Method for Determination of Urinary Pregnane-3-Alpha, 17-Alpha 20-Alpha Triol.* (22965)

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Butler and Marrian(1) isolated pregnanetriol from urine of 2 women with adrenogenital syndrome. Bongiovanni(2) showed that increase in pregnanetriol was the most striking abnormality in urine steroid pattern of children with congenital adrenal hyperplasia. The method for determination of urinary pregnanetriol as proposed by Bongiovanni(3) included enzyme hydrolysis, chromatography on alumina, with final determination of pregnanetriol as sulfuric chromogen. This method has given us rather inconsistent results. Recovery experiments with authentic pregnanetriol showed that the steroid was partially and inconsistently destroyed by adsorption on alumina. Furthermore, when the method was applied to urinary extracts, extraneous material present in the eluate gave rise to color in sulfuric acid and interfered with quantitative determination of the sulfuric acid chromogen.

In the modification described here, urine extract is chromatographed on florisil. The eluate containing pregnanetriol is oxidized with bismuthate according to the method of Norymbersky(4,5) and the pregnanetriol measured as the corresponding 17-ketosteroid.

Materials. All reagents were analytical grade and all solvents redistilled. Beef liver beta-glucuronidase was used for hydrolysis. The preparation of florisil was slightly modified from that of Eik-Nes, Nelson and Samuels(6), in that florisil was cooled to 120°C and kept at this temperature. *Methods. Hydrolysis and Extraction.* Aliquots of either 50 or 100 ml were taken from freshly collected 24-hour urine samples. The pH was adjusted to 4.5 (Hydrion test paper) with glacial acetic acid; acetate buffer (0.5 ml/5 ml of urine) and beta-glucuronidase (300 units/ml) were added and urine incubated at 37°C for 48 hours. The urine was extracted twice with equal volumes of ether and then discarded. The combined ether extracts were washed with 0.1 normal sodium hydroxide until pigment free, and with water until neutral, and then dried under nitrogen. Traces of water were removed with benzene and ethanol. *Chromatography.* A florisil column measuring 1 cm diameter and 8 cm in height and supported by small plug of glass wool, was prepared in benzene. The extract was quantitatively transferred to the column with benzene. Slight heating was occasionally required. Elution was carried out using 75 ml portions of 1, 2, and 4% of ethanol in benzene. The 17-ketosteroids and pregnanediol were eluted in the 2% fraction, and the pregnanetriol is contained in the 4% fraction, while the more polar steroids remain on the florisil column. After chromatography the fraction containing pregnanetriol was dried under nitrogen using temperatures lower than 60°C. The residue was dissolved in 10 ml absolute ethanol from which two 4 ml aliquots were taken, one to be oxidized (A), the other (B) to be dried as unoxidized blank for color reaction. *Oxidation and colorimetry.* The method as described by Norymbersky(4,5) was used to convert pregnanetriol to 17-ketosteroid. Sample A was dried and taken up in 8 ml of 50% acetic acid. One gram sodium bismuthate was added, followed by shaking in the dark for 30 minutes. Ten ml of 6% sodium pyrosulfate ($Na_2S_2O_5$) were added and shaking continued for 15 minutes to reduce the remaining bismuthate. After adding 18 cc of water, the acid solution was transferred to a separatory funnel, extracted twice with ether, which was washed with 5% sodium hydroxide until alkaline, and with water until neutral. Ether was dried over sodium sulfate then transferred to 50 cc tapered centrifuge tube and evaporated. For color development a Holtorff and Koch

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TABLE I. Recovery of Pregnanetriol from Florisil Column. (90 μg pregnanetriol adsorbed on column.)

Recovered in 4% ethanol/benzene, μg	Recovery, %
79	88
77	85
77	85
78	86

(7) micromethod was used (1% metadinitrobenzene, 5 N aqueous KOH). The residue was taken up in 0.4 ml of metadinitrobenzene, 0.2 ml of alkali were added and the sample incubated 60 minutes in the dark. This procedure was carried out simultaneously on the unoxidized sample (B). The amount of 17-ketosteroid obtained from pregnanetriol was calculated by subtracting the Zimmermann value of sample B from that of sample A, using dehydroisoandrosterone as standard, and multiplying by the ratio of molecular weights

$$\frac{\text{(Molecular wt pregnanetriol)}}{\text{(Molecular wt etiocholanolone)}}.$$

Results. Studies were undertaken to determine possible sources of loss of steroid: A. Recovery following chromatography on florisil column: Adequate separation of pregnanetriol from steroids of similar polarity was determined by absorbing 2 mg each of 11β - hydroxyetiocholanolone, pregnane - 3α, 17α, 20α triol-11, 20-dione (tetrahydro E) and 1 mg of pregnanetriol on the florisil column and eluting these by the method described. All the Zimmermann material was eluted with 2% ethanol-benzene solution. No Porter-Silber chromogen was found in the 4% fraction but was eluted in 8% ethanol-benzene. In another experiment pregnanetriol as

TABLE II. Recovery of Pregnanetriol following Oxidation.

