Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 673–679. Pergamon Press. Printed in Great Britain

AN ENZYMATIC METHOD FOR THE DETERMINATION OF HORMONAL STEROIDS IN THE URINE

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(*Received* 21 *December* 1973)

SUMMARY

A method is presented for routine determination of urinary steroids. It is based on the specific enzymatic conversion of the 3α-OH group on the steroid molecule into a 3-keto-group. Quantitation is obtained by measurement of the absorption at 340 nm from NADH, following a simple hydrolysis, extraction and purification. There are, however, disadvantages. Urinary blank values are high and difficult to eradicate without loss of the most polar corticosteroids. For the steroid biochemist the enzymatic method represents an approach that may have a variety of applications.

INTRODUCTION

The number of chemical methods for the determination of the various hormonal steroids in urine and blood is at present very large and is steadily increasing. The advances in this field in recent years have mainly been towards more sensitive and specific procedures for the assay of individual hormones and their metabolites. In the diagnostic work on human beings, these new methods have been valuable tools in the assessment of physiological and pathophysiological states. In the diagnostic situation there remains, however, a definite need for assays of a more encompassing and technically less demanding type, giving information of the total steroid output. Such methods find their application in screening tests for adrenocortical disease, and are used with advantage in conjunction with functional studies where the response to stimulation or suppression of the adrenal cortex is assessed. For this purpose most laboratories today employ modifications of the procedure described by Porter and Silber[1, 2] or that of Norymberski *et al.*[3]. The former has the principal weakness of measuring only steroids with the dihydroxyacetone side-chain, thus excluding part of the total number of metabolites from the adrenocortical secretion. The latter is adequate in this respect, but involves the technical step of oxidation which is subject to interference by reducing substances in the urine such as glucose. The choice of oxidizing agent is critical[4–6]. In our hands the oxidation step has at times been rather erratic. This is easily discovered in a large and regular routine of these assays, but is a definite disadvantage for the more occasional user.

The basic principle of the steroid determinations presented in this paper was introduced by Hurlock and Talalay[7] (see also Talalay[8]). It employs the 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) for the specific oxidation of the 3α-OH group of the steroid molecule after hydrolysis. In comparison with the method originally presented by the above workers, where a partially purified enzyme [containing small, but significant amounts of 3β (or 17β)-hydroxysteroid dehydrogenase (EC 1.1.1.51.)] was used, the enzyme used in this study is a highly purified preparation devoid of any steroid converting enzymes [9, 10]. It is commercially available in a stabilized form.

In practice the procedure is rapid and puts little demand on equipment. It is well suited for automation. It is highly specific and is not subjected to interference by other substances present in the urine in accordance with the nature of enzymatic reactions. Steroid metabolites from testosterone, androstenedione and progesterone will be measured together with the corticosteroids. An interference from these compounds when the method is used for adrenal cortical evaluation in connection with tests in suppression and stimulation must be considered, but only infrequently causes problems in the interpretation. Problems, particularly with regard to the urinary blank value and the recovery of the most polar corticosteroids, will be discussed.

MATERIALS AND METHODS

Abbreviations and trivial names

NAD and NADH	= nicotinamide adeninedinucleotide, oxidized and reduced form, respectively
3α-OH-steroids	= 3α-hydroxysteroids
17 KS	= 17-keto-steroids
17 KGS	= 17-ketogenic steroids
THE	= Tetrahydrocortisone
THF	= Tetrahydrocortisol
DHA	= Dehydroepiandrosterone
Pregnandiol	= 3α,20α-dihydroxy-5β-pregnane
Pregnantriol	= 3α,17α,20α-trihydroxy-5β-pregnane
Cortol-20α	= 3α,11β,17α,20α,21-pentahydroxy-5β-pregnane
Cortol-20β	= 3α,11β,17α,20β,21-pentahydroxy-5β-pregnane
Cortolone-20α	= 3α,17α,20α,21-tetrahydroxy-5β-pregnane-11-one
Cortolone-20β	= 3α,17α,20β,21-tetrahydroxy-5β-pregnane-11-one
DOC	= 17α,21-dihydroxy-4-pregnane-3,20-dione
Metopirone	= (Su-4885) 2-methyl-1,2-bis (5-pyridyl)-1-propanone
Dexametasone	= 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione
Etifollin	= Ethynylestradiol = 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol

