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Plasma cortisone in man: its determination, physiological variation, and significance

J D FEW AND G C CASHMORE

From the MRC Environmental Physiology Unit, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT

SUMMARY In a method for the simultaneous determination of cortisone, cortisol, and corticosterone, the extracted steroids are separated using paper chromatography and assayed by competitive protein binding. Evidence of the reliability of the method, particularly with respect to the determination of cortisone, is presented. Only trivial changes in cortisone level were detected in response to exercise, surgery, and ACTH, even though marked changes in cortisol level were simultaneously recorded. It is suggested that the inertia in plasma cortisone level may be related to its lesser degree of binding to plasma proteins, relative to cortisol, which would permit more rapid hepatic uptake and possible accumulation of a large extravascular pool of cortisone.

Despite advances in our knowledge of steroid metabolism, the role of cortisone in human endocrinology has received little attention. Although earlier reviewers[1,2] had doubts about its existence in human plasma, methods based on competitive protein binding,[3,4] radioimmunoassay,[5] and double isotope dilution analysis[6] all suggest that the concentration of cortisone in peripheral plasma lies in the range 30–150 nmol/l. None of these methods provides a rigorous proof of the existence of cortisone in human plasma, but in aggregate they amount to very strong evidence. Moreover, they are complemented by radiochemical studies in which labelled cortisol has been converted into labelled cortisone both systemically[6–9] and peripherally.[10]

We have previously found that after the administration of ^{3}H-cortisol, both hyperthermia[8,9] and exercise[11] lead to an increase in the ratio ^{3}H-cortisone: ^{3}H-cortisol, which is in contrast to the finding that acute (clinical) stress was without effect upon the cortisone: cortisol ratio.[5]

In order to investigate this discrepancy we have developed a method for the determination of plasma cortisone based upon paper chromatographic separation and competitive protein binding assay using turkey plasma as the source of binding protein. It also proved convenient simultaneously to measure cortisol and corticosterone in appropriate fractions from the chromatogram using human and baboon serum respectively as the source of the binding protein.

Material and methods

Radioactive steroids were purchased from the Radiochemical Centre, Amerhsam, UK; they were stored in ethanol solution at −20°C. Two concentrations were used: 10 μCi/ml, as the ligand in the binding assay, and approximately 150 000 cpm/ml for addition to plasma, to monitor recovery.

Solvents were of analytical reagent grade but were not further purified. Charcoal, Norit A (Sigma Chemical Company), was washed with water to remove fines, and then with alcohol, and finally dried. Dextran-coated charcoal was a 2% suspension of charcoal in a solution containing 0·6 g gelatine and 0·12 g dextran C in 100 ml.

Human plasma used as a source of binding protein was obtained from normal men. Turkey and baboon sera were purchased from Seralab Ltd, Crawley Down, Sussex, UK.

Liquid scintillation counting was performed using a Packard model 3314. Dry samples were dissolved in 0·2 ml ethanol and 5 ml of 0·5% butyl PBD in toluene. Aqueous samples (1 ml) were dissolved in 7 ml of a mixture of 0·5% butyl PBD in toluene (2 parts) and triton X-100 (1 part).

Paper chromatography was performed at about 20°C. Sheets of Whatman No. 1 paper, 230 mm wide, were divided to give eight 20 mm wide lanes; two lanes were used for markers (50 μg of each steroid) and six for samples. Two systems were used: Bush C (toluene-ethyl acetate-methanol-water 180:20:100:100) and E_2B (iso-octane-*t*-butanol-

water 100:50:90). Bush C was equilibrated overnight and developed for 4 hours, and E_2B was equilibrated for 2 hours and developed for 16 hours (overnight).

