*Journal of Steroid Biochemistry*, 1972, Vol. 3, pp. 755–759. Pergamon Press. Printed in Great Britain

SHORT COMMUNICATION

# COMPETITIVE PROTEIN BINDING ASSAY OF CORTICOSTERONE

A. SPÄT* and SÁRA JÓZAN
Department of Physiology, Semmelweis Medical University, Budapest, Hungary

(*Received* 18 *July* 1971)

SUMMARY

Details of a competitive protein binding assay of corticosterone are described. The preliminary TLC separation permits simultaneous estimation of corticosterone, aldosterone and progesterone. "Unstressed" rat plasma corticosterone values were obtained between 0·3 and 9 μg/100 ml.

THE PRINCIPLES for competitive protein binding assay of hormonal steroids were established by Murphy in 1967[1]. Schulster *et al.*[2] described the application of Murphy's method for the assay of corticosterone[B]. Reports on the radioimmunoassay of aldosterone were also published[3, 4]. The purpose of the present work was the adaptation of these techniques for the simultaneous analysis of both corticosterone and aldosterone in extracts obtained from rat plasma or adrenal incubation medium. This requires the separation of corticosteroids prior to the assay. A separation with thin layer chromatography (TLC) and the assay of corticosterone are reported in this paper, the assay of aldosterone is to be reported in a subsequent paper.

*Materials*

Benzene, chloroform, formamide, hexane, methanol, methylene chloride and toluene, all analytical grade, were further purified[5]. Florisil, 60/100 mesh, was also pretreated as described by Rittel *et al.*[6] [1,2-$^3$H]-corticosterone [$^3$H-B]-31·7 Ci/m mol, Radiochemical Centre, Amersham/ was purified by TLC in chloroformacetone-acetic acid (100:40:2·5 by vol.) and tested for purity by paper chromatography with benzene–methanol–water (2:1:1 by vol.) and/or formamide–benzene systems.

*Extraction*

3000–5000 dpm of $^3$H-B in 20 μl of ethanol was added for recovery determination to the plasma samples (0·2–5·0 ml) expected to contain 10–100 ng of corticosterone. Small volume samples were made up to 500 μl with physiological saline solution. During extraction with 5 vol. of $CH_2Cl_2$, 0·02 vol. of 1·25 N NaOH was added. The organic phase was transferred to a second tube and the water phase was extracted twice more with 5 vol. of $CH_2Cl_2$. The combined $CH_2Cl_2$ phase was washed with 0·8 vol. of 0·1 M acetic acid and 1·4 vol. of distilled water. The upper surface of the $CH_2Cl_2$ phase was rinsed twice more with water. After drying with $Na_2SO_4$ the solvent was evaporated either with $N_2$ stream or in a flash evaporator,

*Permanent address: Dept. of Physiology. Semmelweis Medical University, Puskin-u.9., Budapest VIII. Hungary.

depending on its volume. In case of larger plasma volumes, apolar lipids were then removed by partitioning between 70% methanol and toluene-hexane (1 : 9 v/v).

*TLC*

When both corticosterone and aldosterone are to be analyzed in the same sample, the two compounds should be separated before the specific assay. For this reason a TLC system was introduced. 0·3 mm thick Kieselgel G (Merck) layers containing 0·5% Leuchtpigment ZS Super (*Riedel-de-Haen*) were applied. The plates were activated at 110°C for 40 min. The extracts were dissolved in $CH_2Cl_2$-$CH_3OH$ (1 : 1 by vol.) and the chromatogram was run for 13 cm by means of sandwich technique with chloroform–acetone–acetic acid (100 : 40 : 2·5 by vol.). Mean $R_f$ values of aldosterone, B, dexamethasone and progesterone were 0·16, 0·34, 0·51 and 0·68, respectively. The steroids were localized by means of parallelly run standards in U.V. light at 254 nm. Using this system for the separation of aldosterone, blank values of 10–20 pg were usually obtained, as estimated by radioimmunoassay. Since progesterone is also definitely separated, its estimation in the same sample seems to be possible as well. The spots corresponding to corticosterone were eluted in 100 $\mu$l of 50% ethanol and 3 × 1 ml of chloroform. The chloroform phase was evaporated and dissolved in 1 ml of $CH_2Cl_2$-$CH_3OH$ (1 : 1 by vol.). One 0·3 ml aliquot of the eluate was taken into 10 ml of a PPO-POPOP-toluene coctail for recovery determination, two others were taken for the assay.

