Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 717–725. Pergamon Press. Printed in Great Britain.

ENZYMATIC TECHNIQUES IN STEROID ASSAY

M. HÄRKÖNEN*, H. ADLERCREUTZ* and E. V. GROMAN†

* Department of Clinical Chemistry, University of Helsinki, SF-00290 Helsinki 29, Finland
† Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts, U.S.A.

SUMMARY

Enzymatic fluorometric methods to measure steroids at different levels of sensitivity have been developed. After a rapid column chromatography on Sephadex LH-20, cortisol is measured with 3α,20β-hydroxysteroid dehydrogenase using NaOH-induced fluorescence for NAD^+ determination. Cortisol in 100 μl of plasma can be measured (limit of detection 2 pmol). Good correlation with Clark's method has been obtained ($r = 0.92$) and the coefficient of variation of the entire method is 8 per cent. Estradiol and estrone can be measured using 17β-estradiol dehydrogenase (purified from human placenta) coupled to $NADP^+$ (pH 9·8) and NADPH (pH 6·7), respectively. Estradiol is purified from the other reacting steroids (only few occur in plasma) by Al_2O_3-column chromatography. Since the estradiol concentration in non-pregnancy plasma is approx. 1 nmol/l, enzymatic cycling is necessary to measure the NADPH formed in the reaction. Enzymatic dismutation between $NADP^+$ and NADPH can be performed using glucose-6-phosphate dehydrogenase and glutamate dehydrogenase (amplification 10,000-fold). The 6-phosphogluconate formed is measured fluorometrically using 6-phosphogluconate dehydrogenase and $NADP^+$. The limit of detection of the method is 25 fmol of steroid and the coefficient of variation of the entire method is 14 per cent. When the enzymatic estradiol method was compared with a radioimmunologic procedure the correlation coefficient was found to be 0·82.

INTRODUCTION

Twenty years ago Talalay and his co-workers demonstrated that steroids can be determined with enzyme preparations. They used two NAD-linked enzymes purified from *Pseudomonas testosteroni*[1–4], 3α-hydroxysteroid dehydrogenase (E.C.1.1.1.50) and 3β- and 17β-hydroxysteroid dehydrogenase (E.C.1.1.1.51), and assayed certain hydroxy- and oxo-steroids by measuring the accompanying changes in NADH concentration at 340 nm spectrophotometrically. The enzyme reaction was used in both directions and the conditions were optimized to allow determination of about 10^{-9} mol of steroid. The method was applied to steroid determinations in urine in normal subjects and in various diseases[5, 6].

The same procedure was used by Iwata and Yamasaki for bile acids in plasma[7]. By including enzyme cycling and final measurement of ^{14}C-labelled androstenedione by liquid scintillation counting, Carstensen[8] was able to increase the sensitivity of the assay for pure testosterone down to 10^{-13} mol. No results of actual measurements in biological fluids were presented.

Addition of strong alkali to NAD solution produces intense fluorescence, which can be measured with a simple filter fluorometer. If the final assay is carried out by fluorometry and this is done with or without previous enzyme cycling, it is possible to measure steroids at all concentration levels that are of interest in biological work. The present investigation describes the principles of this technique and two applications, determinations of plasma cortisol and plasma estradiol. The results obtained demonstrate that this technique is more sensitive than any procedure hitherto described for the measurement of steroids in biological material.

EXPERIMENTAL

Solvents and reagents

Benzene, chloroform, dichloromethane, diethyl ether (peroxide free), petroleum-ether, methanol and ethanol were all of analytical grade and were redistilled. Water was glass-distilled, deionized, and filtered through activated charcoal and a Millipore filter (the Millipore Super Q System). All other chemical compounds were of analytical grade from Merck AG (Darmstadt, Germany), Boehringer (Mannheim, Germany) or the Sigma Chemical Company (St. Louis, U.S.A.). Dithiothreitol (Cleland's reagent) was obtained from Calbiochem (San Diego, California, U.S.A.).

Reference steroids

Estrone (gift from Schering, Berlin, Germany), estradiol-17β (gift from Schering), estriol, 16-epiestriol, 16-oxoestradiol-17β, 16α-hydroxyestrone, 2-methoxyestrone (Ikapharm, Ramat-Gan, Israel), 15α-hydroxyestrone, 15α-hydroxyestradiol-17β (both gifts from Dr. R. Dorfman), 2-hydroxyestrone (gift from Dr. O. J. Lucis), 2-methoxyestradiol-17β, 6α-hydroxyestradiol-17β (both gifts from M.R.C. Steroid Reference Collection), 2-hydroxyestradiol-17β (gift from Dr. M. M. Coombs), testosterone, 5-androstene-3β,17β-diol (Ikapharm), 5α-androstane-3β,17β-diol (gift from Organon, Oss, Holland), androstenedione (Mann Research Laboratories, New York, U.S.A.), 11-oxoandrostenedione (gift from Organon), dehydroepiandrosterone, aldosterone, cortisol, cortisone, corticosterone, deoxycorticosterone (Ikapharm), 11-deoxycortisol (Schwarz/Mann, New York, U.S.A.), progesterone (Mann Research Laboratories), 17-hydroxyprogesterone (Ikapharm), 11-oxoprogesterone (gift from Organon), 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione (both gifts from Schering, 3β-hydroxy-5β-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one (Ikapharm), 3β-hydroxy-5α-pregnane-11,20-dione (gift from Organon), 20β-hydroxy-4-pregnen-3-one (Ikapharm), 20α-hydroxy-4-pregnen-3-one (The Upjohn Company, Kalamazoo, Michigan, U.S.A.), pregnenolone, 17-hydroxypregnenolone (Ikapharm).

