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CORTICOSTERONE ASSAY – ANALYTICAL METHOD VALIDATION FOR THE ANALYSIS OF CORTICOSTERONE IN RAT SERUM BY HPLC

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ABSTRACT

Plasma/serum corticosterone concentration is one of the most reliable physiological markers of 'stress'since stress is characterized by elevated glucocorticoids. Most of the animal studies in behavioural research involve estimation of plasma/serum corticosterone levels along with behavioural assays. However a vast majority of these studies utilize the radio immuno assay technique which is quick and reliable but quite expensive. In this study, we demonstrate the validation of a method using high performance liquid chromatography coupled with a diode array detector for the determination of serum corticosterone concentrations which is a cost effective and reliable alternative to the routine radio immune assay. The linearity was done at a concentration range of 0 to 1000 ng/ml (R^2=0.996).The percentage recovery was more than 90% and relative standard deviation was less than 8%. The limit of detection was 5ng/ml and limit of quantification was 10 ng/ml. This method has demonstrated reliability and can be used for the routine estimation of serum corticosterone levels for behavioural research.

KEYWORDS: Stress, Corticosterone Assay, HPLC, Behavioural Research.

INTRODUCTION

'Stress' pertains to an experience of negative emotions that occurs due to physiological, biochemical, cognitive and behavioural changes that work towards either escaping from the stressor or making adjustments to its effects.[1] Chronic stress when left untreated leads to serious disorders like insomnia, weakened immune system, high blood pressure, anxiety and muscle pain.[1] It can also play a role in developing major disorders like depression, heart disease and obesity.[1] Two major systems are involved in stress physiology - the Sympathetic-Adreno-Medullary (SAM) system and the Hypothalamic-Pituitary-Adrenal (HPA) axis.[2] Activation of SAM axis is a short term and immediate response to stress. In the long term, the HPA axis is activated. The HPA axis is responsible for the secretion of glucocorticoids. Glucocorticoids act to mobilize energy stores and also to inhibit other physiological systems (e.g reproduction, immune function, growth) in order to conserve energy during the stress response. Glucocorticoids also act on the brain to increase appetite and to increase locomotor activity and food-seeking behaviour, thus regulating behaviours that control energy intake and expenditure.[3] To maintain normal physiological function, glucocorticoids are secreted at a baseline level.[4] During stress, glucocorticoid secretion increases in part to mobilize more metabolic fuel to cope with the stressor, and once the stress is overcome glucocorticoids return to a baseline level.[5] While the major glucocorticoid in humans is cortisol, in rodents it is corticosterone.[6,7] So, the determination of serum corticosterone levels could be a reliable measure for assessing depressive states, in addition to behavioural assays, since depression is associated with elevated serum corticosterone levels.[5]

The currently available methods for the determination of serum corticosterone concentrations from trunk blood is via a commercially available radio immuno assay (RIA) kit.[8] The principle involved in radio immune assay is the measurement of the concentration of antigen molecules using a radioactive label that quantifies the amount of antigen (i.e, hormone) by determining the extent to which it combines with its antibody. The RIA technique involves cross reaction of antiserum with precursors and metabolites of corticosterone, as well as with other endogenous steroids, which may lead to an overestimation of the true levels of corticosterone.[8] The technique is quite expensive and can be used for the analysis of a limited number of samples per kit. However, the versatility of HPLC enables the utilization of this method of analysis for larger groups of samples effortlessly. Though the starting cost of a HPLC method is high, the routine running cost of samples is much lower when compared to radio immune assay.

MATERIALS AND METHODS

An optimised method used by Viljoen *et al.*[8], has been utilized.

Standard: Corticosterone, obtained from Sigma Aldrich.

Internal standard: Dexamethasone, obtained from Sigma Aldrich.

Solvents: Methanol, acetonitrile, and dichloromethane were of HPLC grade and were obtained from Merck while glacial acetic acid is of LR grade and obtained from Fisher Scientific. Distilled water was obtained from Milli Q. Activated decolourising charcoal (charcoal activated) was obtained from Merck.

Animals: Male wistar rats weighing 180-200 g were obtained from National Institute of Nutrition (NIN) and were housed in standard laboratory conditions for 1 week prior to the experiment with 12 h light/dark cycle with food and water *ad libitum*. Chronic variable stress was applied as a stress paradigm with acute exposure to 5 different stressors for 2 weeks as described by Bondi *et al.*[9] Blood sampling was done after the behavioural assays between 4 P.M. and 6 P.M. and serum was separated by centrifugation at 10000 rpm for 15 min. The experimental protocols were approved by the institutional animal ethical committee (IAEC, approval no: 516/PO/c/01/IAEC/3), Andhra University, Visakhapatnam, Andhra Pradesh state.

