Simultaneous Determination of Cortisol and Corticosterone in Human Plasma by Quantitative Paper Chromatography.* (23020)

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Although there are several successful methods for determination of cortisol (hydrocortisone)(1,2) or 17-hydroxycorticosteroids(3,4) in plasma, methods proposed for determination of corticosterone are less satisfactory. Previously described technics have depended either on differential color reactions of only moderate specificity(5) or on use of columns for separation of a "cortisone-like" material (1,6). Application of quantitative paper chromatography to the determination of plasma cortisol in this laboratory(2) suggested that similar methods might be used for determination of corticosterone. A method has been developed for simultaneous determination of cortisol and corticosterone in the same plasma sample.

Procedure. Blood is drawn into heparinized syringes and centrifuged within 15 minutes. A 1 ml portion of a standard ethanolic solution of cortisol-4-C^{14} and corticosterone-4-C^{14} containing 800 counts/minute of each compound is pipetted into 60 ml centrifuge tube and evaporated to dryness. With radioactive steroids now available, this represents about 0.2 μg of each substance. Twenty ml of plasma are added, followed by 0.5 ml of 1 N NaOH. The mixture is stirred and then extracted promptly three times with 20 ml of freshly redistilled chloroform. The pooled chloroform extracts are transferred to 60 ml boiling flask with a small well in the bottom(3) and evaporated to dryness at room temperature. The sides of the flask are washed into the well several times with 1-2 ml of chloroform. Contents of well are then transferred quantitatively with chloroform to a small area at origin of strip of Whatman No. 1 filter paper, previously extracted in Nolan extractor for 72 hours with boiling methanol. A standard spot (approximately 25 μg each of cortisol and corticosterone) is placed on the same line. Spots are placed 3 cm apart, except for the standard, which is placed at greater distance from the sample to avoid overlapping as the spot spreads during chromatography. Chromatography is performed in 2 stages. For the initial step, the paper is inserted in the trough with spots in the dependent position and after at least 2 hours' equilibration in an atmosphere of petroleum ether saturated with 75% methanol, the mobile phase (petroleum ether saturated with 75% methanol) is added to the trough and permitted to wash over the spots and off the paper, which required approximately 4 hours. The paper is then removed from the tank, air dried and reinserted with the steroid spots upward in another tank, equilibrated overnight and developed for 3 hours with toluene saturated with 70% methanol. The paper is allowed to dry and the strip containing the standard spot is then cut off and steroids located with ultraviolet handlamp. With this as a guide, 3 x 4 cm rectangles (for cortisol) and 4 x 7 cm rectangles (for corticosterone) are cut out. Since the spots sometimes tend to streak in direction of flow, the rectangle should be displaced slightly toward the solvent front rather than centered exactly on the standard spot. A blank is provided by cutting out a similar rectangle between cortisol and corticosterone spots. The steroids are eluted from paper by soaking in 4 ml absolute ethanol in test tubes sealed with aluminum-foil-covered corks for one hour at 40°C. The eluate is shaken and then 0.5 ml is taken for estimation of radioactivity and aliquots of the remainder are taken for fluorometric analysis. For alkaline fluorescence, duplicate 0.8 ml portions are pipetted into fluorometer cuvettes, dried *in vacuo,* and read one hour after addition of 0.5 ml of 0.3 N potassium tert butoxide, using 5860 Corning filter in the ex-

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TABLE I. Recovery of Corticosterone.