Buffers

The buffers used were 0·1 M potassium phosphate buffer, pH 6·7, 0·1 M sodium carbonate buffer, pH 9·8, 0·5 M Tris–acetate buffer, pH 8·0 (Trizma–base, Sigma), and 0·2 M Tris–HCl, pH 8·1.

Enzymes

3α,20β-hydroxysteroid dehydrogenase (E.C.1.1.1.53) was obtained from Boehringer.

17β-Estradiol dehydrogenase (E.C.1.1.1.62) was purified from human placenta. Placentas were collected over a 24 h period in 10 mM potassium phosphate buffer (pH 7·2), 5 mM EDTA, and 20% glycerol (buffer A). Homogenization and an initial ammonium sulfate step (0 to 50%) were performed as described by Jarabak[9] except that all manipulations were done in buffer A. The resulting precipitate was suspended in 10 mM potassium phosphate buffer (pH 7·2), 5 mM EDTA, and 50% glycerol (buffer B) divided into 100 ml portions in 250 ml Erlenmeyer flasks. The flasks were then heated with repeated stirring in a water bath at 74–75 °C for 30 min. The pooled contents of the flasks were centrifuged at 2000 *g* for 30 min. The pellet was washed three times with an equal volume of buffer B and the four supernatants were combined. The combined supernatants were next diluted with an equal volume of distilled water giving a final glycerol concentration of 25%. This solution was brought to 25% saturation with solid ammonium sulfate (the pH was maintained at 7·2 with concentrated ammonium hydroxide), stirred for 2 h at room temperature, and the precipitate collected by centrifugation (20,000 *g* for 30 min) was discarded. The remaining supernatant was brought to 50% saturation with solid ammonium sulfate and stirred for 2 h at room temperature. The resulting precipitate was collected by centrifugation (20,000 *g* for 30 min), resuspended in buffer A and dialyzed exhaustively against the same buffer. The remaining purification procedure consisting of four column steps is described by Burns *et al.*[10]. The enzyme was stored in 10 mM potassium phosphate, pH 7·2, containing 5 mM EDTA and 20% glycerol at +4°C.

In the cycling procedure glucose-6-phosphate dehydrogenase (E.C.1.1.1.49), glutamate dehydrogenase (E.C.1.4.1.3) in glycerol and 6-phosphogluconate dehydrogenase (E.C.1.1.1.44) were used. These enzymes were obtained from Boehringer and were purified as described by Lowry *et al.*[11].

Cleaning of glassware

The 3 ml Pyrex tubes used as test tubes and fluorometer tubes were first rinsed with water and then boiled with 0·1 M NaOH for 5 min. The tubes were rinsed with distilled water, boiled for 5 min with 50% HNO_3 and rinsed twice with distilled water. They were then boiled with distilled water for 5 min, rinsed three times with distilled Millipore-filtered water, and air-dried. All the glassware used in the cycling procedure was first treated with warm 0·1 M NaOH for 5 min and then rinsed several times with Millipore-filtered water.

Fluorescence measurement

All fluorometric measurements were made at a volume of about 1 ml in 3 ml Pyrex tubes in a Farrand model A-4 fluorometer. The primary filter was Corning No. 5840 and the secondary filter a combination of Corning Nos. 4303 and 3387 with the latter facing the phototube.

METHOD

Collection and storage of plasma

Peripheral venous blood was drawn into heparinized tubes and centrifuged. The plasma was removed and stored at −20°C before being analyzed.