Equipment: Shimadzu UFLC LC-20AD.

Preparation of Standards

A 100µg/ml stock solution of corticosterone in 20% methanol was prepared afresh in an amber volumetric flask and dilutions were made with distilled water at a concentration range of 10-1000 ng/ml. Another set of standards with a similar concentration range were made with serum for serum standards. Preparation of serum standards involves the collection of blood from healthy rats and all the serum was pooled into one glass beaker. The serum was treated with activated decolourising charcoal to remove the endogenous corticosterone. The suspension was stirred for approximately 90 min. at room temperature and was pipetted into a glass tube and sample was added to a 10×100 mm screw capped glass tube containing 50 µl of the internal standard dexamethasone (1µg/ml).The mixture was extracted with 5 ml of dichloromethane by vortexing it for 2 min after which it was centrifuged at 3000 rpm for 10 min. After centrifugation the upper layer which comprised of either distilled water or serum was removed, and the lower organic layer was transferred to conical tubes and evaporated to dryness under nitrogen at room temperature. The residue was reconstituted with 150 µl of mobile phase. 100 µl of this solution was then injected into the sample injection port.

Sample Preparation

500µl of the water or serum standard or test serum was added to a 10×100 mm screw-capped glass tube containing 50 µl of the internal standard dexamethasone (1µg/ml). The mixture was extracted with 5ml of dichloromethane by vortexing it for 2 min after which it was centrifuged at 3000 rpm for 10 min. After centrifugation the upper layer which comprised of either distilled water or serum was removed, and the lower organic layer was transferred to conical tubes and evaporated to dryness under nitrogen at room temperature. The residue was reconstituted with 150 µl of mobile phase. 100 µl of this solution was injected for analysis.

High Performance Liquid Chromatography conditions:

Stationary Phase	**:**	C_{18} Column
Mobile Phase	**:**	distilled water: acetonitrile: glacial acetic acid (65:35:0.05, v/v)
pH	**:**	4.10
Flow rate	**:**	1ml/min
Sample injection volume	**:**	100 µl
Wavelength	**:**	245 nm
Detector	**:**	Diode Array Detector
Run time	**:**	15 min at a temperature of 24 °C

METHOD VALIDATION[10]

Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using five determinations per concentration. Data from a minimum of nine concentrations over a minimum of three concentration levels covering the specified range (eg. Three concentrations, three replicates of each concentration) is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedures applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using five determinations per concentration. Data from a minimum of nine

concentrations over a minimum of three concentration levels covering the specified range (eg. Three concentrations, three replicates of each concentration) is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

The following equation was used to calculate the % recovery.

$$\% \text{ Recovery} = \frac{\text{Area or height of extracted sample}}{\text{Area or height of unextracted sample}} \times 100$$

Calibration/Standard Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met

(i) The analyte response at the LLOQ should be at least 5 times the response compared to blank response.

(ii) Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy 0f 80-120%

Calibration Curve/ Standard Curve/ Concentration-Response

The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve

1. 20% deviation of the LLOQ from nominal concentration
2. 15% deviation of standards other than LLOQ from nominal concentration
3. At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

RESULTS

Method Validation

Linearity was demonstrated for both the water and serum standard calibration curve. Both the water and serum standards were extracted as described in the extraction procedure to determine the repeatability (precision and accuracy). The recovery for both water and plasma standards following extraction was determined at the concentration range of 0, 10, 500, and 1000 ng/ml.

