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A RAPID RELIABLE METHOD FOR THE ESTIMATION OF PREGNANEDIOL IN HUMAN URINE

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SUMMARY

A method is described for the assay of pregnanediol in pregnancy urine as well as in the urine of men, children and non pregnant women. The analytical procedure comprises Amberlite XAD-2 extraction, hot acid hydrolysis, and automated solid injection g.l.c. of the trifluoroacetate derivatives. For low titer urines an additional alumina column chromatographic step is required. The specificity of the method has been established by g.l.c.–MS; the sensitivity is only limited by the sensitivity of the g.l.c. detector. 0·01 mg pregnanediol/24 h can be determined without difficulty.

INTRODUCTION

During recent years numerous methods for the determination of pregnanediol in human urine have been described (for reviews see [1–31]). Conventional methods involving colorimetric determinations proved to be unsatisfactory with respect to specificity if pregnanediol is excreted in only minute amounts. For this reason gas chromatographic methods are recommended for the analysis of low titer urines [2–5]. Since the liquid injection technique in the course of routine gas chromatographic quantitations is very time consuming, a method was developed which includes automatic solid injection of the samples into the gas chromatograph. In investigations carried out with different pregnanediol derivatives, the best results were obtained with the solid injection technique when the di-trifluoroacetate of pregnanediol was used. Thus, an analytical procedure is described which allows not only pregnancy urines but also low titer urines to be processed in a short time with high reliability.

EXPERIMENTAL

Reference steroids

[1,2-^{3}H]-Pregnanediol (specific radioactivity 50 Ci/mmol, NEN, Boston, Mass.), pregnanediol, 5α-pregnane-3α,20α-diol, progesterone, cholesterol, pregnenolone (E. Merck, Darmstadt), 3α-hydroxy-5β-pregnan-20-one (Ikapharm, Ramat Gan) 5β-pregnane-3α,20β-diol, 5β-pregnane-3β,20β-diol and oestrone 3-methyl ether, (Steraloids, Pawling) were checked for purity by t.l.c. and g.l.c. prior to use. Pregnanediol-3-glucuronide sodium salt (Steroid Reference Collection, London) was recrystallized from 85% aqueous ethanol yielding crystals with m.p. 281–284°C. Pregnanediol-di-trifluoroacetate (5β-pregnane-3α-diol-di-trifluoroacetate) was prepared by treatment of pregnanediol with an excess of trifluoroacetic acid anhydride at room temperature; after 30 min, the reaction mixture was dried *in vacuo* and the residue crystallized twice from ethanol yielding crystals with m.p. 123–124°C. The identity of the compound was established by mass spectrometry.

Chemicals. All solvents used (E. Merck, Darmstadt) were redistilled; the contact of the solvents with any plastic material was carefully avoided. Trifluoroacetic acid anhydride (Schuchardt, München) was redistilled under an atmosphere of dry argon.

Column chromatography. Amberlite XAD-2 (100–200 μ particle size; Serva, Heidelberg) was washed with water and methanol prior to use. Columns (50 × 1 cm) were prepared with a slurry of 7 ml of the purified resin in water containing 1·9 g of Amberlite XAD-2. For the determination of pregnanediol in pregnancy urine, polypropylene columns (0·7 × 10 cm., Bio Rad, Richmond) were filled with 1·5 ml of the resin suspension.

Alumina (Aluminium Oxide W 200; Woelm, Eschwege) was partially deactivated by the addition of distilled water (10 ml) to alumina (100 g). The column was prepared by pouring a suspension of alumina (3 g) in benzene into a glass column (40 × 1 cm.). The elution pattern of pregnanediol was standardized according to Klopper[4] for each stock of deactivated alumina.