Column chromatography

The chromatographic columns are prepared in disposable Pasteur pipettes with a disc of glass fibre

Table 1. Elution pattern for estradiol and other interfering steroids from alumina column (0·5 × 3·5 cm)

Eluent	Estradiol-17β (%)	2-Methoxy-estradiol-17β (%)	2-OH-estradiol-17β (%)	6β-OH-estradiol-17β (%)	15α-OH-estradiol-17β (%)
1. 0·3% EtOH-Benzene (3 ml)	5	73	—	—	—
2. 1·4% EtOH-Benzene (3 ml)	95	27	—	—	—
3. 5·0% EtOH-Benzene (3 ml)	—	—	100	100	100

100% = the total amount of each steroid found in the whole eluate. Estrogen determinations were carried out using gas chromatography.

filter paper grade 934 AH (Reeve Angel, Glifton, U.S.A.) at the lower end of each column. For cortisol separation a 0·5 × 5 cm Sephadex column LH-20 (Pharmacia, Uppsala, Sweden) prepared in the petroleum ether–chloroform mixture is used. In Fig. 1 the separation of various steroids reacting with 3α,20β-hydroxysteroid dehydrogenase and eluted in petroleum ether–chloroform (1:3 v/v) is shown. In the assay the first 6·5 ml is discarded and the following 6·5 ml is used for determination of cortisol.

Steroids reacting with 17β-estradiol dehydrogenase are separated from estradiol on a 0·5 × 3·5 cm Al_2O_3 column. Aluminium oxide (acidic, activity grade 1, Merck) is prewashed with ethyl acetate, reactivated for 1 h at 110°C, and deactivated by addition of 3·75% of distilled water. The columns are packed in benzene and washed with 3 ml of 1·4% ethanol in benzene (v/v) and 3 ml of benzene. In Table 1 the separation of various steroids reacting with 17β-estradiol dehydrogenase is shown. In the assay the column is first eluted with 3 ml of 0·3% ethanol in benzene (containing 2-methoxyestradiol) and then with 3 ml of 1·4% ethanol in benzene (containing estradiol).

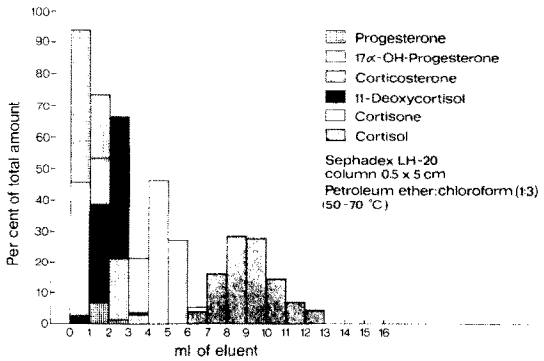

Fig. 1. Elution pattern for cortisol and other steroids from Sephadex LH-20 column (0·5 × 5 cm). Eluting solvent: Petroleum-ether:chloroform. 100% = the total amount of each steroid (^{3}H-labelled) found in the whole eluate.

Assay procedure

Cortisol determination (Fig. 2). Heparinized plasma, 0·5 ml (0·1 ml is enough for a single determination), is extracted twice with 1·5 ml of dichloromethane, the extract is evaporated to dryness in a 5 ml tube and the dry residue is dissolved in approximately 100 μl of petroleum ether (b.p. 50–70°C): chloroform (1:3 v/v). The extract is transferred to a Sephadex LH-20 column and the tube is washed twice with the same volume (100 μl) of the solvent which is transferred to the column. The column is eluted with petroleum ether–chloroform (1:3 v/v); the first 6·5 ml is discarded and the next 6·5 ml is collected in a graduated tube. This fraction is evaporated to dryness and the residue is dissolved in 500 or 250 μl of the eluting solution. Two 100 μl aliquots are then transferred to separate 3-ml Pyrex tubes for duplicate assay. After evaporation of the solvent, 5 μl of ethanol is added to dissolve the

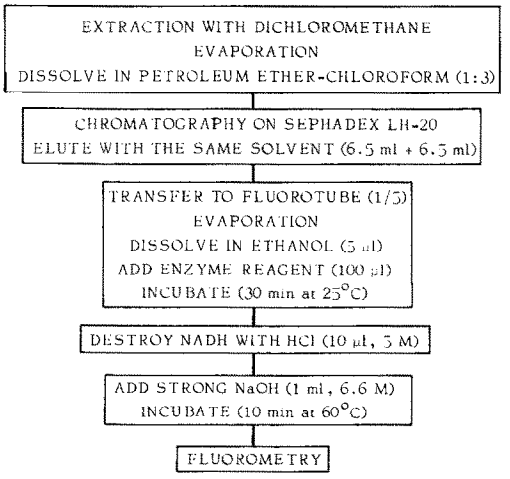

Fig. 2. Flow diagram for enzymatic determination of cortisol in plasma.