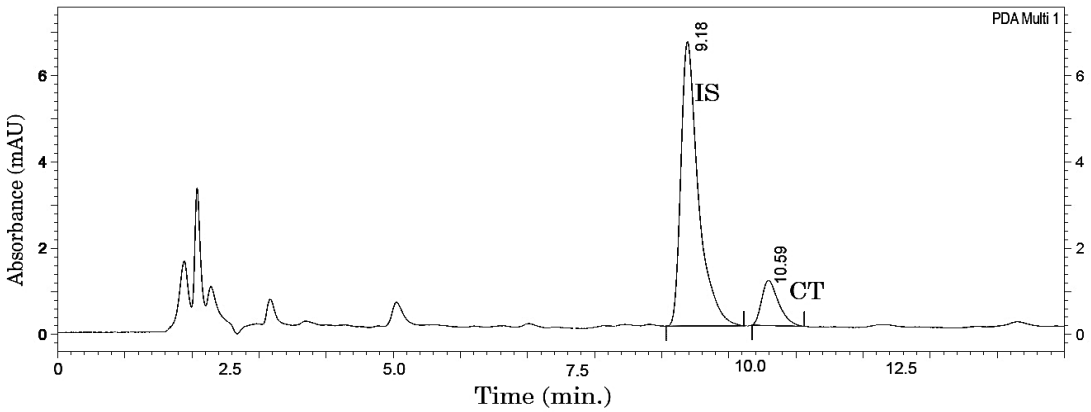

Figure 1: Chromatogram of a 10 ng/ml corticosterone water standard.

Linearity

Linearity was done on the following concentration range: 0, 10, 50, 100, 200, 500, and 1000 ng/ml for both the water and serum standards.

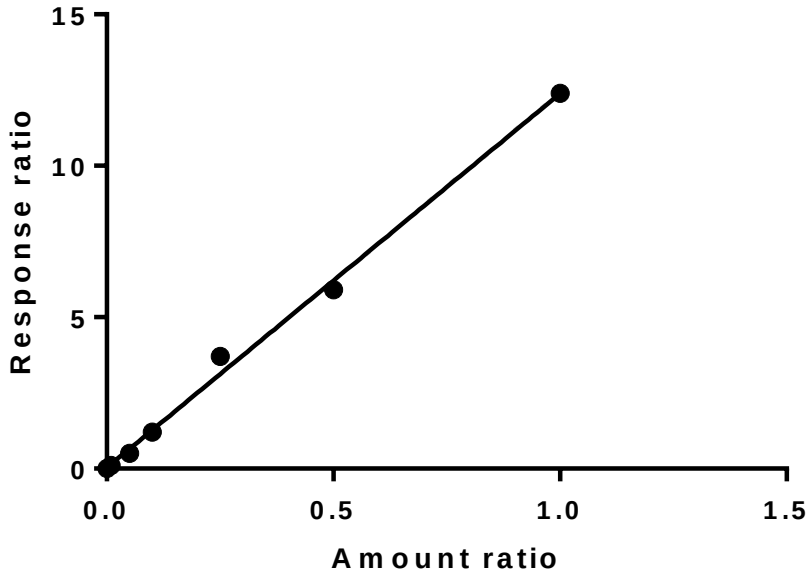

Figure 2: Regression line of the corticosterone water standards.

- The slope of the regression line is 12.35 ± 0.3392
- The coefficient of determination R^2 is 0.9962
- The Y-Intercept is 0.03079 ± 0.1476
- The residual sum of squares is 0.3041

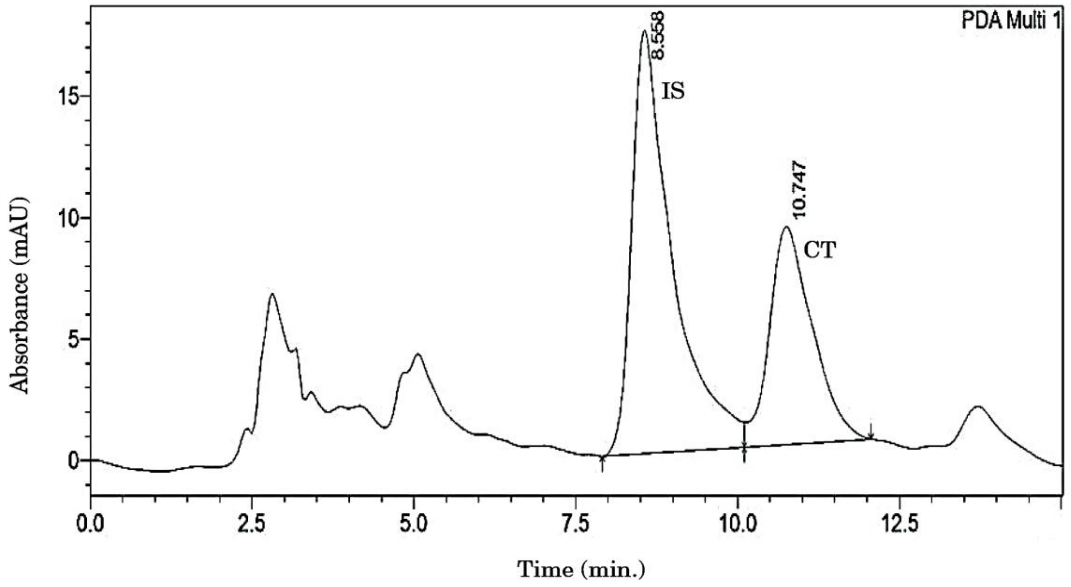

Figure 3: Chromatogram of a 10ng/ml serum standard.