Gas-liquid chromatography. G.l.c. was carried out with a Pye model 104 g.l.c. instrument equipped with a flame ionization detector and a Pye model 106 automatic solid injector (Philips, Hamburg). Operation

conditions: Inlet temperature 320°C, column temperature 220°C, detector temperature 250°C, carrier gas flow 25 ml N_2/min, siliconized glass columns (260 × 0·2 cm.), and stationary phase 3% OV-7 on chromosorb WHP 100/120 (W. Günther Analysentechnik, Düsseldorf). Prior to analysis, steroids were converted to their trifluoroacetates as described below. Peak areas were determined with an infotronics model CRS 204 digital integrator (Techmation, Düsseldorf).

Measurement of radioactivity. An Intertechnique liquid scintillation spectrometer model SL 36 (Deutsche Intertechnique, Mainz) was used for radioactivity measurements. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 0·3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 litre of toluene.

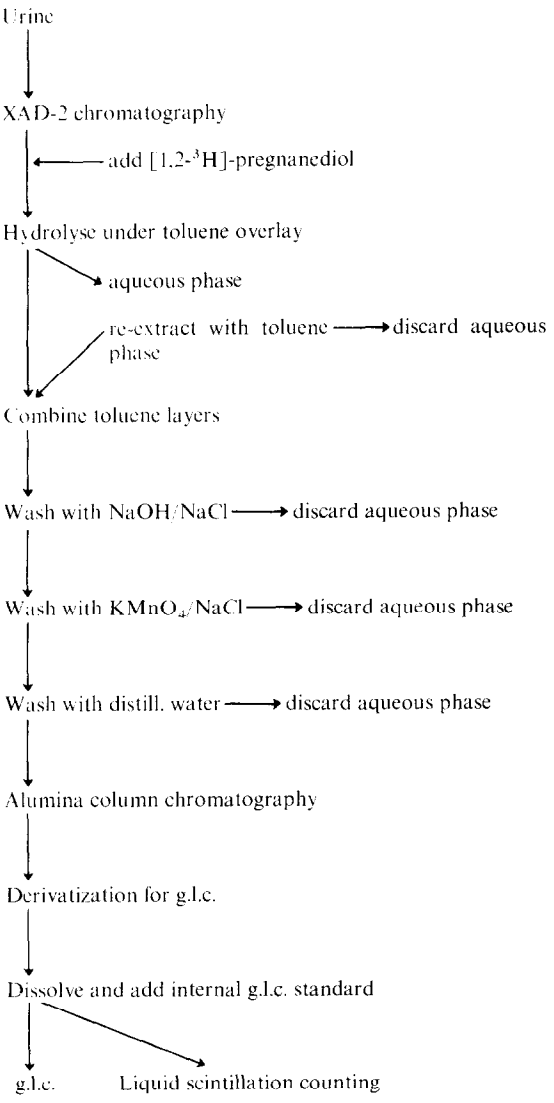

Fig. 1. Flow diagram for the quantitative determination of pregnanediol in non-pregnancy urine.

Gas chromatography–mass spectrometry was carried out using a LKB 9000 instrument (LKB produkter, Bromma) under the operation conditions described previously [6].

Determination of pregnanediol in non-pregnancy urine. A summary of the assay procedure is presented in the flow diagram (Fig. 1).

Portions (5 ml from female, 10 ml from male subjects and children) of 24 h-urine collections were transferred to Amberlite XAD-2 glass columns, elution being carried out with: I. 20 ml of water, II. 15 ml of methanol–water (1:3, v/v), III. 15 ml of methanol–water (3:1, v/v), and IV. 15 ml of methanol–water (9:1, v/v). Eluates I and II were discarded; the eluates III and IV were pooled in one tube and the methanol was removed under a stream of nitrogen. To the aqueous solution, [1,2-^{3}H]-pregnanediol (55 500 d.p.m.), 1 M HCl (10 ml) and toluene (15 ml) were added and the mixture was refluxed for 10 min. After cooling, toluene was separated and the aqueous phase was extracted again with toluene (10 ml). The combined toluene extracts were washed first with 1 M NaOH (5 ml) containing 25% of NaCl, then with 1 M NaOH (5 ml) containing 5% of $KMnO_4$ and finally with 10 ml portions of water until the mixture remained colourless. Sodium sulfate (1 g) was added as desiccant and the toluene was transferred to an alumina column. Chromatography was first carried out with 25 ml of 0·8% ethanol in benzene which were discarded and then with 10 ml of 3% ethanol in benzene. The latter fraction was evaporated to dryness *in vacuo* and the residue was treated with 0·5 ml of trifluoroacetic acid anhydride for 10 min at room temperature. The excess of reagent was then removed *in vacuo* and the dry residue was dissolved in toluene (0·5 ml) containing oestrone 3-methyl ether (25 μg) as internal standard for g.l.c. 50 μl of this solution were subjected to liquid scintillation counting and 25 μl portions were transferred to a solid-sample holder for g.l.c. After removing the solvent in a magazine heater the magazine containing up to 35 solid-sample holders was placed into the injection head of the chromatograph and gas chromatography was started by operating the automatic solid injector controller.