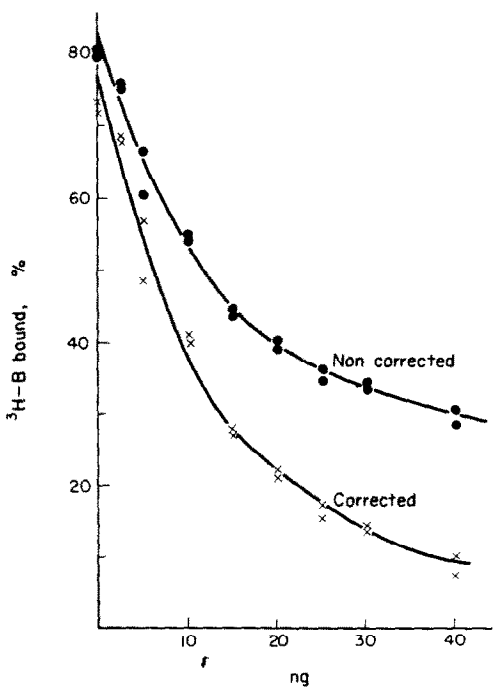


Fig. 1. Calibration curve: corticosterone added (ng) vs. the percentage of bound $^3$H–B. Upper curve: without correction for Florisil adsorption, lower curve: after correction for it.

*Binding assay*

The binding solution contained 2–4 $\mu$Ci of $^3$H-B, 0·4 ml of ethanol and 2 ml of mouse serum (stored at −20°C) per 100 ml. "Protein-free" solution was identical with this but contained no serum. The duplicate sample extracts and duplicate standard samples of corticosterone (0-40 ng) were taken into glass-stoppered centrifuge tubes and evaporated to dryness. 1 ml of binding solution was added to each tube. 1 ml of protein-free solution was added to 2 empty tubes. The tubes were shaken in a water-bath at 38°C for 4 min. After cooling at 3°C for 10 min 43·2 ± 2·4 (S.D.) mg of Florisil (n = 16) was added and the tubes were vigorously shaken for 120 sec. After 5 min. in the ice-bath the supernatant was decanted into a second tube and still in the ice-bath, 0·5 ml of the solution was pipetted into 10 ml of a dioxane containing scintillation cocktail [7] between the 10th and 20th min after adding the Florisil. Tritium activity was *counted* for 10 min in a Packard Tricarb 3320 liquid scintillation spectrometer using automatic external standardization. In order to increase accuracy, the calculation of protein-bound corticosterone (carried out with a Hewlett–Packard 9100 B computer) involved correction for the inefficiency of Florisil to adsorb B, as estimated by the use of protein-free solution [2] and for the dpm counted in the aliquot taken for recovery determination†.

The *standard curve* was constructed by plotting the percentage of bound steroid against the amount of unlabeled standard added (Fig. 1). At computing the amount of corticosterone in the original sample correction was made for blank and recovery.

Following the extraction and purification steps the mean *recovery* of $^3$H-B was 15·50 ± 4·47 (S.D.)% in the 0·3 ml aliquot of the unknown samples (eluted in 1 ml). Samples with recovery below 10% per aliquot had to be discarded twice in 50 analyses.

## RESULTS

*Accuracy* was determined by adding known amounts of corticosterone to 0·5 ml of saline. A linear relationship was obtained between the amount added and quantified in the range of 10–100 ng (Fig. 2). Correlation coefficient attained 0·990 ($p < 0{\cdot}001$), the mean recovery was 92·6 ± 13·6 (S.D.)% (n = 12).

*Precision.* The overall standard deviation for duplicates containing 10 to 100 ng

†At correction for the incomplete adsorption of unbound steroid by Florisil the following symbols were used: T = dpm/0·5 ml of the binding solution; S = dpm/0·5 ml of the supernatant and $S_{pf}$ dpm/0·5 ml of the protein free supernatant. In the protein-free tubes

$$\frac{\text{Free } ^3\text{H}}{\text{Florisil-bound } ^3\text{H}} = \frac{S_{pf}}{T-S_{pf}} = k.$$

Assuming that in the samples $\frac{\text{Free } ^3\text{H}}{\text{Florisil-bound } ^3\text{H}} = k$, then

$$\text{Free} = k\,(T-S) \text{ and Protein-bound} = S-\text{Free} = S-k(T-S) = S(k+1)-kT.$$

Therefore, in the calibration tubes $^3$H-B bound $\% = \frac{S(k+1)-kT}{T} \times 100$ and in the sample tubes:

$$^3\text{H-B bound } \% = \frac{S(k+1)-k(T+R)}{T+R} \times 100,$$

where R signifies the dpm in the aliquot taken for recovery determination.

