Radioassay of Cortisol and Corticosterone by a Modified Competitive Protein-Binding Method

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Abstract □ A competitive protein-binding radioassay using adsorption was evaluated for the individual determination of cortisol and corticosterone. The competitive protein-binding assay using fuller's earth to separate bound and free cortisol was tested and found to offer no specificity for cortisol in favor of corticosterone. The introduction of a carbon tetrachloride/water partitioning procedure to separate corticosterone and cortisol made possible the individual determination of corticosterone and cortisol using the competitive protein-binding radioassay. The modified competitive protein-binding radioassay was evaluated as to precision, accuracy, and specificity; normal values of cortisol and corticosterone in human plasma were determined.

Keyphrases □ Cortisol, corticosterone in human plasma—determination, competitive protein-binding radioassay □ Radioassay, protein-binding—determination of cortisol, corticosterone in plasma □ Transcortin binding—cortisol–corticosterone separation, determination

The determination of cortisol and corticosterone in blood plasma is useful for evaluating the functional capacity of the adrenal cortex and for determining the amount of biologically active corticoid available to the tissues. This information is useful for clinical and investigational work. Murphy *et al.* (1) developed a method for the determination of plasma corticoids using the specific corticosteroid-binding globulin, transcortin. They named the method competitive protein-binding analysis (2). The method utilizes the rather specific binding properties of transcortin, which binds the steroids cortisol, corticosterone, and 11-deoxycortisol. Radioactive corticoid is added to a solution of diluted plasma, where the chief protein exerting steroid-binding effect is transcortin, and allowed to equilibrate. This forms the assay reagent.

Since the binding of the steroid is of a reversible nature, the addition of nonradioactive corticoid to the transcortin solution causes a change in the equilibrium such that the specific activity of the transcortin is decreased. If protein-bound and free steroid can be separated, this change in specific activity of the transcortin can be assessed. A standard curve can be prepared by plotting the activity of the transcortin *versus* the weight of steroid for a series of known weight standards. This can be utilized to determine the quantity of corticoid in an unknown sample from the activity of the transcortin after equilibration with the unknown and removal of the free steroid, providing the unknown does not itself contain any transcortin.

The need for separation has led to several modifications in the method. The separation of protein-bound and free steroid was achieved initially by equilibrium dialysis (1), later by gel filtration (3), and most recently by competitive adsorption (4). These modifications were claimed to have increased the speed, sensitivity, and specificity of the method. In this study, the method of Murphy (4) was tested and some further modifications were made, chiefly to improve specificity.

EXPERIMENTAL

Materials—All radioactive steroid preparations[1] were stored at −15° prior to dilution. The radiochemical purity of the radioactive steroids was checked by TLC and autoradiography. One spot was detected for each compound, indicating that no decomposition had occurred. The steroids used were cortisol-1,2-^{3}H (2 and 32 c./mmole) and corticosterone-1,2-^{3}H (1 and 39.1 c./mmole). The higher specific activity compounds were used in the preparation of the transcortin isotope solution; those of lower specific activity were used in the adsorption and recovery experiments. The transcortin isotope solution was prepared by diluting the desired quantity of plasma with distilled water and then adding the tritium-labeled steroid in a small quantity of ethanol. The solution was then brought to volume with distilled water. Nonradioactive cortisol and corticosterone were obtained commercially[2]. The cortisol was purified by recrystallization from aqueous methanol. Stock solutions of the steroids in redistilled ethanol were made up to a concentration of 10 mcg./ml. and stored at 0°. Working standards were prepared as required in 10-ml. quantities at the concentration of 10 ng./0.1 ml. (ng. = 10^{-9} g.).

Fuller's earth, technical[3], and Florisil[4], 80–100 mesh, were used. The adsorbents were measured in two Plexiglas spoons with capacities of 10.32 ± 1.02 and 59.14 ± 1.58 (*SD*) mg. for fuller's earth, and of 8.38 ± 0.81 and 41.80 ± 0.51 (*SD*) mg. for Florisil. In some cases, weighing of adsorbent was employed.

