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A Radioimmunoassay System for Human Serum Cortisol

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Abbreviations
RIA *Radioimmunoassay*
C-21-hs:BSA *cortisol-21-hemisuccinate:Bovine serum albumin*
PEG *Polyethelene glycol*
NRS *non-immunized rabbit serum*

ABSTRACT

Background: Cortisol (hydrocortisone) is the most potent glucocorticoid produced by the human adrenal cortex with about 55-69 µmol (20-25 mg) released daily, and the rate of release has a pronounced diurnal rythm. The plasma concentration is highest in the early morning around 06:00 (140-700 nmol/L), while the nadir is about midnight (~250 nmol/L). Cortisol acts through specific intracellular receptors and has effects in numerous physiologic systems, including immune function, glucose-counter regulation, vascular tone, substrate utilization and bone metabolism. For accurate detection of this low concentration of cortisol in human serum, Radioimmunoassay (RIA) is used; which is known as the most recognized sensitive microanalytical technique for the determination of the very low concentrations of awide range of substances of biological and medical interest. No other analytical method can reach the low detection limits of assayed analytes attained by RIA realted techniques.Applicability, simplicity , to operate and economy of RIA have paved the way for the last advancement in medical research during the past five decades. **Materials & Methods*:*** Highly purified Hydrocortisone-21-hemisuccinate and Bovine Serum Albumin used to prepare immunogen (cortisol-21-hemisuccinate: BSA), which is immunized into rabbits to produce cortisol polyclonal antibodies, Na ^{125}I (370 MBq/100µl) carrier and reductant free is the radioactive material used to prepare the tracer (Institute of Isotopes Co.,Hungary). All above materials in addition to prepared standards of cortisol used to prepare radioimmunoassay system to assess cortisol level. **Objective:** This work was carried to assess and development the technical as well as the economic feasibility of establishing local radioimmunoassay for human serum cortisol. **Results:** immunogen obtained was of 17 cortisol molecule : 1 BSA molecule ,the dilution of the antibody was chosen to be 1/8000 according to the best displacement percent (76.5%) obtained at this dilution from the the 5th bleed of the 1st rabbit in group A and the prepared tracer was of radiochemical yield percent (58.5%), radiochemical purity (96%)and specific activity (120 µCi/µg) . **Conclusion:** This RIA-technique method for detection of cortisol level in human serum is simple, accurate and can be used as a decisive diagnostic tool for adrenal status.

INTRODUCTION

Cortisol helps control carbohydrate, protein, and lipid metabolism. For example, cortisol increases glucose level in the blood by stimulating gluconeogenesis in the liver and promotes the formation of glycogen (i.e., a molecule that serves as the storage form of glucose) in the liver. Cortisol also reduces glucose uptake into muscle and adipose tissue,

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thereby opposing the effects of insulin [1.2]. Furthermore, in various tissues, cortisol promotes protein and lipid breakdown into products (i.e., amino acids and glycerol, respectively) that can be used for gluconeogenesis [3]. In addition to those metabolic activities, cortisol appears to protect the body against the deleterious effects of various stress factors, including acute trauma, major surgery, severe infections, pain, blood loss, hypoglycemia, and emotional stress [4]. Plasma cortisol level is widely used as an indicator of stress [5].

There are wide variations in cortisol blood levels throughout the day. The typical circadian rhythm of cortisol secretion can be correlated to the activity of the cerebral centres and the pituitary. Cortisolaemia reaches a maximum in the early morning hours, then it shows a sharp decrease during the afternoon and the evening; the lowest level is reached about midnight. It is obvious that cortisol assay on a single blood sample taken at random during the day does not give useful indications about the functional behaviour of the adrenal cortex [6.7].

Moreover, it must be remembered that the blood level of a hormone does not depend only on the amount secreted by the gland cells, but also on the circulating carrier protein, the distribution of the hormone and the clearance rate from the blood due to both hepatic catabolism and renal excretion. Inspite of this, cortisol determination in plasma samples taken at a fixed time (e.g. 8-9 a.m) gives very useful information about gross impairment of adrenals, thus constituting an essential screening tool.

Elevated cortisol levels and lack of diurnal variation have been identified in patients with Cushing's disease (ACTH hypersecretion) [8,9]. Elevated circulating cortisol levels have also been identified in patients with adrenal tumors [1]. Low cortisol levels are found in primary adrenal insufficiency (e.g. adrenal hypoplasia, congenital adrenal hyperplasia, Addison's disease) and in ACTH deficiency [8,2,10] .

