Rev. Biol. Trop., 18(1, 2): 5-15, 1971

Determination of cortisol by competitive protein binding or radiostereoassay*

by

Héctor Mena**,***, Lawrence E. Lundy****

and

M. A. Fenella Greig**

(Received for publication June 10, 1969)

A sensitive technique for plasma insulin assay based on displacement of labeled insulin from its antibody was reported by YALLOW and BERSON in 1960 (9). Subsequently, similar methods based on competitive protein binding have been developed. In 1963 MURPHY and co-workers (6) described a technique involving a dialysis using ^{14}C-cortisol. The time required for this procedure was shortened from two days to two hours when gel filtration using Sephadex was substituted for the dialysis step (2,7). However, the filtration columns required setting up and cooling. Recently (3), a 10-100 fold increase in sensitivity was obtained by the use of tritiated steroids, and the technique was further simplified by replacing the gel filtration step with one involving the precipitation of the unbound fraction by adsorption to insoluble substances (1,4,5).

Using the principles of radiostereoassay suggested by MURPHY (6), we have developed a method for measuring cortisol instead of total corticosteroids as previously done.

MATERIAL AND METHODS

1-2 ^{3}H-cortisol was obtained from New England Nuclear Company, (Boston, Massachusetts, 02118). On arrival the solution was purified by paper

* Worcester Foundation for Experimental Biology, Worcester, Massachusetts 01545.
** Research Fellow, Worcester Foundation for Experimental Biology.
*** Present address: Department of Physiology and Medicine, University of Texas Medical School, San Antonio, Texas 78229.
**** Associate Professor, Department of Gynecology, Jefferson University Medical College, Philadelphia, Pennsylvania 19107.

chromatography using a system of toluene/ethyl acetate 9:1 in 50% methanol-water. It was diluted in ethanol and stored at -10 C. Two batches of labeled cortisol of specific activities of 55 C/mmole and 44 C/mmole were used.

USP Standard Cortisol was obtained from USP Standard Co., and stored in two solutions: a 1 mg/ml solution was used as standard for the thin layer chromatography (TLC) and a 1 μg/ml solution as a working solution to prepare the standard curves.

Acid washed Fuller's earth was obtained from British Drug House Ltd. (Gallard Schlesinger Chemical Mfg. Corp., Carle Place, L.I., N.Y.). 15 mg aliquots of this were used for each determination.

Corticosteroid binding globulin (CBG) was prepared from the plasma of three pregnant women in their last trimester. The plasma was stripped of steroids with charcoal Norit A in a concentration of 50 mg/ml of plasma, filtered twice through Whatman Nº 50 filter paper (Fisher Scientific Co.) and stored in 1 ml aliquots at -10 C.

The scintillation fluid was composed of 42 ml of liquofluor (New England Nuclear Co.), 21 ml of absolute ethanol and 6.5 ml of Biosolv-solubilizer (Beckman Nº 184983) in 1 liter of toluene (spectroanalysed grade).

EXTRACTION: A tracer solution of ^{3}H-cortisol 500-600 cpm in 0.1 ml of ethanol was placed in 15 ml stoppered centrifuge tubes and dried. Unknown plasma in amounts of 0.2-0.5 ml was added. Extraction of this was performed with two 5 ml aliquots of methylene chloride and each tube was shaken in a vortex mixer for 1 minute. The methylene chloride was evaporated under nitrogen, and the sample was reconcentrated by dissolving in 2 ml of acetone and redrying (this last procedure increased the recovery considerably).

THIN LAYER CHROMATOGRAPHY (TLC): The separation of the steroids was performed by TLC using fluorescent silica gel. The system used to develop the TLC was methylene chloride/ethanol 92:8. Cortisone and corticosterone interfere with cortisol in the binding sites of corticosteroid binding globulin. Solutions of these steroids run faster than cortisol in this system and so are easily separated. The speed of the spots can be controlled with the concentration of the alcohol. Two and a half cm of the 2 cm wide silica gel stripes were scraped off the plates and eluted with 7 ml of acetone through disposable pipettes containing acetone washed glass wool. The eluate was collected in 5 ml centrifuge tubes in which the complete binding procedure was performed. The sample was diluted in 2 ml of acetone and a 0.5 ml aliquot was placed in a counting vial to determine the percentage of recovery.

BINDING PROCEDURE: On a standard curve, each set of determinations carried at least four standard replicates and three 0, 10, 20, 40 and 80 ngs replicates. The corticosteroid binding globulin solutions were prepared by diluting the stripped plasma to a 1.5%. Enough ^{3}H-cortisol was added to give a final concentration of 10 ng% of the solution. These concentrations were finally determined as later described.