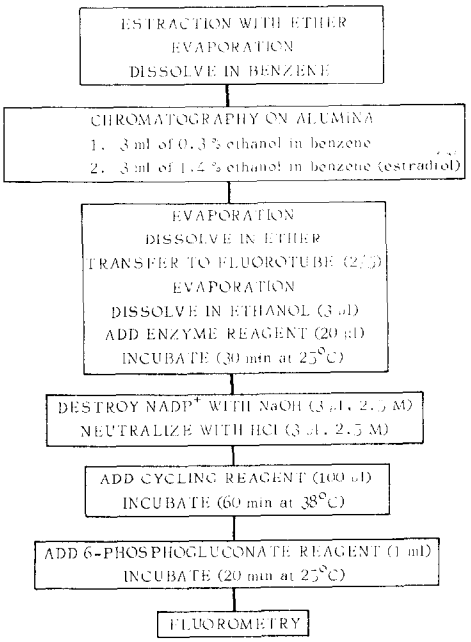

Fig. 3. Flow diagram for enzymatic determination of estradiol in plasma.

dry cortisol extract, and then 100 µl of a reagent containing 0·1 M potassium phosphate buffer, pH 6·7, 0·2 mM dithiotreitol, 0·01 mM NADH (5 mM stock solution of NADH in 0·1 M carbonate buffer, pH 10·6, heated for 10 min at 60° C to destroy contaminating NAD^+), and 25 µg/ml 3α,20β-hydroxysteroid dehydrogenase. The tubes are incubated at room temperature for 30 min and then 10 µl of 5 M HCl is added to destroy NADH[12]. After a few min, 1 ml of 6·6 M NaOH is added from an automatic syringe in dim light and heated for 10 min at 60°C. Under these conditions an intensely fluorescent compound of NAD^+ is formed[13]. The tubes are wiped and the fluorescence measured with a fluorometer.

Estradiol determination (Fig. 3). Heparinized plasma, e.g. 1 ml, is extracted twice with 2 ml of ether, the extract is evaporated almost to dryness and the residue is dissolved in approx. 200 µl of benzene. Thereafter the remaining ether is evaporated completely. The benzene extract is transferred to an alumina column and the tube is washed once with the same vol. (200 µl) of benzene, which is transferred to the column. The column is first eluted with 3 ml of 0·3% ethanol in benzene, which is discarded. Elution is continued with 3 ml of 1·4% ethanol in benzene. This fraction is evaporated to dryness, the residue is dissolved in 250 µl of ether and two 100 µl aliquots are transferred to fluorometer tubes, evaporated to dryness and dissolved carefully in 3 µl of ethanol. To each is added 20 µl of reagent containing 0·1 mM sodium carbonate buffer, pH 9·8, 0·2 mM dithiothreitol, 0·02 mM $NADP^+$ and 3 µg/ml (6 nmol × min^{-1} × ml^{-1}) 17β-estradiol dehydrogenase. The tubes are incubated for 30 min at room temperature (at this enzyme concentration the reaction is over in about 15 min; this can be checked beforehand in a 1 ml fluorometer tube and the concentration adjusted if necessary). Then 3 µl of 2·5 M NaOH is added to destroy $NADP^+$. The tubes are incubated at 60°C for 10 min, then buzzed vigorously with a Vortex mixer and incubated for another 10 min. After the tubes have cooled, the solution is neutralized by addition of 3 µl of 2·5 M HCl. Cycling is performed in 100 µl of cycling reagent[11] containing 0·05 M Tris–acetate buffer, pH 8·0, 5 mM α-oxoglutarate, 1 mM glucose-6-phosphate, 10 mM ammonium acetate, 0·1 mM 5-AMP, 200 µg/ml glutamate dehydrogenase and 15 µg/ml glucose-6-phosphate dehydrogenase. This gives a cycling rate of about 10,000 × h^{-1} at 38°C. After incubation for 1 h the tubes are boiled in a water bath for 2 min and wiped. One ml of 6-phosphogluconate reagent containing 0·02 M Tris–HCl buffer, pH 8·1, 0·1 mM EDTA, 30 mM ammonium acetate, 5 mM $MgCl_2$, 0·03 mM $NADP^+$ and 1·5 µg/ml 6-phosphogluconate dehydrogenase is added. After 30 min at room temperature the fluorescence is read.

Standard curves

Cortisol and estradiol standards were prepared by weighing 18·1 mg cortisol and 13·6 mg estradiol and dissolving the steroids in 10 ml of methanol to produce 5 mM stock solutions.

For calibration of the assay procedure standard solutions were diluted from the stock solutions (cortisol 3·3 × 10^{-6} mol/l, estradiol 6 × 10^{-8} mol/l)

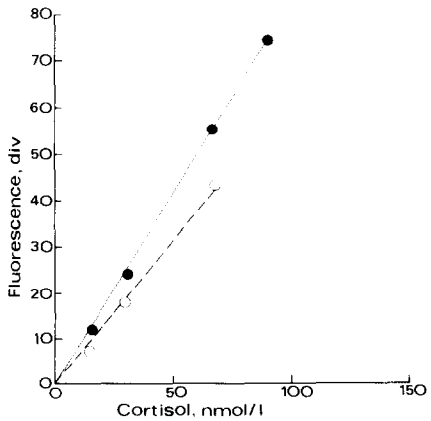

Fig. 4. Cortisol standard curve (●—●) and recovery curve for cortisol added to plasma (○ – – ○).

every time and added in μl amounts for assay. Standard curves for cortisol and estradiol are shown in Figs. 4 and 5. The scale on the ordinate is an arbitrary fluorescence scale in divisions of the microammeter scale and can be adjusted according to the need of sensitivity.

