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# Optimisation of cortisol radioimmunoassay in serum

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## ABSTRACT

An optimised method for accurate radioimmunoassay of cortisol in serum has been developed. The method involved inhibition of binding of cortisol to protein using a TCA/NaOH mixture. The antibodies used were obtained from Mallinkrodt. The separation technique used was that of dextran charcoal which adsorbs free cortisol.

The kinetics of antibody–antigen combination, and the conditions of the experiment including charcoal concentration, antibody dilution, temperature, time of incubation and radioactivity concentration were studied to optimise the standard curve and to obtain high reproducibility; the variance coefficient was evaluated.

Serum samples of different patients were investigated, and the results compared with that of protein binding analysis from 35 normals and 35 hypotensive patients in fasting and non-fasting conditions. Cortisol concentration increased in both instances after ACTH injection.

## INTRODUCTION

Many biologically active substances including polypeptides, steroids and drugs exist in biological fluids at extremely low concentration. Few available chemical techniques are capable of measuring hormones under these conditions. One effective solution to the problem is the use of the high degree of sensitivity and specificity of the antibody reaction as was first realised by Thompson (1941). Several attempts have been made to increase the sensitivity of measurement by Underwood & Williams (1972) and Richardson & Schulster (1972).

The aim of the present work was to develop a sensitive, accurate, simplified and economic radioimmunoassay (RIA) method for the determination of cortisol concentration in human serum. For this purpose we started by comparing the available techniques and kits in a trial to choose the most suitable procedure. Thereafter the study aimed to investigate the various radiobiochemical problems necessary to optimize the selected RIA technique for cortisol and to solve any technical problems. Finally, the validation of the test and its application in clinical endocrinology was investigated.

## EXPERIMENTAL

### MATERIALS

(1) Cortisol standard was obtained from Mallinckrodt Radiopharmazeutika in human serum-free hormone. It was dissolved in 80% ethanol to obtain a standard stock solution of $\mu$g/100 cc.

(2) Serum antibody solution for cortisol (Mallinckrodt) was dissolved in 1 cc of distilled $H_2O$ to obtain a dilution of 1:500.

(3) Cortisol-$^{125}$I was obtained from Mallinckrodt in ethanol at a concentration of 1 $\mu$Ci/cc and specific activity of 50 $\mu$Ci/$\mu$g.

(4) Borate buffer (pH 8·4) was prepared by dissolving 1·88 g $NaB_4O_7 \cdot 10\,H_2O$ + 0·38 g $H_3BO_3$ + 1·4 g EDTA (NaOH) + 1·0 g bovine serum albumin (BSA) dry mixture in 200 cc $H_2O$.

(5) Materials used for separation of bound-free fractions were: (a) Gel adsorption obtained from Amersham, U.K. (Haber *et al.* 1965). (b) Dextran-coated charcoal suspension prepared by mixing 2·5 g charcoal–dextran (10% dextran, 70 cc) in bi-distilled $H_2O$ (Brown *et al.* 1970). (c) Anion exchange resin prepared from Amberlite CG-400 (Frenkel *et al.* 1966). (d) Solid phase technique for coating the test tubes by cortisol antibody before use (Catt & Tregear 1967). (e) Inhibitor for protein binding: a mixture of 20% TCA and 0·5 N NaOH (1:3, v/v) was used for dissociation of cortisol binding in protein as described by Beckers & Cornett (1973) and Megahed *et al.* (1977) for thyroxine and tri-iodothyronine dissociation from protein.

### SUBJECTS

Subjects belonged to two groups: (i) hypotensive patients visiting the Nuclear Medicine Unit, College of Medicine, Cairo University, (ii) Healthy control subjects—students and supporting staff of the same college.

### CORTISOL RADIOIMMUNOASSAY

Cortisol estimation by RIA has been carried out by a modified and optimised technique using dextran-coated charcoal separation (DCC–RIA) in quadruplicate, as follows:

(1) Serum samples were first diluted with ethanol (5:1 v/v), 0·1 cc TCA/NaOH was then added (binding inhibitor solution) and the sample allowed to stand for 1–2 min for cortisol extraction.