All enzymatic determinations were performed with purified 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) from *Pseudomonas testosteroni* (ATCC 11996)[11]. One milligram of the lyophilized enzyme corresponds to 2·5–5·0 U, where 1 U is the amount of enzyme that will reduce 1 μmol 3α-OH-steroid/min at 25°C and pH = 9·5. At this pH the equilibrium constant (K_H) (for the oxidation of androsterone) has been found to be $9{\cdot}2 \times 10^{-9}$ M[12]. This preparation is stable for at least 2 years at room temperature. When dissolved, it is stable *one* day at room temperature (or refrigerated). For longer periods (up to two weeks), it must be kept frozen. The enzyme was dissolved in 0·1 mol/l of hydrazine hydrate pH = 9·5. This compound will combine with the formed 3-keto-steroids, and thus lead to quantitative conversion of the 3α-OH-steroids present in the test solutions.

The assay system. To 2·9 ml of the complete enzyme solution (that is 1 U of enzyme, 0·5 μmol NAD, 10^{-3} M EDTA and 10^{-3} M Cleland's reagent all dissolved in 0·1 M hydrazine hydrate, pH = 9·5) is added 0·1 ml of ethanolic urine extract or steroid standards.

The enzyme 3α-hydroxysteroid dehydrogenase is available with cofactors from Nyegaard & Co. A/S, Oslo as kit "Sterognost-3α".

Steroids used as standards etc. (see text) were purchased from Sigma Chem. St. Louis, USA, Mann Res. Lab., N.Y. USA and Ikapharm, Ramat-Gan, Israel.

Other chemicals were delivered from British Drug Houses Ltd., Poole, England, Koch-Light Lab., Buckinghamshire, England and Difco Laboratories, Detroit, USA.

Urine samples were taken from healthy volunteers and from the daily routine of the Hormone Laboratory, Aker Hospital.

Methods

Ten microlitres of formaldehyde (35% w/v) were added to duplicate samples of 5 ml urine. The samples were shaken briefly and left for some minutes, whereafter 0·15 ml of conc. HCl was added and the urines placed in a boiling water bath for 30 min. After rapid cooling, extractions were performed with ether, 15 ml × 2. The combined extracts were washed with 4 ml of ice cold 0·5 N ammonium hydroxide. The coloured interphase was left with the ether layer, and most of it disappeared during the following wash with 4 ml of distilled water. The purified extract was evaporated to dryness at 50°C under a mild stream of nitrogen in a draught cupboard.

The residues, or evaporated steroid standards were dissolved in 0·4 ml of 96% ethanol. To ensure that all steroid material was dissolved, the ethanol was rolled around at least half the lower part of the tube wall. Four solutions were prepared for incubations at 25°C for 60 min:

(I) 0·1 ml of extract + 2·9 ml of the complete enzyme solution
(II) 0·1 ml of 96% ethanol + 2·9 ml of the complete enzyme solution
(III) 0·1 ml of extract + 2·9 ml of the hydrazine hydrate solution
(IV) 0·1 ml of 96% ethanol + 2·9 ml of the hydrazine hydrate solution.

After 60 min the difference in light absorption at 340 nm between I and II (E_1) and III and IV (E_2) was determined in a spectrophotometer. The specific extinction of NADH, due to steroid transformation was obtained by the subtraction:

$$E_1 - E_2 = \Delta E$$

All measurements were made in pyrex cuvettes with 1 cm light path.

