Chapter 10

A Modified Fluorimetric Method for Corticosterone estimation and its Application to have Estimation in Rat Serum, Tissues and Mitochondria.

Introduction

Corticosterone is the only glucocorticoid hormone of the rat(1,2) The plasma levels of corticosterone show diurnal variation, the levels are highest late in the day or immediately after onset of darkness and are lowest between 6 A M to 7 A M (3-4) There are six episodic bursts of corticosterone (2) The corticosterone levels also change in response to stress (3) Estimation of corticosterone levels in plasma is important for physiological correlates (5,6) It is also recognized now that the concentration of hormones in the target tissue is more important in physiological context than their concentration in the plasma (7)

Silber et al described a flourimetric method for estimation of corticosterone in plasma of rat and other species including monkey and guinea pig (8) Gulemin et al extended this method for physiological validation in terms of plasma corticosterone concentrations (6) However, the method has not been applied for estimation of corticosterone in tissues and in subcellular organelles While the importance of hormone concentrations in the tissues has been recognized, the levels of corticosterone in the tissues and / or subcellular organelles have not been reported

Earlier studies from our laboratory have shown that corticosterone overload in rats resulted in impairment of respiration and uncoupling of rat liver and brain mitochondria (9,10) Therefore it of interest to find out the hormone levels in the tissues and mitochondria With this intension attempts were made to adapt the Silber procedure(8) for estimation of corticosterone in plasma as well as in tissues and mitochondria In the process difficulties were encountered especially with the recovery of the hormone Hence, the procedure was modified which is applicable to

plasma, tissue homogenates as well as subcellular organelles was devised The details of the same are described here

Materials and Methods

Chemicals

Corticosterone was purchased from Sigma All other chemicals were of analytical reagent grade and were purchased locally

Experimental

For extraction of corticosterone, initially the Silber procedure (8) was tried, where 0 1ml of serum or 0 2ml of suitably diluted tissue homogenates or mitochondrial samples were treated with 0 2ml of freshly prepared chloroform methanol mixture (2 1,v/v) to which 1 5ml of petroleum ether (fraction 40-60) was added The sample was vortexed for 30 sec, allowed to stand at room temperature for separation of the phases and the light petroleum ether layer was carefully removed with the help of a Pasteur pipette and discarded The aqueous layer was extracted with 3ml of chloroform The sample was vortexed for 30 sec and subjected to centrifugation at 2000 rpm for 10min and the chloroform layer was carefully removed with the help of a syringe to which a 16 gauge long needle was attached and was transferred to a fresh tube The chloroform extract was then treated with 0 3ml of 0 1N NaOH, vortexed rapidly and the NaOH layer was immediately removed The sample was then treated with 3ml of 30N H_2SO_4 , by vortexing vigorously After phase separation, the chloroform layer on the top was removed with the syringe as described above and discarded After treatment with H_2SO_4 the tubes were kept in dark for 30-60min after

which fluorescence measurements were carried out in a Shimadzu model RF-5000 spectrofluoriphotometer with wavelength setting λ Ex 472 nm and λ Em 523 2 nm Appropriate blanks were kept for individual samples

Other details

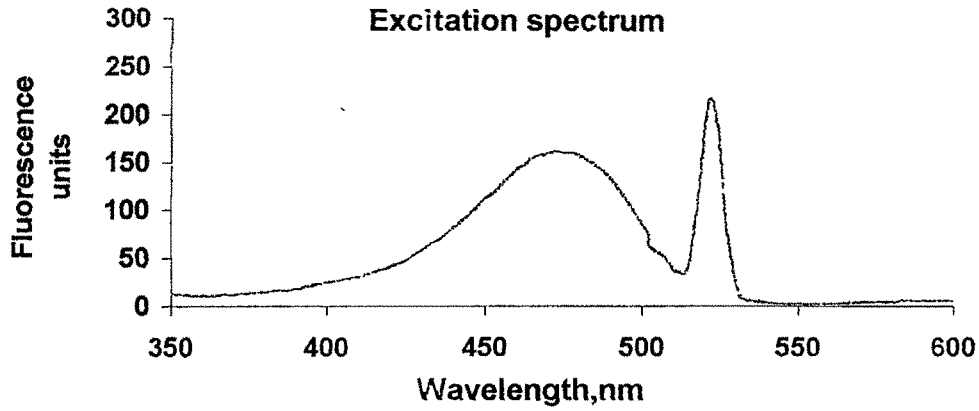
The λ excitation and λ emission were determined in separate experiment with standard corticosterone solution The typical excitation and emission spectra and a standard curve depicting fluorescence yield versus corticosterone concentration are shown in Fig 1

