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A Simple Procedure for Direct Corticosterone Radioimmunoassay in the Rat

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A simple procedure for the radioimmunoassay of corticosterone using tritiated corticosterone has been described for rat. The use of trypsin to hydrolyze serum proteins allowed to eliminate the extraction of the steroid from the serum. A highly significant correlation between direct and conventional (ether extraction) radioimmunoassays was found. Furthermore, the paired «t» test indicated no significant differences between the values obtained with the two methods.

Key words: Radioimmunoassay, Corticosterone, Trypsin hydrolisis.

Radioimmunoassay (RIA) of steroids in blood is more difficult than that of polypeptidic hormones. The two major problems being: (a) cross-reaction between different structurally-related steroid, and (b) presence of circulating proteins having high affinity for steroids which would interfere with hormone binding to the antiserum.

Corticosterone is the major circulating steroid in the rat. Since available antisera are sufficiently specific, cross-reactions do not represent an important problem for corticosterone assay, at least in male rats. However, the presence of binding proteins remains a problem. This could be overcome by organic solvents extraction of corticosterone from serum, but organic, compounds are both expensive and hazardous. Another alternative way is the denaturalization of circulating proteins by means of either methanol or ethanol (2-5), but organic compounds sometimes require prior purification and this is time consuming and laborious.

Recently an elegant method to assay cortisol has been reported (6). It uses trypsin to hydrolyze circulating proteins. The present work describes a simple RIA for rat serum corticosterone using trypsin procedure.

Materials and Methods

Reagents. Absolute ethanol (Merck) was used without any treatment. Dieth-

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yl ether (Carlo Erba) was washed with a solution of $FeSO_4$ in destilled water (10 %) to remove peroxides and it was redestilled before use. 1,2,3,7-^{3}H-corticosterone (Radiochemical Cent., Amersham) S.A. = 80-100 Ci / mmol, and antiserum obtained in rabbits against corticosterone-21-tyroglobulin (Steranti Res. Ltd, London) were used. Each vial of antiserum was redissolved with 2 ml of destilled water and 0.2 ml aliquots were frozen at —20° C. These aliquots were diluted with 2.5 ml of assay buffer just before use. The assay buffer was phosphate 0.01 M, pH = 8.2 containing 0.1 % gelatin (Merck) and 0.9 NaCl (PBGS).

General procedure. Stock solution of unlabelled corticosterone (Merck) was prepared in ethanol and stored at 4° C. Daily standard curve was prepared by adding 0, 12, 25, 50, 100, 200, 400 and 800 pg of corticosterone in 10 μl of ethanol to triplicate glass tubes. Ethanol was evaporated using a nitrogen stream at 40° C. Appropriate amount of steroid free serum was added. Subsequent procedures were similar to those of the samples (see below).

Steroid free serum (SFS) was obtained by adding 1 g activated charcoal (Merck) to 1 ml of rat serum. After vortexing and overnight incubation serum was centrifuged (3,000 rpm, 30 min) and the supernatant stored at —20° C. An aliquot of this treated serum was extracted with dyethyl-ether and its concentration of corticosterone measured by a conventional RIA. No presence of corticosterone was detected.

Trypsin from bovine pancreas (Boehringer) and trypsin inhibitor from egg white (Boehringer) were dissolved in PBGS at different concentrations just before use. 1 μl of serum added to duplicate glass tubes using microsyringes (Unimetrics Co.). 100 μl of trypsin solution was added to both serum samples and standards and the tubes were vortexed. After 60 min incubation at room temparature 100 μl of trypsin inhibitor solution was added, the tubes vortexed and incubated for 30 min at room temperature. Further, 4,000 cpm of tritium labelled corticosterone and corticosterone antiserum each dissolved in 100 μl of PBGS were added. The tubes were mixed and incubated for 30 min at 37° C followed by 60 min at 4° C. Bound and free fractions were separated adding 0.5 ml of charcoal suspension (10 mg/ml) while stirring at 4° C. The tubes, also maintained at 4° C, were vortexed and centrifuged 10 min later (2,500 rpm, 15 min) at the same temperature. The supernatant was transferred to vials containing 7 ml of liquid scintillation counting, and counted for 10 min. General procedure was always the same as described above unless otherwise stated.