Calculation of the results

The results can be calculated according to the following general formula:

$$k \times \Delta \times \frac{\text{total vol.}}{\text{plasma vol.}} \text{ mol/l}$$

k = factor obtained from standard curve (mol × l^{-1} × div^{-1})
Δ = sample reading–blank reading (div)
total volume = total volume in fluorometer tube (μl)
plasma volume = amount of plasma used for determination (μl).

RESULTS

Accuracy

The recoveries of cortisol and estradiol added to plasma samples are shown in Figs. 4 and 5. In the cortisol determination the recovery was 75·5 ± 6·3 (S.D.M.)% and in the estradiol determination 81·1 ± 6·4%. Usually two or three standards added to water (for cortisol determination) and to low estradiol plasma (for estradiol determination) were taken through the whole procedure to correct the values for procedural losses.

Precision

The precision for cortisol determination, calculated from routine duplicate plasma analyses, was 8% (coefficient of variation). The coefficient of variation for the estradiol assay calculated from 10 separate duplicate analyses in pooled female plasma was 14%.

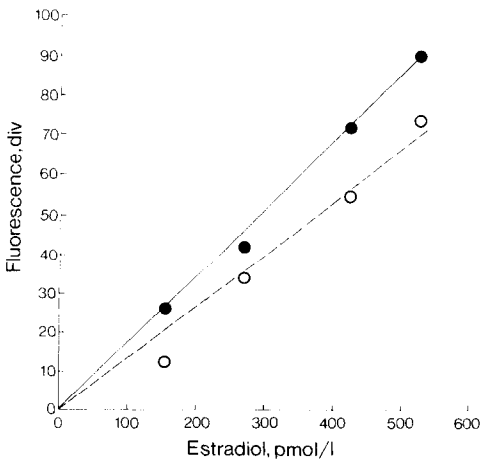

Fig. 5. Estradiol standard curve (●—●) and recovery curve for estradiol added to plasma (○ – – ○).

Sensitivity

When the S.D.M. of blank values are considered, the limit of detection for pure cortisol is approx. 2 pmol and for pure estradiol approx. 25 fmol. In practice, however, the sensitivity of both methods for plasma determinations is less, since the variation in blanks and recoveries are larger than when pure reference compounds are used.

Specificity

The enzymes used in the present study are specific for certain hydroxy- or oxo-groups in the steroid molecule, but several individual steroids will react with them. The specificity also depends on how well the enzymes are purified from other enzymes reacting with steroids. The interference indices for various 3- and 20-oxosteroids in the enzymatic cortisol determination are shown in Table 2. Table 3 shows the relative contributions of those 17-hydroxylated steroids which could interfere with estradiol determination. Not all the steroids listed have been found in human plasma in quantities that would be of any practical

Table 2. Interference index for various 3- and 20-oxosteroids in enzymatic cortisol determination

Steroid	NADH
Cortisol	100
Cortisone	179
11-Deoxycortisol	184
Corticosterone	23
Deoxycorticosterone	165
Aldosterone	0
Testosterone	0
Androstenedione	0
11-Oxoandrostenedione	0
Progesterone	147
17-Hydroxyprogesterone	171
11-Oxoprogesterone	178
5β-Pregnane-3,20-dione	152
5α-Pregnane-3,20-dione	182
3β-Hydroxy-5β-pregnan-20-one	112
3β-Hydroxy-5α-pregnan-20-one	111
3α-Hydroxy-5β-pregnan-20-one	127
3β-Hydroxy-5α-pregnane-11,20-dione	152
20β-Hydroxy-4-pregnen-3-one	0
20α-Hydroxy-4-pregnen-3-one	0
Pregnenolone	141
17-Hydroxypregnenolone	113

The assay conditions were the same as in the cortisol determinations. The final concentration of steroid was 5×10^{-6} mol/l. The values indicate the exact percentage contribution that each of the steroids would make if present at equal concentrations under the conditions used for cortisol determination.

