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THE DETERMINATION OF CORTICOSTERONE CONCENTRATION IN RAT PLASMA BY COMPETITIVE PROTEIN BINDING ANALYSIS

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SUMMARY

A competitive protein binding assay for the determination of corticosterone in rat plasma is described with the criteria of sensitivity, specificity, precision and accuracy evaluated in some detail. Rat plasma corticosterone binding globulin was used as the source of binding protein to assay the mass of corticosterone in ethyl acetate extracts of rat plasma. Steroids other than corticosterone which might interfere with the determination were shown either to be ineffective in displacing the radioligand or were present in such small quantities as to be insignificant in normal rats. The procedure involved the extraction of 50 μl samples of plasma, one fifth of the extract being assayed by means of a standard curve with a range of 0–6 ng. The sensitivity of the assay was 0·77 ng/50 μl (1·54 μg/100 ml) and the precision over the most commonly encountered concentration range, 10·0–20·0 μg/100 ml, was ±0·81 μg/100 ml ($N = 76$).

INTRODUCTION

A METHOD has been described [1] for the determination of cortisol in human plasma using a competitive protein binding (CPB) assay and it has been suggested that this procedure might be adapted for the determination of corticosterone in rat plasma [2]. This determination is fundamental to studies involving the hypothalamic-pituitary-adrenal axis in laboratory rodents and since no simple procedure had been satisfactorily reported until the present study was undertaken the criteria of specificity, precision and accuracy for such a method were examined, using rat plasma corticosteroid binding globulin as the assay protein.

MATERIALS AND METHODS

Materials

All common solvents and reagents were of BDH Chemicals Ltd., Poole, Dorset 'Analar' grade with the exception of ethanol and methanol which were obtained from James Burrough Ltd., London and were of analytical reagent grade. 'Florisil' (laboratory grade, "for chromatographic analysis" 60–100 US mesh) was purchased from BDH. LH-20 'Sephadex' was purchased from Pharmacia Ltd., London.

[1,2,6,7-$^3H_4$]-Corticosterone (specific activity, 106 Ci/mmol) and [1,2-$^3H_2$]-11-deoxycorticosterone (specific activity, 23·6 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. and NEN Chemicals Gmbh., Frankfurt/M, Germany, respectively. Both radioactive steroids were diluted with ethanol to a concentration of approximately 10 μCi/ml and stored at 4°.

18-Hydroxy-11-deoxycorticosterone was a gift from the MRC Steroid Reference Collection, Westfield College, London and all other steroids were purchased from Steraloids, Croydon, Surrey, U.K.

Male Sprague–Dawley rats (280–380g) aged 12–14 weeks were purchased from Fison's Pharmaceuticals Ltd., Loughborough, Leicestershire, U.K.

The assay binding protein was a 0·25% rat plasma solution in distilled water, the plasma being obtained from heparinized rat blood taken by cardiac puncture. The plasma was stored deep frozen at − 15° in portions suitable for one complete day's assay work.

Methods

Measurement of radioactivity. All samples for radioactivity determination were mixed with 10 ml Bray's solution [3] and counted in a Nuclear Chicago Mark II liquid scintillation spectrometer. By means of standards it was found that the efficiency of counting was approximately 30%.

Choice of extracting solvent. Murphy's original method [1] for the determination of cortisol in human plasma involves the use of ethanol for the simultaneous precipitation of plasma proteins and extraction of steroids. In a later publication [2] she noted that extraction of steroids from *rat* plasma by ethanol was unsatisfactory in view of the fact that rat corticosterone binding globulin is incompletely precipitated by this solvent. In an attempt to solve the problem, diethyl ether and ethyl acetate were compared with ethanol for suitability as extracting solvents. "Total removal of binding-proteins which might interfere can be checked by carrying the sample through the assay procedure but omitting the assay protein and comparing the binding value with the result obtained for water treated in exactly the same way" [2]. It can be seen from Table 1 that when this experiment was performed, the binding values from water and pooled rat plasma are comparable when ethyl acetate was the solvent used and indeed ethyl acetate was further recommended by its ease of handling compared to diethyl ether. Recoveries of trace amounts of labelled corticosterone added to plasma samples prior to extraction with ethyl acetate were 90·4 ± 5·3%.

