A Method for the Estimation of 6-Oxygenated Metabolites of Progesterone in Urine

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1. A method is described for the estimation of the 6-oxygenated metabolites of progesterone in urine. After hydrolysis the extract of urine is chromatographed on alumina to obtain a fraction containing mainly the 6-oxygenated metabolites. This fraction is oxidized to convert the metabolites into pregnane-3,6,20-triones, which are estimated as the dinitrophenylhydrazones. 2. The reliability criteria of the method are presented. Normal subjects excrete 0.1–0.6 mg./day, and at the end of pregnancy values of 3.3–11.6 mg./day are obtained.

Pregnanediol (5 β -pregnane-3 α ,20 α -diol), which is the major metabolite of progesterone in the urine, accounts for 5–20% of the administered hormone in humans. The use of labelled progesterone has shown that up to 10% of the administered dose of hormone may be excreted as metabolites that are more polar than pregnanediol (cf. Harkness & Fotherby, 1963; Romanoff, Morris, Welch, Grace & Pincus, 1963). That some of these polar metabolites contain an oxo or hydroxyl group at position 6 (the 6-oxygenated metabolites) was suggested by the isolation of three of the isomeric 3,6-dihydroxy-pregnane 20-oxo steroids from urine by Dobriner and his colleagues (cf. Fieser & Fieser, 1959) and the demonstration of the presence of pregnane-3,6,20-triols in the urine of pregnant women (S. Kamyab & K. Fotherby, unpublished work). Further, radioactive 3 α ,6 α -dihydroxy-5 β -pregnan-20-one was isolated from urine by Harkness & Fotherby (1961) after the administration of [4-¹⁴C]progesterone to an ovariectomized-adrenalectomized woman. To assess the importance of the 6-oxygenated steroids in the metabolism of progesterone the method described below was developed for the estimation of these metabolites in urine. In this method the 3,6-dihydroxypregnane 20-oxo steroids and the pregnane-3,6,20-triols in the extract of urine are separated from most of the urinary steroids by chromatography on alumina. By oxidation with chromic acid the 6-oxygenated metabolites are converted into the pregnane-3,6,20-triones, which are then separated from other oxidation products on a second alumina column and estimated by the formation of the dinitrophenylhydrazone.

EXPERIMENTAL

Materials

Ethanol (absolute) was refluxed with KOH pellets and twice distilled. Benzene (A.R.) and light petroleum (b.p. 80–100°) (A.R.) were redistilled. Chromic acid reagent was prepared by dissolving 1.8 g. of CrO₃ in 2.4 ml. of conc. H₂SO₄ and making up to 10 ml. with water. The dinitrophenylhydrazine reagent contained 250 mg. of dinitrophenylhydrazine (A.R.) in 50 ml. of ethanol and 0.8 ml. of conc. HCl. Benedict's reagent contained 2.4 g. of CuSO₄·5H₂O, 17.3 g. of sodium citrate and 10 g. of Na₂CO₃ in 200 ml. of water. Alumina (Hopkin and Williams Ltd., Chadwell Heath, Essex; MFC, purified, neutral, Brockman activity I) was not deactivated for the first column; the activity of different batches was checked with 3 α ,6 α -dihydroxy-5 β -pregnan-20-one to ensure that it was of the right activity. For the second column 15 ml. of water was added to 100 g. of the alumina and the mixture stirred until all lumps had been broken down. The activity of the alumina was then checked with 5 α -pregnane-3,6,20-trione. Water-saturated solvents were used for eluting the steroids from the second columns, which were run at about 20°. For the paper chromatography of the pregnanetriones and compounds of a similar polarity the system light petroleum–benzene–methanol–water (4:1:4:1, by vol.) was used, and the solvent system benzene–chloroform–methanol–water (4:1:4:1, by vol.) was used for the paper chromatography of the pregnanetriols, the 3,6-dihydroxypregnane 20-oxo steroids and steroids of similar polarity.

Methods

Hydrolysis and extraction. With urine collections obtained from women during the second or third trimester of pregnancy a 25 ml. sample of urine was usually sufficient for the estimations; with urine collections from pregnant

subjects during the first trimester and non-pregnant subjects it was usually necessary to use 100 ml. of urine.