Radioimmunoassay (RIA) is known as the most recognized sensitive microanalytical technique for the determination of the very low concentrations of a wide range of substances of biological and medical interest. No other analytical method can reach the low detection limits of assayed analytes attained by RIA realted techniques. Applicability, simplicity , to operate and economy of RIA have paved the way for the last advancement in medical research during the past five decades [11].

MATERIALS AND METHODS

(I) Materials:

Highly purified Hydrocortisone-21-hemisuccinate, analytical standard : H2882, Fluka, Bovine Serum Albumin-Fraction V, lyophilized powder Purity ≥ 98% :85040C, Sigma-Aldrich,USA, complete and incomplete freunds adjuvant, chloramine-T, sodium metabisulphite ,polyethelyne glycol 8000(PEG), Goat anti-rabbit IgG (whole molecule) purchased from Sigma-Aldrich, USA, NaI^{125} (370 MBq/100µl) carrier and reductant free (Institute of Isotopes Co.,Hungary).

Chemicals: All chemicals used in these experiments were provided from Sigma Chemical Co. of high quality and purity.

(II) Methods:

Preparation of Hydrocortisone-21-hemisuccinate : BSA

Hydrocortisone-bovine serum albumin conjugate (C-21-hs:BSA) was prepared by the mixed anhydride procedure according to Erlanger et al., 1957,1958 [12.13] with some modifications using Highly purified Hydrocortisone-21-hemisuccinate and Bovine Serum Albumin at the C-21 position

Production of cortisol polyclonal antibodies:

Production of cortisol polyclonal antibody was undertaken through immunization of eight male mature white New-Zealand rabbits weighing 2-3 kg with the prepared antigen. Rabbits classified into two groups the first group comprised six rabbits (R1-R6) and the second group comprised two rabbits (R7-R8). They were kept under the same hygienic conditions, well balanced diet and water supply. The local production of polyclonal antibody was carried out through primary and boosters immunizations.

For primary immunization: each rabbit in the first group received 1000 µg of immunogen in 1 ml emulsion. While, each rabbit in the second group received 200 µg of the immunogen in 1 ml emulsion.

For boosters: each rabbit in the first group received 500 µg of the immunogen in 0.5 ml emulsion. While, each rabbit in the second group received 100 µg of the immunogen in 0.5 ml emulsion.

For emulsion preparations Freund's adjuvant complete (FAC) was used for primary injection and Freund's adjuvant incomplete (FAI) was used for booster doses (Jacobs, 1974, Goding, 1986, Pillai and Bhandarkar, 1998)." (26,27,28) [ ]

Emulsification was performed using Hamilton double hub syringes connected to each other with narrow metallic tubing. The aqueous solution was placed in one syringe and the adjuvant in the other one. The aqueous solution was forced through the tube into the oil in other syringe; then, the mixture was forced back and front until a stable water-in-oil emulsion was formed. Immunization takes place according to the following schedules :

Five injections were administrated, one primary and four booster injections, at 4 weeks interval and Blood samples were collected three weeks after each injection

Each bleed tested for cortisol antibody. Anti-sera obtained were assessed in terms of: titer, displacement, immunoresponse.

Preparation of radio-iodinated cortisol tracer:

The preparation of radiolabelled Hydrocortisone-21-hemisuccinate: BSA using radioactive ^{125}I was carried out according to Midgley et al;1969 [14] and Jeffcoate et al.,1972 [15] with slight modification using chloramine-T method as described by Hunter and Greenwood 1962 [16].

Preparation of cortisol standards

10 mg of highly purified Cortisol were dissolved in 10 ml of ethanol to get a concentration of 1 mg/ml. A stock solution of 1 µg/ml was prepared using steroid assay buffer. The serial dilutions were made to obtain different concentrations ranged from 0.5 - 60 µg/dl using steroid assay buffer. Prepared standard were kept at 4 ^{o}C.

Preparation of double- antibody liquid phase RIA system

Radioimmunoassay of cortisol was carried out as follow: 100 µl of standards or unknown samples and 100 µl of cortisol tracer were incubated with 100 µl anti-cortisol for 3h at R.T . The separation of bound and free fractions was carried out by incubation with 700 µl of precipitating agent which contains 100 µl 2nd antibody (goat anti-rabbit IgG) ,100 µl non-immunized rabbit serum (NRS) and 500 µl polyethylene glycol (PEG) (8000,12%) into all assay tubes [17] . the supernatant was decanted after centrifugation and the bound fraction was counted using gamma counter.