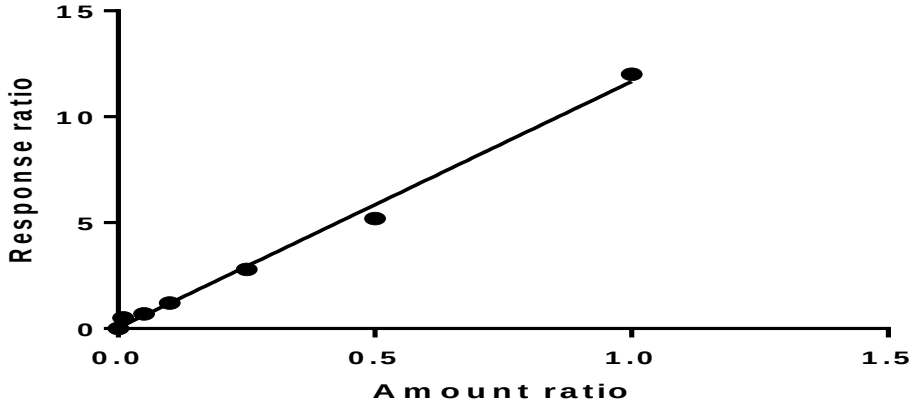

Figure 4: Regression line of the corticosterone serum standards.

- The slope of the regression line is 11.63±0.4126
- The coefficient of determination R^2 is 0.9937
- The Y-Intercept is 0.02730 ± 1.795
- The residual sum of squares is 0.3700

Repeatability (Precision and Accuracy)

The precision and accuracy results gave percentage relative standard deviation values less than 8. The values were given in Table 1& 2 and Fig 5.

Table 1: Recovery of analyte (Corticosterone) and internal standard (Dexamethasone) from water standards.

Conc.	Extracted Peak Response		Unextracted Peak response	
	Cort	**IS**	**Cort**	**IS**
10 ng LQC	313426	2516845	325492	3042154
	321462	2736922	338646	3124668
	314773	2467331	351645	3123922
		Mean % Recovery = 93.49 S.D = 2.494 % CV = 2.7 %		
500 ng MQC	212885500	41275092	239873122	47523224
	232812440	42848546	234782130	45632115
	225424360	44200854	245621140	46542002
		Mean % Recovery = 93.23 S.D = 5.35 % CV = 5.7 %		
1000 ng HQC	334406094	27506699	335014254	28506699
	324255688	25529861	344532601	29654203
	314635772	26885463	339568420	29138407
		Mean % Recovery = 95.55 S.D = 3.83 % CV = 4.13 %		

Cort: corticosterone; IS: Internal standard; CV: coefficient of variation; LQC: low quality control.; MQC: medium quality control.; HQC: high quality control.

- Mean % Recovery of analyte at LQC, MQC,& HQC = 94.09%
- Mean % CV of analyte = 4.13%
- Mean % Recovery of IS at LQC, MQC,& HQC = 88.87%
- Mean % CV of IS = 6.67%

Table 2: Recovery of analyte (Corticosterone) and internal standard (Dexamethasone) from rat serum

Conc.	Extracted Peak Response		Unextracted Peak Response	
	Cort	**IS**	**Cort**	**IS**
10 ng	417083	764523	484980	875056
LQC	398468	704936	443165	856015
	428114	849765	431522	768124
	Mean % Recovery = 91.71%			
	S.D = 6.79			
	% CV = 7.4%			
500 ng	325283011	62178960	382463417	66864233
MQC	384200654	66857123	368233541	75997561
	337542310	68886185	378564203	71427203
	Mean % Recovery = 92.84%			
	S.D = 10.138			
	% CV = 10.92%			
1000 ng	445301472	38076226	514032629	44620887
HQC	497614063	42485820	522533610	45409338
	423764831	34964094	463219457	39024385
	Mean % Recovery = 91.11%			
	S.D = 4.32			
	% CV = 4.74%			

Cort: corticosterone; IS: Internal standard; CV: coefficient of variation; LQC: low quality control; MQC: medium quality control; HQC: high quality control