No. of exp.	Initial plasma conc., μg/100 ml	Corticosterone added, μg/100 ml	Recovery (%)		
			Chemical	Radioactive	Corrected
4	.29	13.8	52.3 ± 7.0	78.3 ± 6.4	82.1 ± 4.1
6	.00	9.8	72.3 ± 3.0	81.3 ± 5.8	88.8 ± 6.0
3	.38	9.8	79.9 ± 4.2	65.6 ± 2.7	115.7 ± 4.3
3	.29	9.8	74.8 ± 2.8	66.8 ± 5.3	106.3 ± 4.4
9	.00	9.8	61.2 ± 8.3	73.1 ± 9.7	82.6 ± 9.9
25	—	—	67.9 ± 9.2	74.3 ± 7.2	90.9 ±13.4

Corticosterone was added to plasma immediately before extraction.

citing beam and 2418 Corning filter for emitted light(7). For sulfuric acid fluorescence, two 0.3 ml portions are pipetted into fluorometer cuvettes. 0.7 ml of concentrated sulfuric acid is added to each fluorometer tube, and fluorescence is read after one hour in Farrand fluorometer, using 3389 and 5113 Corning filters in the exciting beam and 3486 Corning filters for the emitted light(8). Both types of fluorescent measurements can be made on each sample if necessary. For each steroid a standard curve is also determined, using 0.5, 1 and 2 μg of cortisol, and 0.05, 0.1 and 0.5 μg of corticosterone. The 0.5 ml aliquots intended for radioactive measurements are pipetted into 30 ml optically clear vial and the ethanol evaporated to dryness. The residue is then dissolved in 15 ml of scintillation mixture (0.4% 2, 5-diphenyl-oxazole and 0.03% 1,4di(2[5-phenyloxazole]) benzene in toluene) and placed in a liquid scintillation counter (Technical Measurements Corp. Model LP2A) where it is counted for 5 minutes. The counter has an efficiency of 65% and a probable error of counting, under these circumstances, of 2%. With the quantities used, about 100 counts/minute are observed in the aliquot of paper eluate. Plasma concentration is calculated from the observed fluorescence after subtracting the paper blank. The figure is corrected for radioactive recovery, and the quantity of steroid added as radioactive tracer is subtracted from the total (2).

Corticosterone and cortisol are separated in a satisfactory manner by the second (toluene/70% methanol) chromatography; however, if this separation is performed without preliminary purification of the plasma extract, large amounts of spontaneously fluorescing material contaminate the area in which corticosterone is found. By use of preliminary chromatographic purification with petroleum ether/75% methanol, the fluorescent contaminant is washed off the paper without any alteration in position of either corticosterone or cortisol. The justification for introducing the radioactive correction has already been discussed(2). As a result of the use of this correction, added cortisol is completely recovered. Recovery of corticosterone is presented in Table I. Recovery is not quite so good as for cortisol(2), perhaps in part because cortisol travels a shorter distance and therefore the spot has less tendency to spread. In the original studies of plasma cortisol concentration, the steroid was determined by measuring its fluorescence in potassium butoxide(7). This method has the advantage of high specificity for compounds containing the 4-ene, 3-one configuration, but it is somewhat less sensitive than the sulfuric acid fluorescence described by Sweat(8). The blank fluorescence by Sweat's method also proved to be somewhat lower than with the alkaline reagent. A comparison was therefore made between the results obtained by the 2 methods, and no significant difference was found. Since maximum sensitivity is needed for determining minute quantities of corticosterone in human plasma, the sulfuric acid method may be preferable. The sensitivity of this method has been increased approximately 50% by reading the fluorescence in a 7:3 sulfuric acid:ethanol mixture rather than the 9:1 mixture recommended by Sweat(8). The altered sulfuric acid concentration does not modify characteristics of the excitation or

TABLE II. Cortisol and Corticosterone Concentrations in Plasma of Normal Subjects.

	Cortisol			Corticosterone		
	♂	♀	Total	♂	♀	Total
	μg/100 ml			μg/100 ml		
No.	16	13	29	16	13	29
Mean	11.4	8.8	10.2	1.8	.8	1.3
S.D.	3.7	2.9	3.6	3.0	1.6	2.5
Median	11.6	9.0	10.3	.6	* .0	.2
Range	5.9–17.7	4.0–13.1	4.0–17.7	.0–4.0	.0–5.0	.0–5.0

* Failure to find corticosterone indicates that less than 0.1 μg/100 ml of plasma is present.

emission spectra from those originally described. Fluorescence reaches a stable plateau within an hour and remains stable for several hours thereafter.

