi derivative).

derivative of estriol has been reported earlier[22] and is therefore not shown in this report. In addition, the specificity of the method was evidenced by the identical behaviour of estriol and estetrol with that of the reference standards in two column chromatographic systems and in two TLC systems (Table 2).

Table 2. $R_f$ values of the estriol and estetrol in extracts from pregnancy urine and those of the reference standards in TLC systems A and D of Lisboa *et al.*[15]

| | TLC system A | | TLC system D | |
|---|---|---|---|---|
| | Reference standard | Urinary extract | Reference standard | Urinary extract |
| $R_f$ for estriol | 0·64 | 0·63 | 0·67 | 0·66 |
| $R_f$ for estetrol | 0·44 | 0·44 | 0·48 | 0·47 |

*Accuracy of the method*

Because conjugated estetrol is not commercially available, it has not been possible to determine recoveries after the whole procedure. To be sure that all urinary estetrol is eluted in peaks I and II[12] after gel filtration, fractions were collected before peak I and after peak II, the last fraction being eluted with aqueous ammonia (conc. ammonia: water 1 : 10 v/v), and analysed. In the fraction before peak I there was no estetrol and in the fraction after peak II there were only negligible amounts (< 1·5% of total) of this steroid adsorbed on the Sephadex. This indicates that practically all the estetrol in pregnancy urine is present in peaks I and II after gel filtration. The loss seems to be the same as with estriol, less than 1–2%. No free ethyl acetate-extractable estetrol or estriol could be detected in pregnancy urine. It can therefore be concluded that estetrol is excreted in the urine only as a conjugate. The nature of the conjugation is not known. It was therefore assumed that it is conjugated like other estrogens and therefore both sulphatase and β-glucuronidase containing *Helix pomatia* extract was used for the hydrolysis. In some experiments, the results of which are presented in Table 3, acid hydrolysis was employed, and it was found that after boiling for 60 min with 15 vol.% HCl the recoveries obtained were 10–15% less than after enzymic hydrolysis of the same urine samples. Therefore acid hydrolysis can also be used in analysing estriol and estetrol by this method. The effectiveness of the hydrolysis with *Helix pomatia* extract was further studied by acid hydrolysis of the aqueous residue remaining after ethyl acetate

Table 3. Mean values of 10 determinations of estriol and estetrol in pregnancy urine, using enzymic or acid hydrolysis and the percentual recovery of the two steroids after acid hydrolysis as compared to enzymic hydrolysis

| | Enzymic | Acid | $\frac{\text{Acid}}{\text{Enzymic}} \times 100$ |
|---|---|---|---|
| Estriol | 14·2 | 11·8 | 85 |
| Estetrol | 0·89 | 0·82 | 92 |

extraction of the free estrogens released by enzymic hydrolysis. No detectable amounts of estetrol were found with this procedure, which suggests that complete hydrolysis of the estetrol conjugate(s) was effected by the enzyme. To ascertain the efficiency of recovery, known amounts (5–50 μg) of estetrol reference standard and (1–100 μg) estriol reference standard were added to gel-filtered and hydrolysed male urine. The losses occurring during gel filtration and hydrolysis are thus not included in these figures. For estetrol the mean recovery of 21 determinations was 88·7 ± 7·8% (range 77–102%). For estriol the experiments (18 determinations) gave a mean recovery of 94·6 ± 5·7% (range 91–104%).

*Precision of the method*

Table 4 shows the coefficients of variation for estriol and estetrol calculated from duplicate determinations.

Table 4. Precision of the method expressed as the coefficient of variation of duplicate determinations

| | Coefficient of variation (%) | Number of duplicates |
|---|---|---|
| Estriol | ±5·7 | 20 |
| Estetrol | ±8·4 | 22 |

*Quantitative determination in pregnancy urine*

The results of 85 quantitative determinations of estetrol and estriol in normal pregnancy urine are shown in Table 5. Surprisingly, estetrol could be found as early as the ninth week of pregnancy. Therefore these samples were subjected to combined GLC-MS analysis using a SE-30 column. The spectra obtained

Table 5. Determination of estriol and estetrol in urines of normal pregnant women from the 9th week of pregnancy to term

| Weeks of pregnancy | Estriol mg/24 hr | Estetrol mg/24 hr | Number of determinations |
|---|---|---|---|
| 9–16 | 0·34–3·30 | 0·02–0·40 | 15 |
| 17–24 | 2·83–14·0 | 0·10–1·19 | 22 |
| 25–32 | 3·60–28·8 | 0·30–1·74 | 18 |
| 33–40 | 7·65–50·4 | 0·45–3·80 | 30 |

clearly demonstrated that the compound detected and estimated by the method was estetrol even in early pregnancy. Similarly, the estriol fractions were analysed by combined GLC-MS in early pregnancy. The investigation revealed that the compound determined was indeed estriol.

## DISCUSSION

There is no specific and sensitive method for the quantitative determination of estetrol in pregnancy urine. The method presented here has the advantage that

it determines the excretion of estetrol and estriol simultaneously and also gives the possibility to determine estrone, estradiol-17$\beta$ and 11-dehydroestradiol-17$\alpha$. The specificity has been carefully elucidated at both high and low steroid levels. The analysis by combined GLC-MS showed that the symmetrical GLC peaks were free from impurities, e.g. homogeneous. Both accuracy and precision are fairly good.

The method was developed especially for determination of estetrol in the urine during the second half of pregnancy, because foetal well-being is a special focus of clinical interest towards the end of pregnancy. The method can also be used in early pregnancy, but the final determination has to be carried out with two different liquid phases (one polar, the other nonpolar) in order to check possible interference by nonsteroidal compounds (drugs) at these low levels of excretion. However, it was demonstrated by combined GLC-MS that the determination of estetrol in early pregnancy urine is as specific as in the later stages if the subjects are not taking any drugs. In this report the accuracy and precision were not determined for the steroids in fraction 2 obtained after chromatography on silica gel (estrone, estradiol, and 11-dehydroestradiol-17$\alpha$). However, it was found that after column chromatography on partially deactivated alumina of the methyl ether derivatives of the three estrogens the gas chromatograms obtained with the TMSi derivatives of the steroids are free from interfering impurities.

The methodological studies demonstrated that, after gel filtration of normal pregnancy urines, acid and enzymic hydrolysis give comparable results for both the steroids measured. Acid hydrolysis saves time and can be recommended, if the method is used for clinical purposes. In our experience a well trained technician can carry out 8 to 12 determinations in twelve hours.

However, the number of determinations carried out in different periods of pregnancy is too small for any definite conclusions with regard to the excretion of estetrol throughout pregnancy. Our preliminary observations suggest that the slope of the excretion of estetrol differs from that of the three classical estrogens, which would indicate a different origin or metabolism for this steroid or its precursors.

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