- Mean % Recovery of analyte at LQC, MQC,& HQC = 91.88%
- Mean % CV = 7.69%
- Mean % Recovery of IS at LQC, MQC,& HQC = 91.60%
- Mean % CV of IS = 8.91%

Acceptance criteria

1. For accuracy measurement, the mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%.
2. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

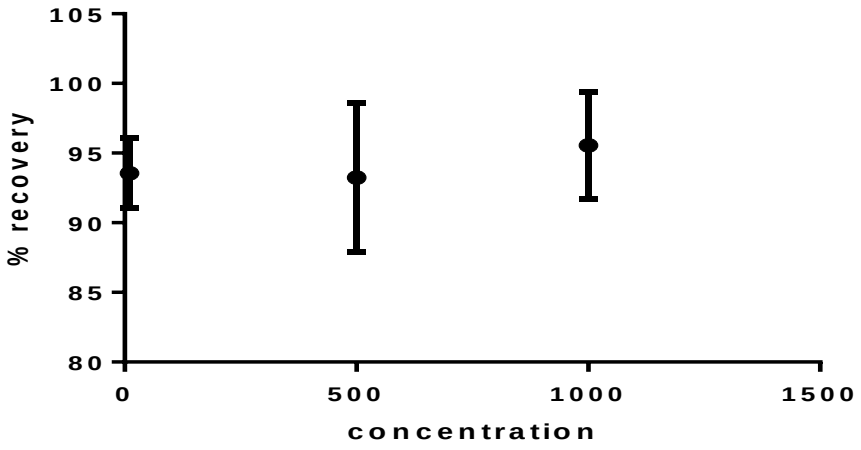

Figure 5: Plot representing accuracy and precision of serum standards at 3 concentration levels. The whiskers with up-tics and down- tics represent standard deviations

Limit of detection

1. The limit of detection for this method was 5 ng/ml.
2. Non instrumental method was used to determine the limit of detection. The minimum level at which the analyte could reliably be detected was determined.
3. Concentrations were prepared in the range of 0, 1, 5, 10, 50, 100, 200, 500, and 1000 ng/ml and were run in triplicate at each concentration level.
4. The analyte could be reliably detected at a concentration of 5 ng/ml.

Limit of quantification

1. The limit of quantification was 10 ng/ml.
2. Non-instrumental method was used to determine the limit of quantification based on the criteria that LOQ should be atleast 5 times the response compared to blank response.
3. The same concentration range that was used to determine linearity was used to determine LLOQ and each concentration was run in triplicate.
4. The precision and accuracy of the measurement were given in **Table 1.**

DISCUSSION

The linearity for both the water and serum standards were excellent and the repeatability was less than 15 %.The recovery was greater than 90%. The limit of detection was 5ng/ml and limit of quantification was10ng/ml. This validated hplc method for the detection of serum corticosterone has been successfully employed in our lab for the detection of serum corticosterone concentrations in behavioural research.

CONCLUSION

Since the results of the corticosterone assay were comparable to the results of the behavioural assay in both the control and stress induced animals, this method has been proved as a reliable and robust method for the routine analysis of serum corticosterone levels in behavioural research.

REFERENCES

1. Khan S, and Khan RA. Chronic Stress leads to Anxiety and Depression. Annals of Psychiatry and Mental Health, 2017.
2. Rizzo DC. Fundamentals of Anatomy & Physiology. second edition. Thomson Delmar Learning, 2006.
3. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. Hormones and Behaviour, 2003; 43: 2-15.
4. Bonier F, Martin PR, Moore IT, Wingfield JC. Do baseline glucocorticoids predict fitness? Trends in Ecology & Evolution, 2009; 24: 634-642.
5. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews, 2000; 21(1): 55-89.
6. Romero LM. Physiological stress in ecology: Lessons from biomedical research. Trends in ecology & Evolution, 2004; 19(5): 249-255.
7. Martinez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nature Reviews Cancer, 2011; 11(3): 161-176.
8. viljoen FP. An Optimized Method For The Analysis of Corticosterone In Rat Plasma By UV-HPLC. Medical Technology SA, 2012; 26(2): 39-42.
9. Bondi CO, Rodriguez G, Gould GG, Frazer A, and Morilak DA. Chronic Unpredictable Stress Induces a Cognitive Deficit and Anxiety-Like Behavior in Rats that is Prevented by Chronic Antidepressant Drug Treatment. Neuropsychopharmacology, 2008; 33: 320-331.
10. FDA. Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services Food and Drug Administration (FDA), 2001; 1-22.