The scintillation fluid used was Bray's solution (naphthalene, 60 g.; diphenyloxazol, 4 g.; 1,4-di[2(5-phenyloxazolyl)]benzene, 0.2 g.; methanol, 100 ml.; ethylene glycol, 20 ml.; and dioxane sufficient to make 1 l.). Counting was done using a Picker Nuclear Liquimat 110 or 220 liquid scintillation counter equipped with ^{137}Cs external standard. Quench correction was done by the external standard count method for the Liquimat 110 and the channel ratio of the external standard method for the Liquimat 220. A digital PDP-8L computer was used in conjunction with the Liquimat 220 for quench correction. A program that computed the quench-corrected activity in disintegrations per minute and the reciprocal of activity in milliseconds per disintegration was used.

Plasma samples for analysis were collected in tubes containing ethylenediaminetetraacetic acid as anticoagulant. The samples were centrifuged at once at 2000 r.p.m. The plasma was collected and stored at −15° until analysis was performed. Steroid-free serum was obtained by dialyzing serum, which had been diluted 1 in 10 with physiological saline, against physiological saline. The saline was changed frequently during the 66.5-hr. dialysis period. After dialysis, the serum was freeze-dried and reconstituted to its original strength.

Methods—*Determination of Adsorption of Free Steroids*—For the determination of adsorption of free steroids, a measured quantity of adsorbent was added to 1 ml. of a solution of tritiated steroid which had been cooled to at least 10° for 15 min. The tubes were shaken for 2 min. on a horizontal mechanical agitator, cooled for 10 min. in an ice bath, and centrifuged. The supernatant, containing the unadsorbed steroid, was removed with a Pasteur pipet. One-half milliliter of the solution was added to 10 ml. of Bray's solution,

[1] Amersham-Searle, Des Plaines, Ill.
[2] British Drug Houses, Toronto, Ontario, and Nutritional Biochemicals Corp., Cleveland, Ohio, respectively.
[3] Fisher Scientific Co., Pittsburgh, Pa.
[4] Floridin Co., Tallahassee, Fla.

and the sample was counted. From the difference between initial activity and the activity remaining after treatment with adsorbent, it was possible to calculate the percentage adsorption of steroid. This method is essentially that described by Murphy (4).

Determination of Protein-Bound Steroid—To determine the percentage of steroid bound to protein, paired solutions (1 ml.) of tritiated steroids, one with and one without protein, were prepared. The tubes were warmed to 45° for 5 min. to promote rapid equilibration of the steroid with the protein. The samples were then cooled to 0° for 10 min. A measured quantity of adsorbent was added, and the tubes were shaken for 2 min. The tubes were returned to the ice bath for 15 min., then centrifuged, and returned to the ice bath; finally, the supernatant was removed. One-half milliliter of this supernatant was added to 10 ml. of Bray's solution, and the sample was counted. The samples without protein contained unadsorbed steroid in the supernatant, while the samples containing protein had unadsorbed steroid plus protein-bound steroid in the supernatant. The difference in the percentage of original activity remaining in the protein and nonprotein samples represents the percentage of steroid bound to protein.

Preparation of Standard Curves and Assay of Samples—Standards were prepared in duplicate in the desired range; usually, 10 standards were used for each determination of the standard curve. One milliliter of transcortin-isotope solution was added to each of the evaporated standards and samples. These were mixed and heated to 45° for 5 min. After cooling in an ice bath for 15 min., the adsorbent was added. The mixture was shaken for 2 min. on a horizontal agitator, cooled 10 min., and then centrifuged. The supernatant was collected and recentrifuged if the adsorbent was fuller's earth. One-half milliliter of the supernatant was added to 10 ml. of Bray's solution, and the samples were counted twice for a sufficient time to give 1% counting error with 2 σ statistics. A plot of milliseconds per disintegration *versus* nanograms of steroid in the standard was prepared, or a linear regression equation (5) describing this relationship was computed. The graph or equation was used to determine the quantity of steroid in the sample from the reciprocal of activity of the sample.