(2) Disposable polystyrene tubes were set up containing 100 $\mu$l (0·1 cc) of cortisol standards (0, 1, 2, 4, 8 and 16 $\mu$g%) or 0·1 cc of cortisol serum extract.

(3) Borate buffer (3·0 cc), 0·02 cc of $^{125}$I-cortisol solution (0·02 $\mu$Ci/tube), 0·02 cc of cortisol antibody solution (dilution 1:500) and 0·02 cc of BSA were added.

(4) The mixture was agitated and then incubated at room temperature (20–28°C) for 90 min.

(5) Dextran charcoal was maintained in suspension by a magnetic stirrer, then 0·5 cc (6 mg/tube) was added to each test tube. Tubes were rotated in a rotator for 45 min and centrifuged at 3000 rpm for 15 min.

(6) The supernatants were transferred to fresh, clean test tubes (bound fraction B).

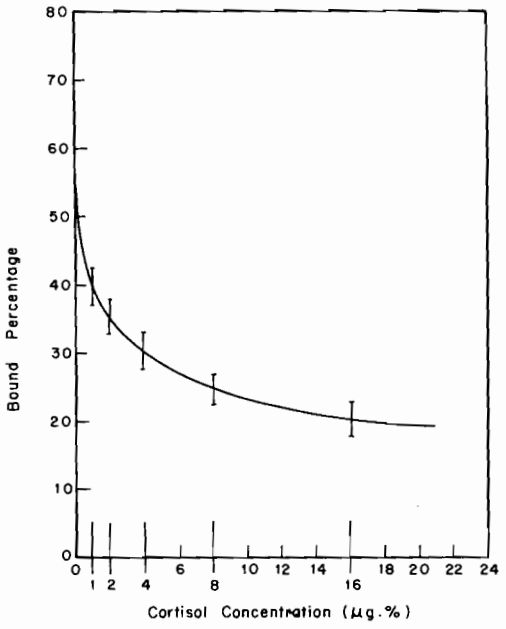


**Fig. 1.** Cortisol RIA standard curve of the optimized dextran charcoal technique.

(7) The free fractions (charcoal pellet) and bound fractions B were counted.

(8) Bound or bound/free fraction or bound percentage was plotted against cortisol concentration and used for the standard curve. The cortisol concentration of the tested samples was then read directly from the standard curve, as presented in Fig. 1.

## PRELIMINARY TESTS

*1. Selection of suitable cortisol RIA technique*

In order to evaluate the sensitivity, reproducibility and accuracy of the available techniques for cortisol RIA we performed each procedure twenty times using pooled sera of normal individuals. The pooled sera were diluted 5:1 with ethanol for cortisol extraction. The following techniques were compared:

*(A) Mallinckrodt RIA cortisol (ion exchange resin) (Mallinckrodt-Radiopharmazeutika 1976)*

In this method 0.1 cc cortisol standard or serum extract, 3 cc borate buffer, 0·02 cc radiotracer solution, 0·02 cc antibody solution (1: 500 dilution) and 0·02 cc BSA were incubated in a polystyrene tube for 2 hr at room temperature (20–28°C). Separation of the free from the bound fraction was carried out by adding a strip of ion exchange resin (Mallinckrodt). The reaction mixture was rotated for 45 min, the ion exchange strips were removed gently and counted for the free fraction, and the remaining solution counted for the bound fraction. The results are presented in Fig. 2 and Tables 1 and 2.

*(B) Cortisol RIA using thyroxine binding globulin (TBG) chemical blocking agents (TCA/NaOH) (Beckers & Cornett 1973)*

In this experiment 0·1 cc of TCA/NaOH mixture was used as a chemical blocking agent