Optimization of the liquid-phase RIA system:

To optimize the liquid phase RIA system under investigation the optimum assay conditions were performed by studying four important factors: temperature, incubation time, sample volume, and separating agents.

Effect of temperature: The effect of temperature on the local liquid phase RIA for cortisol using three different temperature degrees (4 ^{o}C , Room Temp. 25 ^{o}C " and 37 ^{o}C) for 3 hours was studied . **Effect of incubation time** The effect of incubation time on the local liquid phase RIA of cortisol was carried out through out 24 hrs, ranged from one hour to 24 hrs, all at 37 ^{o}C. **Effect of sample volume** Different sample volumes were tested **Effect of separating agents** Different dilutions of goat anti-rabbit IgG (2nd antibody) , non-immune rabbit serum and different concentrations of PEG-8000 were studied for the best displacement

Standard curve representation: standard curve for Cortisol represented by measuring the B/B0 value for each standard value from zero to 60 µg/dl.

Validation of cortisol radioimmunoassay system: To assure the validity and reliability of the proposed assay some performance characteristics studies, included sensitivity, specificity, precision, accuracy (recovery and dilution tests) must be studied

RESULTS

For the polyclonal antibodies produced, the data obtained from studying titer and displacement for each bleed of each rabbit showed that the antisera obtained from bleeding five of rabbit one in group A (R1) gave the highest displacement percent (76.5 %) with binding (43.2%) at dilution 1:8000 which is chosen to complete the assay using it.

For the tracer prepared, the radiochemical yield percent of the prepared tracer was found to be 58.5% (Fig. 1), also the radiochemical purity percent was determined on paper electrophoresis and it was found to be 96% (Fig. 2) , the specific activity of the prepared tracer was 120 µCi/µg.

Optimum conditions detected are summarized in (Tab. 1)**For the temperature:** The highest differences in percent binding between the studied concentrations were proved using temperature at 37 ^{o}C. **For incubation time:** The data obtained for 3 hrs incubation are optimum. **For sample volume:** The highest differences in displacement between the concentrations of cortisol were demonstrated using 100 µl as a sample volume. **For separating agents:** Results showed that the optimum dilutions were 1:80, 1:200 for 2nd antibody and non-immune rabbit serum respectively and 500 µl of 12% concentration of PEG-8000

These optimum conditions used to draw an optimum standard curve (Fig. 3) which is used to identify concentrations of unknown samples of cortisol.

Validation of the liquid-phase RIA system

Sensitivity: A detection limit of 0.11 µg/dl has been obtained by assaying 20 replicates of the zero standard. The sensitivity has been determined as the concentration corresponding to mean count per minute minus its double standard deviation (Tab. 2).

Precision: It is a statistical index of the ability of an assay to yield the same result when the assay is repeated on the same sample through two methods: intra-assay precision (within run) which was determined by taking samples and running these samples multiple times in the same assay and inter-assay precession (Run to run) which was determined by measuring the same samples in multiple assays (Tab.3,4)

Accuracy: Accuracy assessment of the assay under investigation was carried out throughout recovery and dilution tests (parallelism)

Recovery test: The recovery test measures the concentration in human samples before and after adding known samples of pure analyte (cortisol) the recovery data of this study (Tab. 5) are in good agreement with

the data of Pilliai and Bhandarkar 1998 [18] and El-kolaly et al.2006 [19] who stated that the recovery of the assay should be 100.±15%.

Dilution test : The results in (Tab. 6) reveal the concentrations of three human samples undiluted and at various dilutions in the matrix of the assay to assess the linearity of the assay. Edwards, 1996 [20] reported that non-linearity indicated inaccurate calibration or an inappropriate matrix or both.

Specificity (cross-reactivity): Specificity is expressed as cross reactivity and interference with high concentrations of related hormones such as (progesterone, Estradiol, Testesterone) (Tab. 7)

DISCUSSION:

Measurement of cortisol hormone in serum is an important tool for the diagnosis of adrenal status.

We use radioimmunoassay method in this study because it is an elegant technique used in measurement of very low concentrations of specific compounds in the presence of excess of other materials

The present study was motivated by many factors as the commercial importance, the kit manufacturers don't disclose the details of the technique and also there is a lack of literature in respect to details of these technique. So, local preparation and evaluation of reagents and establishment of the liquid phase RIA technique becomes necessary.