Preparation of Plasma Samples for Assay—Removal of transcortin from the plasma samples was achieved by alcohol precipitation. Two-tenths of a milliliter of plasma was diluted with 0.1 ml. of water; then 1 ml. of 95% alcohol was added, and the sample was mixed for 2 min. After centrifugation the supernatant was collected. Another milliliter of alcohol was added to the precipitate, and the mixing and centrifugation were repeated. The combined alcoholic supernatants were evaporated at reduced pressure in a freeze-drying apparatus.

To separate cortisol and corticosterone, the residue from the alcohol supernatant was dissolved in 2 ml. of water and partitioned against 20 ml. of carbon tetrachloride by mechanical mixing for 2 min. The corticosterone favors the carbon tetrachloride, while the cortisol remains in the water. The carbon tetrachloride was backwashed with 2 ml. of water, and the original water was backwashed with 20 ml. of carbon tetrachloride. The backwash phases were discarded. The water was evaporated in a freeze-drying apparatus; the carbon tetrachloride was evaporated at 45° in a stream of air. The samples were then ready for assay, as described previously.

RESULTS

Adsorption of Free Steroid—Experiments to determine the adsorption of free cortisol by fuller's earth were conducted using concentrations of cortisol from 1 to 40 ng./ml. and weights of fuller's earth from 10.3 to 118.3 mg. For cortisol concentrations of 5–40 ng./ml. and fuller's earth in weights of 41.3–118.3 mg., adsorption of free cortisol was constant at 85.9 ± 4.4% (n = 22). Decreasing either adsorbent or steroid weight below these ranges gave less adsorption. The adsorption of free corticosterone by Florisil was studied using concentrations of 1.4–36 ng./ml. of corticosterone and 8.4–120 mg. of Florisil. Percentage adsorption was constant for all concentrations of corticosterone tested. For 8.4 and 10 mg. of Florisil, adsorption was 81.6 ± 3.7% (n = 16); for 16.8–120 mg. of Florisil, adsorption was 88.2 ± 2.4% (n = 36).

Separation of Bound and Free Steroid—Using 5% human plasma as the source of transcortin, the percentage of protein-bound cortisol was determined when weights of fuller's earth from 10.3 to 118.3 mg. were used to separate bound and free cortisol (Table I).

Table I—Influence of Fuller's Earth on Protein-Bound ^{3}H-Cortisol[a]

Cortisol Weights, ng.	Weight of Fuller's Earth, mg. 10.3	20.6	41.3	69.5	90.1	118.3
10.4	36.1[b]	45.6	43.0	37.6	34.0	21.8
13.5	34.2	41.2	45.0	42.0	30.9	18.0
22.0	12.4	25.4	20.6	—	18.5	13.5

[a] Source of transcortin was 5% human plasma; concentrations of cortisol were 10.4, 13.5, and 22 ng./ml. [b] Expressed as percent protein-bound; each value of percent bound is the average of two determinations, two at each steroid concentration; values of percent protein-bound cortisol which are underlined are not significantly different at the 5% level by Duncan's multiple-range test (5).

Three concentrations of cortisol were used (10.4, 13.5, and 22 ng./ml.); however, the trend of adsorbent effect on protein binding was the same for all concentrations of steroid, although the percentage of protein-bound steroid decreased as the concentration of cortisol increased due to increased displacement of radioactive steroid from transcortin.