(a) Enzymic hydrolysis. Urine was adjusted to pH 4.7 with acetic acid, 0.1 vol. of 5M-acetate buffer, pH 4.7, and 1000 units of β -glucuronidase/ml. were added and the mixture was well shaken. After incubation for at least 18 hr. at 37° the urine was extracted with 2 vol. of chloroform, the chloroform extract was washed once with 0.1 vol. of 0.1N-NaOH and twice with 0.1 vol. of water, dried with Na_2SO_4 and a sample (40 or 160 ml., depending on the volume of chloroform used) was evaporated to dryness.

(b) Acid hydrolysis. Conc. HCl (4 or 16 ml.) was added to 25 or 100 ml. samples of urine respectively and the urine hydrolysed for 30 min. in an open container in a boiling-water bath. After cooling, the urine was extracted, the extract washed and a sample evaporated to dryness as described under (a).

First chromatographic separation. The dry residue was dissolved in 5 ml. of benzene and transferred by means of a capillary pipette to a column of alumina (3 g.) prepared in benzene. The flask that had contained the residue was washed with a further 5 ml. of benzene, which was also added to the column. When the benzene had almost passed through the column 30 ml. of 3% (v/v) ethanol in benzene was added to the column and the eluate discarded. The 6-oxygenated steroids were then eluted from the column with 30 ml. of 5% (v/v) ethanol in benzene and the eluate evaporated to dryness in a warm-water bath under a stream of air.

Oxidation. The residue from the first column was dissolved in 1 ml. of acetone and 2 drops of chromic acid solution were added. The tubes were shaken to mix the reagents and left to stand for 5 min. Water (10 ml.) was then added and the solution extracted with 20 ml. of chloroform. The aqueous phase was removed and the chloroform washed once with 4 ml. of 0.1N-NaOH and once with 4 ml. of water. The chloroform was dried with Na_2SO_4 and a sample (15 ml.) evaporated to dryness in a warm-water bath under a stream of air.

Second chromatographic separation. The residue from the oxidation was dissolved in 5 ml. of benzene-light petroleum (9:1, v/v) and transferred as described above to a column of deactivated alumina made up in benzene-light petroleum. The tube that had contained the residue was rinsed with a further 5 ml. of the solvent, which was also added to the column. A further 40 ml. of solvent was passed through the column and the eluate discarded. The pregnane-3,6,20-trione was then eluted from the column with 20 ml. of 1% (v/v) ethanol in benzene. Two 9 ml. samples of the eluate for duplicate determinations were evaporated to dryness at about 60° in a stream of air. For estimations on urine specimens from non-pregnant subjects, when low values were expected for the 6-oxygenated metabolites, the colour reaction was performed on an 18 ml. sample of the eluate.

Colour reaction. To the dry residue 0.5 ml. of dinitrophenylhydrazine solution was added, and the tubes were stoppered and placed in a water bath at 60° for 10 min. A reagent blank and a standard of 25 μg . of 5 α -pregnane-3,6,20-trione were also included. Benedict's reagent (1 ml.) was then added to each tube and the unstoppered tubes were placed in a boiling-water bath for 10 min. After cooling 2 ml. of water was added and the dinitrophenylhydrazone extracted with 10 ml. of chloroform. The chloroform was

washed once with 2 ml. of water and dried with Na_2SO_4 . The extinctions of the chloroform solutions were read at 330, 365 and 400 μm and the corrected extinction at 365 μm was calculated by using the formula of Allen (1950).

RESULTS

Hydrolysis and extraction

For studying the extraction of 3 α ,6 α -dihydroxy-5 β -pregnan-20-one and 5 β -pregnane-3 α ,6 α ,20 β -triol from aqueous solutions, 50 μg . of each steroid in 0.2 ml. of ethanol was added to 10 ml. of water and the solution extracted with 2 vol. of benzene, chloroform or ethyl acetate. The extracts were chromatographed on paper and the paper was sprayed with phosphomolybdic acid solution. Benzene failed to extract completely either of the compounds, and chloroform extracted all of the 20-oxo steroid and about 95% of the pregnane-triol. Ethyl acetate would extract the triol completely, but for convenience chloroform was used.