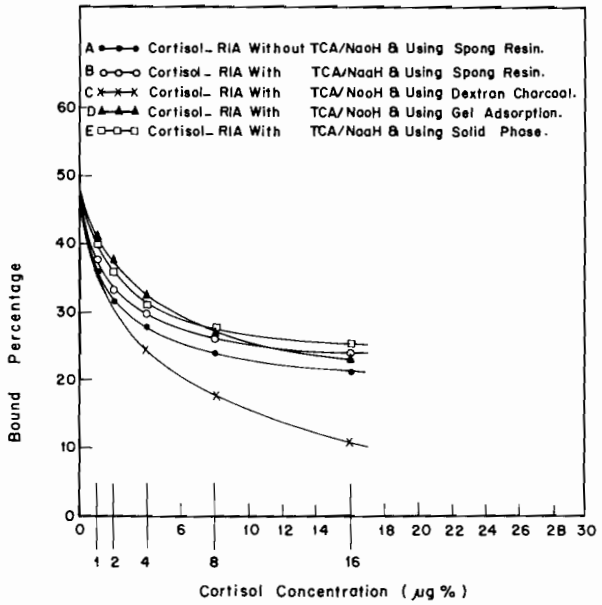


**Fig. 2.** Bound percentage versus cortisol concentration for different RIA techniques.

**Table 1.** Cortisol concentration in μg/100 cc (mean ± S.D.) for pooled sera of healthy subjects (fasting level) applying different RIA techniques

| | | Cortisol concentration (μg/100 cc) (mean ± S.D.) | | | | |
|---|---|---|---|---|---|---|
| Tested samples | No. of assays | Tech. (A) | Tech. (B) | Tech. (C) | Tech. (D) | Tech. (E) |
| Healthy subjects (fasting level) | 20 | 10·4 ± 0·4 | 9·7 ± 0·3 | 9·4 ± 0·3 | 10·6 ± 0·8 | 11·2 ± 1·6 |
| Variance coefficient | | 0·038 | 0·031 | 0·032 | 0·075 | 0·143 |

**Table 2.** Reproducibility of different RIA techniques in comparison with the approved clinical diagnosis

| | | Reproducibility percentage values | | | | |
|---|---|---|---|---|---|---|
| Tested samples | No. of samples | Tech. (A) | Tech. (B) | Tech. (C) | Tech. (D) | Tech. (E) |
| Healthy subjects (fasting level) | 20 | 98 | 97 | 98 | 86 | 80 |

for dissociation of the hormone from the protein. Subsequently the same procedure as for Mallinckrodt cortisol RIA was followed. The results are presented in Fig. 2 and Tables 1 and 2.

*(C) Cortisol RIA applying dextran-coated charcoal (DCC) (free fraction adsorption) (Megahed* et al. *1977)*

This technique was carried out by incubating, in polystyrene tubes, 0·1 cc of cortisol standards or serum extract and TCA/NaOH solution, 3 cc borate buffer, 0·02 cc antibody solution (1:500) and 0·02 cc BSA at room temperature for 2 hr. Then the separation of free fraction was carried out by addition of 0·5 cc DCC (10 mg/cc), rotation for 45 min and centrifugation. The supernatant was then separated from the charcoal pellet (free fraction) and the two fractions were counted. The results are presented in Fig. 2 and Tables 1 and 2.

*(D) Cortisol RIA applying sponge resin (differential migration by molecular weight) (Amersham Radiochemical Centre 1976)*

The experiment was carried out as above (C), but the separation of free from bound fraction was performed using sponge resin (Amersham), 50 mg/sample, instead of charcoal suspension. The results are presented in Fig. 2 and Tables 1 and 2.

*(E) Solid phase cortisol RIA (adsorption of bound fraction) (Catt & Tregear 1967)*

Applying the solid-phase technique, the test tubes were first coated by cortisol antibody using 2 cc of diluted antibody (1:500) instead of 1:500 in borate buffer (pH 8·4) containing 25% BSA for 24 hr. The tubes were then emptied and washed with saline (1 cc) in readiness for the cortisol RIA. Incubation of 0·1 cc serum extract or cortisol standard and 3 cc borate buffer was then completed as in (C). After incubation, the supernatant was counted in fresh clean test tubes for free fractions while the bound fractions were counted in the original test tubes. The results are presented in Fig. 2 and Tables 1 and 2.

From the results it is apparent that the most sensitive technique was that using dextran charcoal (C). It was therefore selected for optimisation.