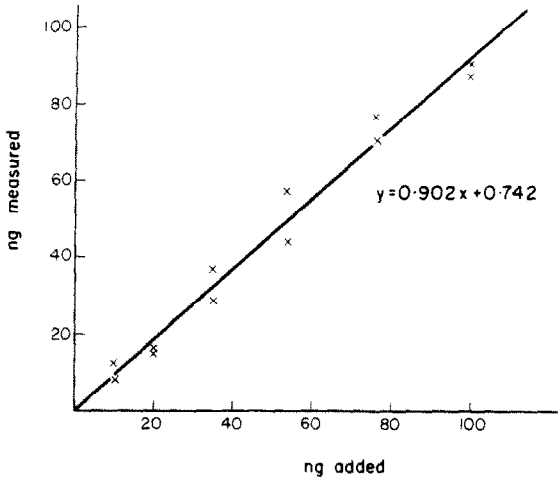


Fig. 2. Study of accuracy: corticosterone added vs. corticosterone quantified.

of standard corticosterone was ±5·24 ng (coefficient of variation: 10·71%). Precision was further examined by repricate determinations of corticosterone in different plasma pools. 5 samples from a pool with a mean corticosterone concentration of 30·20 μg% had a coefficient of variation of 12·88%. 4 determinations of a pool with a mean concentration of 3·46 μg% had a coefficient of variation of 3·76%. Duplicate determination from a third pool, containing 0·21 μg per 100 ml, exhibited a coefficient of variation of 4·07% (Fig. 3).

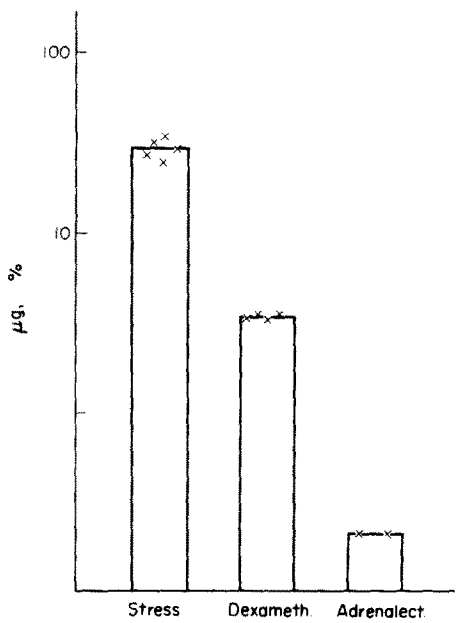


Fig. 3. Study of precision. Plasma-pool obtained from male rats after acute formalin treatment, after acute dexamethasone treatment and 24 h. after bilat. adrenalectomy, resp.

*Blank.* Physiological saline was used for blank. The mean blank was 1·92 ± 2·08 (S.D.) ng (n = 12), irrespectively of the size of the sample between 0·5 and 5 ml. *Sensitivity* or the smallest amount significantly different from zero (after the correction of the reading for blank) was computed on the basis of the upper confidential limit of the blank and the lower confidential limit of the regression curve in Fig. 2 and may be set at 2·4 ng.

*Specificity.* In order to obtain a high specificity, mouse serum was used as a source of binding protein[1]. Cortisol and cortisone, intervering with this binding protein, are not contained in rat samples, while the minute amounts of progesterone are separated by the TLC applied.

"*Normal*" values. Intact male rats (200 g b.w.) of the same cage were decapitated at one min intervals at 10.00 a.m. Their plasma corticosterone level attained 3·0, 3·9, 8·4, 15·0 and 19·8 μg per 100 ml. This increase probably reflected the stress caused by disturbing the animals. A repeated examination on another day failed to exhibit such a phenomenon. Corticosterone levels attained this time 5·7, 0·3, 5·5 and 1·0 μg per 100 ml of plasma.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. T. Garzó (Dept. of Medical Chemistry) for his help in liquid scintillation measurements and for his criticism of this paper, to Mr. B. Kanyár and Miss J. Szutrély (Dept. of Biophysics) for writing the computer program and to Drs. J. Sturcz, T. Harza (Dept. of Physiology) and Zs. Ács (Inst. for Exptl. Medicine) for their advice and criticism.

REFERENCES

1. Murphy B. E. P.: *J. clin. Endoc.* **27** (1967) 973.
2. Schulster D., Tait S. A. S. and Mrotek J.: *Endocrinology* **86** (1970) 487.
3. Bayard F., Beitins I. Z., Kowarski A. and Migeon C. J.: *J. clin. Endocr.* **31** (1970) 1.
4. Mayes D., Furuyama S., Kem D. C. and Nugent C. A.: *J. clin. Endocr.* **30** (1970) 682.
5. Bush I. E.: *The Chromatography of Steroids.* Pergamon Press, Oxford (1961) p. 347.
6. Rittel W., Hunger A. and Reichstein T.: *Helv. Chim. Acta* **35** (1952) 434.
7. Bruno G. A. and Christian J. E.: *Anal. Chem.* **33** (1961) 1216.