The percentage of protein-bound steroid was constant for weights of 10.3–69.5 mg. of fuller's earth and decreased at 118.3 mg. The decrease at 118.3 mg. indicates that at higher weights of adsorbent, some protein-bound steroid is probably adsorbed after dissociation of the complex. For corticosterone (0.88 ng./ml.) using 0.5% mouse plasma as the transcortin source, the percentage of protein-bound steroid was determined using 8.4–125.4 mg. of Florisil (Table II). Above 20 mg. Florisil, there was very little protein binding; 8.4 and 10 mg. gave substantial binding but were different from each other in the percentage bound.

To determine how much adsorbent to use for separation of bound and free steroid in the assay procedure, the results of the adsorption and protein-binding experiments were considered. An optimum amount of adsorbent was described as that which gave maximum removal of free steroid but also maximum protein binding, thus giving greatest sensitivity. Since a range of weights fulfilled these criteria for fuller's earth, a weight (59.1 mg.) in the middle of the range was chosen so that slight errors in measuring the adsorbent would have little influence on the assay. For Florisil, only the second part of the criteria was met in choosing 8.4 mg. as the weight.

Standard Curves—For cortisol, a standard curve was prepared using 5% human plasma as the transcortin source, ^{3}H-cortisol (4.65–11.05 μc./100 ml.) as the labeling agent, and 59.1 mg. fuller's earth as the adsorbent. The range employed was 0–40 ng. For ease of comparison, all data were corrected to 6 μc./100 ml. The standard curve which resulted when milliseconds per disintegration was plotted *versus* nanograms of cortisol was described by the equation $Y = 0.0381X + 1.203$, where Y = milliseconds per disintegration and X = nanograms. The standard deviation of the slope of this line, where 68 values were used to determine it, was 0.00213 (6). The standard deviation of an X value estimated using this equation was determined as 4.57, where the true value of X = 10 ng. and duplicate estimates are made (6).

For corticosterone, mouse plasma and human plasma were tried as sources of transcortin, both ^{3}H-cortisol and ^{3}H-corticosterone were used as labeling agents, and both Florisil (8.4 and 41.8 mg.) and fuller's earth (59.1 mg.) were used as adsorbents. Table III shows the data on the standard curves obtained using the various combinations of transcortin, labeling agent, and adsorbent for cortisol and corticosterone.

Table II—Influence of Florisil on Protein-Bound ^{3}H-Corticosterone[a]

Weight of Florisil, mg.	8.4	10	20	41.8	83.6	125.4
Percent protein-bound corticosterone	46.6	30.9	13.6	9.0	8.1	5.4

[a] Source of transcortin was 0.5% mouse plasma; concentration of corticosterone was 0.88 ng./ml.; each value of percent protein-bound corticosterone is the average of two determinations; values of percent bound which are underlined are not significantly different at 5% level by Duncan's multiple-range test.

Table III—Standard Curves

System	Steroid	Transcortin Source	Labeling Agent	Adsorbent	Equation	Range, ng.	n[a]	s_b[b]	cv_b[b]	s_x[c]
1	Corticosterone	0.5% Mouse plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0619X + 1.19$	0–5	6	0.0061	9.9	0.42
2	Corticosterone	0.5% Mouse plasma	^{3}H-Corticosterone	Florisil, 41.8 mg.	No slope	0–40	10	—	—	—
3	Corticosterone	3% Mouse plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0079X + 0.994$	0–20	10	0.0011	14	2.5
4	Corticosterone	1% Human plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0732X + 1.11$	0–10	39	0.010	14	1.5
5	Corticosterone	1% Human plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0378X + 0.914$[d]	0.5–2.5	6	0.0029	7.7	0.13
6	Corticosterone	1% Human plasma	^{3}H-Corticosterone	Fuller's earth, 59.1 mg.	$Y = 0.374X + 1.69$	0–5	8	0.016	4.3	0.18
7	Corticosterone	1% Human plasma	^{3}H-Cortisol	Fuller's earth, 59.1 mg.	$Y = 0.0678X + 1.31$	0–10	10	0.0036	5.3	0.48
8	Corticosterone	2.5% Human plasma	^{3}H-Corticosterone	Florisil, 8.4 mg.	$Y = 0.0267X + 1.03$	0–20	18	0.0025	9.4	3.0
9	Cortisol	5% Human plasma	^{3}H-Cortisol	Fuller's earth, 59.1 mg.	$Y = 0.0381X + 1.20$	0–40	68	0.0021	5.5	4.57