*2. Optimisation assays*

The modifications examined deal with the following aspects:

*(i) $^{125}$I-cortisol activity.* The selected technique of DCC–RIA has been carried out different concentrations of $^{125}$I-cortisol (0·01, 0·02, and 0·04 $\mu$Ci/tube). The test was performed in quadruplicate for 0·1 cc extracted serum or 0·1 cc standard cortisol with 0·1 cc TCA/NaOH.

From the results it appeared that 0·02 $\mu$Ci/tube could be considered as the best (Figs 3 and 4) since it used the smallest quantity and produced the best range.

*(ii) Cortisol antibody dilution.* Cortisol antibody in dilutions of 1:200, 1:250, 1:333, 1:500, 1:670, 1:1000, 1:2000 and 1:5000 was incubated for 2 hr at room temperature with 0·02 $\mu$Ci $^{125}$I-cortisol. Then 0·5 cc coated charcoal (10 mg/cc) was added to precipitate free labelled antigen and the supernatant counted for the antibody-bound hormone. The bound antibody count was plotted against the antibody dilution (Fig. 5). Applying the DDC–RIA technique with optimum $^{125}$I-cortisol activity for three groups of different antibody dilutions 1:250, 1:500 and 1:1000 in borate buffer, the results are shown in Fig. 6. Results indicate that the 1:500 dilution can be considered as the most sensitive. This agrees with the findings of Ekins (1974).

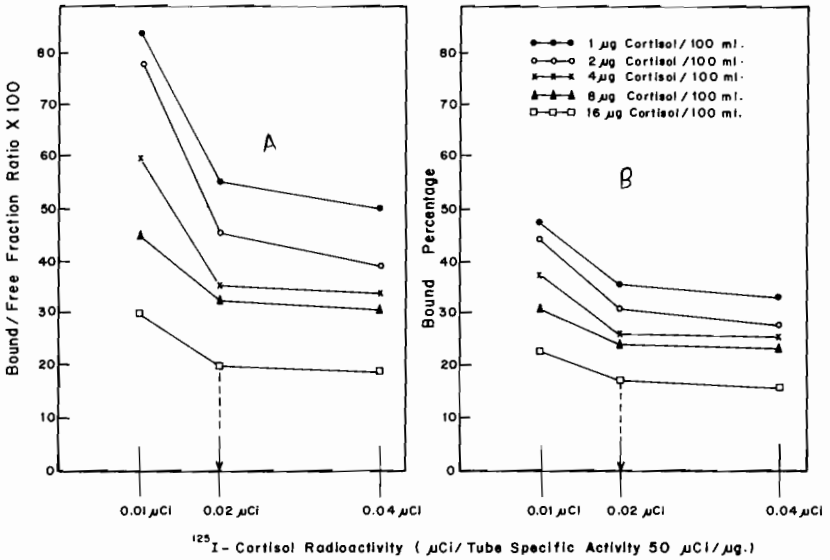


**Fig. 3.** Changes in $^{125}$I-cortisol activity for DCC.-cortisol RIA. (A) Bound/free ratio versus $^{125}$I-cortisol activity. (B) Bound percentage versus $^{125}$I-cortisol activity.

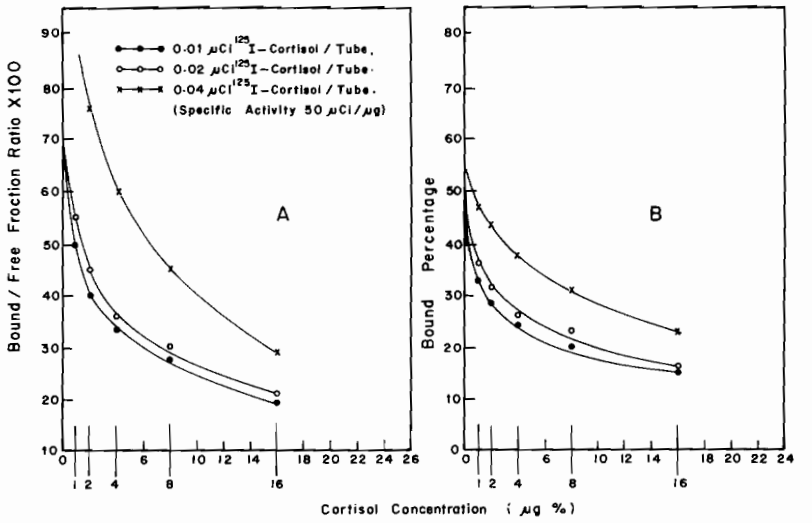


**Fig. 4.** Standard curve for DCC.-cortisol RIA obtained at different radioactivity. (A) Bound/free ratio versus cortisol concentration. (B) Bound percentage versus cortisol concentration.

