A simplified method for the quantitative determination of pregnanediol in urine

For many years the determination of pregnanediol (5β-pregnane-3α,20α-diol) has been used to estimate corpus luteum function, pregnanediol being the most important metabolite of the corpus luteum hormone. The methods employed usually comprise the following steps: (1) hydrolysis of the pregnanediol glucuronide, the form in which most of the pregnanediol is excreted in the urine, (2) extraction of the free pregnanediol, (3) separation of the pregnanediol from other compounds simultaneously extracted and (4) some sort of quantitative (or semiquantitative) estimation of the separated pregnanediol.

In some of the early methods, step (3) was omitted and the extract was estimated gravimetrically. As the extract from the urine contains many different compounds (including other closely related corpus luteum metabolites) this method was not exact. The methods were greatly improved when separation of the extracted compounds was carried out by column chromatography (KLOPPER, MICHIE AND BROWN[1]). Since elution of pregnanediol from the column without being contaminated by other compounds, especially the other chemically related corpus luteum hormone metabolites, is very difficult, it was found to be more advantageous to carry out the separation by thin-layer chromatography (TLC). By this method it is possible to localize the pregnanediol spot precisely and separate it from any other compound. WALDI[2] has developed a method using this principle, which gives a semiquantitative estimation of the amount of pregnanediol in urine and proposes the method as a pregnancy test. STARKA AND MALÍKOVÁ[3] have described a method for the quantitative assay of urine pregnanetriols using TLC followed by elution of the pregnanetriols from the spot and a colorimetric estimation of the eluted pregnanetriols with sulphuric acid as described by FOTHERBY AND LOVE[4].

The TLC method may be simplified by carrying out the sulphuric acid reaction directly on the material scraped from the plate. In this way it is possible to make a quantitative estimation of any of the urine pregnanediols and pregnanetriols, both in pregnancy urine and non-pregnancy urine from any part of the menstrual cycle.

Procedure

Hydrolysis with hydrochloric acid and extraction with cyclohexane is carried out according to WALDI[2]. 100 ml urine or less is used depending on the pregnanediol content of the urine.

The extract is evaporated to dryness and the residue dissolved in $^1/_2$ ml chloroform. 50 μl of the solution is chromatographed on silicagel with chloroform–acetone (90:10) as solvent. Simultaneously, on the same plate standards (*e.g.* 30 and 50 μl) of a 0.1 % (w/v) solution of pure pregnanediol in chloroform are run. After the solvent front has moved 10 cm (in an S-chamber[5]), the plate is dried and sprayed with distilled water. The pregnanediols are visible as white spots on a grey background. The urinary pregnane-3α,20α-diol is localized by means of the R_F values of the standards. The spots of pregnanediol are marked with a pencil point and the plate is dried. When dry, the area of the layer containing the pregnanediol is scraped off and the material introduced into a glass tube; 3 ml concentrated sulphuric acid is

added and mixed with the material. After standing for 10 min at room temperature and centrifuging, the optical density of the supernatant is measured in a spectrophotometer at 430 mμ. A blank is prepared from a similar area of the silicagel layer as the spots, but without any pregnanediol or evidently coloured compounds on it. The spots of the standard solution of pregnanediol are treated in the same way as the urinary extracts. With the optical density as ordinate and the concentration of pregnanediol as abscissa a curve is drawn which should be rectilinear. The content of pregnanediol in the spots from the urinary extracts is estimated by means of this standard curve. The whole procedure from hydrolysis to photometry may be carried out on 2–3 urine specimens (in triplicate) by one technician in about 6 hours.

Discussion

The ability of TLC to separate compounds chemically closely related to pregnanediol from the latter is illustrated in Table I, where the R_F values of the compounds examined are given, and in Fig. 1 the TLC of the compounds is shown.

