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AN OPTIMIZED METHOD FOR THE ANALYSIS OF CORTICOSTERONE IN RAT PLASMA BY UV-HPLC

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ABSTRACT

Animal models are useful in the study of stress disorders in that they offer the possibility of stimulating a human condition under controlled conditions in a simpler, more readily understandable system. Stress-related activation of the hypothalamic-pituitary-adrenal (HPA) axis characterized by an increase in plasma corticosterone (CT) levels in the rat is an important manifestation of the physiological stress response. Current available methods for the determination of peripheral corticosterone concentrations from trunk blood, is via a commercially available radio immunoassay (RIA) kit. The aim of this study was to optimize and validate a sensitive, specific and cost-effective high performance liquid chromatography (HPLC) method for the determination of CT levels in plasma of rats. A 500 µl plasma sample was extracted with 5 ml dichloromethane and analyzed by HPLC coupled to a diode array detector at 245 nm. The standard curve was linear over a concentration range of 10 - 500 ng/ml (r^2=0.996). The percentage recovery was above 85%, the relative standard deviation was less than 7% and the limit of quantification was 10 ng/ml. Results from this method were compared with values obtained from a RIA method and the values were in close proximity of each other. We conclude that the current HPLC method that was optimized and validated is suitable for use in subsequent studies in rats.

KEYWORDS

Corticosterone, rats, plasma, HPLC.

INTRODUCTION

Stress is a common important risk factor in an array of diseases and it involves the same hormones that ensure survival during a stressful period[3]. Stress in the practical sense of the word can be defined as anything that causes an increased secretion of glucocorticoids[3]. The main target of glucocorticoids is the brain[3]. Determination of corticosterone (CT) levels in our laboratory was historically performed by means of commercially available radioimmunoassay techniques (RIA) which is expensive, qualitative and limited in the number of samples that can be done per RIA-kit. The RIA techniques involves cross reaction of antiserum with precursors and metabolites of CT, as well as with other endogenous steroids, and may lead to an overestimation of the true levels of CT[2]. Since the focus of our research is anxiety and stress-related disorders, routine analysis of CT is imperative, however, making use of an RIA-kit is impractical and expensive. Current HPLC instruments with an autosampler lend themselves effortlessly to processing and automation of larger groups of samples[2]. Although the starting costs of a HPLC method is high, the routine running cost of samples is much lower than compared to RIA assays[2].

Corticosterone is produced in the zonae fasciculate and the glomerulosa of the adrenal cortex[4, 5]. It is a corticosteroid type, 21 carbon steroid hormone[4]. Being the dominant glucocorticoid in rodents it is, however, less abundant in humans[5]. In rodents it is involved in the regulation of fuel metabolism, immune reactions and stress response[4] and is of the same importance as that of cortisol in humans. It can thus be used as an indicator of adrenal function[6]. Stress has a marked effect on circulating CT levels and under stressful situations such as temperature change, experimental stimuli or unusual routine it causes a rapid increase in the levels of these molecules[6]. Increases in plasma levels of CT (glucocorticoids) is a reliable indication of HPA axis activation[7]. In stressful situations neuroendocrine response mainly involves the HPA axis, which results in an increase in glucocorticoid levels as well as corticotrophin-releasing and adrenocorticotropin hormone levels[7]. Determination of CT levels in plasma, as a validation of the stress response in animals and particularly in studies focusing on anxiety, depression, fear, post traumatic stress disorder (PTSD), is commonly done[9-11].

MATERIALS AND METHODS

The method of Wong *et al.* 1994[2] was revised and optimized. The raw materials for corticosterone as the standard and dexamethasone (DX) as internal standard were obtained from Sigma-Aldrich. Distilled water was obtained from a Milli-Q® Reagent Water System. Both the acetonitrile and dichloromethane was of HPLC grade and obtained from Merck, as well as the glacial acetic acid. Activated decolorizing carbon (charcoal activated) was also obtained from Merck. Male Sprague-Dawley rats (180 ± 20 g) bred and housed at the North-West University's animal centre were obtained for the animal studies. During the time of the study the rats were kept on a natural 12 hour light/dark cycle with free access to food and water and they were housed 6 per cage[9]. Time-dependent sensitization stress (TDS) was applied as a stress paradigm and in short comprises of an acute exposure to three different stressors and 7 days later a re-exposure

to one of the stressors as described by Liberzon and colleagues (1997). All the TDS experiments took place between 08h00 and 12h00 in the morning whereafter they were left undisturbed for 7 days post the restress procedure and sacrificed for the plasma corticosterone and other neurochemical assays[9]. Approval of the study protocol was granted by the Animal Ethics Committee of the North-West University (Ethics approval number 04D06) [9]. All animals were treated according to the code of ethics in research as laid down by this Animal Ethics Committee[9].