METHOD

Into 150 × 25 mm stoppered tubes were dispensed 20 μl (3000 cpm) each of [^{3}H] corticosterone, [^{3}H] cortisone, and [^{14}C] cortisol followed by 1 ml plasma, 1 ml 0·05 M NaOH, and 10 ml dichloromethane. The tubes were gently mixed for 20 minutes, and when the two phases had separated clearly the aqueous layer was removed. The organic layer was washed with 2 ml volumes of 0·1 M acetic acid and of water, dried over sodium sulphate, and evaporated to dryness. The residues were transferred to the paper chromatograms using methanol and chloroform. The Bush C system was used routinely. When the papers were dry the markers were located under the ultraviolet lamp, and the three steroid bands were cut out from the sample strips parallel to the positions of the markers. The steroids were eluted with 3 ml of 5% ethanol for 30 minutes. Duplicate 0·5 ml portions were transferred to 75 × 12 mm polystyrene tubes for binding, and 1 ml was evaporated to dryness in a counting vial for estimation of the recovery.

Some details of the binding assays are given in Table 1. The binding reagents were prepared by diluting the appropriate volume of serum to 100 ml with distilled water followed by the addition of 5 μCi (0·5 ml) of the appropriate ligand. As the final volume is 1·0 ml the final dilution is half that shown in Table 1. The sets of tubes were kept in the refrigerator overnight (16 hours) and then placed in an ice bath. Dextran-coated charcoal (1·0 ml) was added to each tube, which was vortex mixed and centrifuged at 2000 *g* for 10 minutes. One millilitre of supernatant was taken for counting.

Appropriate standards were run with each set. For cortisol and corticosterone, time for 10^4 counts was linearly related to the weight of steroid, and results were computed using a linear regression equation. For cortisone a quadratic regression equation fitted the data more closely and was used routinely as described previously.[12] A typical cortisone calibration curve is shown in Figure 1.

Table 1 *Composition and characteristics of the binding reagents*

	Cortisone	*Cortisol*	*Corticosterone*
Source of plasma	Turkey	Human	Baboon
Concentration (%)	2	5	0·5
Ligand*	[^{3}H] cortisone	[^{3}H] cortisol	[^{3}H] corticosterone
Range (pmol)	0–300	0–1500	0–150
Zero binding (%)	35	80	65
Calculation	Quadratic	Linear	Linear

*All used at 5μCi/100 ml.

Results

VALIDATION OF THE METHOD

Only the validation of the cortisone assay will be described in detail. Comparable results have also been obtained for cortisol and corticosterone.

Precision

Forty-five samples with cortisone concentrations in the range 36–190 nmol/l were analysed in duplicate to give a within-set coefficient of variation of 8·0%. Eight samples were also analysed in duplicate, in different sets, to give a between-set coefficient of variation of 14%.

Accuracy

Non-radioactive cortisone was added to plasma samples and to water, equivalent to 25–165 nmol/l. A linear regression of quantity of cortisone recovered (y) on quantity of cortisone added (x) was calculated. The slope of this regression equation is therefore the recovery of cortisone and was 0·80 and 0·85 for plasma and 0·93 for water (ie, 80%, 85%, and 93% respectively). For the plasma samples, but not for water, the slopes were significantly different from unity, suggesting the presence in plasma of material that interferes with the binding reaction.

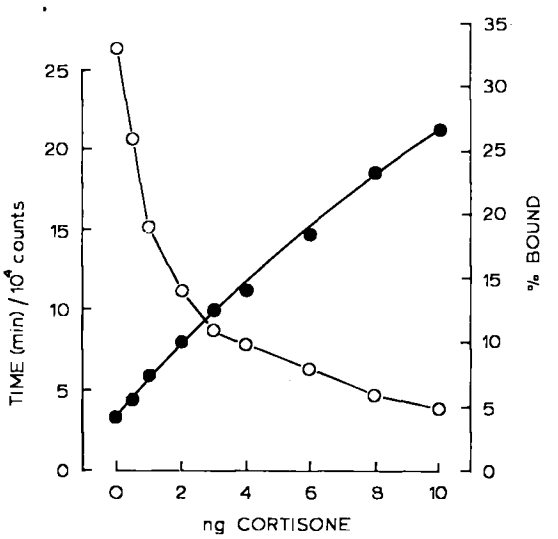

Fig. 1 *Typical calibration curve for the cortisone assay. Solid symbols representing actual data points (min. 10^{-4} counts) closely fit the associated curve, which is the computed quadratic regression. The same data are expressed as % bound by the open circles.*