Calculations

The following formula was used:

mg 3α-hydroxysteroid/24 h

$$= \frac{\Delta E \times \bar{M}_w \times D \times 0{\cdot}4 \times 3}{6{\cdot}22 \times 5 \times 0{\cdot}1 \times 1000}$$

where:

D = total amount of urine excreted/24 h (diuresis)
3 = volume of the reaction mixture (cuvette volume)
6·22 = the molar extinction coefficient for NADH
0·4 = total extract volume
0·1 = the part of the ethanol extract added to the assay mixture
5 = volume of the urine sample which was extracted
$\overline{M}_w$ = the molecular weight of the urinary steroids vary from 288 (C_{19}-series) to 368 (reduced C_{21}-steroids).

For calculations of the *milligram* amount of steroid in the urine, it is necessary to use an approximative average molecular weight value. For this 335 was chosen. With insertion of known values, the above formula can be given an abbreviated form:

mg 3α-hydroxysteroid excreted/24 h

$$= \Delta E \times D \times 0{\cdot}13.$$

The misrepresentation of true values resulting from the introduction of an approximative molecular weight can be avoided advantageously by expressing the results in μmol/24 h:

μmol 3α-hydroxysteroid excreted/24 h

$$= \Delta E \times D \times 0{\cdot}39.$$

17-Ketogenic steroids (17 KGS) were determined by the method of Norymberski *et al.*[3] as modified by Diczfalusy *et al.*[4]. The Zimmermann reaction was performed using the organic base, N-benzyltrimethyl ammonium methoxide according to Bongiovanni *et al.*[13]. The 17 KGS result obtained by this method is the difference between the total steroid content of the urine determined by the Zimmermann reaction after oxidation with sodium bismutate, and the 17 KS content determined directly in the same specimen.

RESULTS

Velocity measurements

Since the maximum velocity for the enzyme towards the different substrates varies considerably[5] the time needed for a complete oxidation of two typical steroids —androsterone and THE—was measured at a substrate concentration of 50 μg/3 ml assay volume for both. As a consequence of the results, presented in Fig. 1, all incubation procedures were performed for 60 min at 25°C before readings of optical density at 340 nm were made.

Linearity of assay and volume of urine extract

The linearity of the assay with variations of the extracted urine volume was shown in three different urines. Aliquots varying from 0·0125 to 0·2 ml were added to the complete enzyme solution. The results obtained in one of these tests are shown in Fig. 2.

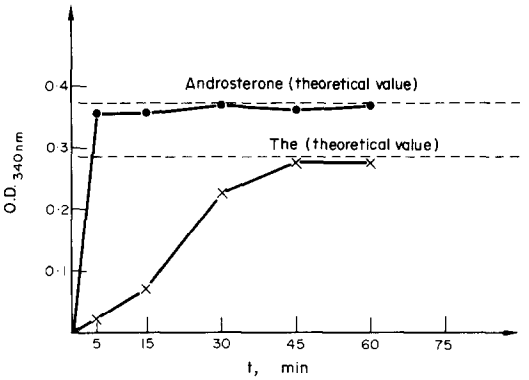

Fig. 1. The time needed for complete oxidation of androsterone (—·—) and THE (— × —) by 3α-HSD. Both substrates were tested at a concentration of 50 μg/3 ml assay volume at pH 9·5 and t = 25°C. Each value is the mean of three determinations.

Assay of pure steroids

The steroids listed in Table 1 were dissolved in 96% ethanol. With few exceptions (noted in the table), the concentrations were arbitrarily chosen to 100 μg/3 ml assay volume. This corresponds, in the urine tests, to 20 mg/l of urine. The recovery values and ranges are given in the table. All determinations were performed in sixplicate.

Four steroids were arbitrarily chosen, androsterone, etiocholanolone, THE and THF for the establishment