By following this procedure as described by Silber (8) the major difficulty that was encountered was that the phase separation was not clear, in that a foggy interphase appeared after treatment with petroleum ether and the volume at the foggy interphase varied depending on the sample used for extraction This raised a doubt as to whether the recovery would be quantitative and also whether the different samples were affected differently

In the light of the above an alternate simplified procedure was followed where after mixing the sample with chloroform methanol it was extracted directly with 3ml of chloroform After vigorous vortexing, the sample tubes were centrifuged and clear organic phase was removed, treated with NaOH as above and finally with H_2SO_4 The remaining procedure was the same as described above

The efficacy of the two extraction procedures i e as per Silber and the present method as described above was evaluated by recovery experiments For these experiments the

Fig.1


Excitation spectrum
A
Fluorescence units
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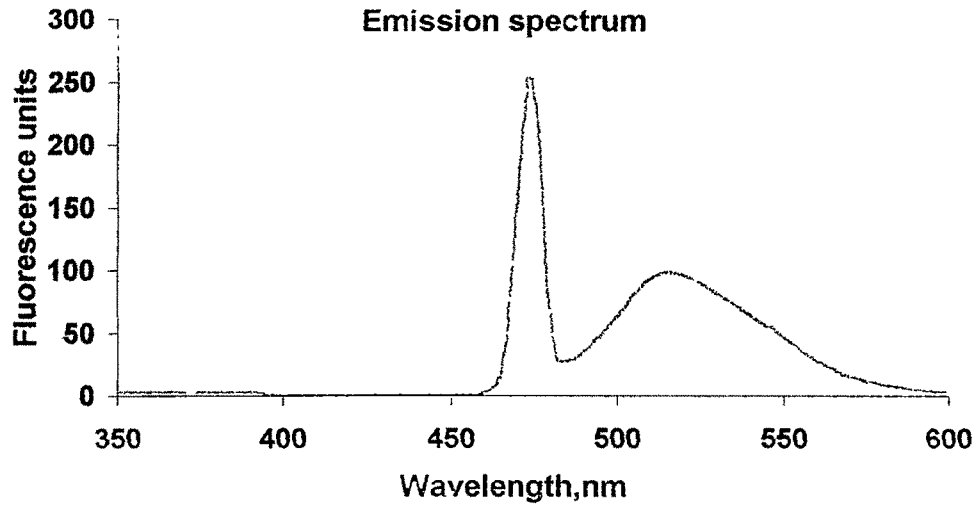
Wavelength,nm


Emission spectrum
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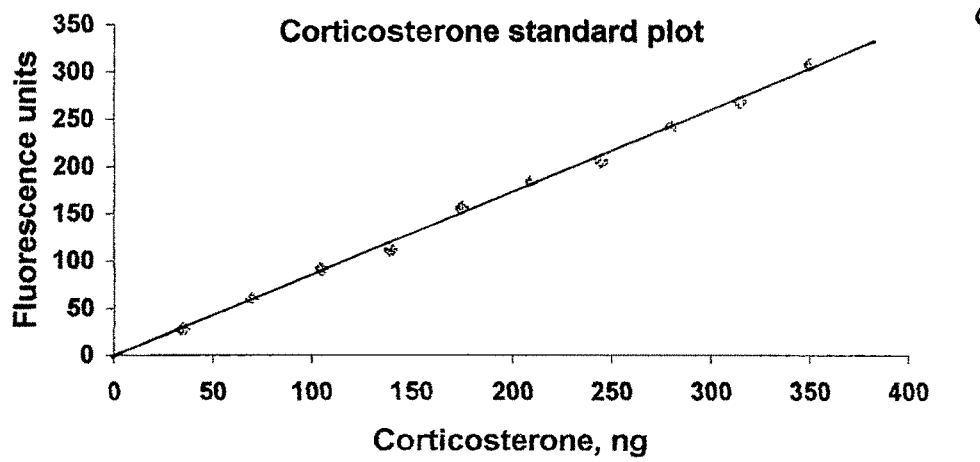
Corticosterone standard plot
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Fluorescence units
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250
300
350
400
Corticosterone, ng

biological samples containing 60-250ng of corticosterone were taken in three sets of tubes To one set of tubes 100ng of corticosterone (dissolved in chloroform) was added and in the second set of tubes 200ng of corticosterone was added The sample in which no corticosterone was added exogenously served as control for calculation of percentage recovery

Results and Discussion

The data on recovery experiments by the two procedures i e Silber (8) and the presently described direct chloroform extraction method are given in Table 1