Determination of pregnanediol in pregnancy urine. Portions of 1 ml of 24 h-urine collections from pregnant women were passed through an Amberlite XAD-2 polyethylene column. Elution was carried out with: I. 5 ml of water, II. 3 ml of methanol–water (1:3, v/v) III. 4 ml of methanol–water (3:1, v/v) and IV. 4 ml of methanol–water (9:1, v/v). Eluates III and IV were pooled in one tube and extracted with *n*-hexane (5 ml). The *n*-hexane layer was discarded and the aqueous-methano-

lic phase was evaporated under a stream of nitrogen. After the addition of [1,2-^{3}H]-pregnanediol (55·500 d.p.m.), 1 M HCl (2·5 ml) and toluene (4 ml), the mixture was boiled for 10 min. The mixture was then cooled and after removing the toluene layer, the aqueous phase was again extracted with toluene (4 ml). The combined toluene layers were washed first with 1 M NaOH (2·5 ml) containing 25% of NaCl, then with 1 M NaOH (2·5 ml) containing 4% of $KMnO_4$ and finally with 5 ml portions of water until the mixture remained colourless. The organic layer was then evaporated under a gentle stream of nitrogen. Without further purification on alumina, the dry residue was directly treated with trifluoroacetic acid anhydride (0·5 ml) and liquid scintillation counting and g.l.c. were processed as described above.

Calculation of the results. The amount of pregnanediol (PD) excreted within 24 h was calculated using the following formula:

$$\text{mg PD/24 h} = \frac{R_{pa} \times V_t \times A_s \times M_s}{V_s \times A_E \times C_{pa}},$$

R_{pa} = Ratio of the peak areas of pregnanediol-di-trifluoroacetate (PDTFA) and oestrone methyl ether (E_1Me)

$$= \frac{\text{peak area PDTFA}}{\text{peak area } E_1\text{Me}}$$

V_t = Volume of the 24 h-urine collection

V_s = Volume of the urine sample analysed

A_s = Radioactivity of [1,2-^{3}H]PD added as internal standard

A_E = Radioactivity recovered in the final extract

M_s = Amount of estrone methyl ether added as g.l.c. standard (usually 0·025 mg)

C_{pa} = Coefficient of the relative FID responses of PDTFA and E_1Me, determined by plotting R_{pa} against the corresponding PD values in a calibration curve

RESULTS

Specificity

A gas chromatographic record obtained after analysing 5 ml of early luteal-phase urine (3 mg of pregnanediol/24 h) is shown in Fig. 2a. The peak assumed to be pregnanediol-di-trifluoroacetate was identical with authentic material in respect to relative retention time (0·59; relative to the retention time of E_1Me) and peak shape and peak width (0·6 min). Neither addition of pregnanediol or pregnanediol glucuronide to the