Therefore, the aim of the present study was oriented to optimize the procedure used for determination of cortisol level in human sera. The research plan of this work was designed to achieve the following:

Preparation of the immunogen (Hydrocortisone-21-hemisuccinate: BSA) this is because cortisol has haptenic nature, so, it must be linked by accepted ratio to protein which can provoke the immune system of the host animal.

We choose BSA because of its availability and fast solubility in partial organic/aqueous solvent system [21]

The ratio of attachement of cortisol residue to BSA was in the acceptable range (17 molecule cortisol / 1 molecule BSA)[11.12] .

Preparation of the basic reagents of cortisol assay which comprised of: polyclonal antibodies, radiolabelled cortisol tracer and cortisol standards.

results obtained in the step of production of polyclonal antibodies are in good agreement with many reports [18,22]

At the same time the preparation of ^{125}I-Cortisol tracer of high radiochemical purity and immunoreactivity is essential for the development of sensitive, precise and accurate radioimmunoassay technique

The results of the present work revealed that ^{125}I-Cortisol tracer yield was 58.5% and purity 96 % with specific activity 120 µCi/µg. therefore, the chloramines-T oxidation method was found to be the suitable and fast technique which give high yield, stable and pure tracer

Third component was cortisol standards which was prepared and calibrated to be used for preparing the standard curve

as the main components were prepared, the development of double antibody radioimmunoassay system for the measurement of cortisol in human serum was performed by studying four important factors (temperature, incubation time, sample volume and precipitating agents)

and to assure the validity of the local double antibody RIA technique, some performance characteristics studies were carried out. As : sensitivity, precision (intra and inter- assay), accuracy (recovery and dilution tests) and specificity

CONCLUSION

In conclusion, the technical simplicity of this sensitive, precise and accurate method may suggest that this cortisol-RIA technique should be suited for routine laboratory uses and can be used effectively as a decisive diagnostic tool for adrenal status.

Table(1) : Optimum conditions used to draw the standard curve was:

Dilution of antibody	**Incubation temperature**	**Incubation time**	**Sample volume**
1/8000 of R1 bleed 5	37 C	3 h	100 µl
PEG concentration	**PEG volume**	**Second antibody**	**Non-immune rabbit serum**
12 %	500 µl	1: 80	1:200

Table(2) : The sensitivity of cortisol liquid phase RIA system

Cpm (mean-SD)	**Cpm (mean-2SD)**	**B/Bo %**	**Apparent Concentration (µg/dl)**	**Approximate Sensitivity(µg/dl)**
10239 – 112.95	10239 - 225.9	95.1	0.11	0.11

Table(3) : Intra-assay precision for Cortisol using liquid phase RIA system

Samples	**Intra-assay**		
	Mean (µg/dl)	**SD (µg/dl)**	**CV%**
Low	2.35	0.14	6.1
Normal	8.1	0.29	3.6
High	13.2	1.10	8.4

Table(4) : Intra-assay precision for Cortisol using liquid phase RIA system

Samples	**Intra-assay**		
	Mean (µg/dl)	**SD (µg/dl)**	**CV%**
Low	2.58	0.20	7.6
Normal	7.95	0.55	6.9
High	13.4	1.26	9.4

Table(5) : Recovery assessment for cortisol using liquid phase RIA system.

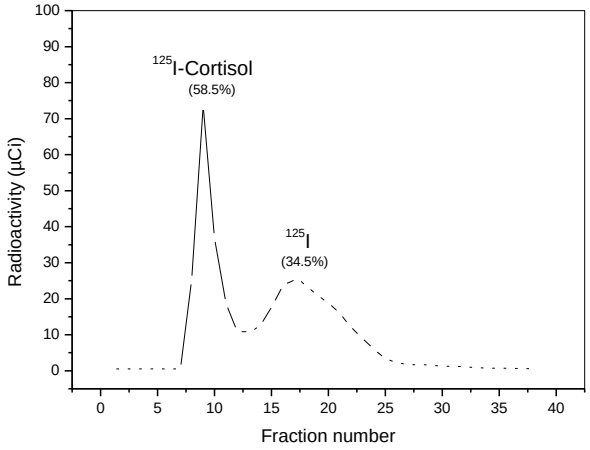
Sample	Endogenous (µg/dl)	Added (µg/dl)	Expected (µg/dl)	Observed (µg/dl)	Recovery (%)
1	2.47	1.92	2.20	2.18	99.25
		3.84	3.16	3.50	110.98
		7.68	5.08	4.65	91.7
2	8.14	1.92	5.03	4.40	87.48
		3.84	5.99	6.10	101.81
		7.68	7.91	7.31	92.41
3	13.31	1.92	7.62	7.30	95.82
		3.84	8.58	7.67	89.49
		7.68	10.50	9.14	87.08