In the initial experiments enzymic hydrolysis was used to release the steroids from their conjugates. The results, however, were inconsistent, and there was a tendency for the amount of 6-oxygenated steroids that were extractable from urine to increase as the urine became older, suggesting that enzymic hydrolysis was incomplete and that bacterial contamination of the urine was assisting the hydrolysis. For these reasons hydrolysis with acid was tried. Table 1 shows a comparison of the amount of 6-oxygenated steroids liberated by acid and by two β -glucuronidase preparations, one from ox liver (Ketodase) and one from the

Table 1. *Efficiency of enzymic and acid hydrolysis of conjugates of 6-oxygenated steroids*

Urine sample	Amount of 6-oxygenated metabolites released by hydrolysis (mg./24 hr.)		
	With enzyme preparation		
	Ketodase	Limpet	With acid
A	3.65	4.2	4.7
B	11.0	13.2	13.3
C	3.2	4.4	5.7
D	8.5		9.0
E	2.9		4.4
F	2.6		4.6
G	4.3		6.2
H	4.7		5.6

limpet. Although with some urines the amount of 6-oxygenated steroids released by acid and enzyme were the same, in most cases enzymic hydrolysis gave lower values. The use of acid hydrolysis also had another advantage: many of the steroids present in urine that might give rise in subsequent stages of the method to products interfering in the colour reaction were eliminated. Thus 5 β -pregnane-3 α ,17 α ,20 α -triol was completely destroyed, as were also most of the 16-hydroxy steroids; less than 25% of 3 β ,16 α -dihydroxy-5 β -pregnan-20-one survived the hydrolysis. Any 11 β -hydroxy steroids in urine were dehydrated, and interference in the method from any compounds likely to give rise to androstane-3,11,17-triones or pregnane-3,11,20-triones was minimized. In model experiments with 3 α ,11 β -dihydroxy-5 β -androstane-17-one it was shown that under the hydrolysis conditions 80–85% of this steroid was converted into the $\Delta^9(11)$ compound. However, acid hydrolysis also caused changes in the 6-oxygenated metabolites. If 3 α ,6 α -dihydroxy-5 β -pregnan-20-one was hydrolysed with acid and the products were oxidized and chromatographed on paper, two spots giving a positive Zimmermann reaction of about equal intensity were seen. The more polar of these had R_F similar to that of 5 β -pregnane-3,6,20-trione. The less polar was presumably 5 β ,17 α -pregnane-3,6,20-trione, since the formation of 17 α -pregnanes from the normal compounds by heating with acid has been described by Lieberman, Dobriner, Hill, Fieser & Rhoads (1948). In the solvent system benzene–light petroleum–methanol–water (4:1:4:1, by vol.) 3 α ,6 α -dihydroxy-5 β -pregnan-20-one and the 17 α -isomer apparently have the same R_F (0.38), since chromatography of the residue obtained by boiling the former steroid with acid showed the presence of only one compound and this had the same R_F as the standard steroid.

First chromatographic separation

The purpose of the initial chromatography of the urine extract on alumina was to obtain as far as possible a fraction containing the 3,6-dihydroxy-pregnane 20-oxo steroids and the pregnane-3,6,20-triols and which did not contain any steroids likely to interfere in the subsequent stages of the method. In this respect it was essential to exclude from the fraction containing the 6-oxygenated steroids the 3 α -hydroxy-5 α - and 3 α -hydroxy-5 β -androstane-11,17-diones, and any 11 β -hydroxy-17-oxo steroids that were present in extracts of enzyme-hydrolysed urine or that had escaped dehydration during acid hydrolysis. The androstane-3,11,17-triones produced from 11-oxygenated steroids during the chromic acid oxidation cannot be separated from the

pregnane-3,6,20-triones on the second alumina column, and would therefore have interfered in the estimation. By passing through the column 30 ml. of 3% ethanol in benzene it was possible to elute most of the steroids known to occur in urine that might be oxidized to a triketone compound in the subsequent oxidation. Thus with pure steroids it was shown that this volume of 3% ethanol in benzene would elute the 3 α -hydroxy-5 α - and 3 α -hydroxy-5 β -androstane-11,17-diones completely and more than 90% of 3 β ,16 α -dihydroxy-5 α - or 3 α -hydroxy-5 β -pregnan-20-one, 5 α - or 5 β -pregnane-3 β ,16 α ,20 β -triol and 3 α ,11 β -dihydroxy-5 β -androstane-17-one.