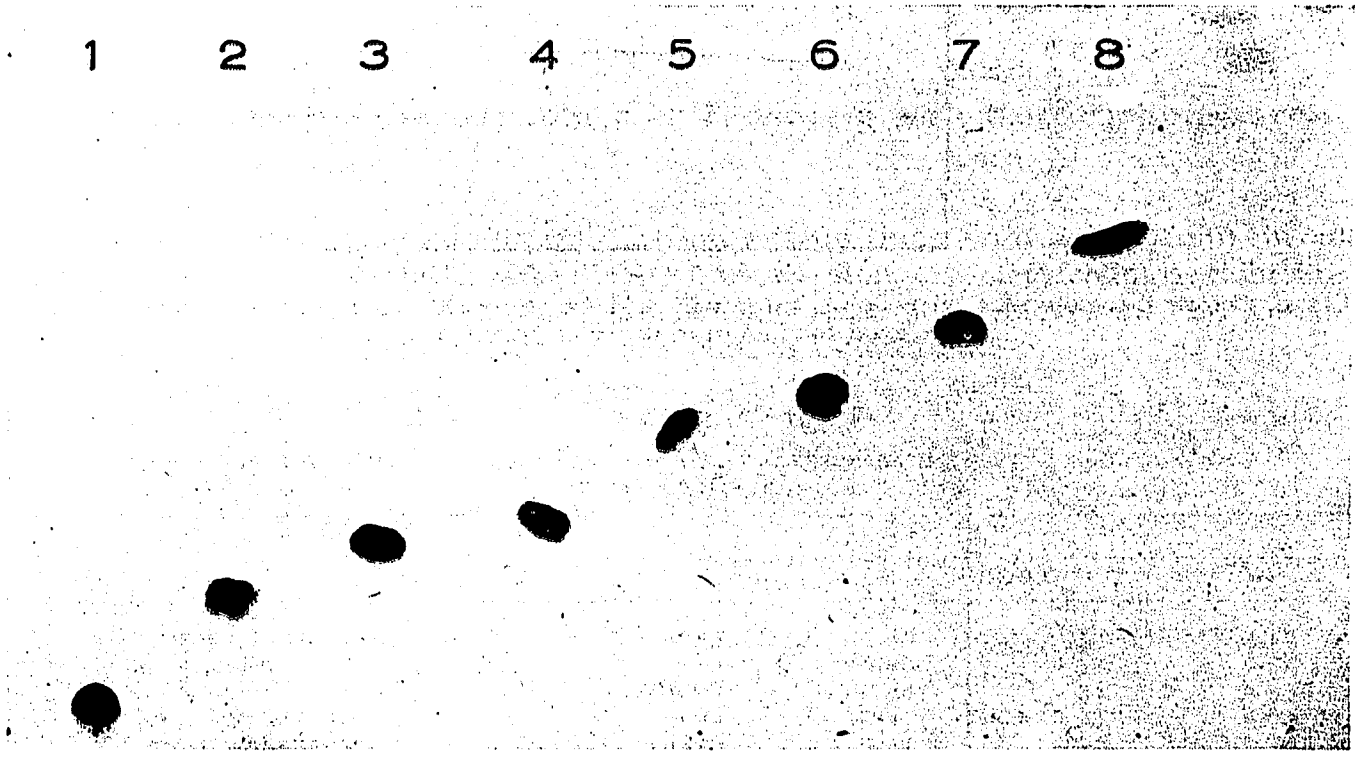

Fig. 1. Steroids related to pregnanediol. Solvent: chloroform–acetone 90:10 on silicagel. Colour development on the plate with sulphuric acid and by heating. For the compounds, see Table I.

The effect of time on the colour development with concentrated sulphuric acid was investigated (Fig. 2) and it was found that the colour development was complete after 10 min and did not change measurably during 40 min; Fig. 3 shows that with a development time of 10 min the Lambert-Beer law was obeyed.

At a level of 1–4 mg/l urine the results are expressed as the mean of duplicate determinations ± 0.2 mg, and the 95 % confidence limits calculated from duplicate determinations on 18 urine samples were 0.14 mg.

TABLE I

THE R_F VALUES OF VARIOUS STEROIDS RELATED TO PREGNANEDIOL

	Steroid	*R_F values*
1	5β-Pregnane-3α,6α-diol-20-one	0.03
2	Pregnanediol (5β-pregnane-3α,20α-diol)	0.17
3	5α-Pregnane-3β,20α-diol	0.26
4	5α-Pregnane-3β,20β-diol	0.29
5	5β-Pregnan-3α-ol-20-one	0.39
6	5α-Pregnan-3β-ol-20-one	0.43
7	5β-Pregnan-3β-ol-20-one	0.55
8	5β-Pregnane-3,20-dione	0.65

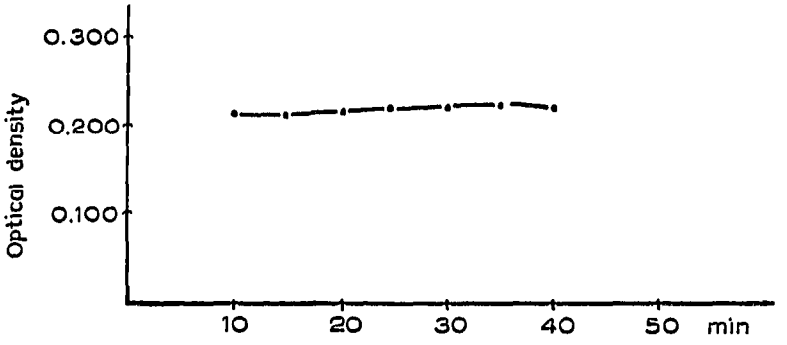

Fig. 2. Colour development with conc. sulphuric acid on the plate. Optical density as ordinate related to time in minutes as abscissa.

