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TITLE PAGE

ANNUAL PROGRESS REPORT

February 1, 1962 through January 31, 1963

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HAZLETON LABORATORIES, INCORPORATED

MEASUREMENT OF 17-HYDROXYCORTICOSTEROIDS IN URINE  
DEVELOPMENT OF A METHOD FOR THE MEASUREMENT OF  
PREGNANEDIOL IN URINE

Contract No. DA-49-193-MD-2318

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The progress of the development of a method for the analysis of urinary pregnanediol is described. The urine is subjected to enzymic hydrolysis, solvent extraction and the pregnanediol is determined by gas-liquid chromatography.

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# HAZLETON LABORATORIES, INCORPORATED

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January 29, 1963

## ANALYSIS OF URINARY 17-HYDROXYCORTICOSTEROIDS

The determination of 17-hydroxycorticosteroids<sup>(2)</sup> in 545 samples of human urine and 77 samples of monkey urine was carried out for Walter Reed Army Institute of Research in conjunction with the research program on stress.

## DEVELOPMENT OF A METHOD FOR THE MEASUREMENT OF PREGNANEDIOL IN URINE

The purpose of this research was to develop a direct, sensitive method for the qualitative and quantitative analysis of urinary pregnanediol.

## INTRODUCTION

The first reported methods for the measurement of pregnanediol were based on the gravimetric analysis of sodium pregnanediol glucuronide crystallized from the butanol extract of urine<sup>(9 and 10)</sup>. This procedure is subject to severe limitations since the sensitivity is no greater than 15 mg. per 24 hours and compounds other than sodium pregnanediol glucuronide are present in the measured precipitate. Subsequent methods

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employed the acid or enzymic hydrolysis of pregnanediol glucuronide, extraction of the free metabolite, purification by column or paper chromatography, and spectrophotometric measurement of the sulfuric acid-pregnane-diol chromogen. These methods are very time consuming and require extraordinary analytical technique in order to maintain a high degree of accuracy and specificity<sup>(1, 3, 5, 6, 7, 8, 9, 12, and 13)</sup>.

In view of these facts, the possibility of measuring pregnane-diol by gas chromatography was considered. By this means a compound can be separated from a complex mixture rapidly and in a very pure state. The identity of the unknown compound can be determined by comparison of the retention time of the unknown compound to that of the standard compound. The quantity of unknown compound is determined by measuring the area under the curve and relating this area to a standard curve of the known compound. The sensitivity achieved by this method is 1  $\mu$ g.

#### MATERIALS

An F&M Gas Chromatograph, Model 500, was equipped with a hydrogen flame ionization detector. Helium was used as the carrier gas at a flow rate of 70 ml/min. Hydrogen gas and air at flow rates of 21 and 40 ml/min were used in the detector cell. The temperatures of the injection port and detector block were maintained at 275 and 300° C.



The solid supports, Chromosorb W and Gas Chrom P, were prepared by the method of Horning et al.<sup>(4)</sup> and coated with neopentyl glycol succinate, XE 60 Nitrile Silicone Polymer, or SE 30 Silicone Rubber Gum.

#### METHOD

An aliquot of urine representing from six- to 72-hour output was mixed with one half volume of acetate buffer (pH 4.5, ionic strength, 2 M), 400 units of ketodase per milliliter of original urine, and 1250 units of penicillin per milliliter of original urine. The mixture was incubated at 46° C. for 16 hours. The samples were then extracted three times with an equal volume of chloroform. The combined extracts were washed three times with one-third volume of 1 N NaOH, two times with one-third volume of 1 N HCl, and two times with one-third volume of distilled water. The extract was evaporated at 60° C. under reduced pressure in a rotary evaporator. The residue was dissolved in a small amount of chloroform, filtered through sodium sulfate, and transferred quantitatively to a 2-ml. graduated vial. One microliter of extract was injected into the gas chromatograph.

Standard solutions of increasing concentrations of 5 $\beta$  pregnane 3 $\alpha$ , 20 $\alpha$  diol in ethanol were prepared. One microliter of each solution was injected into the gas chromatograph in order to obtain a standard curve at the time of analyses of the unknown samples.