As can be noted the amount of corticosterone in the serum sample as estimated by the Silber procedure was found to be 17ng whereas by the direct chloroform extraction method showed the content to be 40ng The recovery by the Silber procedure was in the range of 57-69% whereas direct chloroform extraction method gave almost 100% recovery

Results on the corticosterone content in the brain homogenate and mitochondria are also given in Table 1, from which it is evident that once again the corticosterone content of the homogenate was found to be much lower by the Silber procedure than that obtained by the chloroform extraction method However, these values for mitochondria were comparable Nevertheless, the recovery in the Silber procedure was in the range of 65-80% whereas the chloroform method gave 86-99% recovery

For the liver samples, the corticosterone content in the tissue homogenates as well as mitochondria was comparable by both the procedures (Table 1) However, once again

Table 1. Recovery experiment for corticosterone estimation with petroleum extraction and chloroform extraction methods.

Group	Silber method			Present Procedure		
	Corticosterone content of the sample (ng)	% Recovery		Corticosterone content of the sample (ng)	% Recovery	
		A	B		A	B
Serum	16.66 ± 1.60	56.87 ± 9.82	69.37 ± 7.12	40.15 ± 3.58	100.40 ± 11.30	94.91 ± 6.39
Homogenate						
Liver	201.56 ± 16.47	65.20 ± 12.17	69.09 ± 8.22	230.20 ± 23.06	91.15 ± 4.30	83.10 ± 5.44
Brain	92.06 ± 12.50	64.34 ± 9.41	80.04 ± 8.84	137.21 ± 12.21	86.06 ± 4.00	98.8 ± 5.30
Heart	271.13 ± 23.82	82.67 ± 14.81	77.94 ± 10.57	243.91 ± 17.13	96.03 ± 18.44	106.76 ± 7.23
Mitocondria						
Liver	256.75 ± 35.92	71.30 ± 15.24	69.60 ± 9.84	257.92 ± 23.94	87.60 ± 10.75	76.06 ± 6.74
Brain	177.64 ± 17.22	72.24 ± 9.11	67.37 ± 5.69	175.37 ± 13.42	97.61 ± 7.80	92.56 ± 4.57
Heart	151.86 ± 28.03	80.33 ± 14.40	80.97 ± 13.01	205.19 ± 18.95	86.50 ± 10.14	91.95 ± 7.11

0.1ml serum or tissue / mitochondrial samples containing 65-250ng corticosterone were taken for extraction and recovery experiments as described. For recovery experiments, 100 or 200ng corticosterone dissolved in chloroform was added to the individual samples.

The sample to which corticosterone was not added served as the control for computing recovery.

Results are expressed as mean ± SEM of 10 independent observations for each individual experiment.

the recovery in the Silber procedure was low, in the range of 65-70% whereas the recovery values by the chloroform extraction procedure were in the higher range i e 76-91%.

For the heart samples, the Silber method underestimated the mitochondrial corticosterone content and the recovery by this procedure was found to be 78-83%; the recovery values by the chloroform method were in the range of 87-107% (Table 1) The overall results thus emphasize that recoverywise the modified direct chloroform extraction method gave better results.

After standardization of the recovery procedures, attempts were made to quantitate corticosterone content in the serum, tissue homogenates and mitochondria employing both the procedures These results are given in the Table 2 As is evident, consistent with the data in the Table 1, the Silber method underestimated the corticosterone content in serum and tissue homogenates, although these values for the mitochondria were comparable by both the methods However, it is important to note that the extent of variation was much higher in the Silber method, which is consistent with the recovery data shown in Table 1 It is also interesting to note that underestimation of corticosterone content by Silber procedure was noted especially for serum and brain and liver homogenates, for mitochondria the values were comparable It would therefore seems that high protein as well high lipid contents of the samples used for extraction posed a problem of phase separation and foggy interphase formation in the Silber procedure This in turn would severely affect the recovery

From the results presented (Table 2) it is also apparent that the tissues as well as the mitochondria contain significant amount of corticosterone Thus the content of corticosterone was the highest (86 μg / g tissue) in the brain, than in the liver and heart Although the content of corticosterone in the latter two tissues was comparable (Ca 29 μg/g tissue) It was only about 33-34% of that in the brain The picture for mitochondria was very different Highest amounts were present in the brain mitochondria (1 μg / mg protein) followed by the heart mitochondria (0.3μg / mg protein) The liver mitochondria contained the least amount (0 1 μg / mg protein) (Table 2)

The values for serum concentration of corticosterone obtained by the chloroform extraction reported here (Table 2) are somewhat higher compared to those reported by Sapolsky et al (3) but are in close agreement with the values reported by others (8, 11,12) Two reasons may account for the differences These experiments were carried out on Charles-Foster strain of rats It is possible that this strain has intrinsic higher values of corticosterone than the other strains used by different researchers (3,11-12), we could not get from literature the values for Charles Foster strain with which we could compare