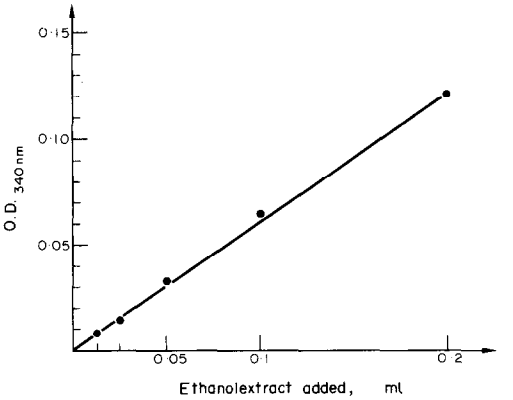

Fig. 2. Linearity of assay of the enzymatic method using varying amounts of urine extract. Assay volume (3 ml) consisting of: x ml of ethanolic urine extract added to (3·0 − x) ml of 3α-hydroxysteroid dehydrogenase in 0·1 M hydrazine hydrate, pH = 9·5 ("Sterognost-3α"). Readings were made after 60 min of incubation at 25°C. Each value is the mean of three determinations.

Table 1. Assay of pure steroids dissolved in 96% ethanol

Steroids tested	Recovery (%)	Range (%)
Androsterone	96	88–108
11β-Hydroxy-Androsterone*	92	77–98
11-Keto-Androsterone*	92	77–94
Etiocholanolone	98	83–102
11β-Hydroxy-Etiocholanolone	94	81–97
11-Keto-Etiocholanolone	93	81–96
Tetrahydrocortisone	90	83–97
Tetrahydrocortisol	91	83–98
Cortol-20α	92	88–96
Cortol-20β*	98	96–101
Cortolone-20α	91	77–98
Cortolone-20β*	98	92–101
Pregnanediol	90	74–95
Pregnanetriol	80	66–94

* Tested with 30 μg/3 ml assay volume, all others tested with 100 μg/3 ml assay volume. All determinations were performed in sixplicate.

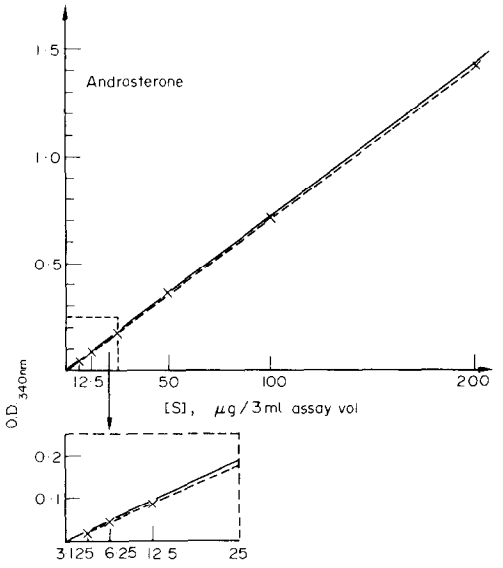

Fig. 3. Enzymatic assay of different concentrations of androsterone. The steroid was added to the assay system as 0·1 ml of ethanolic solution. Readings were made after 60 min of incubation at 25°C. Each value is the mean of three determinations: ·——· theoretical values, ·– – – · measured values.

of standard curves in concentrations varying from 1·56 to 200 μg/3 ml assay volume. These steroids were added as ethanolic solutions (0·1 ml) to the complete enzyme solutions (2·9 ml). After 60 min at 25°C, readings were made at 340 nm in the usual manner. The results for androsterone are presented in Fig. 3.

The response was linear for all four standard steroids over the whole range.

Recovery from urine

Androsterone in various amounts was added to urine samples (5 ml) and subjected to the entire procedure. The results are shown in Fig. 4. The results obtained for etiocholanolone, 11β-hydroxy-etiocholanolone, 11-keto-etiocholanolone, 11β-hydroxy-androsterone and 11-keto-androsterone were similar to those for androsterone.

The recovery of added THE, THF, cortol-20α and cortolone-20α varied from 50 to 75%. The loss occurred mainly with the alkaline and water washing of the ether extract. The polar steroids were closely associated with the coloured interphase between the ether and water layer. It was also found by the use of different organic solvents (ether, methylene chloride and ethyl acetate) that the compounds giving high blank values and the polar steroids were extracted to the same extent. A preservation of the polar steroids in the organic solvent thus resulted in an increased blank value in the final assay. Additional refinement—and loss of simplicity—would be necessary in order to improve on this situation.