Assay procedure. Duplicate aliquots (50 μl) of each plasma sample were extracted with 500 μl ethyl acetate by vigorous mixing on a Vortex mixer. A portion

Table 1. Comparison of solvent suitability for extraction of corticosterone in the competitive protein binding method

Sample	Counts/min in final supernatant*		
	Ethanol	Diethyl ether	Ethyl acetate
Water (50 μl)	781	860	809
	776	845	822
Pooled rat plasma (50 μl)	855	849	810
	845	825	794

*Figures refer to counts observed in final supernatant following processing of duplicate extracts through the method described below except that a solution of [1,2,6,7-^{3}H$_4$]-corticosterone in distilled water (approximately 20,000 counts/min/ml) was used instead of the assay protein solution.

(100 μl, equivalent to approximately 10 μl of plasma) of the supernatant was dried in an assay tube (7·5 cm × 1·2 cm dia) by means of a stream of air at 40°. A triplicate series of tubes containing various standard quantities of corticosterone in ethanolic solution were prepared and the ethanol was removed in a stream of air at 40°. The assay protein solution was prepared by addition of 200 μl of the stock ethanolic solution of [1, 2, 6, 7-$^3H_4$]-corticosterone to 50 ml of 0·25% rat plasma. A portion (1 ml) of this mixture containing 0·103 ng (approx. 20,000 counts/min) of [1,2,6,7-$^3H_4$]-corticosterone was pipetted into each assay and standard tube. Each tube was then agitated on a Vortex mixer and warmed at 40° for 5 min to effect solution of the steroids. After cooling at 4° for at least 10 min, 80 mg 'Florisil' was added by means of a plastic spoon[1] to enable the separation of "bound" and "free" fractions. The mixture was again agitated on a Vortex mixer (exactly 60 s) and the tubes centrifuged briefly in a refrigerated centrifuge to sediment the 'Florisil' thoroughly. An aliquot (500 μl) of the supernatant was then removed for determination of radioactivity.

RESULTS

Criteria for the determination of corticosterone by CPB assay

A typical standard curve is shown in Fig. 1. In order to test the *precision* and *accuracy* of the method, a single large pool of rat plasma was sub-divided into three batches. The first was used as a control; to the second and third batches corticosterone was added in quantities equivalent to 10 μg/100 ml and 20 μg/100 ml respectively. Aliquots (50 μl) of each batch were then analysed for corticosterone and the results are given in Table 2. The difference between the

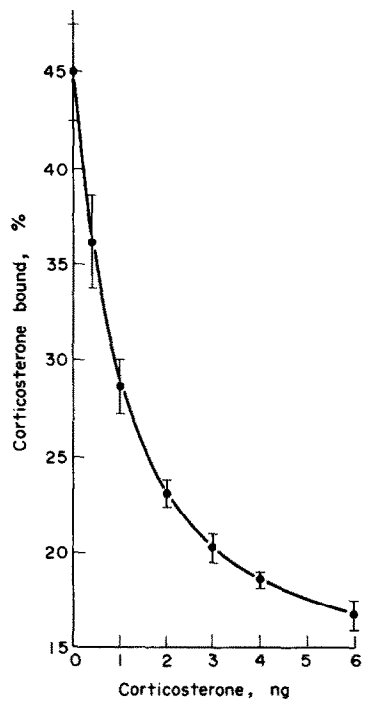

Fig. 1. Typical standard curve used in the determination of corticosterone by CPB. The vertical bars represent one standard deviation from the mean.

Table 2. Measurement of corticosterone in pooled rat plasma-precision and accuracy data

Sample	Volume (no. of assays)	Corticosterone (μg/100 ml) [Mean ± S.D.]
Water blank	50 μl (6)	0·5
Pool	50 μl (10)	15·7 ± 1·08*
Pool + 10 μg/100 ml	50 μl (10)	24·9 ± 0·78
Pool + 20 μg/100 ml	50 μl (10)	36·8 ± 2·24

*Standard deviations are equivalent to coefficients of variation of ±6·9% ±3·1% and ±6·1% respectively.

control and the samples were 9·2 and 21·1 μg/100 ml representing recoveries of 92 and 105·5% respectively. Blank values were acceptably low, equivalent to 0·5 ± 0·00 μg/100 ml.

The criteria of assay precision were further examined broadly as described by Brown *et al.* [4]. A wide range of concentrations was encountered throughout the series of experiments and the differences between duplicate values (d) was used to obtain estimates of the standard deviation (s) over the concentration ranges shown in Table 3 by means of the formula $s = \pm\sqrt{\Sigma d^2/2N}$, where N is the number of duplicate determinations made [5]. The fiducial ranges (F.R.) were calculated by the formula F.R. $= M \pm ts\sqrt{n}$ where M is the mean value, t is Student t value ($P = 0{\cdot}01$) for the number of duplicate determinations and n in this case is the number of determinations, i.e. 2.