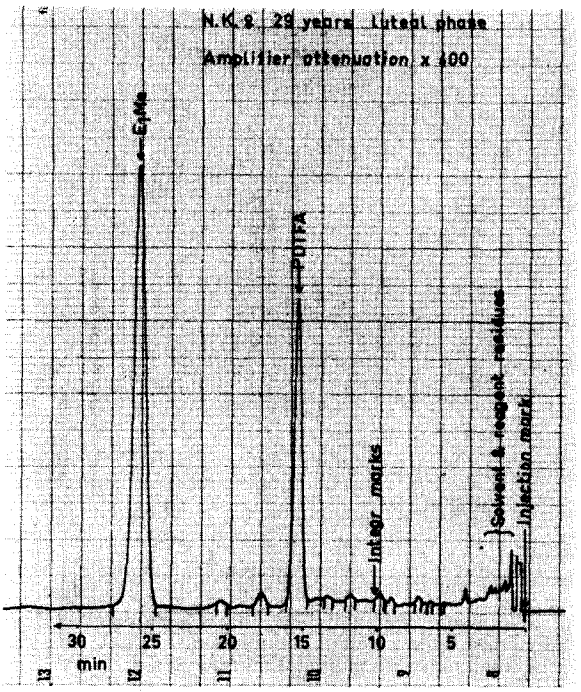

Fig. 2a. g.l.c. of pregnanediol-di-trifluoroacetate (PDFTA) obtained after analysis of 5 ml of a luteal phase urine following the method described for non-pregnancy urine. The conditions for g.l.c. are described in the text. E_1Me = estrone methyl ether.

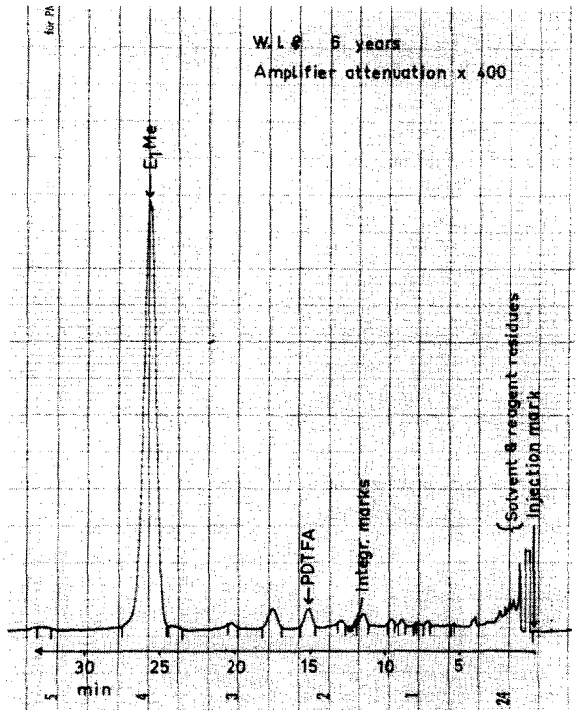

Fig. 2b. g.l.c. of pregnanediol-di-trifluoroacetate (PDTFA) obtained after analysis of 10 ml of urine from a 6 yr. old girl following the method described for non-pregnancy urine.

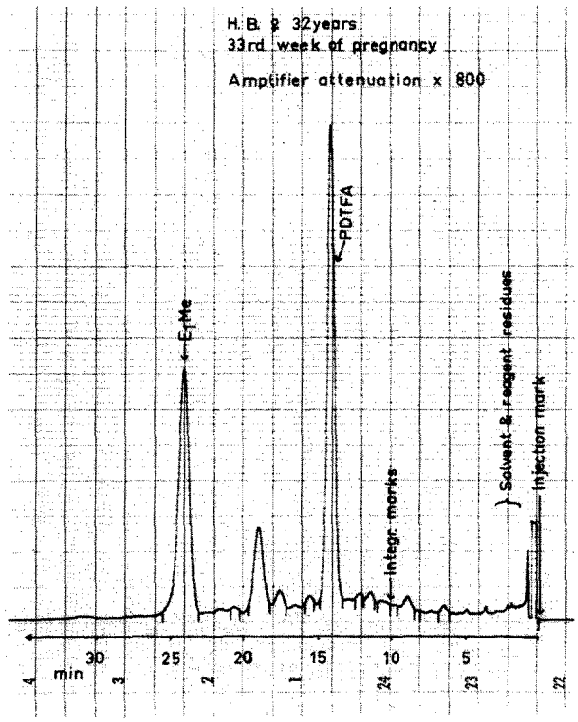

Fig. 2c. g.l.c. of pregnanediol-di-trifluoroacetate (PDFTA) obtained after analysis of 1 ml of urine from a pregnant woman following the method described for pregnancy urine.