Observations. When the technic was applied to the study of plasma from normal volunteers, the concentration of corticosterone was very low (Table II). The cortisol concentration is not significantly different from that previously reported(2). Although distribution of cortisol is approximately normal, the large discrepancy between mean and median of the corticosterone concentration emphasizes the significant skewing of distribution.

Plasma cortisol and corticosterone concentrations were measured in several patients convalescent from diseases not primarily affecting the adrenal glands during stimulation with adrenocorticotrophic hormone. The pituitary hormone was administered intravenously at a steady rate of 5 units/hour for 5 hours, and plasma drawn for analysis just before and at termination of the infusion. The results are seen in Table III. In every instance except one, the corticosterone concentration rose after ACTH.

TABLE III. Effect of ACTH on Plasma Cortisol and Corticosterone Concentrations, in 12 Subjects.

Cortisol, μg/100 ml		Corticosterone, μg/100 ml	
Initial	Final	Initial	Final
11.6	24.1	*.0	.1
21.2	46.8	.0	2.4
32.0	56.3	.0	.0
14.8	39.9	1.3	8.7
14.5	50.1	.7	9.7
18.7	32.0	.5	5.3
7.8	28.8	.1	4.8
14.1	30.1	2.8	8.6
10.2	39.4	1.4	23.4
15.3	39.5	.9	7.7
19.3	32.5	2.5	11.9
17.5	40.3	.0	6.0

* See footnote to Table II.

Discussion. Corticosterone has been isolated and identified in plasma from the human adrenal vein(1,9-11) and breakdown products of the steroid have been found in human urine(12). Presumably, therefore, appreciable amounts of this substance are present in the peripheral plasma of man. Previous studies(1,5-7) have reported widely varying quantities of steroid, partly perhaps because of inadequate purification of the plasma extract before measurement of the hormone, and perhaps also in part because of unpredictable variability of recovery of the steroid. The present method utilizes a paper chromatography system of high resolution for purification and corrects for losses incurred during analysis by radioactive controls of each determination.

The identity of the steroids measured as "cortisol" and "corticosterone" can not be established absolutely. In regard to the former, however, the chromatographic characteristics combined with fluorescence in alkali indicate that it is a Δ^4, 3-ketosteroid of characteristics similar to cortisol or cortisone. The fact that fluorescence in sulfuric acid is equal to that in alkali, eliminates cortisone as a quantitatively important component of this spot. The "corticosterone" spot, similarly, might also include 11-dehydrocortisone, but the correspondence between alkaline and acid fluorescence indicates that no significant quantities of the 11-ketonic steroid are present. In view of the definite identification of cortisol and corticosterone in human adrenal vein blood, and the failure to find appreciable quantities of other Δ^4, 3-ketosteroids which might be difficult to separate in the chroma-

TABLE IV. Variability of Cortisol/Corticosterone Ratio.

Subject	Date	Cortisol, μg/100 ml	Corticosterone, μg/100 ml	Cortisol / Corticosterone	Remarks
B.	9/21/56	8.4	10.4	.8	Upper resp. infection
	11/ 9	11.6	.8	14.5	Normal
R.	9/21	19.2	19.4	1.0	48 hr post-dental extr.
	11/ 2	13.1	.0	*(131)	Normal
	11/ 9	14.6	1.2	12.7	Acute urticaria
J.	10/ 8	18.7	1.0	18.7	
	10/28	21.2	.0	*(212)	1 wk on depot ACTH
	11/ 5	13.1	.6	21.8	2 " " " "

* This ratio was computed assuming that a corticosterone conc. of .0 actually represents a conc. of .1 μg/100 ml.

tographic system used for this analysis, it seems highly probable that the substances being studied are indeed cortisol and corticosterone.