One ml of the CBG-isotope solution was added to each tube of the standard curve and of the unknown samples, and these were shaken on a

vortex mixer. The samples were incubated at 45 C for 5 min and then cooled at 8 C for 10 min. Fifteen mg of fuller's earth were added and each was shaken briefly on a vortex mixer, and a further 2 min on a rotating agitator. The tubes were then cooled at 8 C for another 10 min. After centrifugation for 2 min at 2500 rpm, 0.5 ml of the supernatant was placed in a counting vial. Ten ml of scintillation fluid were added to the vial and counted in a Liquid Scintillation Counter (Nuclear Chicago Mod. 720) with an efficiency of 15%.

RESULTS

CONSTRUCTION OF STANDARD CURVE: The standard curves were constructed with reference points at 0, 10, 20, 40 and 80 ngs. The effect of the plasma concentration on the sensitivity of the standard curves is shown in Fig. 1. With a lower concentration of CBG, a steeper curve is obtained, and the effect of albumin binding is eliminated. The effect of a different concentration of ^{3}H-cortisol on the same concentration of plasma is shown in Fig. 2. These studies show that the most sensitive curve and highest binding is obtained with 1.5% plasma and 10 ngs% of ^{3}H-cortisol. The standard deviation (SD) of the zero reference point was 1.93%.

BLANKS: No interference was found with the reagents which were used. The difference from zero for acetone was 0.98%; for the methylene chloride 1.74%; for the glass wool filling the disposable pipettes 0.43%; and for the silica gel 0.24%. The blank of the method, determined by running distilled water, gave a difference from zero of 0.28%. All the differences were less than the SD.

RECOVERIES: Extraction using methylene chloride proved to be the best for corticosteroids (4). After extraction with two 5 ml aliquots of methylene chloride the recovery was 88.66 ± 7.07 (SD). The recovery from the TLC following the extraction was 50.32 ± 9.2 (SD).

PRECISION OF ANALYSIS: For the analysis of the precision of the method, plasmas with low, medium, and high levels of cortisol were tested. These were obtained, respectively, from a patient who was ovariectomized and treated with dexametasone; from a normal patient; and from a patient treated with cortisol. The precision obtained in the analysis of these plasma pools was high (5). The standard deviation expressed as a percentage of the mean was obtained by repeated determinations on each plasma. The results are shown in Table 1.

TABLE 1

Precision of analysis of cortisol in plasma pools

Number of determinations	level of plasma	Plasma cortisol in ng/ml mean	SD	SD as % of the mean
6	low	9.2	± 1.3	13.0
9	medium	120.0	± 18.5	15.0
9	high	257.0	± 38.0	14.7

DETERMINATIONS FROM WATER: Several determinations were carried out using known amounts of USP Standard Cortisol. The coefficient of correlation was calculated from the following formula:

$$r: \frac{\text{covariance}}{\text{geometric mean of variance}} : 0.997$$

with a level of significance of less than 0.001. Fig. 3 shows the results of the water determinations.

DETERMINATIONS OF NORMAL VALUES: The circadian excretion of cortisol produces a large variation of normal values. Between 9:00 and 10:00 AM the values were 9.87 ± 3.15 (SD) μg of cortisol per 100 ml of plasma (range 5.15 to 14.9 μg%). In the evening (22:00 to 24:00) the values were undetectable when using up to 0.5 ml of plasma.

OTHER DETERMINATIONS: Some determinations were carried out on patients in various physiological conditions. The results were as follows:

Pregnancy	23.3 μg/100 ml plasma
Estrogen administration	37.4 μg/100 ml plasma
Newborn	3.7 μg/100 ml plasma

DISCUSSION

Two new steps have been introduced into the assay of corticosteroids by the protein binding technique as described by MURPHY (4). This has lengthened the procedure but increased the specificity of the method. The first step is extraction with methylene chloride, and the second, thin layer chromatography in a methylene choride/ethanol system. The use of fuller's earth in suitable amounts as an adsorbent proved to be of high specificity for cortisol, because the adsorbent virtually removed most of the other steroids, rendering them unable to compete with ^{3}H-cortisol for binding sites on corticosteroid binding globulin. The technique of cooling the samples is easier than the same procedure with the Sephadex columns. The whole binding procedure with fuller's earth is faster than dialysis or dextran-coated charcoal methods.