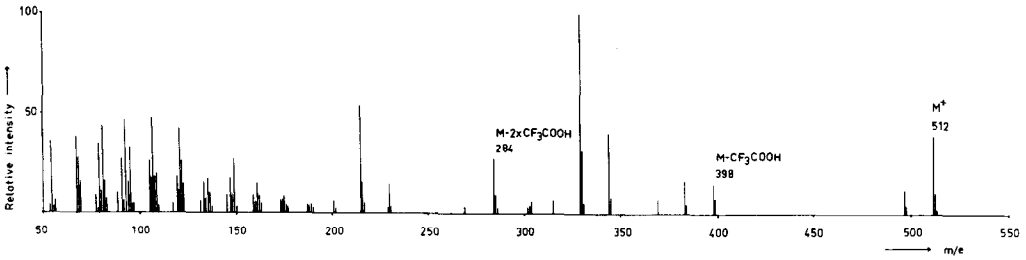

Fig. 3. Mass spectrum of the di-trifluoroacetate derivative of pregnanediol isolated from a luteal phase urine following the method described for non-pregnancy urine.

urine specimen nor addition of pregnanediol-di-trifluoroacetate to the final residue altered the gas chromatograms qualitatively. The amount of the urinary pregnanediol analysed was not influenced by the addition of 5α-pregnane-3α,20α-diol, progesterone, cholesterol, pregnenolone or 3α-hydroxy-5β-pregnane-20-one, since these steroids are separated from pregnanediol by the chromatographic procedures of the method. 5β-Pregnane-3α,20β-diol, 5β-pregnane-3β,20α-diol, and 5β-pregnane-3β,20β-diol are definitely separated from pregnanediol by g.l.c.

The identity of the gas chromatographic peaks was also confirmed by mass spectrometry. The mass spectrum of the di-trifluoroacetate derivative of pregnanediol isolated from female urine was identical with that of authentic material (Fig. 3). A contamination of the pregnanediol peak by pregnenediols can be excluded. Even minor impurities could be easily recognized in the mass spectrum as a peak at m/e 510.

Precision. 10 Replicate samples of a pool of luteal phase urine were analysed. The pregnanediol excretion was found to be 5·69 ± 0·09 mg PD/24 h (mean ± S.D.). The same procedure showed a child's urine to contain 0·050 ± 0·003 mg PD/24 h (mean ± S.D.) and a pregnancy urine (33rd week of gestation) to contain 64·9 ± 1·8 mg PD/24 h (mean ± S.D.).

When analysing an early luteal-phase urine on 12 consecutive days, the standard deviation of the "day-to-day precision" was calculated to be ±0·085 mg PD/24 h with a mean of 3·13 mg PD/24 h.

Accuracy. To 14 aliquots (5 ml) of a normal male urine, different amounts of PD ranging from 0 to 30 μg were added after Amberlite XAD-2 chromatography. These specimens were processed as described above for nonpregnancy urines; the mean recovery of the PD added was 100·5%, the standard deviation being ±3·1%.

In another series of experiments increasing amounts of pregnanediol glucuronide sodium salt ranging from 0 to 60 μg were added to sixteen aliquots (5 ml) of the same male urine prior to the Amberlite XAD-2 extraction and subsequently pregnanediol was determined. The results are comparable with those of the first experiment: The mean recovery was 95·9%, the standard deviation ±5·8%.

Sensitivity. The sensitivity of the method described herein is mainly limited by the sensitivity of the flame ionization detector. Amounts as small as 0·01 mg PD/24 h could be determined with no difficulty when using the method described for non-pregnancy urines.

Results from urine. The methods described herein are used for the routine analyses of the pregnanediol excretion in urine obtained from children, men and women. The ranges of the urinary excretion of normal subjects are listed in the Table. Some typical g.l.c. records are demonstrated in Fig. 2a–c.