The *sensitivity* of the assay, or the lowest concentration distinguishable from zero is $ts/\sqrt{n}$ and by means of the data obtained for the lowest range (0·0–2·4 μg/100 ml) shown in Table 3 the sensitivity is 1·54 μg/100 ml. (In fact this range was not encountered during the determinations of corticosterone through-

Table 3. Estimates of precision of rat plasma corticosterone measurement expressed as estimates of the standard deviation (s) of results from their means (from duplicate determinations), and the fiducial ranges at the various concentration levels.

Range (μg/100 ml)	$s(est)$[1]	Fiducial Range ($p = 0{\cdot}01$)[2]
0·0– 2·4	± 0·73 (14)	M ± 1·54
2·5– 4·9	± 1·04 (14)	M ± 2·19
5·0– 9·9	± 1·26 (29)	M ± 2·46
10·0–19·9	± 0·81 (76)	M ± 1·51
20·0–39·9	± 1·11 (69)	M ± 2·08

[1]Estimated standard deviation $(s) = \sqrt{\Sigma d^2/2N}$, where d is difference between duplicates and N is the number of determinations.

[2]Fiducial range $= M \pm ts/\sqrt{n}$, where M is the mean value, and n is the number of determinations/assay, i.e. 2.

out this study and was examined by dilution of a suitable number of plasmas in order to find the ultimate sensitivity of the method).

The *specificity* of the method was assessed by determination of a series of values for corticosterone concentration before and after chromatography of plasma ethyl acetate extracts on LH-20 "Sephadex"[6]. The columns were 29 × 1 cm in size and were equilibrated in and developed with a mixture of methylene chloride:methanol (98:2, v/v). [1,2,6,7-$^3H_4$]-Corticosterone, 11-dehydrocorticosterone and [1,2-$^3H_2$]11-deoxycorticosterone standards were chromatographed on the column to ascertain their elution volumes and the results are shown in Fig. 2. The corticosterone peaks were detected by determination of the radioactivity content in 1 ml fractions and the 11-dehydrocorticosterone peak by measurement of the fraction contents using displacement of [1,2,6,7-$^3H_4$]-corticosterone in the CPB assay. It can be seen that when the fraction 23–29 ml is taken, there is little interference from the other steroids tested. These steroids together with 18-hydroxy-11-deoxycorticosterone were assessed for their ability to displace [1,2,6,7-$^3H_4$]-corticosterone from the binding protein (Fig. 3). Corticosterone and 18-hydroxy-11-deoxycorticosterone were respectively the most and the least effective displacing compounds.

An aliquot (400 μl and/or 200 μl) of a series of plasmas was extracted with 1 ml ethyl acetate containing approximately 1000 counts of [1,2,6,7-$^3H_4$]-corticosterone. A major portion (500 μl) of the extract was taken to dryness and chromatographed on LH-20 "Sephadex". The appropriate fraction (23–29 ml) of the eluate was collected and an aliquot (3 ml) taken for determination of radioactivity. Further aliquots (2 × 0·6 ml) of the eluate together with 2 × 50 μl aliquots of the original ethyl acetate extract were analysed in the usual way by competitive protein binding. The results are shown in Table 4 and it can be seen that:

(i) There is no consistent difference between values obtained for chromatographed and unchromatographed plasmas;

(ii) the greatest discrepancies tend to occur with low titre plasmas;

(iii) there is good agreement between values obtained at the 200 and 400 μl plasma volume levels.

These results suggest that there is little interference from other steroids in the

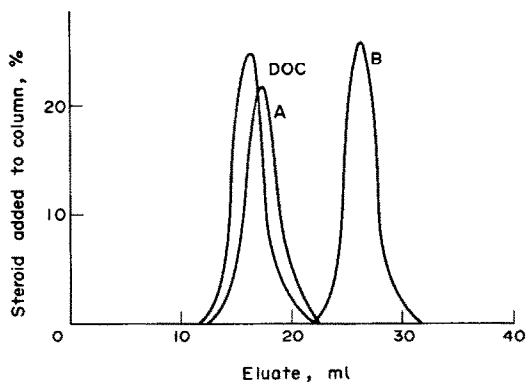

Fig. 2. Separation of 11-deoxycorticosterone (DOC) and 11-dehydrocorticosterone (A) from corticosterone (B) on a column of Sephadex LH-20 (29 × 1 cm) using solvent system, methylene chloride:methanol (98:2, v/v).