Advantage has been taken of the increase of corticosteroid binding globulin in the blood in the last months of pregnancy. Pregnancy plasma has been used in a low concentration of 1.5%, after stripping it of steroids. This procedure gave a steeper standard curve than had been obtained previously, and the low concentration diminished the effect of non-specific albumin binding. In consequence, the SD is lower and the precision higher than reported by NUGENT (8) and MURPHY (4). Apart from great sensitivity, the particular advantage of these methods is the virtual absence of effects due to non-steroidal substances (synthetic steroids, androgens, estrogens, tranquilizers, vitamins, etc.) making special preparation of reagent and special handling of glassware unnecessary (4).

SUMMARY

A method to measure plasma cortisol has been developed using the technique of competitive protein binding or radiostereoassay. Thin layer chromatography developed with methylene chloride/ethanol proved to be the best method for the separation of the different corticosteroids. Separation of the bound steroids was made by adsorption with fuller's earth.

The standard deviation for the zero reference point of the standard curve was 1.93%. No interference was found with the method at the hormone levels measured. The precision of the method was high, and the mean SD at different levels was 14.2%. The coefficient of correlation in water determinations was r: 0.997. Values in normal plasma were found to be 9,87±3.15 (SD) μg of cortisol per 100 ml of plasma.

RESUMEN

Se ha desarrollado un método para determinar cortisol plasmático utilizando los principios de la técnica del "competitive protein binding" o "radiostereoassay". La cromatografía de capa fina (TLC) se desarrolló con un sistema de cloruro de metilo/etanol que demostró ser el mejor para la separación de los distintos corticosteroides. La separación de los esteroides ligados a globulinas y libres se hizo por medio de adsorción con tierra de batán La reacción immunológica fue controlada con ^{3}H-cortisol y la cantidad correspondiente a la fracción ligada a la globulina fue medida en un contador centelleante de líquido (beta).

La desviación estándard para el punto cero de referencia en la curva estándard fue de 1.93%. El método en sí no presentó ninguna interferencia con la determinación de los niveles de la hormona. La precisión del método fue alta, con una desviación estándard media a diferentes niveles de cortisolemia de 14.2%. El coeficiente de correlación en determinaciones en agua fue de r: 0.997. El valor en plasmas normales fue de 9.87±3.15 (DE) μg de cortisol por 100 ml de plasma.

LITERATURE CITED

1. De Moor, P. & W. Heyns
   1968. Cortisol binding affinity of CBG as studied by competitive adsorption. *J. Clin. Endocr.*, 28: 1281.

2. Doe, R. P., R. Fernández, & U. S. Seal
   1960. Significance of the concentration on non-protein bound plasma cortisol. *J. Clin. Endocr.*, 20: 1484.

3. Murphy, B. P.
   1964. Applications of the property of protein binding to the assay of minute quantities of hormone and other substances. *Nature (Lond.)*, 201: 679.

4. MURPHY, B. P.
1967. Some studies of the protein binding of steroids and their application to the routine micro- and ultramicro measurment of various steroids in body fluids by competitive protein binding radioassay. *J. Clin. Endocr.*, 27: 973.

5. MURPHY, B. P.
1968. Clinical evaluation of urinary cortisol determinations by competitive protein binding radioassay. *J. Clin. Endocr.*, 28: 343.