DISCUSSION

The method summarized in Fig. 1 is entirely adequate for the determination of pregnanediol, even at

Table 1. Excretion of pregnanediol in urine of normal subjects. Determinations of pregnanediol were carried out following the method described for non-pregnancy urines

Subjects	No. of subjects studied	No. of determinations	Pregnanediol excreted (mg/24 h) mean	range
girls aged 2–6 years	6	48	0·068	0·041–0·11
men	6	12	0·89	0·64–1·15
women (follicular phase)	12	46	0·91	0·53–1·25
women (luteal phase)	16	52	5·20	3·28–7·73

the very low concentrations found in the urine of children. A prerequisite to obtain clear g.l.c. records and a high reliability of the method was the use of Amberlite XAD-2 column chromatography [7] for the pre-purification of urine. By fractionated elution of the Amberlite XAD-2 column with aqueous methanol of different concentrations a satisfactory separation of pregnanediol glucuronide from urinary impurities was achieved and the pregnanediol conjugate was nearly quantitatively recovered.

Further steps in the working-up procedure were based upon the method described by Klopper[4] involving hot acid hydrolysis under toluene overlay, alkaline wash, $KMnO_4$ oxidation and the first chromatography step on alumina. However, it was shown that the second alumina chromatography after formation of the pregnanediol derivative [4] was not necessary for this method; this was due to the efficient pre-purification by Amberlite XAD-2 chromatography.

Accuracy and precision were ensured by the use of tritium-labelled pregnanediol to correct procedural losses and by the use of estrone methyl ether as internal standard for g.l.c. On the other hand, high sensitivity and specificity were accomplished by carefully selecting the g.l.c. conditions: the combination of silicone OV-7 as stationary phase and of trifluoroacetates proved to be very useful. Instead of acetates or trimethylsilyl ethers, which are often used for g.l.c., the trifluoroacetates were preferred because they exhibit shorter retention times and produce sharp gas chromatographic peaks [8]; furthermore trifluoroacetates are stable against air and moisture and therefore were favoured for the solid injection technique. Using this solid injection technique, excellent g.l.c. records were obtained in all cases without solvent tailings (Fig. 2a–c) which often are observed following liquid injection. Since the g.l.c. recorder returns to the base-line after each peak, the quantitative evaluation of the peak areas can be carried out by means of an automatic electronic integrator which also enables automatic overnight runs.

For the determination of pregnanediol in pregnancy urine, the method could be simplified without decrease of reliability. Thus, when an extraction with *n*-hexane is carried out to remove interfering material, alumina column chromatography can be omitted.

The particular advantages of these methods are not only based on specificity but also on practicability. Thus, one technician is able to carry out 12 determinations of pregnanediol in non-pregnancy urines within 2 days while only 1 working day is necessary for the analysis of 12 pregnancy urines.

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REFERENCES

1. Wyss H. I.: Oestriol- und Pregnandiolausscheidung in der zweiten Schwangerschaftshälfte, Verlag H. Huber, Bern (1970) p. 108.
2. Hammerstein J. and Zielske F.: *Z. Anal. Chem.* **243** (1968) 272–288.
3. Bush I. E.: *Adv. Clin. Chem.* **12** (1969) 57–139.
4. Klopper A.: In *Methoden der Hormonbestimmung* (Edited by H. Breuer and H. L. Krüskemper) Georg Thieme Verlag, Stuttgart (1974, in press).
5. Klopper A., Wilson G. R. and Shearman R. P.: *J. Obstet. Gynaec. Br. Commonw.* **77** (1970) 531–535.
6. Hoppen H. O. and Siekmann L.: *Steroids* **23** (1974) 17–34.
7. Shackleton C. H. L., Sjöval J. and Wisén O.: *Clin. Chim. Acta* **27** (1970) 354–356.
8. Voelter W., Jung G., Breitmaier E., Bouchon G. and Bayer E.: *Anal. Chim. Acta* **53** (1971) 185.