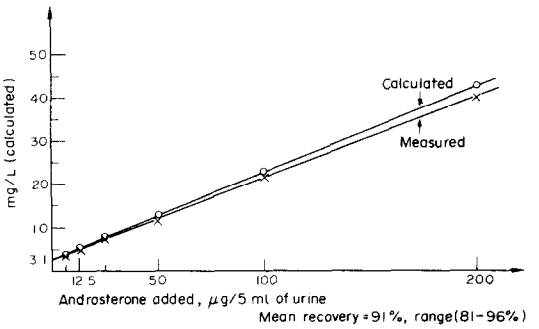

Fig. 4. Recovery of androsterone added, in different amounts, to 5 ml samples of urine, before hydrolysis and extraction. Each value is the mean of five determinations.

Table 2. Duplicate variation in routine urinary determinations

3α-OH steroids				17 ketogenic steroids			
n	Mean	Deviation	Per cent	*n*	Mean	Deviation	Per cent
44	9·6	0·81	8·5	32	8·8	1·07	12·1
24	25·5	1·37	5·4	29	21·7	2·32	11·9

Precision

A comparison of the precision as judged by duplicate variation of 3α-OH-steroid and the 17 ketogenic steroids was performed. The method is shown in Table 2.

Specificity

Urines from each of six different donors were divided into four batches for duplicate determination in 5 ml samples. The first duplicate set served as control (A). To the second were added 100 μg of DHA per sample (B); to the third 100 μg of estriol (C) and to the fourth 100 μg of glucose (D). The steroid determination in these urines gave the following results, respectively. Mean ± S.D.: A: 12·6 ± 5·1, B: 12·8 ± 4·7, C: 12·7 ± 5·0, D: 12·6 ± 4·9.

It is evident that the enzyme system is not influenced by 3β-OH-steroids, estriol or glucose. At a later occasion ascorbic acid at a concentration of 10 μg/ml urine was similarly tested and found to be without influence.

Sensitivity

As seen from the standard curve (Fig. 3) amounts of steroids of the order of 6 μg were clearly distinguishable from zero. With the standard extraction volume of 5 ml and assuming 100 per cent extraction efficiency this corresponds to 1·2 mg of steroid per 1000 ml, or with a small 24 h urine volume, proportionately less. In practice, using a simple extraction and purification procedure for the steroids in the urine, this very favourable situation is to some extent modified. Mainly, the reduction of sensitivity is caused by unspecific chromogens extracted with the steroids and giving colour at 340 nm with hydrazine hydrate. Accepting a lower limit of sensitivity for the steroids at the double of the photometer readings of this "urinary blank", the lower limit of sensitivity is 2·5–3 mg per 1000 ml.

Practicability

The 3α-hydroxydehydrogenase method has been used routinely in our laboratory for more than 1 yr. One trained laboratory technician can easily perform 100 tests in duplicate per week.

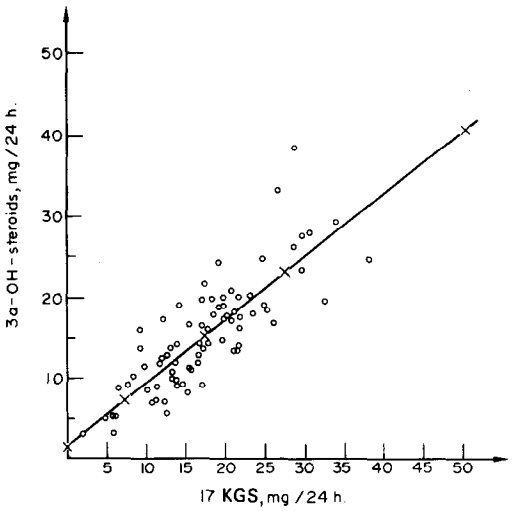

Fig. 5. Relation between the excretion of 3α-OH-steroids and of 17 KGS in 78 subjects.

Urinary 3α-OH-steroid values

3α-OH-steroids and 17 KGS were determined in 78 randomly selected urines from both sexes of patients with various diseases. The results are presented in a scatter diagram (Fig. 5). The coefficient of correlation was calculated to be 0·82.