Parallelism between plasma samples and cortisone standards

Larger volumes of plasma (5 ml) were extracted, without prior addition of labelled steroids, analogously to the routine method. The cortisone zone of the chromatogram was eluted with 6 ml 5% ethanol, and various volumes were assayed for cortisone. A linear regression of weight of cortisone on volume of eluate was computed for each sample (n = 4). In each case the correlation was better than 0·995. Similar results were also obtained for cortisol and corticosterone. Deviations from linearity of the type previously described for a simple plasma cortisol assay[12] were not detected. Conversely, when 0·5 ml and 2·0 ml volumes of plasma were analysed, the measured cortisone concentration was not significantly different from that when 1·0 ml was analysed.

Specificity

The binding protein present in turkey serum is not specific for cortisone (Table 2) so that the specificity of the method is dependent upon the efficient isolation of cortisone by paper chromatography. When the region of chromatograms between cortisol and corticosterone was divided into 10 mm lengths there was good agreement between the profile of cortisone determined by competitive protein binding and that of the added ^{3}H-cortisone (Fig. 2). However, this could conceal interference by the dihydrocortisols, which weakly cross-react with cortisone and are not separated from it by the B5 chromatogram (Table 2). When cortisone eluted from B5 chromatograms was rechromatographed in the E_2B system it was clearly separated from the dihydrocortisols (Fig. 3). No cortisone-like material, as measured by competitive protein binding, ran parallel with either of these compounds, and the profiles of cortisone (competitive protein binding) and ^{3}H-cortisone were again superimposable. Moreover, the additional E_2B chromatogram did not significantly alter the measured cortisone concentration (56 ± 9·0 *v* 62 ± 8·6 nmol/l; n = 4).

It is clear from Table 2 that the corticosterone fractions will certainly be contaminated with cortexolone. Fortunately, this latter compound only cross-reacts to about 10% of its real value in the corticosterone assay and so would not seriously contribute to the apparent corticosterone levels. The cross-reactivity of cortisol acetate is essentially zero.

Water blanks (1 ml water substituted for plasma) give negligible values in all three assays. Plasma, drawn at 1000 from a subject who had received 0·5 mg dexamethasone the previous evening and at 0700, gave values for cortisol, cortisone, and corticosterone of 70, 8, and 10 nmol/l, respectively.

Table 2 *Chromatographic mobilities and binding properties of some steroids*

	$R_{cortisol}$		
	Bush C	*E_2B*	*%Cross reaction*
Cortisone	1·62	1·20	100
5α-Dihydrocortisol	1·60	1·40	38
5β-Dihydrocortisol	1·70	1·50	20
Aldosterone	1·00	0·73	0
Prednisone	1·37	1·10	180
Cortisol	1·00	1·00	
Prednisolone	0·80	1·00	
Tetrahydrocortisone	0·82	1·50	
Corticosterone	2·55		
Cortexolone	2·60		
Cortisol Acetate	2·35		

APPLICATIONS OF THE METHOD

Normal values

Because we use male subjects almost exclusively for our physiological studies, most of our data on cortisone are derived from men only. The cortisone concentration of plasma drawn from subjects at rest is in the range 30–80 nmol/l while the cortisone/cortisol ratio is in the range 10–30%.