Secondly, most of the values reported in the literature are based on the estimation of corticosterone by RIA (13-16) RIA detects the unbound hormone, the steroids are known to be bound Incomplete dissociation could give underestimation of the contents In the present method, direct extraction with chloroform ensured complete recovery of the hormone (Table 1) Besides, the fluorimetric method is specific for corticosterone and marginal interference only by cortisol has been reported (12)

Table 2 Corticosterone content in serum, tissue homogenates and mitochondria

Sample	Silber Method	Present Procedure
Serum	16 66 ± 1 60	40 15 ± 3 58
Homogenate		
Liver	25 55 ± 2 08	29 20 ± 2 28
Brain	51 60 ± 7 80	85 80 ± 7 70
Heart	21.07 ± 3 89	28 45 ± 1 80
Mitochondria		
Liver	0 11 ± 0 01	0 10 ± 0 01
Brain	0 98 ± 0 13	0.96 ± 0 07
Heart	0 28 ± 0 04	0 33 ± 0 03

The experimental details are as described in the text Corticosterone content in the serum is expressed as μg / dL Corticosterone content in the tissues is given as μg / g tissue while that in the mitochondria is given as μg / mg protein

Results are given as mean ± SEM of 10 independent experiments

However, the only glucocorticoid of the rat is corticosterone, cortisol is absent due to 17 α hydroxylase deficiency (2) Hence the possibility of interference by cortisol is ruled out Therefore the data (Tables 1 and 2) represent the true corticosterone content of the system under consideration

It has been reported that the mitochondrial matrix volume is about 1 2 µl/ mg protein (17) and the mitochondrial protein content in three tissues is in the range of 50 to 65 mg / g tissue(17,18) It is also reported that the aqueous volume of the cell is about 4 times that of the mitochondrial matrix volume (18,19) Based on this information the total content and concentration of corticosterone in the mitochondrial compartments can be computed By subtracting the mitochondrial content of corticosterone from the total cellular content, the content of corticosterone in the cellular compartment can also be computed These computed values are given in the Table 3

As can be noted, the liver, brain and heart mitochondria contained significant proportion (17, 56 and 75% respectively) of the tissue corticosterone content It can be noted that the concentration of corticosterone in serum is 1 16mM Compared to this, the concentrations in the tissue cytosol were significantly low and ranged from 0 065 to 0 3mM (Table 3) As against this, the computed concentrations of corticosterone in the mitochondria significantly high (10 to 20 times high) especially in the brain (2 3 mM) mitochondrial corticosterone concentration in the liver and heart were 0 24 and 0 79 mM respectively (Table 3)

It is well establish that corticosterone in the serum is bound to CBG (2) Therefore the concentration of free corticosterone in the serum would be significantly low compared

Table 3 Computed content and concentration of corticosterone in tissue cytosol and in mitochondria

Sample	Corticosterone content		Concentration, mM	
	Silber method	Present Procedure	Silber method	Present Procedure
Serum	16 66 ± 1 60	40 15 ± 3 58	0 481±0 046	1.16±0 103
Cytosol				
Liver	20 05 ± 1 63	24 23 ± 1.89	0.243 ± 0 020	0 29 ± 0.030
Brain	2 60 ± 0 39	37 82 ± 3 39	0 031 ± 0 005	0 12 ± 0 011
Heart	2 87 ± 0 33	7 00 ± 0 44	0 027 ± 0 005	0 07 ± 0 004
Mitochondria				
Liver	5 50 ± 0 05	5 00 ± 0 05	0 26 ± 0 004	0 24 ± 0.004
Brain	49 01 ± 6 51	48 00 ± 3.50	2 36 ± 0 310	2 31 ± 0 170
Heart	18 22 ± 0 12	21 45 ± 0 21	0 67 ± 0 101	0 79 ± 0 072

The experimental conditions and calculations are as described in the text Results are given as mean ± SEM of 10 independent observations Corticosterone content in cytosol and in mitochondria are expressed as µg / g tissue weight and in serum as µg / dl

Summary

The Silber procedure for extraction and estimation of corticosterone was modified to avoid foggy interphase formation resulting after petroleum ether extraction The homogenate was extracted directly in chloroform Recovery studies revealed that consistent 100% recoveries with the modified procedure compared to poor recovery and variable results in Silber procedure The method was appeared to examine the corticosterone content in rat tissues such as liver, brain and heart as well as in the mitochondria from these tissues The results show that the tissues and mitochondria accumulate significant amounts of corticosterone in a tissue specific manner

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