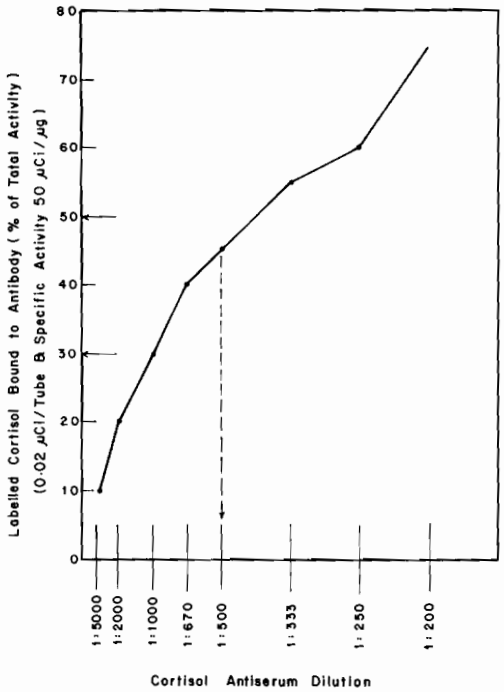


**Fig. 5.** Influence of cortisol-antibody dilution on the binding sites of labelled cortisol in DCC.-cortisol RIA. (The titre is 1 : 500).

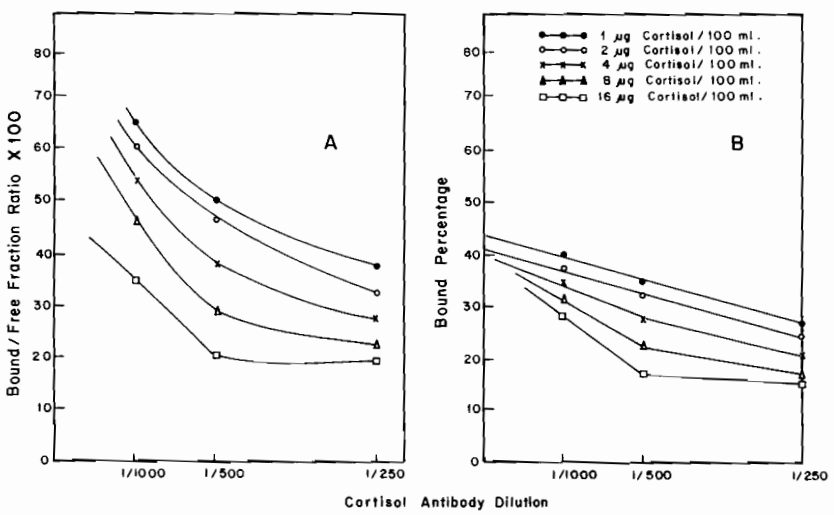


**Fig. 6.** Changes in cortisol-antibody dilution for DCC.-cortisol RIA. (A) Bound/free ratio versus cortisol-antibody dilution. (B) Bound percentage versus cortisol-antibody dilution.

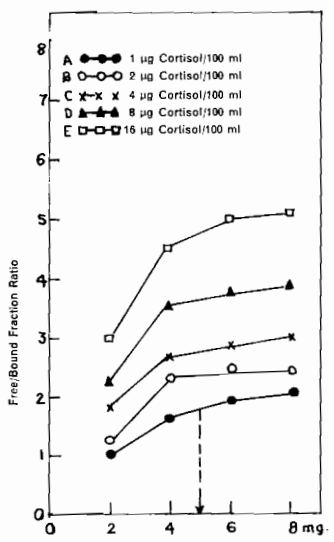


Fig. 7.