Table 3. Interference index for various 17β-hydroxylated steroids in enzymatic estradiol determination

Steroid	$NADP^+$	NAD^+
Estradiol-17β	100	100
Estriol	0	4·3
16-Epiestriol	0·3	1·7
16-Oxoestradiol-17β	0·6	0·9
2-Methoxyestradiol-17β	76	71
2-Hydroxyestradiol-17β	32	16
6α-Hydroxyestradiol-17β	89	79
15α-Hydroxyestradiol-17β	91	76
Testosterone	0	0
5-Androstene-3β,17β-diol	0·5	0·7
5α-Androstane-3β,17β-diol	0	0

The assay conditions were the same as in the estradiol determinations except that the concentration of pyridine nucleotide was 0·1 mM (see Method). The final concentration of steroid was 5×10^{-6} mol/l. The values indicate the exact percentage contribution that each of the steroids would make if present at equal concentrations under the conditions used for estradiol determination.

significance in the assay. For cortisol determination progesterone, deoxycortisol, cortisone and corticosterone should be separated from cortisol. To our knowledge no other interfering 17β-hydroxylated steroids than those listed in Table 3 are present in plasma in significant amounts. The chromatographic procedures described were developed to separate cortisol and estradiol from all known interfering endogenous steroids and therefore increase the specificity of the methods considerably.

When the enzymatic cortisol method was compared with the procedure of Clark *et al.*[15] using as a test series plasma from 18 patients, the value 0·36 ± 0·05 μmol/l (mean ± S.E.M.) was obtained with the enzymatic method and 0·36 ± 0·06 μmol/l with the method of Clark *et al.* Regression analysis gave the formula $y = 1{\cdot}02 . x - 0{\cdot}01$, where y is the method of Clark *et al.* and x the method described. The correlation coefficient, r, was, 0·92. In this series two samples showed cortisol values less than 0·01 μmol/l with both methods.

Cortisone separates almost completely from cortisol (Fig. 1) and because its concentration is only about one tenth of that of cortisol its contribution to the cortisol value is negligible.

The estradiol concentration in pooled female plasma was determined with the enzymatic method and with a highly specific radioimmunoassay method[14]. The enzymatic method gave the value 0·54 nmol/l and the RIA method 0·43 nmol/l. Plasma estradiol levels in one subject throughout one menstrual cycle were also determined; a typical estradiol pattern was obtained and is shown in Fig. 6.

In addition, a test series of 18 female plasma samples were analyzed with both methods; the enzymatic method gave a mean of value of 0·67 ± 0·05 nmol/l (mean ± S.E.M.) for the series and the RIA method 0·52 ± 0·09 nmol/l. Five male plasma samples were also analyzed; the enzymatic method gave the value of 0·17 ± 0·03 nmol/l and the RIA method 0·17 ± 0·01 nmol/l. Regression analysis for all determinations gave the formula $y = 1{\cdot}04 . x - 0{\cdot}14$ where y is the RIA method and x the enzymatic method. The correlation coefficient was 0·82. Thus, the enzymatic method gives somewhat higher values for female plasma estradiol than the RIA method. A similar difference was in fact also found when the mass fragmentographic method was compared with the RIA method using pregnancy plasma samples[14]. The reason for this difference between the RIA method and the enzymatic or mass fragmentographic method is not known.

DISCUSSION

The main advantage of using steroid assay techniques that combine enzymes with fluorometry is their high sensitivity, the linearity of the standard curve over a wide range of concentrations, the speed of assay, and the inexpensive nature of the reagents and equipment as compared to those required for other methods of comparable sensitivity and specificity. The specificity is comparable to that of radioimmunological (RIA) techniques, because only a limited group of steroids will react with the enzyme and interfering compounds are relatively easy to detect and may be eliminated by conventional techniques. Mass fragmentography (MF) adequately applied to steroid analysis is even more specific, but calls for highly qualified personnel and very expensive instrumentation, and the method is time-consuming as compared with other procedures.

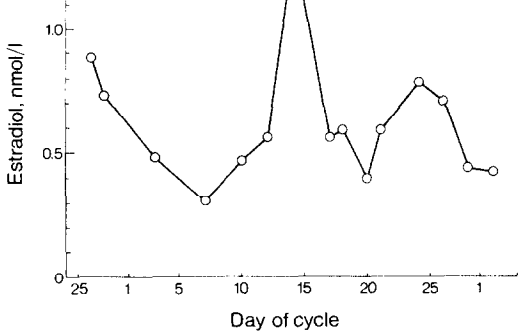

Fig. 6. Estradiol concentration in plasma during a normal menstrual cycle.

The same can be said of gas chromatographic (GC) techniques utilizing electron capture detection; the methods are complicated and technically difficult to perform. In enzymatic fluorometry the final step of the assay is extremely rapid; 100 tubes can be read in 20 min as compared with about 1000 min for liquid scintillation counting in radioimmunoassay and approximately as long for sample analysis in mass fragmentography. If enzymatic estradiol analysis has to be carried out in a single day, one technician can complete 20 duplicate assays. However, if the determinations are made in two steps, extraction and chromatography can be carried out on the first day and the final analysis and calculation of results the following morning. In this way 50 samples can be analyzed in duplicate in one and a half days.