PREPARATION OF STANDARDS

A 100µg/ml stock solution of CT was prepared in 20% methanol, in an amber volumetric flask and stored in a refrigerator. Blood of healthy rats were collected in heparin blood tubes and centrifuged. All the plasma was pooled into one glass beaker. The plasma was treated with activated decolorizing carbon to remove the endogenous CT[2]. The suspension was stirred for ±90 minutes at room temperature where after it was pipetted into a glass tube and centrifuged at 3000 rpm for 10 minutes. The top layer of plasma was filtered through a 0.45-µm Millipore filter to remove all the carbon particles. Two sets of the concentration range 10-500 ng/ml were made, one with distilled water for water standards and another with the activated decolorizing carbon treated plasma for plasma standards.

SAMPLE PREPARATION

500µl of the water or plasma standard or test plasma was added to a 10 x 100 mm screw-capped glass tube containing 50µl of the internal standard Dexamethasone (1µg/ml). The mixture was extracted with 5ml of dichloromethane by vortexing it for 2 minutes where after it was centrifuged at 3000 rpm for 10 minutes. After centrifugation the upper layer which comprised of either distilled water or plasma 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 150µl of mobile phase. The 150µl solution was transferred into inserts in vials and placed in the autosampler.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CONDITIONS

The mobile phase for this chromatographic procedure consisted of the following: distilled water; acetonitrile and glacial acetic acid (65:35:0.05, v/v)[2] with a pH between 4.10 and 4.20. The flow rate was set at 1.0 ml/min and the sample injection volume was 100µl. The eluent was monitored at a wavelength of 245 nm by the diode array detector. The run time for each sample was ± 15 minutes in a room where the temperature was controlled at 24°C and ± 20 minutes in a room controlled at a lower room temperature.

METHOD VALIDATION

Linearity was done on the following concentration range: 10, 25, 50, 100, 200 and 500 ng/ml. Both the water and plasma 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 and the same concentration range as for the linearity was used.

To calculate the recovery the next equation was used:

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

RESULTS

Chromatograms

Figure 1 depicts a chromatogram of a corticosterone (CT) water standard.

Figure 2 depicts a chromatogram of a rat's plasma sample.

Method Validation[8]

Linearity

Linearity was demonstrated for both the water standard calibration curve (r^2=0.997) and the plasma standard curve (r^2=0.996).

Repeatability (Precision and Accuracy)

The precision and accuracy results gave percentage relative

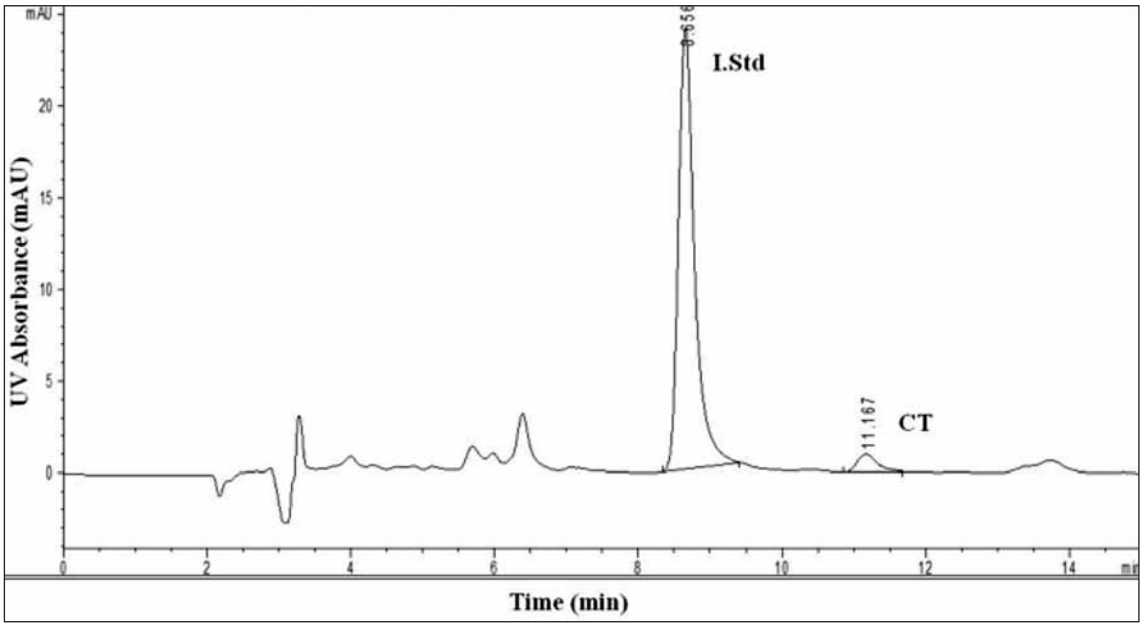

Figure 1: 10 ng/ml Corticosterone (CT) Water standard.

standard deviation values less than 7.0% for all the standards of the concentration range (refer to method validation).

Recovery of the extraction method

The recovery of the extraction method was between 86-100% and 93-100% for the water and the plasma standards respectively for the concentration range used (refer to method validation).