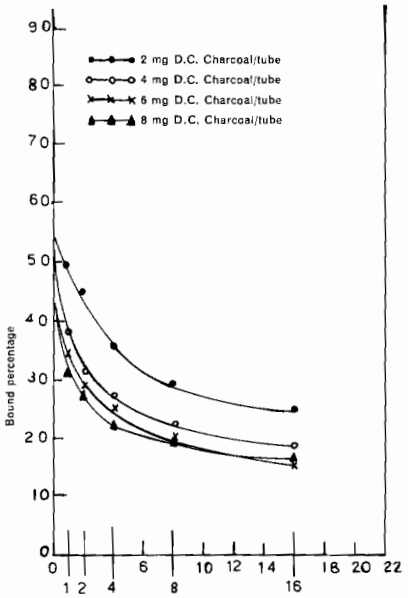


Fig. 8.

**Fig. 7.** Changes in dextran charcoal concentration for DCC.-cortisol RIA. Free/bound ratio versus charcoal concentration.

**Fig. 8.** Standard curve for DCC.-cortisol RIA obtained at different charcoal concentrations, bound percentage versus cortisol concentration.

*(iii) Dextran charcoal suspension concentration.* The dextran charcoal technique with fixed conditions of $^{125}$I-cortisol concentration and antibody dilution was performed using different concentrations of charcoal suspensions (2, 4, 6 and 8 mg/tube) for a cortisol standard. The results (Figs 7 and 8) show that 4, 6 and 8 mg concentrations are nearly equivalent; however, 6 mg/tube results in a steeper slope than the other concentrations.

*(iv) Time and temperature of incubation.* The DCC–RIA technique was carried out for different incubation times (30, 60, 90, 120 and 150 min) at room temperature, since results of previous studies have shown that when the antiserum antigen reaction is allowed to proceed to equilibrium, the equilibrium reaction is much more rapid at room temperature than at 4°C (International Atomic Energy Panel 1974). Figs 9 and 10 show the equilibrium process at different incubation times (90, 120 and 150 min). The standard curve of 90 min exhibited the best slope covering a wide range of cortisol concentrations.

The optimised technique for DCC–RIA of cortisol was carried out in 35 volunteers, in comparison with the competitive protein binding analysis (Cortipac kit, Amersham Radiochemical (1976). The results are presented in Fig. 11 and Table 3.

In another clinical use for cortisol estimation, the optimised DCC–RIA cortisol was performed to evaluate the effect of intramuscular injection of ACTH on cortisol levels for 35 hypotensive patients both at fasting and at 5 p.m. The results are presented in Fig. 12.

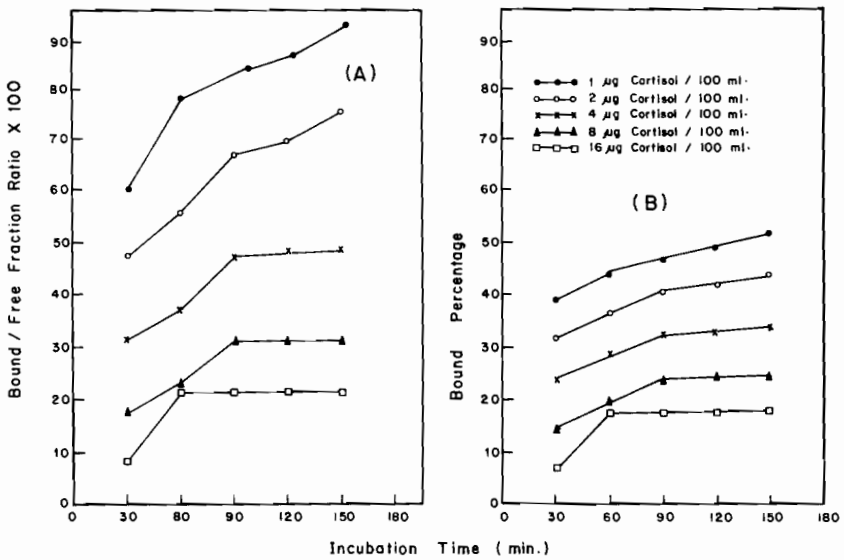


**Fig. 9.** Changes in incubation time for DCC.-cortisol RIA. (A) Bound/free ratio versus incubation time. (B) Bound percentage versus incubation time.