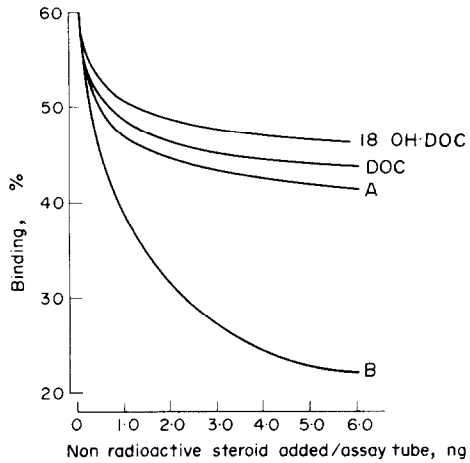

Fig. 3. Standard curves for 18-hydroxy-11-deoxycorticosterone (18-OH.DOC), 11-deoxycorticosterone (DOC), 11-dehydrocorticosterone (A) and corticosterone (B).

Table 4. Measurement of corticosterone in rat plasma extracts before and after chromatography on 'Sephadex' LH-20

Sample No.	Plasma Vol. (μl)	Corticosterone (μg/100 ml) Chromat.[1]	Unchromat.[2]	Difference (%)	
1	200	3·4	5·5	−38·2	
2	200	6·6	6·8	−3·0	
3	200	6·8	5·0	+36·0	
4	200	9·0	9·3	−3·2	
5	200	16·3	16·0	+1·9	S.D. ±
6	200	16·5	17·8	−7·3	6·27%
7	200	17·6	15·5	+13·5	
8	200	24·5	22·8	+7·5	
9	200	25·8	23·3	+10·7	
10	200	32·0	32·5	−1·5	
11	200	5·3	5·0	+6·0	
	400	5·5	6·0	−8·3	
12	200	11·3	11·8	−4·2	
	400	10·7	10·3	+3·9	
13	200	12·7	12·3	+3·3	S.D. ±
	400	12·1	12·7	−4·7	1·61%
14	200	13·2	13·8	−4·4	
	400	13·9	14·5	−4·1	
15	200	14·5	14·3	+1·4	
	400	13·6	13·7	−0·7	

[1]Analyses performed on eluates from "Sephadex" LH-20 columns.
[2]Analyses performed on crude extracts of plasma.

determination of corticosterone by the CPB method described. In common with the experience of many others, greatest inaccuracy of measurement occurs when determinations are made at the extremities of a standard curve.

DISCUSSION

The problem was to achieve a *simple* method of proven sensitivity, accuracy, specificity and precision for the determination of corticosterone concentrations in rat plasma. Previous workers in this field[7] have described a method for the measurement of corticosterone levels in the mouse using dog plasma as their source of binding protein. They found rat plasma unsuitable for their purpose since the "index of precision" [8] obtained with the rat plasma was greater (i.e. less favourable) than that taken from the dog. Throughout this study, however, rat plasma appeared to give satisfactory results as a source of binding protein for the purposes required and was more readily available. It was not possible to compare our standard curve data directly with that of Grad and Khalid[7] since the information required was not quoted in sufficient detail and in any case a comparison of the λ'*s* is only meaningful in certain circumstances and hence of little *practical* value. Vermeulen and Verdonck[9] expressed the precision of their standard curves for the determination of plasma testosterone by CPB as the coefficient of variation of the apparent concentration at each nominal concentration of testosterone in n replicate curves. In this manner, it is simple to compare standards of precision between methods or even laboratories. The coefficients of variation in the present study were 4·3, 2·2, 1·5, 1·3, 3·0 and 2·2%[$n = 24$] at respectively 0·4, 1·0, 2·0, 3·0, 4·0 and 6 ng of corticosterone in the assay mixture. Table 3 shows the estimates of the standard deviation of assays in practice as well as their fiducial ranges ($p = 0{\cdot}01$) and it was concluded that adequate precision had been achieved.

Steroids which might be expected to be present in rat plasma and interfere with the assay were either eliminated by the chromatographic step (Fig. 2) or did not bind to any great extent with the assay protein (Fig. 3). However, the chromatographic step seemed to be unnecessary in view of the results shown in Table 4 which revealed that the process made little difference to the values obtained and those that were observed could be accounted for in terms of experimental error. The present procedure therefore contrasts with some previous methods which have required a tedious paper[10] or thin-layer chromatography purification[11] of the corticosterone fraction.

The observed sensitivity of 1·54 μg/100 ml plasma was more than adequate for the range of values encountered; and the standard of accuracy as manifest by the recovery of radioinert corticosterone added to pool (Table 2) and individual samples (Table 4) was satisfactory.

The foregoing data thus indicate that a reliable method for the determination of corticosterone in rat plasma with all the required criteria can be established using materials readily available in most laboratories.

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