An indication of the normal means and ranges is presented in Table 3.

Figures 6 and 7 present the use of the 3α-OH assay in function tests involving the steroid producing glands. The results conform closely to a pattern which might be predicted on the basis of the present knowledge of steroid metabolism and the specificity of the assay. It is to be noted that the metopirone tests were performed on patients with suspected hypopituitarism, the control values therefore tended to be lower than normal, in contrast to the results from the hirsute females.

Table 3. Urinary excretion of 3α-OH-steroids in healthy subjects of varying age and sex. Each determination was performed in duplicate

Age	Number	Mean (mg/24 h)	Range (mg/24 h)
Males			
14–35 days	5	0·4	0·3–0·7
20–30 years	5	22·2	16·0–27·5
50–60 years	5	15·0	10·0–18·4
Females			
20–30 yr	5	16·3	10·8–24·4
50–60 yr	5	15·1	7·2–16·2

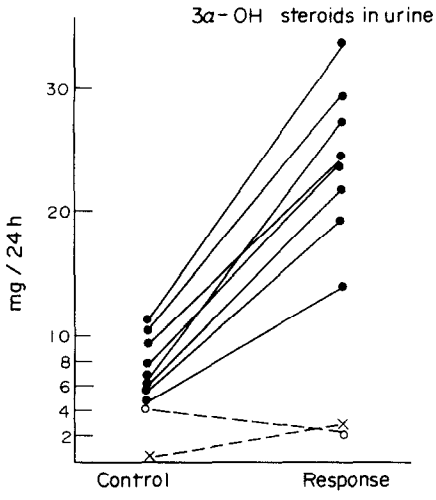

Fig. 6. *Metopirone*: metopirone was given at a dose of 5·5 g distributed over 24 h to eight patients (ages from 20 to 63 yr, mean 45 yr) of suspected, but disproved pituitary failure and two cases (broken lines) of proven hypopituiarism. The fully drawn lines connect the values obtained before the metopirone administration and during the 24 h following this medication.

DISCUSSION

The presented procedure is aimed at being a simple, rapid and reliable methodological choice to fulfil the needs of large routines of adrenal screening and studies with adrenal stimulation and suppression. For the laboratories with a large turnover, the basic method has definite possibilities for automation. It might also be useful for the laboratory that only occasionally has need of determination of urinary steroids, that the enzymatic end-point assay is very similar to that used in a number of analyses outside the endocrine field. Meticulously purified solvents and reagents – as generally used in the steroid laboratory – are not critical.

In order to preserve the rapidity and simplicity, the presented method employs acid hydrolysis, extraction with ether twice, and washing of the extract with ammonium hydroxide and water. This obviously is a minimum that carries with it certain draw-backs. The urinary hydrazine hydrate "blank" value affecting the lower level of sensitivity has been alluded to earlier. Repeated experiments with the addition of pure steroids have shown an excellent recovery (90–100%) of those with a low polarity. The more polar steroids, such as THF and THE, cortols and cortolones, are recovered only to the extent of 50–75 per cent. In the practical use in the study of responses to ACTH, Metopirone and Dexamethasone, this has not been a great detraction. Nor have these problems unduly affected the value of the method in the screening for adrenocortical disease. In this respect all methods measuring total steroid output have limitations[14].

Improvements with regard to the urinary "blank" value can be obtained by substituting the acid hydrolysis with hydrolysis using β-glucuronidase and sulphatase. The gentle enzymatic hydrolysis furthermore is less destructive to the steroids than the boiling with hydrochloric acid. This change in procedure is, however, time consuming and involves increased expenses.