When more than 30 ml. of 3% ethanol in benzene was used the 3 α ,6 α -dihydroxy-5 β -pregnan-20-one began to be eluted. After passing 30 ml. of 3% ethanol in benzene through the column this steroid could be eluted with 30 ml. of 5% (v/v) ethanol in benzene (mean recovery 98%, six experiments); however, the mean recovery of the pregnane-3,6,20-triol was slightly lower (85%, six experiments). When extracts of enzyme-hydrolysed urine were used this second fraction also contained most of the 5 β -pregnane-3 α ,17 α ,20 α -triol present in the urine. To show that there was no loss of the 17 α -pregnane isomer during the first chromatographic separation samples of the 3 α ,6 α -dihydroxy-5 β -pregnan-20-one were boiled with acid and further samples were boiled with acid and chromatographed on alumina as described above. There was no difference between the recoveries of the boiled and extracted standard and those of the samples that had also been chromatographed.

Oxidation and second chromatographic separation

For the oxidation the method of Bowers, Halsall, Jones & Lewin (1953) employing chromium trioxide in acetone was used. The time required for oxidation of 3 α ,6 α -dihydroxy-5 β -pregnan-20-one was not critical; the recovery after oxidation for 5, 10, 15, 30 or 60 min. was found to be quantitative even in the presence of a urine extract. Similarly, when eluates from the chromatography of urine extracts were oxidized the same values were obtained after oxidation times of 5, 10 or 30 min., and 5 min. was therefore chosen as a convenient oxidation time. The oxidation of 3 β ,16 α -dihydroxy-5 α - or 3 β ,16 α -dihydroxy-5 β -pregnan-20-one or of 5 α - or 5 β -pregnane-3 β ,16 α ,20 β -triols was somewhat variable. Most of the compounds appeared to be destroyed, although small amounts of two compounds giving reddish-brown colours in the Zimmermann reaction on paper chromatograms were often observed. These two compounds accounted for only a small proportion of the 16-hydroxy steroid oxidized.

5 β -Pregnane-3 α ,17 α ,20 α -triol was completely converted into 5 β -androstane-3,17-dione. Compounds of the above types that would be present in eluates from extracts of enzyme-hydrolysed urine were therefore eliminated during the oxidation.

The mixture obtained after oxidation was chromatographed on deactivated alumina to separate the pregnane-3,6,20-triones from the oxidation products of the pregnane-3,16,20-triols and 5 β -pregnane-3 α ,17 α ,20 α -triol. The latter two groups were eluted from the column with 40 ml. of benzene–light petroleum (9:1, v/v), and the pregnane-3,6,20-trione and its isomers were then eluted with 1% ethanol in benzene. This fraction would also have contained any 5 α - or 5 β -androstane-3,11,17-trione and 5 α - or 5 β -pregnane-3,11,20-trione resulting from the oxidation step.

Colour reaction

Although the Zimmermann reaction was of use in detecting the pregnanetriones on paper chromatograms, it was of less use for the quantitative estimation of these compounds. The absorption spectra of the complex formed by 5 α -pregnane-3,6,20-trione in the Zimmermann reaction was rather flat with a maximum at 490 m μ , and the corrected extinction at 490 m μ given by 50 μ g. of the steroid was only about 0.1. A more sensitive means of estimating the pregnanetrione was the application of the method of Reich, Sanfilippo & Crane (1952) with dinitrophenylhydrazine. The dinitrophenylhydrazone of the steroid gave a peak extinction at 365 m μ , and the corrected extinction at 365 m μ was obtained by measuring the extinctions at 330, 365 and 400 m μ and using the correction formula described by Allen (1950). The corrected extinction of 25 μ g. of 5 α -pregnane-3,6,20-trione was about 0.35.

In investigating the reaction it was found that a

higher extinction was obtained by forming the dinitrophenylhydrazone at 60°, rather than overnight at room temperature as recommended by Reich *et al.* (1952). The results presented in Table 2 show that 10 min. at 60° was sufficient for complete reaction; this time was selected as being the most convenient but was obviously not critical. The relationship between the corrected extinction at 365 m μ and the steroid concentration of 5 α -pregnane-3,6,20-trione is linear. With most urines duplicate values agreed whether calculated by using the extinction at 365 m μ or the corrected extinction at this wavelength. In practice, however, the corrected extinction has been used for the calculation of the results, since with about 20% of the urine samples the duplicate values at 365 m μ did not agree, although the corrected extinctions did. The colour reaction was also carried out with other steroids and the results are shown in Table 3. The reaction provided a simple sensitive means of estimating ketone compounds, particularly the saturated diones and triones in the pregnane series. It appears that the 17 α -hydroxy group slightly depresses colour formation at the C-20 oxo group. The increase in extinction with increase in the number of reactive ketone groups in the molecule suggests that all three oxo groups of pregnane-3,6,20-trione react.