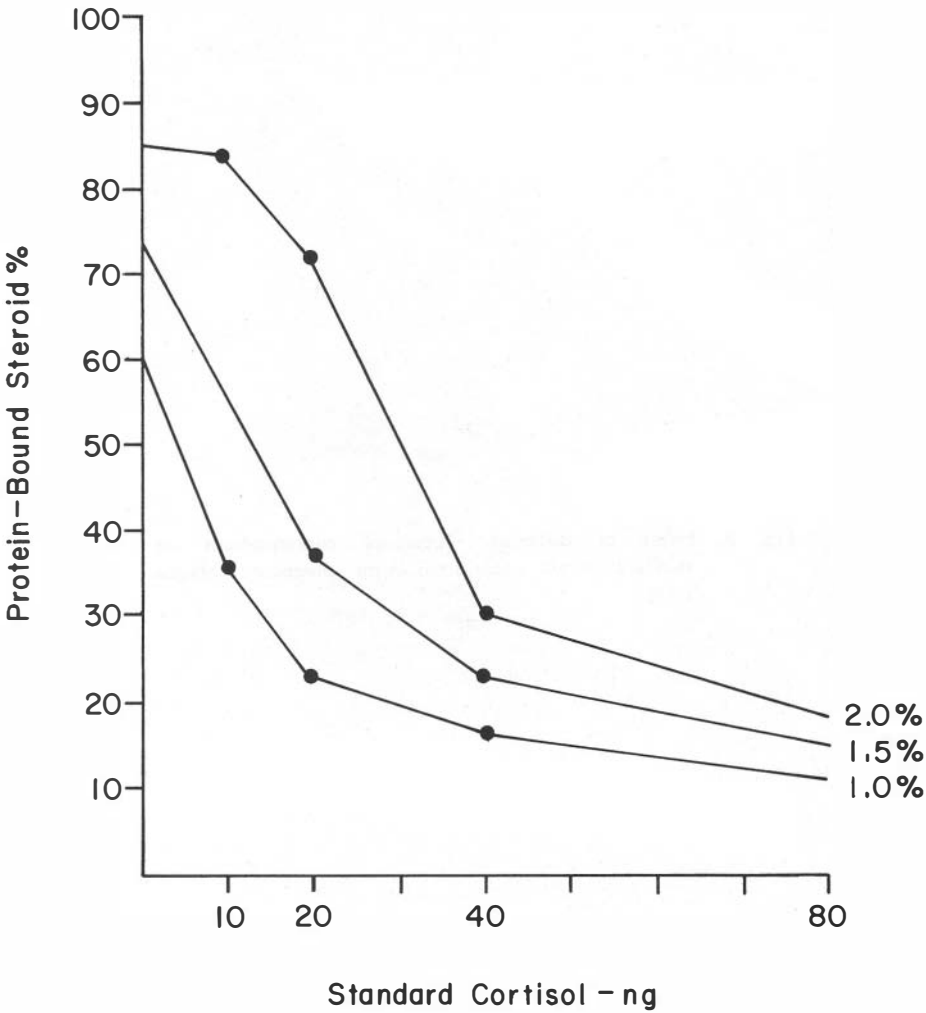
6. MURPHY, B. P., W. ENGELBERTH, & C. J. PATTEE
1963. Simple method for the determination of plasma corticoids. *J. Clin. Endocr.*, 23: 293.

7. MURPHY, B. P., & C. J. PATTEE
1964. Determination of plasma corticoids by competitive binding analysis using gel filtration. *J. Clin. Endocr.*, 24: 919.

8. NUGENT, C. A., & D. M. MAYES
1966. Plasma corticosteroids determined by use of CBG and dextran-coated charcoal. *J. Clin. Endocr.*, 26: 1116.

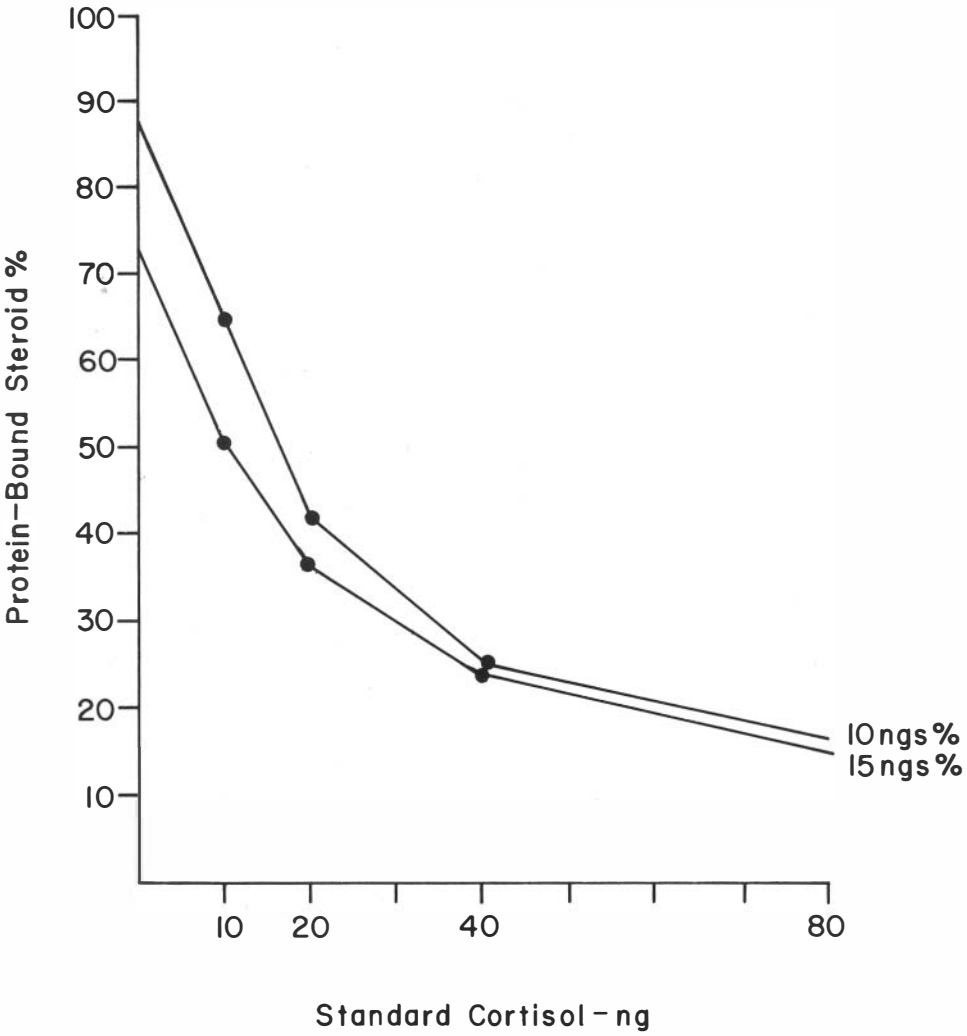
9. YALLOW, R. S., & S. A. BERSON
1960. Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.*, 39: 1157.