The direct enzymatic assay without addition of strong alkali has a sensitivity of about 10^{-10} mol. If strong alkali is added, the sensitivity increases 10-fold to 10^{-11} mol. GC with electron capture detection has a sensitivity of about 10^{-12} mol[16], and RIA and MF techniques have detection limits between 10^{-11} and 10^{-13} mol. Enzymatic assay with enzyme cycling can be carried out at a level of 10^{-14} mol. With double cycling it is theoretically possible to increase this sensitivity a further 10,000-fold.

Such enzymatic techniques do, however, present certain technical difficulties. Problems are encountered in handling samples of minute volume in the first steps of the analysis, and dissolving the steroid in the assay buffer is a major problem. Some purification of the sample is always necessary: we use rapid and simple chromatographic procedures in Pasteur pipettes.

Unfortunately, only one pure steroid enzyme is commercially available (3α,20β-hydroxysteroid dehydrogenase from Boehringer), and this at present prevents the use of these techniques for the determination of a wider range of steroids. However, several of the dehydrogenases reacting with specific oxidoreducible functions in steroids are easy to purify, as was demonstrated already by Talalay[4]. The criteria for the usefulness of these enzymes is not their absolute physical purity but that the preparation does not contain other enzymes which react directly with the steroids or their enzymatic products present in the sample. The group specificity of these enzymes has certain advantages over absolute specificity, because after appropriate chromatography several of the steroids can be determined with the same enzyme, e.g. 11-deoxycortisol and cortisol with 3α,20β-hydroxysteroid dehydrogenase. This is sometimes useful, e.g. in the Metyrapone® B test. Presumably, at least the following steroids of biological interest can be assayed after purification of the appropriate enzymes: Cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, estradiol-17β, 2-methoxyestradiol-17β, 6α-hydroxyestradiol-17β, 15α-hydroxyestradiol-17β, estrone, 2-hydroxyestrone, 2-methoxyestrone, 15α-hydroxyestrone, 16α-hydroxyestrone (see Table 4) and several synthetic steroids, such as megestrol acetate after de-acetylation. Work on such methods is in progress.

We are quite aware that the estradiol assay may not be directly applicable to routine laboratory analyses, because of the trickiness of the enzymatic cycling procedure. However, if the method is in the hands of a skilful technician familiar with this type of procedure, the assay system can be used for routine determinations. These techniques are suitable for quantitative histochemical procedures on freeze-dried sections[17] and could be used to study the distribution and concentrations of steroids, e.g. in the central nervous system.

Table 4. Interference index for various 17β-oxosteroids in enzymatic estrone determination

Steroid	NADPH	NADH
Estrone	100	100
2-Hydroxyestrone	72	94
2-Methoxyestrone	72	114
15α-Hydroxyestrone	104	136
16α-Hydroxyestrone	95	29
Androstenedione	9	16
Dehydroepiandrosterone	20	3

The assay conditions were as follows: 0·1 M phosphate buffer, pH 6·7, 1×10^{-5} M NADPH or NADH and 2·5 μg/ml 17β-estradiol dehydrogenase. The final concentration of steroid was 5×10^{-6} mol/l. The values indicate the exact percentage contribution that each of the steroids would make if present at equal concentrations under the conditions used for estrone determination.

Acknowledgements—We would like to thank Mrs. Anja Manner for skilful technical assistance and Drs. O. J. Lucis and M. M. Coombs, N. V. Organon, Oss, Schering, Berlin, the Upjohn Company, Kalamazoo, and M.R.C. Steroid Reference Collection, London for generous gifts of steroids. This work was supported by the Ford Foundation, New York.

REFERENCES

1. Talalay P., Dobson M. M. and Tapley D. F.: *Nature* **170** (1952) 620–621.
2. Talalay P. and Dobson M. M.: *J. biol. Chem.* **205** (1953) 823–837.
3. Hurlock B. and Talalay P.: *J. biol. Chem.* **227** (1957) 37–52.
4. Talalay P.: *Methods of Biochemical Analysis*, VIII (1960) 119–143.
5. Hurlock B. and Talalay P.: *Proc. Soc. exp. Biol. Med.* **93** (1956) 560–564.
6. Hurlock B. and Talalay P.: *Endocrinology* **62** (1958) 210–215.

7. Iwata T. and Yamasaki K.: *J. Biochem.* **56** (1964) 424–431.
8. Carstensen H.: *Nature* **212** (1966) 1604–1605.
9. Jarabak J.: *Methods in Enzymology* **15** (1969) 746–752.
10. Burns D. J. W., Engel L. L. and Bethune J. L.: *Biochemistry* **11** (1972) 2699–2703.
11. Lowry O. H., Passonneau J. V., Schulz D. W. and Rock M. K.: *J. biol. Chem.* **236** (1961) 2746–2755.
12. Lowry O. H., Passonneau J. V. and Rock M. K.: *J. biol. Chem.* **236** (1961) 2756–2759.
13. Lowry O. H., Roberts N. R. and Kapphahn J. I.: *J. biol. Chem.* **224** (1957) 1047–1064.
14. Onikki S. and Adlercreutz H.: *J. steroid Biochem.* **4** (1973) 633–639.
15. Clark B. R., Rubin R. T. and Poland R. E.: *Biochemical Medicine* (1971) 177–179.
16. van der Molen H. J.: *Ann. clin. Res.* **2** (1970) 279–288.
17. Lowry O. H.: *J. Histochem. Cytochem.* **1** (1953) 420–428.