Table (6): Dilution test for cortisol using liquid phase RIA system**.**

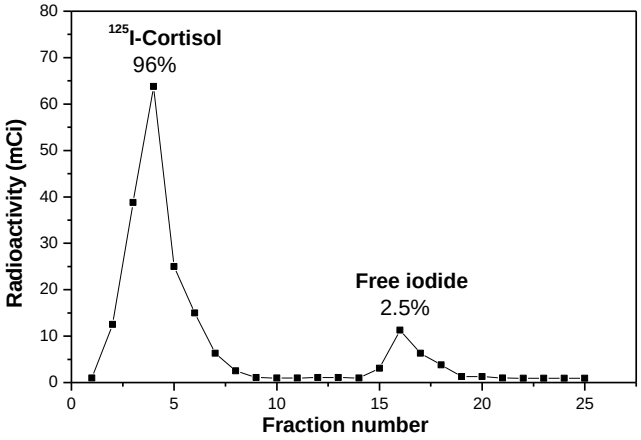
Sample	Endogenous (µg/dl)	Dilution factor	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1	2.47	1:2	1.24	1.10	88.68
		1:4	0.62	0.53	86.45
		1:8	0.31	0.24	76.3
		1:16	0.15	0.12	78.24
2	8.14	1:2	4.07	4.09	100.5
		1:4	2.04	1.93	94.97
		1:8	1.02	1.08	106.61
		1:16	0.51	0.55	107.47
3	13.31	1:2	6.66	6.43	96.67
		1:4	3.33	3.06	92.08
		1:8	1.66	1.42	85.55
		1:16	0.83	0.70	84.55

Table(7): specificity (cross-reactivity) of the anti-cortisol using liquid phase RIA system.

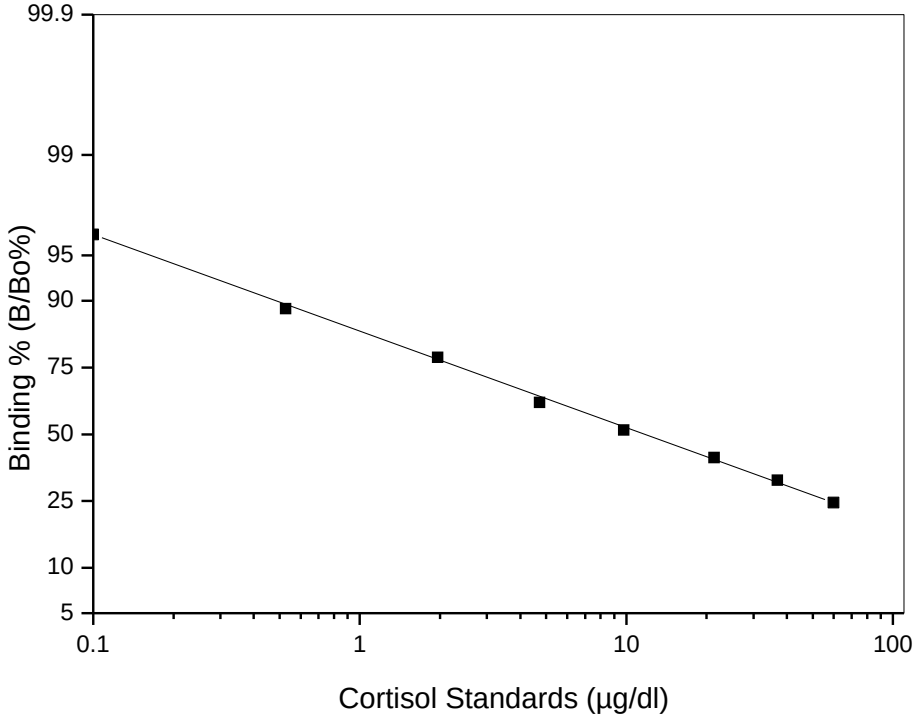
Steroid	Cross Reactivity %
Cortisol	100
Testosterone	0.17
Progesterone	0.13
Estradiol	0.04



Figure(1): Elution pattern of radioiodination mixture of cortisol and purification of ^{125}I- cortisol tracer using gel chromatography on sehadexG-25



Figure(2): Electrophoretical pattern of radiochemical purity of produced ^{125}I-Cortisol tracer



Figure(3): Optimized standard curve for cortisol using liquid phase RIA system.

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