Pregnanetriol, μg	Pregnanetriol calculated from recovered etiocholanolone, μg	Recovery, %
48	44	92
96	91	95
28	28	100
80	64	80

TABLE III. Total Recoveries of Pregnanetriol (5 Normal Subjects).

Material, ml	Endogenous pregnanetriol	Added pregnanetriol, μg	Recovery of added pregnanetriol, %
H_2O, 50		90	82
Urine, 50	70	90	85
Idem	65	90	70
"	83	90	73
"	0	90	83
"	15	90	83

determined by its sulfuric acid chromogen, was recovered as indicated in Table I. B. Recovery following oxidation procedure: Varying amounts of crystalline pregnanetriol were converted to etiocholanolone by the oxidation procedure. The pregnanetriol equivalent recovered is shown in Table II. C. Total recoveries from water and from urine are shown in Table III. A series of urines from 12 normal females, 4 normal males and one patient with adrenogenital syndrome was also examined (Table IV).

Conclusion. A method for determination of pregnanetriol is presented. The method seems to be reproducible with adequate recoveries and specificity. Recoveries average 78.5%. Specificity is assured by chromatographic purification, oxidation, and determination of pregnanetriol as ketogenic steroid. The spectrum of material from the florisil column in sulfuric acid was authentic with the one obtained from pure pregnanetriol (Fig. 1). In the oxidized sample the Zimmermann color obtained was purple with distinct peak at 520 mμ (Fig. 2). The un-

TABLE IV. Excretion of Pregnanetriol from Normal Patients 21 to 31 Years Old.

	Pregnanetriol per 24 hr, mg	Pregnanetriol per 24 hr, mg
Normal ♀	1.5	.54
	1.3	.39
	1.3	.80
	.0	1.67
	1.1	1.01
	1.36	1.6
Normal ♂	2.4	
	1.3	
	1.4	
	2.3	
Adrenogenital syndrome (♀)	16.	

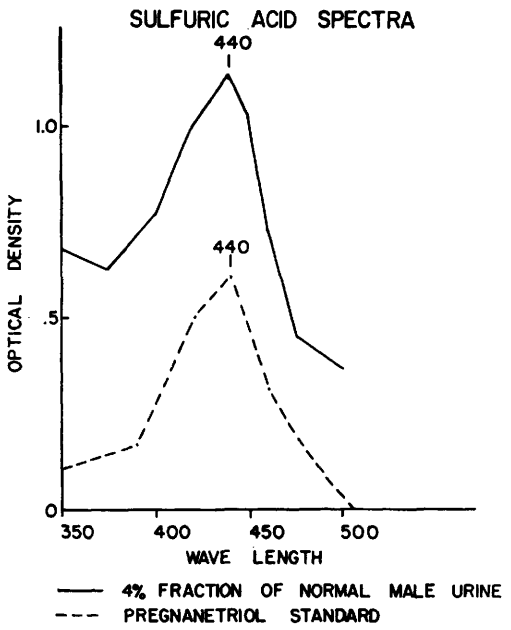

FIG. 1.

oxidized sample showed brown color with the Zimmermann reagent and gave a linear absorption curve.

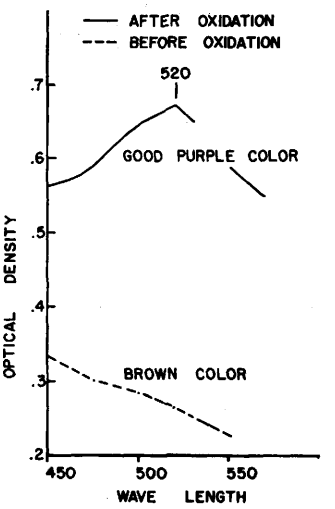

FIG. 2. Spectrum of oxidized and unoxidized 4% fraction following Zimmermann reaction (normal male urine).

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Blood 5-Hydroxytryptamine (Serotonin) Levels after Reserpine and Electroshock Therapy.* (22966)

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Recent evidence appears to lend credence to the postulate of Gaddum(1) and Woolley and Shaw(2) that 5-hydroxytryptamine (5-HT, serotonin) is involved in the function of the nervous system. Shore, Silver, and Brodie have shown that animals treated with the tranquilizing drug, reserpine, excrete large quantities of 5-HT metabolite, 5-hydroxyindoleacetic acid(3), and that brain and other body stores of such animals become depleted of their 5-HT(4,5). They have demonstrated further that this 5-HT-releasing activity is limited only to those Rauwolfia alkaloids with tranquilizing action(6).

It seemed of interest to determine blood levels of 5-HT in human subjects after treatment with reserpine; at the same time we

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