An investigation of the photometric extinction of the hydrazine hydrate urinary "blank" shows that it is linear over the range from 290 to 350nm. For the evaluation of the total output from the adrenal cortex

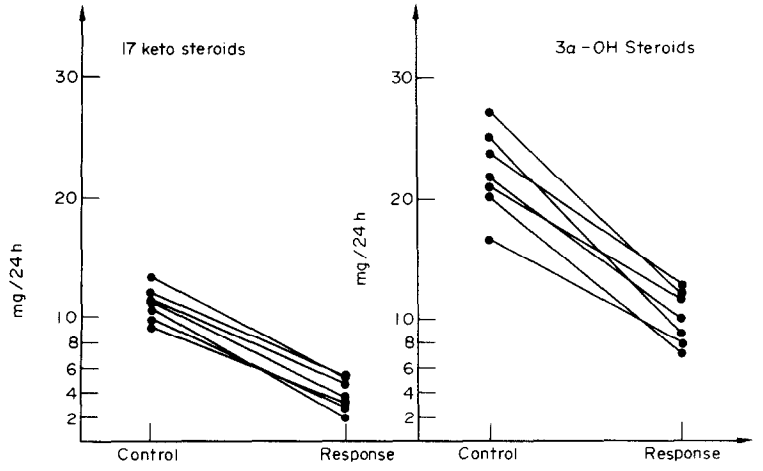

Fig. 7. *Etifollin-Dexamethasone*: seven hirsute females, aged 18–33 yr, mean 24 yr, were given ethynylestradiol 0·15 mg g.i.d. for 6 days; in addition dexamethasone 0·5 mg g.i.d. was administered for the last 2 days. The figure shows the urinary steroid values before this treatment and on the last day of the combined treatment.

the method is quite suitable as the 3α-OH group occurs in all metabolites from adrenocortical steroids apart from dehydroepiandrosterone, pregnenolone, 17α-hydroxy-pregnenolone and estrogens. The small quantity of cortisol, cortisone, aldosterone and DOC found in the urine will not be included in the measurement.

When the method is used in order to get an evaluation of the functional state of the adrenal cortex, it is necessary to consider instances when significant amounts of 3α-OH metabolites from steroids of extra-adrenal origin occur. The major sources of error are androgens from the gonads, and progesterone from corpus luteum or placenta.

In the female the normal gonad produces insignificant amounts of androgens. In the male, testosterone and androstenedione from the testicle contribute only in the order of 30% of the total androsterone-etiocholanolone found in the urine. This does not significantly detract from the 3α-OH-steroid determination in adrenal evaluation.

The very small number of normal cases included in our study (Table 3) does not allow any establishment of normal ranges. The values presented are, however, in the same range as those presented by Hurlock *et al.*[7], which in their case also were obtained from a very small series of measurements.

In instances of hirsutism, or with other signs of virilisation in the female, the associated adrenal-ovarian pathophysiology is complex. The method will register the increased output of androgens metabolized to 3α-OH-steroids. More refined methods, and the use of stimulation and suppression, will be necessary for further clarification with ragard to the underlying pathology. The 3α-OH-steroid method will be of value as a screening procedure in this situation.

With regard to progesterone metabolites, they will disturb the adrenal evaluation during the second half of the menstrual cycle and during pregnancy. It is possible to consider, or make allowance for, the relatively slight quantity of pregnanediol in the urine during the latter half of the cycle in woman with regular menstruation. Admittedly this is not ideal. Without refinement in the form of specific extraction or other separation of the pregnanediols the method is not suited for adrenal evaluation in pregnancy. Experience with the method for more than 1 yr in the ordinary routine use of our laboratory has shown that it is indeed simple, rapid and reliable when care is taken during the extraction. Quantitatively there has been a significant improvement as the same personnel can take care of at least 50% more analyses with the combination 17 KS/3α-OH-steroids than with the combination 17 KS/17 KGS.

The specific determination of the 3α-hydroxy group may be of value as a method for identification in analytical steroid work. We have had no experience as yet with this particular application.

In conclusion a steroid assay based on an enzymatic principle of end-point determination is presented. The method has advantages with regard to specificity and ease of operation, but also serious disadvantages with high urinary blank values which are difficult to eradicate without loss of the most polar corticosteroids. This last problem is a major one and difficult to solve with preserved simplicity of the extraction procedure. For the steroid biochemist the enzymatic method represents an approach that may have a variety of applications.

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