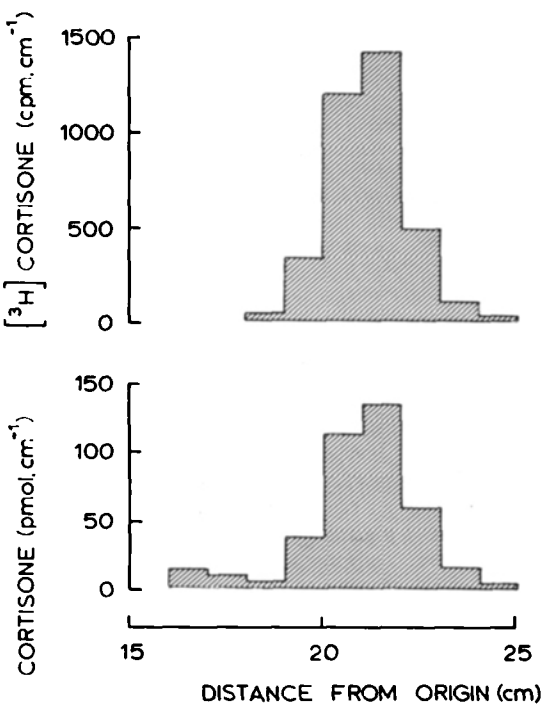

Fig. 2 *Coincidence of [^{3}H] cortisone and cortisone determined by competitive protein binding on Bush C chromatogram.*

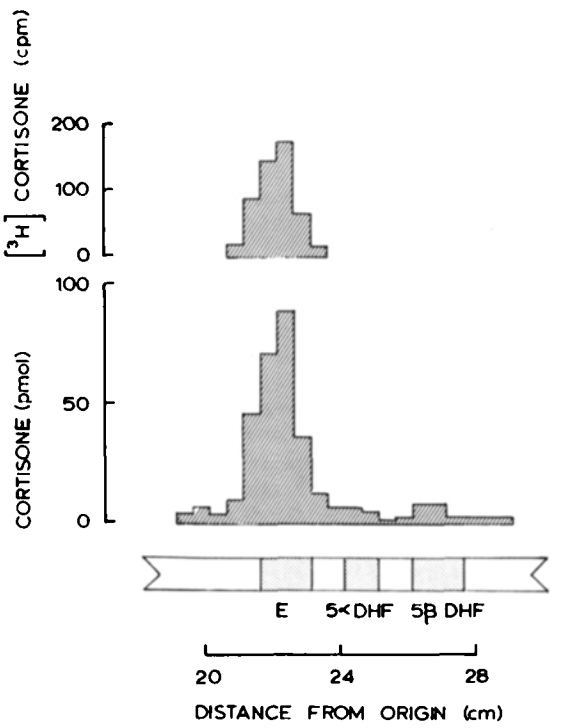

Fig. 3 *Coincidence of [³H] cortisone and cortisone determined by competitive protein binding when the cortisone eluate from a Bush C chromatogram was rechromatographed in the E_2B system. The shaded areas in the lower strip indicate the positions of cortisone (E), 5α-dihydrocortisol (5αDHF), and 5β-dihydrocortisol (5β DHF) on guide strips stained with blue tetrazolium.*

Effects of exercise

Figure 4 shows the typical plasma cortisone and cortisol responses of a 24-year-old man walking on a treadmill at 6·4 km/h. The gradient was increased in 3% increments every 10 minutes; the final load required about 90% of the subject's maximum power output and was exhausting. Despite the brisk rise in cortisol level during the higher work loads, and in the early recovery period, there were only trivial changes in cortisone concentration. Figure 5 shows the plasma cortisone, cortisol, and corticosterone responses to running on a treadmill for 4 hours, at about 70% of maximum aerobic power output, by a highly trained athlete. Even in response to this powerful physiological stimulus the cortisone concentration increased by only about 40 nmol/l. However, the fact that the plasma cortisone concentration was linearly related to the duration of running ($r=0{\cdot}84$; $P<0{\cdot}005$) encourages us to believe that the increase was significant. In three similar experiments, rises of 40 and 50 nmol/l were recorded from two subjects, while the third showed no significant rise in plasma cortisone. In all three experiments marked rises in cortisol and corticosterone were recorded.