Fig. 1. Effect of different plasma concentrations on standard curves constructed with USP cortisol 15 ng%.


100
90
80
70
60
50
40
30
20
10
Protein-Bound Steroid %
2.0%
1.5%
1.0%
10
20
40
80
Standard Cortisol – ng

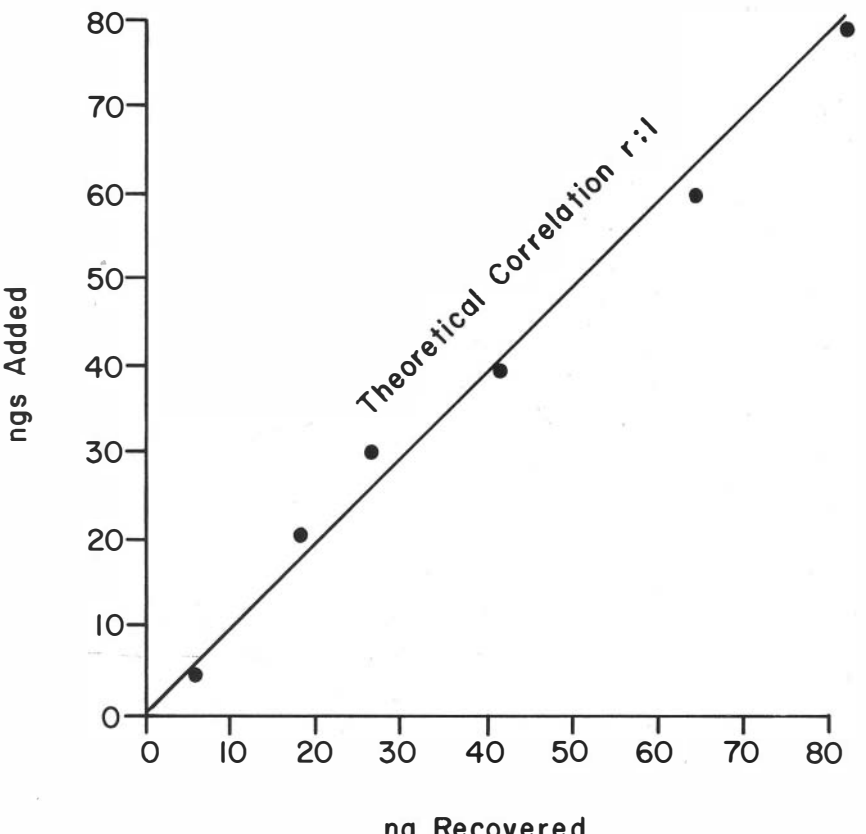
1

Fig. 2. Effect of different ^{3}H-cortisol concentrations on standard curves constructed with pregnancy plasma 1.5%.


100
90
80
70
60
50
40
30
20
10
Protein-Bound Steroid %
10
20
40
80
Standard Cortisol - ng
10 ngs%
15 ngs%

2

Fig. 3. Correlation of USP Standard cortisol determinations in water.


80
70
60
50
40
30
20
10
0
ngs Added
Theoretical Correlation r:1
0
10
20
30
40
50
60
70
80
ng Recovered

3