Evaluation of the method

The recovery of 3 α -6 α -dihydroxy-5 β -pregnane-20-one added in ethanol to either water or urine after hydrolysis is shown in Table 4. The recovery

Table 2. *Effect of varying time and temperature of reaction on the formation of the dinitrophenylhydrazone of 5 α -pregnane-3,6,20-trione*

The colour reaction was performed on 25 μ g. of 5 α -pregnane-3,6,20-trione as described in the Experimental section, except for variations in time and temperature of the reaction. The results are given as means $\pm$ s.d., with the numbers of observations in parentheses.

Time	Temp.	Corrected $E_{365m\mu}$
18 hr.	Room temp. (about 18°)	330 $\pm$ 7 (8)
60 min.	60°	366 $\pm$ 14 (11)
10 min.	60°	362 $\pm$ 10 (8)

Table 3. *Extinction values of steroid dinitrophenylhydrazones*

The colour reaction described in the Experimental section was performed on 25 μ g. of each steroid. Extinction values per μ mole were calculated from the corrected extinction at 365 m μ and are the means of at least four estimations.

	Corrected $E_{365m\mu}/\mu\text{mole}$
3 β -Hydroxyandrost-5-en-17-one	1.49
3 α -Hydroxy-5 α -androstan-17-one	1.47
3 β -Hydroxypregn-5-en-20-one	1.41
3 α -Hydroxy-5 β -pregnan-20-one	1.24
3 α ,6 α -Dihydroxy-5 β -pregnan-20-one	1.53
3 α -Hydroxy-5 β -androstane-11,17-dione	1.19
3 β ,17 α -Dihydroxypregn-5-en-20-one	1.08
3 α ,17 α -Dihydroxy-5 β -pregnane-11,20-dione	1.06
5 α -Pregnane-3,20-dione	2.68
3 β -Acetoxy-5 α -pregnane-6,20-dione	2.52
5 α -Pregnane-3,11,20-trione	2.78
5 α -Pregnane-3,6,20-trione	3.84

Table 4. Recovery of 3 α ,6 α -dihydroxy-5 β -pregnan-20-one

The steroid in 0.2 ml. of ethanol was added to 25 ml. of water or urine after hydrolysis. The results are given as means $\pm$ S.D., with the numbers of observations in parentheses.

Fluid used	Amount of steroid added (μ g.)	Recovery (%)
Water	50	88 $\pm$ 4.3 (6)
Urine from normal humans	50	90 $\pm$ 7.2 (6)
Urine from pregnant subjects	50	84 $\pm$ 16.6 (9)
	100	80 $\pm$ 2.6 (6)

of 5 β -pregnane-3 α ,6 α ,20 β -triol was lower than that of the 20-oxo steroid, owing to a loss of about 15% in the first chromatographic separation, and to an increased lability of the pregnanetriol to oxidation; recoveries of pregnanetriol have been in the range 60–65%. These values do not take into account any losses that occurred during hydrolysis. The precision of the method has been calculated (Snedecor, 1952) from the difference between the results of duplicate determinations of a series of 34 estimations and was found to be 6%.

The values found for the excretion of 6-oxygenated metabolites by 15 normal males was in the range 0.1–0.4 mg./day, and for ten normal females it was in the range 0.1–0.6 mg./day. The amounts excreted by pregnant subjects were much greater: 16 women in the eleventh to sixteen week of pregnancy excreted 0.5–2.4 mg./day and at the end of pregnancy the range was 3.3–11.6 mg./day (25 subjects). In these subjects the ratio of the excretion of 6-oxygenated metabolites to pregnanetriol varied in the range 0.08–0.25:1.

For urines that are expected to contain more than 2 mg. of 6-oxygenated steroids/day a 25 ml. sample of the 24 hr. urine collection is sufficient for the estimation. The sensitivity of the method can be increased by using a larger volume of urine, by using an 18 ml. sample of the eluate from the second chromatographic separation instead of a 9 ml. one or, instead of measuring the extinction of the chloroform extract of the dinitrophenylhydrazones, by evaporating the extract to dryness and measuring the extinction after dissolving the residue in a small volume of solvent.