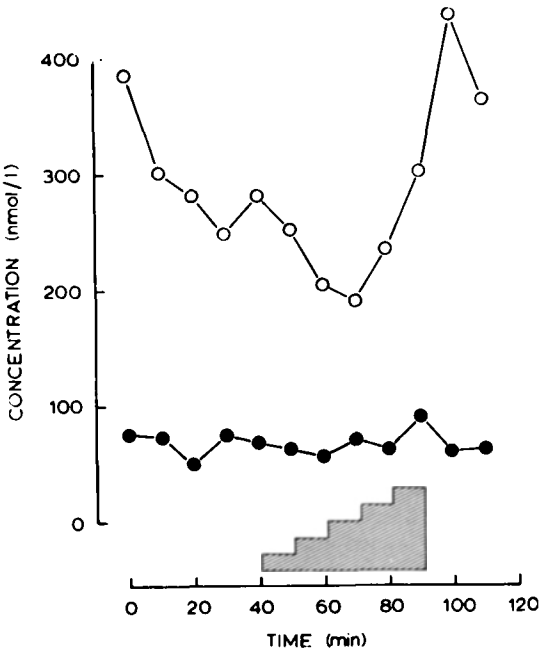

Fig. 4 *Plasma cortisone (●) and cortisol (○) responses to treadmill exercise at 6·4 km/h. The treadmill gradient was initially zero and was increased by 3% increments every 10 minutes until a gradient of 12% was reached; this is shown schematically by the shaded block.*

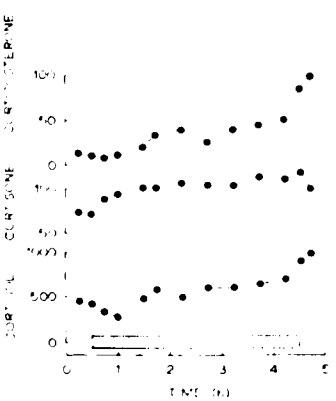

Fig. 5 *Plasma corticosteroid levels during 4 hours of treadmill running by a well-trained athlete. The shaded bar indicates the period of running. Plasma corticosteroid values are in nmol/l.*

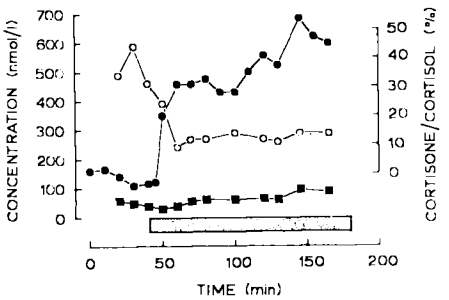

Fig. 6 *Effect of* β^{1-24} *ACTH infusion on plasma levels of cortisol* (●) *and cortisone* (■). *Cortisone:cortisol ratio* (O)

Table 3 *Effect of major surgery on plasma concentrations (nmol/l) of cortisone (E) and cortisol (F)*

	Patient					
	M		*K*		*F*	
	E	*F*	*E*	*F*	*E*	*F*
24 h preop	44	250	50	323	61	300
3 h postop	39	1070	44	1300	63	500
48 h postop	39	270	44	315	75	545

Blood was also obtained from 10 other athletes within 5 minutes of completing a 4-hour outdoor run. These samples yielded mean (±SD) values for cortisone and cortisol of 120 ± 22 nmol/l and 980 ± 345 nmol/l respectively. These cortisone levels are significantly ($P<0.001$) higher than we find in resting subjects. Because of the circumstances of these runs it was not possible to obtain samples immediately before the runs but several of the subjects had been found to have cortisone levels within the normal range on other occasions.

ACTH stimulation

Figure 6 shows the changes in plasma cortisone and cortisol levels in response to a continuous infusion of β^{1-24}ACTH. Though the cortisone level increases by only about 60 nmol/l, it is linearly related to the duration of ACTH infusion ($P<0.005$).