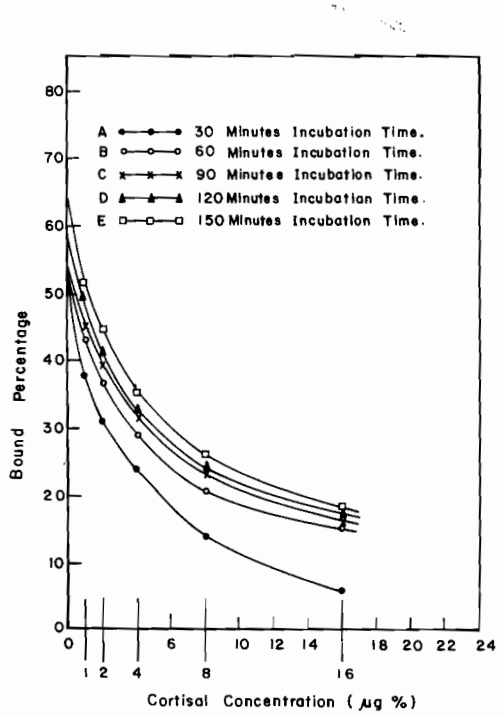


**Fig. 10.** Standard curve for DCC.-cortisol RIA obtained at different incubation times.

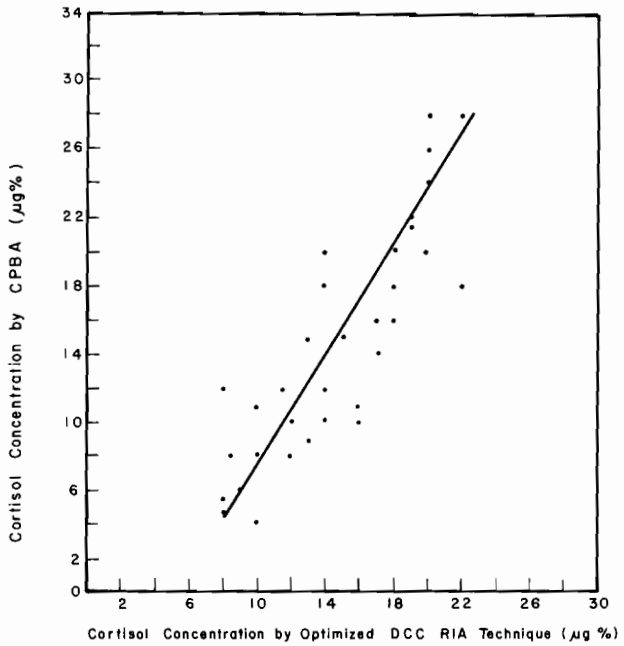


**Fig. 11.** Cortisol concentrations measured by the optimised DCC.-cortisol RIA technique compared with those from competitive protein-binding analysis.

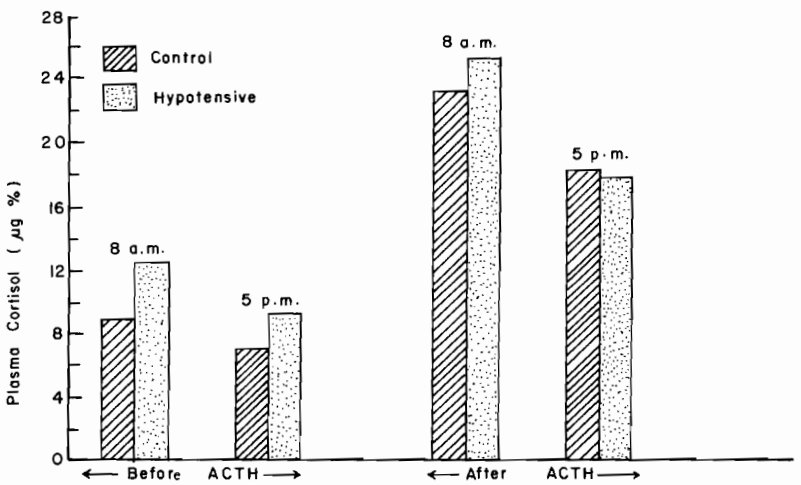


**Fig. 12.** Levels of plasma cortisol in both control and hypotensive groups before and after ACTH using the optimised DCC-cortisol RIA.