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## RESULTS

Table No. 1 - Retention time of pregnanediol

| <u>Column</u>                         | <u>6' SE 30</u><br><u>175° C.</u><br><u>min.</u> | <u>3' XE 60</u><br><u>235° C.</u><br><u>min.</u> | <u>3' NGS</u><br><u>220° C.</u><br><u>min.</u> |
|---------------------------------------|--------------------------------------------------|--------------------------------------------------|------------------------------------------------|
| Pregnanediol standard                 | 4.2                                              | 7.2                                              | 8.0                                            |
| Major peak from human pregnancy urine | 4.8                                              | 7.2                                              | 8.0                                            |

Table No. 2 - Recovery of pregnanediol from chloroform extract of urine

| <u>Sample</u> | <u>Pregnanediol</u><br><u>Added</u><br><u>mg.</u> | <u>Pregnanediol</u><br><u>Measured</u><br><u>mg.</u> |
|---------------|---------------------------------------------------|------------------------------------------------------|
| 1             | 0.0                                               | 0.75                                                 |
| 2             | 0.5                                               | 0.9                                                  |
| 3             | 1.0                                               | 1.3                                                  |
| 4             | 4.0                                               | 3.9                                                  |

Conditions: 3' XE 60, 250° C.



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Table No. 3 - Recovery of pregnanediol from urine hydrolysate

| <u>Sample</u> | <u>Pregnanediol<br/>Added</u><br>mg. | <u>Pregnanediol<br/>Measured</u><br>mg. |
|---------------|--------------------------------------|-----------------------------------------|
| 1             | 0.0                                  | 5                                       |
| 2             | 0.0                                  | 2.3                                     |
| 3             | 5.5                                  | 6.0                                     |
| 4             | 5.5                                  | 5.7                                     |
| 5             | 11.0                                 | 9.2                                     |
| 6             | 11.0                                 | 11.4                                    |
| 7             | 16.5                                 | 15.0                                    |
| 8             | 16.5                                 | 23.0                                    |

Conditions: 3' NGS, 235° C.