Surgical stress

Table 3 shows the effect of major surgical stress (partial gastrectomy) on the levels of cortisone and cortisol. Even though the expected rise in cortisol was observed in each patient, changes in cortisone level were not detected.

Discussion

The method described in this paper differs from previous plasma cortisone competitive protein binding methods which utilise thin-layer chromatography[3 5] or columns of LH-20[4] for the isolation of the cortisone, and by using turkey serum rather than that of chicken[3] or adrenalectomised dogs[4] as the source of the binding protein. Although the cited papers[3 4] contain insufficient data to permit a detailed comparison with the present method, particularly with respect to the binding assay, they yielded values comparable with those described above. This concordance, together with our own validation, suggests that the values reported in this paper are of the right order of magnitude.

When we consider the results obtained with the method, the most striking feature is the inertia of the plasma cortisone level compared to simultaneous changes in cortisol level. Similar findings have been reported by Newsome *et al.*[4] and Srivastava *et al.*,[5] who also discussed the phenomenon thoroughly but did not reach any conclusions.

It appears to be generally accepted that the major source of plasma cortisone is the peripheral conversion of cortisol, and that the liver converts cortisone back to cortisol. It was suggested by Srivastava *et al.*[5] that perhaps the capacity for peripheral oxidation of cortisol to cortisone was limited, so that the fractional conversion of cortisol to cortisone would decrease as the concentration of cortisol rose. Certainly this view is compatible with most of the facts, though these authors did note some anomalies. In the absence of any understanding of the physiological role of peripheral conversion of cortisol to cortisone this suggestion is difficult to assess.

Alternatively, the lower degree of binding of cortisone to plasma proteins, compared with the binding of cortisol,[13] may be of significance. This would lead to cortisone being more efficiently extracted by the liver than is cortisol, which is in accord with the known higher metabolic clearance rate[6] and shorter half-life of cortisone.[14] This effect would be reinforced as cortisol levels rose, leading to displacement of cortisone from binding sites and yet higher rates of hepatic extraction, analogously to that of aldosterone.[15] It is noteworthy that during prolonged, heavy exercise, in which a small elevation of cortisone was detected, there is usually a reduction in liver blood flow.[16] The lower degree of plasma binding of cortisone could also lead to its diffusing out of the plasma into extravascular sites more rapidly and extensively than does cortisol. If there is a large extravascular pool of cortisone, in rapid equilibrium with plasma cortisone, then it would tend to buffer changes in the plasma level. Some support for this possibility comes from several observations that cortisone concentration does not fall in response to acute decline in cortisol level (eg, Fig. 4).

It is clear from Fig. 4 that cortisol concentration is the major determinant of changes in the cortisone: cortisol ratio. In this example, and in others not illustrated, if the cortisone level is essentially constant, then the cortisone: cortisol ratio can only increase if the cortisol level is falling. This is in contrast to observations after the intravenous injection of ^{3}H-cortisol, in which exercise resulted in increases of both plasma cortisol level and the ^{3}H-cortisone:^{3}H-cortisol ratio. The finding, now, that plasma cortisone level is held remarkably constant despite simultaneous rises (and falls) in plasma cortisol level, suggests that radiochemical findings of an increase in the cortisone: cortisol ratio in response to exercise may have been an artefact due to deviation from steady-state conditions.

The physiological role of the interconversion of cortisol and cortisone is still not clear. One possible effect would be to facilitate the mobility of corticosteroid on account of the lower degree of protein binding of cortisone. Another role might be to act as a non-active reserve of corticosteriod available for conversion to cortisol, though at present it is not clear whether cortisone can be converted to cortisol peripherally, nor what factors control the cortisone: cortisol ratio at the tissue level. In both these roles this interconversion of corticol and cortisone would be complementary to the protein binding of cortisol, a phenomenon that is well known but little understood.

In conclusion, we suggest that the cortisone in human plasma should not be regarded as an unimportant by-product, but that it may be a clue to the further understanding of the role of cortisol in human physiological processes.

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