Since the pregnane-3,6,20-trione is estimated by forming the dinitrophenylhydrazone and measurement of the resultant colour, the specificity of the method depends on the purification achieved during the chromatographic separations. Evidence that the fraction eluted with 1% ethanol in benzene from the second column does in fact only contain pregnane-3,6,20-trione is presented below.

Paper chromatography of the eluate from the second column. The Zimmermann reaction with either 5 α - or 5 β -pregnane-3,6,20-trione on paper was characteristic in that on gentle heating a blue-grey spot was formed that after about 30 min. faded to a brownish-grey colour and after about 24 hr. had almost completely disappeared. One possible contaminant in this fraction, namely androstane-3,11,17-trione, had the same R_F as 5 α -pregnane-3,6,20-trione, but in the Zimmermann reaction gave an intense violet colour that did not fade after 24 hr. Thus by chromatographing a mixture of androstane-3,11,17-trione and 5 α -pregnane-3,6,20-trione it was possible to detect small amounts of the former steroid in the presence of the latter.

On a number of occasions 25 and 100 ml. samples of urine collected from normal males, from normal females during the follicular and luteal phases of the menstrual cycle and from women at various stages of pregnancy have been processed as detailed in the Experimental section with the exception that, instead of the colour reaction being done on the final eluate, the eluate has been chromatographed on paper. These papers were then sprayed with the Zimmermann reagent and, in all urines hydrolysed by enzyme, only a single spot was seen and this had the same R_F and the same characteristics in the Zimmermann reaction as 5 α -pregnane-3,6,20-trione. In extracts of urine that have been hydrolysed by acid two blue-grey spots were seen after spraying with the Zimmermann reagent and heating, and both faded in a similar manner to that of authentic 5 α -pregnane-3,6,20-trione. One of these had the same R_F as authentic 5 α -pregnane-3,6,20-trione and the other was slightly less polar. As indicated above this less polar spot was undoubtedly the 17 α -steroid, and it could also be produced by boiling 5 α -pregnane-3,6,20-trione with acid. The possibility that the second spot might be a dehydration product of a 6-hydroxy steroid was eliminated by comparing the products of the reduction with sodium borohydride of both authentic 5 α -pregnane-3,6,20-trione and the less polar spot. Paper chromatography showed that similar products were produced, whereas had dehydration occurred the reduction product of the artifact would have been much less polar than pregnane-3,6,20-triol.

None of the extracts obtained from processing 25 ml. samples of pregnancy urine contained any trace of androstane-3,11,17-trione. Occasionally a trace of this steroid was found in the eluate from the second column when 100 ml. of pregnancy urine and enzymic hydrolysis were used; however, even under these conditions when the columns were possibly overloaded contamination with androstane-3,11,17-trione would not have caused an error in the estimations greater than 5%.

Sodium borohydride reduction of the eluate from the second column. With enzyme- or acid-hydrolysed urine the residue from the eluate of the second chromatographic separation and 50 μ g. samples of 5 α -androstane-3,11,17-trione, 5 β -androstane-3,11,17-trione, 5 α -pregnane-3,11,20-trione, 5 α -pregnane-3,6,20-trione and 5 β -pregnane-3,6,20-trione were reduced for 30 min. with sodium borohydride in ethanol and chromatographed. On spraying the paper chromatograms with phosphomolybdic acid solution two spots were obtained from the reduction of the urine residue and these spots had R_f values (0.38 and 0.46) similar to those of the two spots obtained by reduction of 5 α -pregnane-3,6,20-trione. Reduction of 5 β -pregnane-3,6,20-trione gave a major spot with R_f 0.42 and two minor spots (R_f 0.35 and 0.47). Both 5 α -androstane-3,11,17-trione and 5 β -androstane-3,11,17-trione on reduction gave one major spot each with R_f 0.39 and 0.47 respectively; minor products with R_f 0.47 with the former compound and R_f 0.39 and 0.50 with the latter compound were also observed. Reduction of 5 α -pregnane-3,11,20-trione gave three very faint spots, all of which ran in front of the pregnane-3,6,20-triols.