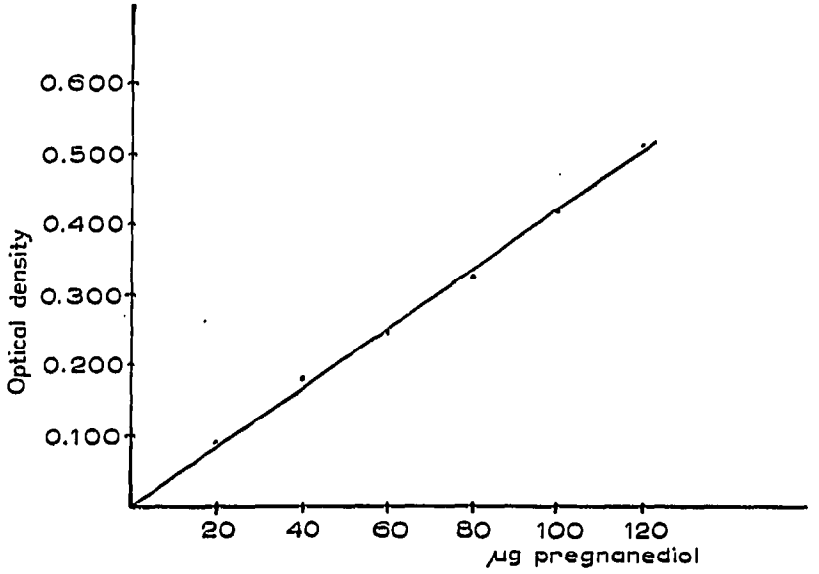

Fig. 3. Optical density as ordinate related to pregnanediol concentration as abscissa. Reaction with concentrated sulphuric acid for 10 min.

The content of pregnanediol in the morning urines of fertile, non-pregnant women was found to be about 1 mg/1000 ml in the pre-ovulatory phase and about 6 mg/1000 ml in the late post-ovulatory phase.

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Dünnschichtchromatographische Trennung von Ketocarbonsäuren

Prinzip

Die Ketocarbonsäuren wurden mit 4-Oxo-2-thion-thiazolidin (Rhodanin) in die entsprechenden Rhodaninderivate übergeführt, die sich auf Acetylcellulose-Schichten trennen lassen. Untersucht wurden (angeordnet nach steigender Zahl der Kohlenstoffatome), die in Tabelle I angegebenen Ketocarbonsäuren.

TABELLE I

R_F-WERTE DER RHODANINDERIVATE DER KETOCARBONSÄUREN IN VERSCHIEDENEN ELUTIONSMITTELN

	R_F			
	I	*II*	*III*	*IV*
α-Ketopropionsäure	0.55	0.22	0.32	0.51
Oxalessigsäure	0.54	0.22	0.32	0.51
α-Ketobuttersäure	0.67	0.34	0.44	0.59
α-Ketoisovaleriansäure	0.71	0.47	0.53	0.65
α-Keto-*n*-valeriansäure	0.73	0.51	0.57	0.70
γ-Keto-*n*-valeriansäure	0.59	0.26	0.41	0.54
α-Ketoglutarsäure	0.29	0.04	0.09	0.27
α-Ketocapronsäure	0.78	0.65	0.68	0.78
α-Keto-D-gluconsäure, Kaliumsalz	0.68	—	0.58	0.67
α-Ketoisocapronsäure	0.78	0.70	0.67	0.77
α-Ketooenanthsäure	0.83	0.79	0.77	0.85
α-Ketocaprylsäure	0.87	0.83	0.85	0.91
Phenylglyoxylsäure	0.65	0.54	0.47	0.59
α-Ketopelargonsäure	0.74	0.62	0.50	0.75
α-Ketophenylpropionsäure	0.60	0.47	0.66	0.65
4-Hydroxyphenylbrenztraubensäure	0.67	0.42	0.49	0.71
Reagenz			0.60	0.70