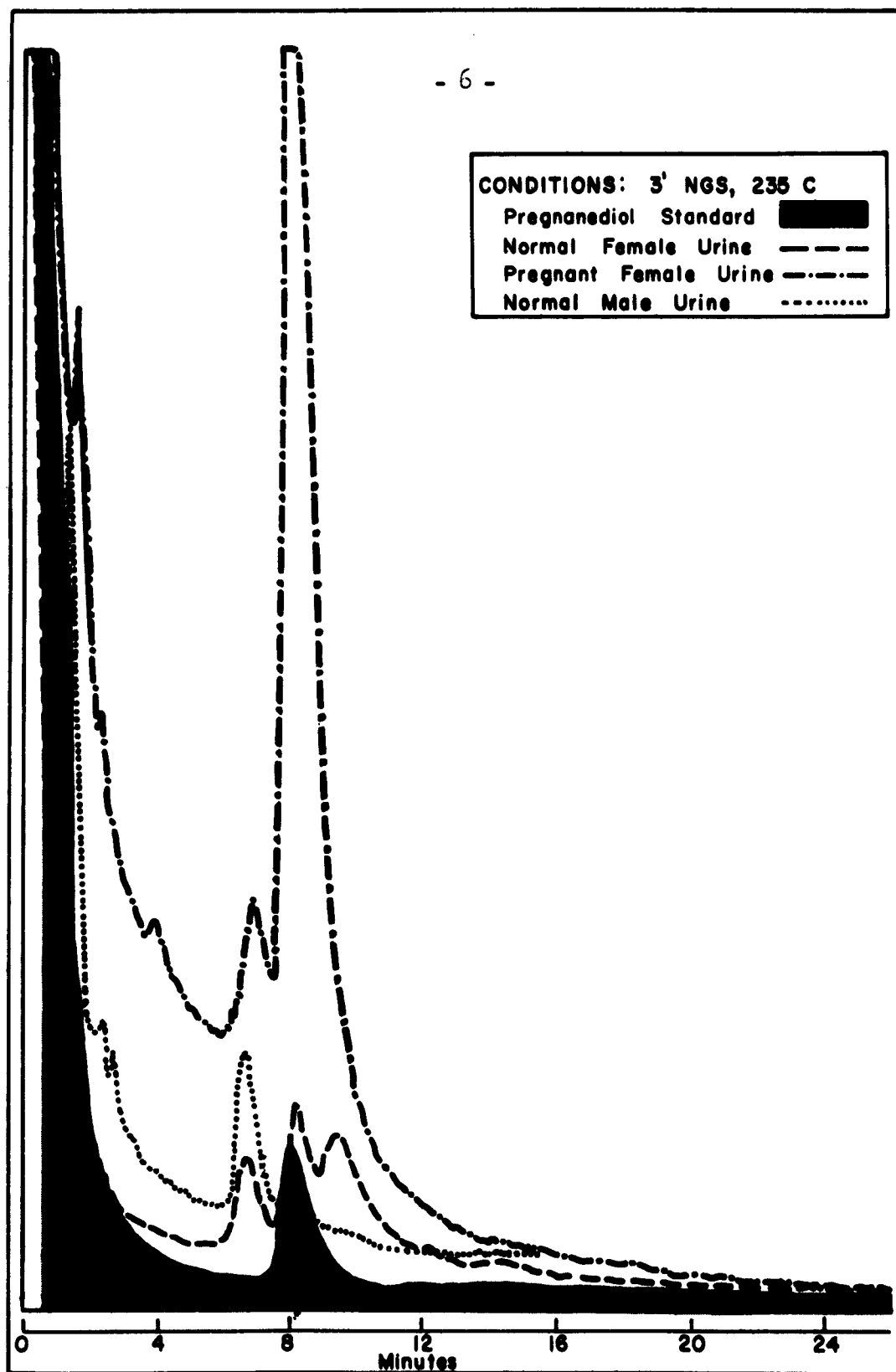


Figure No. 1 Composite Chromatogram of Human Urine Extract

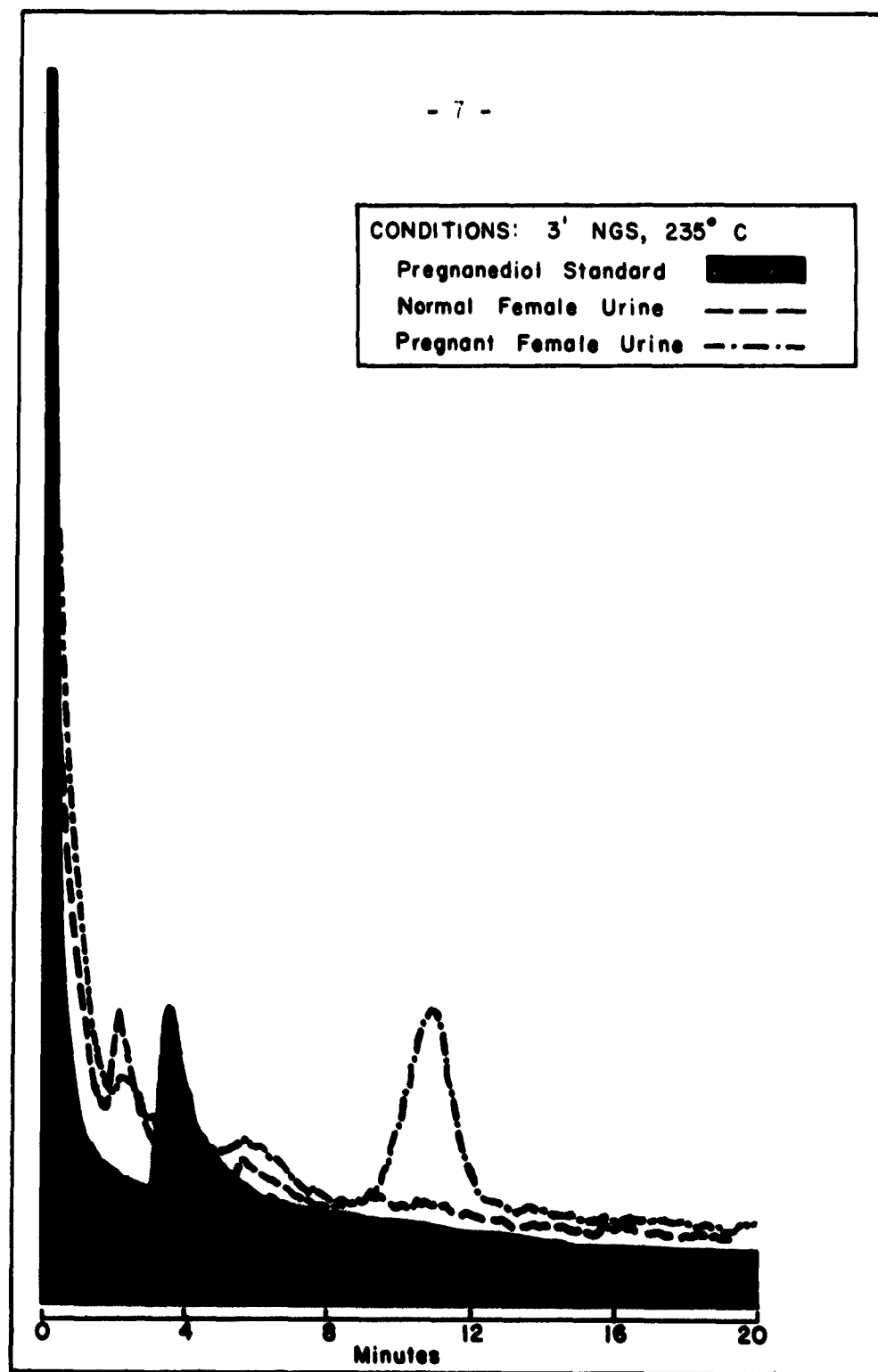


Figure No. 2 Composite Chromatogram of Monkey Urine Extract

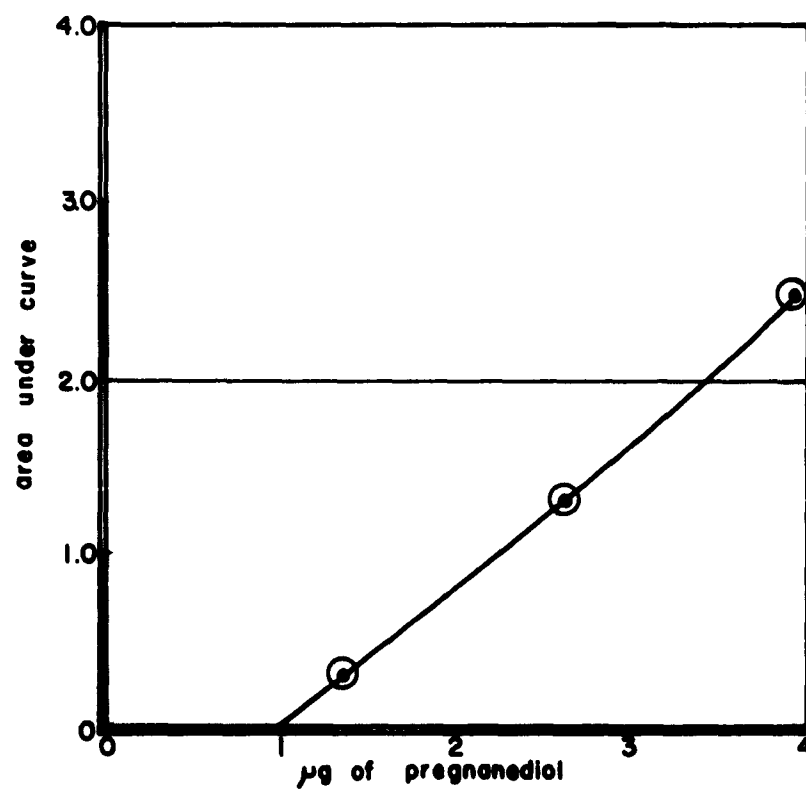


Figure No. 3 — Standard Curve of Pregnanediol on NGS

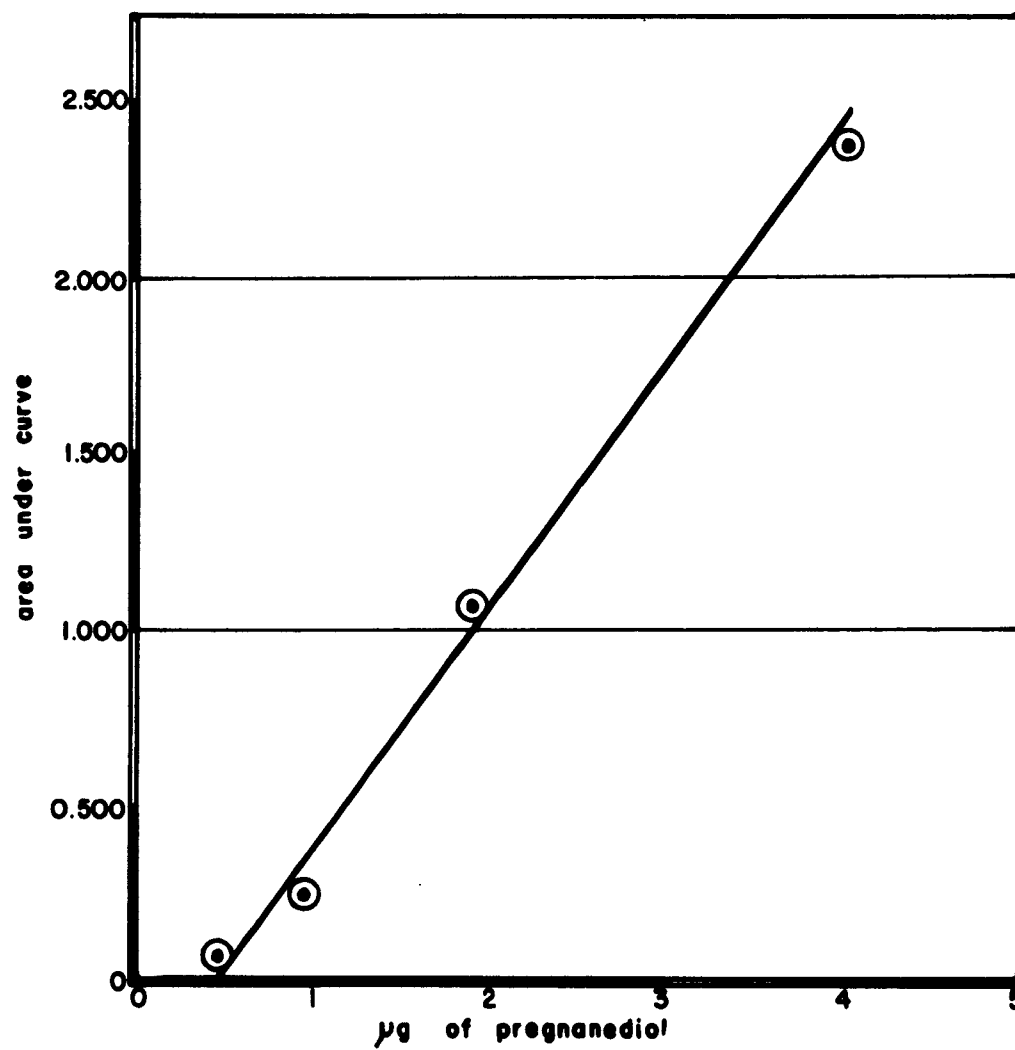


Figure No. 4 — Standard Curve of Pregnanediol on XE 60



## DISCUSSION

From the data presented in Figure No. 1 and Table No. 1, it appears that a compound is extracted from human female urine which has the same retention time as the standard 5 $\beta$  pregnane 3 $\alpha$ , 20 $\alpha$  diol. Additional evidence contributing to the identity of the compound is the large increase in the area of the peak obtained from chromatography of an extract of pregnancy urine. Analysis of an equivalent amount of normal male urine failed to reveal the presence of pregnanediol, indicating the necessity of increasing the sensitivity of the method. Perhaps this can be achieved by using a larger aliquot of urine for hydrolysis, removing the background material by purification of the extract, or improving the resolution of the peaks by altering the operating parameters of the gas chromatograph.

Examination of the standard curves shows that there is a linear relationship between the area under the curve and the micrograms of pregnanediol in the range of 1 to 5  $\mu$ g. However, the recovery studies showed considerable variation between duplicate samples. This may be due to a change in column characteristics from substances in the urine extract. Thus in order to obtain precise measurement of the compound, it may be necessary to incorporate an internal standard which will reflect any variation due to the method itself.

The results obtained from analysis of the monkey urines (see Figure No. 2) indicate that metabolism of progestational compounds in the monkey is different from that in the human. It appears that a compound similar to pregnanediol, but of greater polarity, is excreted by the female monkey.



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#### SUMMARY

The progress of the development of a method for the analysis of urinary pregnanediol is described. The urine is subjected to enzymic hydrolysis, solvent extraction and the pregnanediol is determined by gas-liquid chromatography.

Submitted by Edward A. Garlock  
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