In this way it was possible to distinguish 5 α -pregnane-3,6,20-trione from the other steroids tested except for 5 α -androstane-3,11,17-trione.

Elution of pregnanetriolone from paper chromatograms. The possibility of the final fraction containing substances with R_f different from that of the pregnane-3,6,20-trione that might interfere in the colour reaction was eliminated by dividing the eluate from the second column into two halves, chromatographing each on paper and spraying one half with the Zimmermann reagent. The unsprayed part of the paper was then divided into three parts. The first consisted of the area of paper containing the pregnane-3,6,20-trione, the second part contained the compounds more polar than the 3,6,20-trione and the third part contained any compound less polar than the 3,6,20-trione. These three separate parts of the paper were then eluted with ethanol and the colour reaction was performed on the residues obtained by evaporation of the ethanol. The intensity of colour obtained from the other parts of the paper was never more than 10% of that given by the pregnane-3,6,20-trione area.

Absorption spectra of the eluate from the second column. Further proof of the specificity was obtained by measuring the absorption spectra of the solution obtained by treating the residue from the eluate of the second column with the Zimmermann reagents. 5 α -Pregnane-3,6,20-trione gave a rather flat curve with a broad peak at 490 $m\mu$, whereas 5 α -androstane-3,11,17-trione gave a curve with a sharp peak at 520 $m\mu$. The spectrum of the eluate from the second column showed a curve

similar to that obtained with pregnane-3,6,20-trione and without the presence of even a shoulder at 520 $m\mu$.

DISCUSSION

The use of labelled progesterone has shown that compounds that are more polar than pregnanediol may be quantitatively important metabolites of the hormone (Harkness & Fotherby, 1963; Romanoff *et al.* 1963). One component of this polar fraction undoubtedly contains metabolites of progesterone containing an oxo or hydroxyl group at position 6 (cf. Fotherby, 1964). No estimates of the amounts of 6-oxygenated metabolites of progesterone excreted in urine have previously been made and the role of 6-hydroxylation in the metabolism of progesterone remains to be elucidated. The use of radioactive progesterone for the measurement of the polar fraction has only a limited application and the development of a chemical method seemed desirable. The method described above provides a simple and satisfactory way of measuring the urinary excretion of the metabolites of progesterone oxygenated at position 6. It has been shown to measure the pregnane-3,6,20-triols and the 3,6-dihydroxypregnane 20-oxo steroids, and it would possibly also measure the other dihydroxy mono-oxo isomers. It would not, however, measure the pregnane-3,6,20-triones or the monohydroxydioxo compounds, although preliminary experiments indicate that only very small amounts of these steroids are present in urine; in these experiments the urine extract was reduced with sodium borohydride before the first chromatographic separation to convert all the 6-oxygenated steroids into pregnane-3,6,20-triols.

The conversion of the 6-oxygenated compounds into pregnane-3,6,20-triones and the application of the dinitrophenylhydrazone reaction provides a sensitive means of estimating the 6-oxygenated metabolites. The method is also specific whether hydrolysis by enzyme or acid is employed; the latter method is to be preferred since it is not only more convenient but also leads to the destruction of a number of steroids likely to give rise to products interfering in the colour reaction. In checking the specificity it was shown that, when the eluate from the second column was reduced with sodium borohydride, the chromatographic behaviour of the resulting pregnane-3,6,20-triols was the same as that of the pregnanetriols obtained by reduction of 5 α -pregnane-3,6,20-trione but different from that obtained from the 5 β -pregnane-3,6,20-trione. This finding might suggest that most of the 6-oxygenated metabolites of progesterone in urine had the 5 α -configuration, a finding consistent with the fact that two of the 3,6-dihydroxypregnane

20-oxo steroid isomers isolated from urine had the 5α -configuration. However, more work is necessary before the $5\alpha:5\beta$ ratio of the metabolites can be ascertained with certainty.

Although it has been assumed in the present paper that the 6-oxygenated compounds arise solely from the metabolism of progesterone, the possibility must be borne in mind that some of the compounds measured might arise from 6-hydroxy- or 6-oxo-progesterone secreted by the ovary, the placenta or the adrenal; hydroxylation of progesterone at position 6 has been shown to occur with ovarian, placental, adrenal, foetal and liver tissue (cf. Fotherby, 1964). The contribution of these compounds to the 6-oxygenated metabolites in urine is at present under investigation.

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