Limit of detection & Limit of quantification

The limit of detection for this method was 5 ng/ml and the limit of quantification was 10 ng/ml.

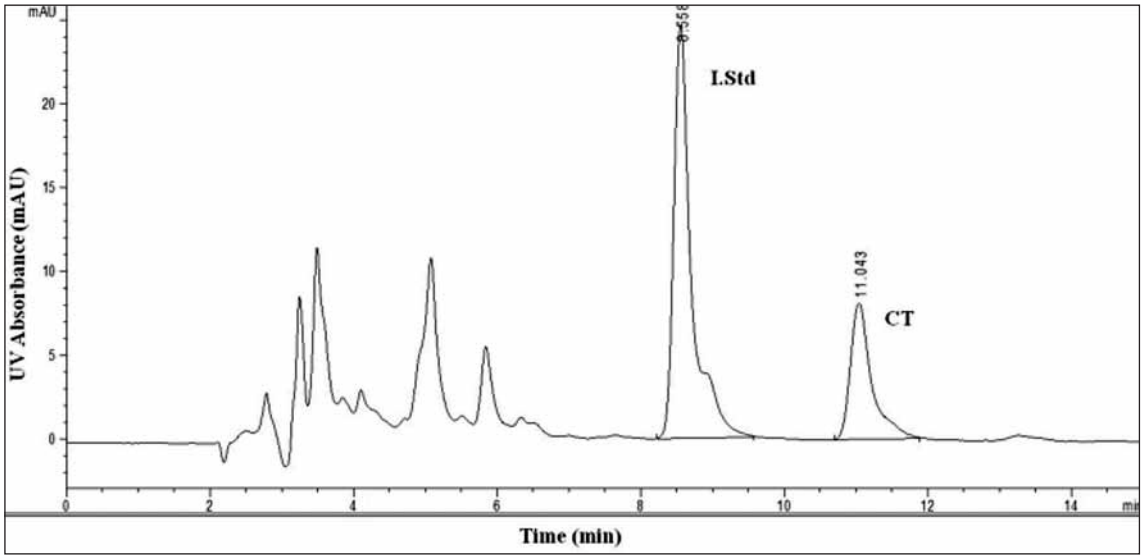

Figure 2: Chromatogram of a rat's plasma sample with a CT concentration of 214ng/ml.

Table 1: The new optimized HPLC method compared with RIA method

Ten Rat Samples (5 Control & 5 Test samples	Results obtained through RIA method (ng/ml)[9]	Reults obtained through new HPLC method (ng/ml)
Control 1	101	99
Control 2	103	117
Control 3	201	164
Control 4	229	190
Control 5	243	214
Test 1	581	563
Test 2	693	717
Test 3	772	679
Test 4	800	812
Test 5	1019	985

Table 2: Comparison between the old method and new optimized method

Parameters changed	Method of Wong *et al.* 1994[2]	The New Method
1. Sample volume	500µl	500µl
2. Column	LiChrospher 100 RP-18	Synergi Luna C18
3. Extraction phase	15ml Dichloromethane	5ml Dichloromethane
4. Extraction procedure	Shaked for 15 minutes	Vortexed for 2 minutes
5. Internal Standard	Dexamethasone – 4µg/ml	Dexamethasone – 1µg/ml
6. Wavelength	254nm (UV Detector)	245nm (Diode Array Detector)
7. Data acquisition	SP 4290 Integrator (Spectra-Physics)	Chemstations (Agilent)
8. Wash phases	2 Wash phases with NaOH and distilled water	No wash phase
9. Reconstitute volume after evaporation	250µl	150µl
10. Injection volume on the HPLC column	Not mentioned	100µl

ANIMAL STUDY: COMPARISON BETWEEN THE NEW HPLC METHOD AND A RIA METHOD

As mentioned, in the original animal study the plasma corticosterone values were determined by means of a RIA method and in order to compare the values from the RIA method and the HPLC-method, a sample of the plasma of 5 control rats (no stress procedure) and 5 test rats (stressed in the TDS procedure) were taken from the original study[9] covering the whole concentration range of CT levels found in plasma of control and stressed rats.

DISCUSSION

Utilizing the diode array spectra of corticosterone, obtained by using a diode array detector, the best wavelength for CT was found to be 245 nm. The linearity for both the water and plasma standards was very good and the repeatability was below 15% with the recovery above the required limits[8]. The application of the HPLC-method to control and stressed rats' plasma samples were compared to the results obtained by the RIA technique and it was found that it compared very well (Table 1). This new HPLC-method, optimized from the method of Wong *et al.* 1994[2], was validated in our laboratory and proved to fulfill all the necessary criteria.

CONCLUSION

From the evidence found in this study it can be concluded that this method give results similar to those found by the RIA technique and is a highly suitable and much less expensive method to be utilized in measuring plasma CT levels in rodents. While it proved to give comparable results in both control and stressed animals, this method can be used as a valuable indication of the stress response as found in anxiety and stress-related disorders.

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