Bush(13,14) has recently reviewed information regarding the relative concentrations of cortisol and corticosterone in human plasma. The range of cortisol/corticosterone reported by various observers ranges from 0.9 to 10.0. In our observations the mean ratio was 7.9; however, in the many instances where no corticosterone could be detected, the ratio is meaningless. Although the ratio did not change much in patients receiving ACTH, it was not constant in any given individual whose plasma was analyzed on several occasions (Table IV). This seems to speak against the hypothesis advanced by Bush that the ratio is an inherent quality of the individual and suggests that the pattern of secretion may be altered by the environment. Recent observations(15,16) that corticosterone may be cleared from the plasma much more rapidly than cortisol suggest that the plasma concentrations may not necessarily reflect rates of secretion of the 2 substances by the adrenal, but may be the resultant of the respective rates of secretion and removal.

Summary and conclusions. A method is presented for measuring cortisol and corticosterone simultaneously in a single specimen of plasma. The steroids are purified by paper chromatography, eluted and measured by fluorometry. Losses incurred during analytical procedure are compensated by application of an isotope dilution correction. The normal range of corticosterone in human beings is 0 to 5 μg/100 ml, with a mean of 1.3 ± 2.5 μg/100 ml. The normal range for cortisol is 4.0 to 17.7 μg/100 ml, with a mean of 10.2 ± 3.6 μg/100 ml.

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Insulinase-Inhibitory Activity of Protein Hydrolysates.*† (23021)

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During studies of the identity of the factor (or factors) in liver extracts which inhibits activity of insulinase(1), it became apparent that the greatest concentration of this factor occurred in fractions reported by others to be rich in strepogenin activity(2). Since hydrolysates of insulin and a variety of other proteins are also rich in strepogenin activity (2,3), the insulinase-inhibitory activity of such hydrolysates was studied.

Methods. The various proteins used were hydrolyzed for 3 hours with concentrated hydrochloric acid (4 g protein/100 ml acid) at 37°C, neutralized and evaporated to dryness in flash evaporator. The hydrolysates were kept in the dry state until immediately before use.¶ The effect of the presence of various concentrations of the protein hydrolysates on rate of degradation of I^{131} labeled insulin by liver extract was determined by the procedure described previously(4). Inhibitory activity of the 3-hour acid hydrolysates of proteins was computed on the basis of quantity of nitrogen necessary to produce a 50% inhibition of insulinase activity of the extract. Since an hydrolysate of insulin had the greatest inhibitory activity, the activities of other hydrolysates were expressed as percentage of activity of the insulin hydrolysate. The effect of prolonged acid hydrolysis on insulinase-inhibitory activity of crystalline insulin, bovine plasma albumin and casein was determined by treating these proteins as above, with concentrated hydrochloric acid for 72 hours at 37°C. Aliquots were removed at the beginning and at various designated intervals during hydrolysis, neutralized and lyophilized. The quantity of each hydrolysate, in terms of nitrogen, necessary to produce a 50% inhibition of insulinase activity of a liver extract was determined. Maximum insulinase-inhibitory activity of an hydrolysate was attributed to that aliquot which produced a 50% inhibition with the smallest quantity. All other assays were expressed in terms of percentage of aliquot with maximum activity. In addition, tryptic and chymotryptic digests of insulin, bovine plasma albumin, crystalline lysozyme and casein were prepared. The proteins were dissolved or dispersed in distilled water, heated at 100°C for 3 minutes, cooled and adjusted to pH 7.8. A 0.5% solution of each protein was incubated under toluene for 12 hours at 37°C with an amount of crystalline trypsin or crystalline chymotrypsin equal to 1% of weight of the protein. pH was maintained during incubation period by addition of alkali. At the end of incubation period, the reaction mixtures were adjusted to pH 3, heated to 100°C, cooled, neutralized and lyophilized. The action of hydrolysates on degradation of I^{131} labeled insulin was determined as above using the equivalent of 5 mg of protein. The data were expressed in terms of activity of an equivalent amount of a 3-hour acid hydrolysate of crystalline insulin. The type of inhibition produced by some of the hydrolysate of proteins was determined from studies of the effect of single concentration of each hydrolysate on action of

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