Statistic analysis. They were done either with the paired «t» test or with ANOVA.

Results and Discussion

Effect of different concentrations of trypsin and trypsin inhibitor on tritiated corticosterone binding to the antiserum. Different amounts (0, 50, 100, 200, 400 and 800 μg) of trypsin were added to quadruplicate tubes without SFS. Subsequently 0, 75, 125, 250, 500 and 1,000 μg of trypsin inhibitor were added respectively. Antiserum bound fraction was unaltered by the presence of different amounts of both trypsin and trypsin inhibitor.

Effect of different periods of incubation with trypsin on the antiserum-bound fraction. Two μl of SFS was

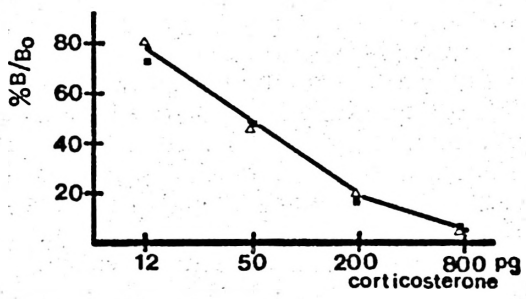

Fig. 1. *Effect of different amounts of steroid free serum on the standard curve: 0 (△), 2 (●), or 5 (■) μl.*
No significant differences were found.

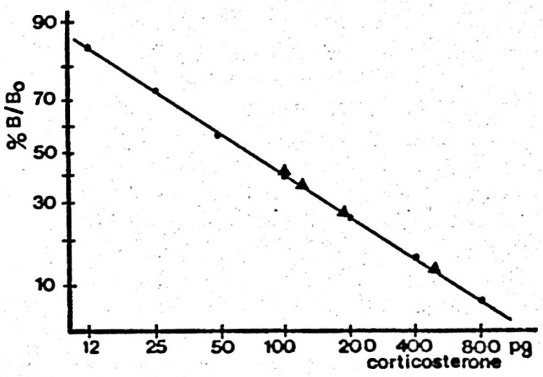

Fig. 2. *Parallelism between the standard curve (●) and the displacement provoked by different amounts of serum (▲): 0.5, 1,2 or 5 μl.*
The standard curve is represented in logitlog paper.

added to all the tubes. Three additional tubes without SFS were included and processed in the same manner. 200 μg of trypsin was added and incubated for 25, 50 or 80 min. Further, 250 μg of trypsin inhibitor was added and incubated for 30 min. No significant effect of trypsin incubation period was found. This indicates that 25 min trypsin incubation was sufficient to hydrolyze serum proteins.

Effect of the presence of SFS on the standard curve. Three standard curves were constructed by adding 0, 2 or 5 μl of SFS to standard tubes. 200 μg of trypsin and 250 μg of trypsin inhibitor were used. The results (fig. 1) indicate that all three standard curves were essentially similar. 200 μg of trypsin was used, as we previously found (data not shown) that this amount was sufficient to wholly hydrolyze proteins present in 5 μl of serum.

Characteristics of the RIA. Sensitivity: 12 pg of corticosterone might be significantly ($p < 0.001$) distinguished from zero point of the standard curve.

Accuray: Different amounts of serum were assayed to show the parallelism between the standard curve and the samples. An excellent parallelism was found (fig. 2). Recovery of different amounts of corticosterone added to serum was satisfactory as table I shows.

Precision: Intra- and inter-assay coefficients of variation are expressed in table I. Intra-assay coefficients of variation were calculated using three serum pools. Inter-assays variation of a serum pool was also calculated.

Specificity: Cross-reactions of this antiserum as provided by Steranti are indicated in table II. Some cross-reaction were also calculated in our laboratory using the present method, and they are also presented in table II.

