Factors Affecting the Estimation of Pregnanediol in Human Urine

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The chromatographic and spectrophotometric features of the estimation of pregnanediol in urine has been re-evaluated. Spectral data for pregnanetriol and pregnanediol and critical features for the preparation of the activated alumina chromatographic column are given. Pregnanediol excretion patterns of 4 successive menstrual cycles in a female of reproductive age is given.

The aim of this communication is to reveal some findings relevant to the estimation of pregnanediol in urine encountered during clinical and metabolic study with a new progestational agent, 6α-methyl-17-acetoxy progesterone* (Fig. 1).

Several closely related steroids of the pregnane series such as pregnanediol, pregnantriol, pregnanetetrolone, pregnanediolone, and their allo isomers are excreted in the urine as metabolites of steroids derived from secretory products of various endocrine glands; e.g., ovaries, testes, adrenals, and placenta. All are “recovered” as a group during the solvent extraction and isolation step of the procedure after hydrolysis of the conjugated steroid.

With the use of beta-glucuronidase (1) in place of hydrochloric acid for deconjugation, there is little chance for conversion of the triol compound, for example, to a compound with specific pregnanediol spectral characteristics, probably as a result of bonding of the pregnanetriol at C16 position (Fig. 2).

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* Distributed as Provera by the Upjohn Company.
However, while it introduces obvious desirable features such as reduction of nonspecific background absorbancy or artifacts as a result of acid-catalyzed epimerizations or rearrangements, it introduces a set of conditions that can also lead to erroneous results in the estimation of the pregnanediol content of the urine.

Since we were interested in determining whether 6α-methyl-17 acetoxy progesterone differed in any way from progesterone in its metabolism or interaction with other hormones, it was necessary to assay the urine for pregnanetriol and for other relevant steroids. We applied the methods of Bongiovanni and Clayton (2) as modified by Bongiovanni and Eberlein (3).

Because we had some authentic pregnanetriol* available, we took the precaution of standardizing the procedure with known amounts of both the diol and triol forms.

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Although the chromatographic technics with partition gradient elution previously described (2) appeared to resolve the pregnanetriol from mixtures of pregnanediol and pregnanetriol satisfactorily (95–97 per cent recovery of the triol), we invariably ended with more (112–118 per cent recovery) pregnanediol than we could account for, regardless of the ratios or absolute amounts of both compounds. It was this consistent "excess" recovery of pregnanediol over the calculated amount that led us to resurvey the chromatographic and spectrophotometric features as well as the recovery from urine.

**Results**

It would be expected that introduction of an additional hydroxyl group at C17 of the pregnanediol molecule would produce spectral changes that would provide a means of distinguishing them in the presence of each other by the usual spectrophotometric procedure for binary compounds (4). Examination of the observed absorbancies (O.D.) of several combinations of mixtures of the two compounds at their corresponding wavelengths shows that this is not the case (Curves 3 and 4, Fig. 3).

While the shape of the curves for the diol and triol alone are quite similar, from 420 m\(\mu\) to 590 m\(\mu\), the color value or extinction coefficient (\(E_{\text{max}}\)) for pregnanetriol is almost 5 times that for pregnanediol in the region of the spectrum at 420–440 m\(\mu\).

A mixture of equal amounts (0.05 mg. pregnanediol–0.05 mg. pregnanetriol) in concentrated sulphuric acid caused a decrease in the O. D. of the pregnanetriol at its \(\lambda_{\text{max}}\), accompanied by a shift of the \(\lambda_{\text{max}}\) for pregnanediol to that of pregnanetriol with 0.25 mg. of pregnanediol (a ratio of 1:5, respectively). Reacting with sulphuric acid caused a marked increase in the O.D. reading of pregnanediol to twice that of a similar quantity of the diol alone, with a slight shift to the shorter wave length of the spectrum (Curve 4).

In addition, the "hold-up" volume of the mobile phase solutions, benzene and ethanol in benzene, is influenced by the degree of hydration of the previously activated alumina as a result of heating at 400°. Thus the degree of inactivation by addition of water and its incorporation onto the alumina is critical. If the column is too "dry" or too "wet," fractionation of the two added compounds of interest is inadequate, with an overlap of either into the phase of the other.
Consequently, unless there is complete separation of the two compounds, the higher color value of the pregnanetriol will account for the exaggeratedly higher recovery values for the pregnanediol. That the phenomenon described above can not be duplicated by admixtures of pregnanediol and its isomer allopregnanediol in concentrated H₂SO₄ may readily be seen in Fig. 4, (6) when the O.D. of pure compounds of the two are plotted against wave number. In fact $E_{\text{max}}$ and $\lambda_{\text{max}}$ are identically linear in the 240–400 m$\mu$ region and almost linear at the 420–500 m$\mu$. Indeed the allo form has a better $\lambda_{\text{max}}$ (superior peaking) at 425 m$\mu$ than pregnanediol.
**Fig. 4.** Spectrophotometric distribution of optical densities at various wave lengths of 0.5 mg. pregnanediol and 0-5 mg. allopregnanediol. Sample (0.005%) in concentrated H₂SO₄, read 20 min. after preparing solution. Reference cell contained concentrated H₂SO₄. Lot numbers are Sigma Chemical Company products.
Urinary Preparations

What might happen when urines are enzymatically hydrolyzed and separation of the diol and triol are incomplete or when the urine is acid hydrolyzed, as in the Klopper et al. (5) method, is strikingly demonstrated in Fig. 5. In this case, the urines were hydrolyzed with beta-glucuronidase for a survey of pregnanediol during 4 successive normal menstrual cycles, without chromatographic fractionation of the recovered steroids. The graphic picture of the absorption intensities at 420–425 m\(\mu\) plotted against the days of the cycle almost speaks for itself. One can perhaps account for the abnormal irregularity of the progesterone metabolism as reflected in the saw-tooth configuration of the plotted chromogen values (Curve 3, Cycle 3). One can also account for the significantly elevated values on a basis of the quantitative definitions applied previously above, since the preparations were not chromatographed and, in the presence of the psychosomatic disturbance of adrenocortical function, the pregnanetriol augmented the color value of pregnanediol with its additive effects.

Fig. 5. Pregnanediol excretion pattern (Somerville-Duhoff method) of 4 successive menstrual cycles in a female of reproductive age. First, second, and fourth cycles had a sixteenth day ovulation; third cycle had a twelfth day ovulation. Arrow indicates 3 successive days of emotional disturbance bordering on hysteria during urine collection, third cycle.
Since the subject was not receiving medication, the fact that she ovulated on the twelfth day during the third cycle instead of on the sixteenth day as she had done for the preceding two cycles and the subsequent cycle, is not clinically significant, as such variations are well within the normal shifts in ovulation that often occur. It is evident that a significant change in estrogen and progesterone ratio took place after the fourteenth day, as manifested by a departure of the pregnanediol curve (third cycle) from the others (Fig. 5, Curves 1, 2, and 4). It is the contention of the investigators that, had not the psychic disturbance ensued, the plot of the daily pregnanediol values for the third cycle would have followed the same pattern as the others.

References
6. Unpublished data.