**Table 3.** The mean cortisol concentration in normal subjects applying the modified dextran-coated charcoal RIA technique in comparison with the classical competitive protein binding assay (CPBA) cortisol test.

| No. of subjects | Condition | Cortisol conc. using CPBA ($\mu g\%$) (mean ± S.D.) | Cortisol conc. using RIA ($\mu g\%$) (mean ± S.D.) |
|---|---|---|---|
| 35 | Fasting | 6·46 ± 1·60 | 9·00 ± 1·40 |
| 35 | At 5 p.m. | 4·84 ± 1·11 | 7·00 ± 0·71 |

## DISCUSSION

Radioimmunoassay, protein binding assay and other saturation assay techniques have, in the past decade, made an explosive impact upon endocrinology and other areas of medicine, by permitting accurate measurement of small concentrations of biologically potent compounds. The major advantages of RIA for hormones are the high specificity exhibited by antisera, the extreme sensitivity, and the possibility of handling large numbers of samples without extraction.

An increasing number of manufacturers are making available the reagents necessary for a rapidly widening range of substances in the form of kits. A faulty kit may provide an inaccurate result and can be hazardous to the wellbeing of a patient. It is therefore important to evaluate the sensitivity, reproducibility and accuracy of the available techniques for cortisol RIA. The results revealed that the most sensitive technique was that of DCC–RIA which has a reproducibility of 98% and a variance coefficient of 0·032.

The DCC–RIA cortisol was selected for optimisation and various modifications were introduced, involving antigen stripping, quantity of labelled hormone, antibody dilutions, separation methods and condition of incubation.

The optimised coated charcoal RIA for cortisol was performed on serum from 35 healthy subjects giving values from 7 to 20 $\mu g\%$ with a mean concentration of 9·0 ± 1·4 $\mu g\%$. These values showed correlation between our optimised assay and the competitive protein binding assay using the Cortipac kit (Amersham Radiochemical Centre 1976).

Cortisol estimation was also carried out in the present study in one clinical application in order to evaluate the effect of intramuscular injection of ACTH on the cortisol level in hypotensive patients by estimation of cortisol at 8 a.m. (fasting) and 5 p.m., before and after ACTH injection. In both instances cortisol concentration increased in response to ACTH. The clinical findings of the results will be discussed in another publication.

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# تحسين التقديرات المناعية الاشعاعية لهرمون الكوريتزول في الدم

ياقوت مصطفى مجاهد
قسم النظائر المشعة ،
هيئة الطاقة الذرية ،
الدقي ، القاهرة

جابر علي زيادة  محمود محفوظ  محمد عبد الرزاق  شوقي الحداد
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جامعة القاهرة ، القاهرة

## خلاصة

في هذه الدراسة تم التوصل إلى طريقة حديثة حساسة وسهلة لتعيين تركيز هرمون الكورتيزول في الدم بواسطة القدرات المناعية الاشعاعية للكورتيزول . تناولت الدراسة أولا تعيين تركيز الكورتيزول في الدم لمجموعة عينات من الاصحاء المتطوعين بواسطة الطرق الاشعاعية المناعية المختلفة والمتداولة حاليا في معامل الغدد الصماء بالعالم وذلك لتحديد أنسب هذه الطرق .

بعد التوصل إلى أن طريقة استخدام الفحم المغطى بالديكستران هي أحسن الطرق لفصل الهرمون المرتبط بالأجسام المضادة عن الهرمون الحر في المحلول ، تناولت دراستنا تغيير الظروف المختبرية المختلفة التي تؤدي إلى زيادة فاعلية هذه الطريقة . وقد استخدمت الطريقة المحسنة لتعيين تركيز الهرمون في عينات مختلفة من المرضى قبل العلاج وبعده ، وقورنت النتائج بنتائج الطرق الاشعاعية الأخرى . وقد بلغت درجة حساسية الطريقة الجديدة ٩٨٪ .