Some serum samples were analyzed both with the present method and with a

Table I. *Some aspects of the direct RIA for corticosterone in the rat.*
Means ± SEM are represented. Number of samples analyzed, 5.

Recovery of unlabelled corticosterone			Intra-assay coefficients of variation (C.V.) *	
Added	Measured	Recovery (%)	Pool (μg/dl)	%
25	25.8± 6.2	103±25	9.1	3.9
100	103.2± 9.8	102±11	19.4	5.0
400	353.4±18.2	89±5	44.4	4.3

* Inter-assay CV = 12.1 %.

Table II. *Cross-reactions* of this antiserum as calculated at B/B_0 = 50 %*
Values are expressed in %.

Steroid	Steranti Res.	Our method
Progesterone	17.0	28.3
11-Deoxycorticosterone	27.0	25.3
20α-Hydroxyprogesterone	3.5	—
Cortisol	7.5	6.8
Testosterone	4.5	7.4
Aldosterone	4.6	6.9
11-Deoxycortisol	4.2	—

* Only steroids having higher cross-reactions are indicated.

conventional RIA with dyethyl-ether extraction. No significant difference between both methods was found using the paired «t» test. In addition, a highly significant coefficient of correlation was observed (fig. 3).

The results show that trypsin method might be successfully used to assay circulating corticosterone in the rat using tritiated corticosterone. The commercial antiserum use has high cross-reactivities with some steroids; for instance, cross-reaction with progesterone could be especially important in estrous female rats. However, it is not a major problem since higher specificity antiserum might be obtained at present (1).

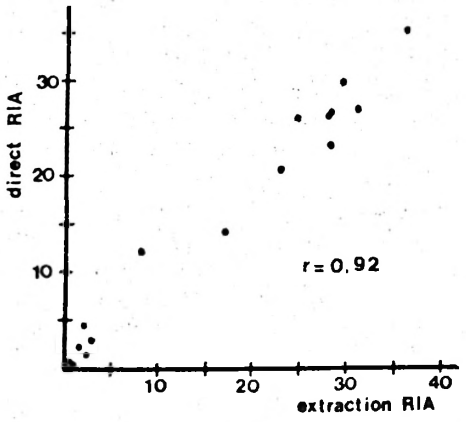

Fig 3. *Correlation between serum corticosterone values obtained with both conventional (extraction) and direct (trypsin) radioimmunoassays.*
The Pearson coefficient of correlation was higly significant ($p < 0.001$, $n = 15$).

The present method does not require solvent extraction, so that it has some advantages: a) It avoids the hazards of organic compound manipulations. b) It eliminates the blank effect of some solvents in spite of being of analitical grade. c) The great simplification of sample manipulation allows to process 30 samples in duplicate in one day. In addition, it reduces the coefficients of variation. Thus, in our laboratory conventional RIA using the same reagents has higher coefficients of variation than those obtained with direct RIA.

Some direct methods previously reported gave values of serum corticosterone higher than those obtained after extraction (2, 3). Our RIA procedure showed a highly significant correlation with conventional RIA, and no statistically significant differences between the values obtained using both procedures were found.

Although the use of tritiated corticosterone is considered to have some disadvantages due to the cost scintillation cocktail and a lower specific activity than ^{125}I-corticosterone, it possesses much more stability and long half life. This method could be applied to both labelled hormones using 1-5 μl of serum depending on expected values.

Resumen

Se describe un método simple para analizar corticosterona en rata por radioinmunoanálisis usando hormona tritiada. El uso de trispsina permite la hidrólisis de la transcortina y otras proteínas con afinidad por la corticosterona, eliminando así la necesidad de una extracción previa de la hormona con disolventes orgánicos. Se observa una alta correlación positiva con un radioinmunoanálisis convencional y no existen diferencias significativas entre los resultados obtenidos por uno u otro método. El análisis directo de corticosterona permite una gran rapidez en el procesamiento de las muestras y necesita cantidades mínimas de suero o plasma (1-5 μl).

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