DISCUSSION

Breuer:

I think this is a very elegant approach of determining steroids in plasma, but I have just a few questions. You mentioned that substances interfering with the assay could be easily removed. Anybody who has done measurements with NADP and NADPH knows how difficult it is to remove substances which react with NAD and NADH or NADP and NADPH in plasma and as you know there are high blank values if the concentrations of substrates are very low. The second point is: you mentioned that some purification of the enzymes is necessary. Could you specify what you mean by "some" purification because I think this is a very important point. You showed a very nice slide indicating the increase in extinction of fluorimetry for NADH and NADPH. Have you done this measurement also with plasma extracts? This appeared to me to be a measurement with pure compounds but I would be very interested to see a slide with substances extracted from plasma. The last question is: when you say you chromatographed estradiol on alumina, do you use free estradiol? As you know it was rather difficult to chromatograph free estradiol on alumina and to get total recovery; that was the reason why Jim Brown introduced the 3-methyl ether.

Harkonen:

First of all, "some purification" meant the purification of the steroids not the enzymes. The enzyme must always be very pure, e.g. the 17β-estradiol dehydrogenase has been highly purified in Dr. Engel's laboratory. Talalay also purified these enzymes. Concerning your question on blank values from NADH or NADPH; we destroy the substrate pyridine nucleotide by acidic or alkaline treatment and then we measure the product only. The blank is not high. In the cycling procedures, the only blank which you have is the cycling blank, the impurities in plasma do not contribute at this stage.

Breuer:

I just want to make clear that there are many substances, "substrates", in plasma which react with the pyridine nucleotides and I wonder whether you get rid of all these substances before you do the actual assay between the steroid and the pyridine nucleotide because if you determine small or minute amounts of a substrate with NADP or NADPH you get what we call a very high blank due to other substrates.

Harkonen:

First, the enzymes are specific for certain steroids and do not react with other substrates. In addition, we do column chromatography before the enzymatic reaction is carried out. In the final slide, I showed all the measurements done in plasma extracts for cortisol and for estradiol. For cortisol we used 100 μl of plasma and for estradiol we usually can measure it in 100 μl but for practical purposes we usually take 1 ml and divide it so that we take 500 μl for measurement because then we get a very good signal to noise ratio (sample/blank). Dr. Adlercreutz would like to answer the question on the estradiol separation.

Adlercreutz:

We have always worked with alumina oxide despite the fact that many people think that this is very difficult. We found that if you buy the alumina, acid alumina from Merck, and try to make chromatography it doesn't work very well, but if you take the alumina and wash it with ethyl acetate and reactivate it overnight at 110°C and then you add the water and de-activate again, then the recovery is much better. Even the ketolic estrogens can be analysed as free, non-methylated or non-derivatised. If you have very polar estrogens, you must add 1% water to the chromatographic eluent and then you get a much better recovery.

Korenman:

It seems to me that the hormone that we've had the most trouble with that it might be the best to work out a measurement by this technique would be estriol. I wonder whether you have been able to adapt this enzymatic assay system to estriol?

Harkonen:

Estriol doesn't react with this enzyme.

Korenman:

I mean derivatised.

Harkonen:

We haven't tried to convert estriol to a compound reacting with this enzyme. We would like to work with several other steroids because if we get enzymes for the first reaction step then we can apply the cycling system to a general procedure.

Müller:

I still would like to know a bit more about the blank of your method. Could you tell me what kind of cortisol concentration you measure in the plasma of a totally adrenalectomized patient treated with dexamethasone? A second related question is: How do synthetic steroids such as prednisone or dexamethasone interfere with your assay?

Harkonen:

We have measured these steroids only in plasma of untreated

human subjects and then we got these good correlations between the "reference" method and the new method. We have not studied prednisone and dexamethasone treated subjects.

Exley:

Have you considered that enzyme immunoassay may be a better way of attacking this problem? In enzyme immunoassay, the free ligand competes with enzyme bound ligand for the antibody sites. This method would be probably far more specific than your enzymatic technique since one could use specific antisera and obtain the same sort of specificity as the present fairly specific radioimmunoassay methods.

Harkonen:

We haven't thought of doing it yet but may consider it now after your comment.

Exley:

The enzyme recycling technique would apply equally well to the enzyme immunoassay technique as it does to the enzymatic method.

Harkonen:

A very good comment.