[a] Number of values used in determining the regression equation. [b] s_b = standard deviation of slope, b (6); cv_b = coefficient of variability for slope = $(s_b/b) \times 100$. [c] s_x = standard deviation of X where duplicate estimates of X are made using the regression equation (6). For corticosterone, X = 2 ng.; for cortisol, X = 10 ng. [d] In this case, steroid standards were added to steroid-free plasma.

Specificity—Specificity for cortisol or corticosterone individually was tested by assaying ethanol samples, containing known amounts of cortisol and/or corticosterone, and plasma samples, with and without additional amounts of cortisol and corticosterone. When plasma was used, it was necessary initially to precipitate proteins from the plasma with alcohol as described previously. Since initial experiments (Table IV) indicated that there was no specificity for either cortisol or corticosterone using various combinations of adsorbent and plasma, physical separation of the two steroids was needed to improve the specificity. Results of the specificity studies are shown in Table IV.

The 5% human plasma system, using 59.1 mg. of fuller's earth as the adsorbent, did not give specificity for cortisol; corticosterone was detected to the extent of 1.81 ± 1.08 mcg./mcg. of corticosterone in the sample. To improve the specificity for cortisol, carbon tetrachloride/water partitioning was employed. The water phase was assayed for cortisol. Comparison of sets of samples containing plasma only and the same plasma with additional amounts of corticosterone was done to see if interference of corticosterone was still detectable. In most cases, the added corticosterone did not significantly elevate the estimate of cortisol.

Assay of corticosterone using 1% human plasma and either 8.4 mg. Florisil or 59.1 mg. fuller's earth as the adsorbent did not provide a system specific for corticosterone; cortisol was detected to an approximately equal extent as corticosterone. Application of carbon tetrachloride/water partitioning to recover the corticosterone in the carbon tetrachloride resulted in some difficulties. When assay of the carbon tetrachloride residue for corticosterone was done using System 4, 6, or 7, negative amounts of steroid were detected; that is, the value of milliseconds per disintegration for the plasma samples fell below the zero point of the standard curve. This elevated activity in these samples could be due to a decrease in adsorption of steroid or an increase in protein binding. Ethanol precipitation of protein did not give complete deproteinization, as shown by a positive ninhydrin test. When the ethanol supernatant was treated with an excess of ammonium sulfate and filtered, the supernatant was free of protein. This ethanol supernatant was evaporated and partitioned as described previously. The carbon tetrachloride was

Table IV—Specificity of Competitive Protein-Binding Radioassay

Assay System	Sample	Number of Samples	Carbon Tetrachloride/Water Partition Step Used	Added Steroid, mcg./100 ml. Cortisol	Added Steroid, mcg./100 ml. Corticosterone	"Found" Steroid, mcg./100 ml. Cortisol	"Found" Steroid, mcg./100 ml. Corticosterone	Interference[a]
5% Human plasma, ^{3}H-cortisol, 59.1 mg. fuller's earth	Ethanol[b] solution of steroid	2	No	10	1	13.06 ± 0.59	—	3.06 ± 0.59
		2	No	10	5	15.54 ± 0.27	—	1.11 ± 0.06
		2	No	10	10	20.66 ± 1.78	—	1.07 ± 0.18
		2	No	10	20	30.83 ± 2.34	—	1.04 ± 0.11
		2	No	0	1	3.33 ± 1.78	—	3.33 ± 1.78
		2	No	0	5	8.96 ± 1.48	—	1.79 ± 0.30
		2	No	0	10	12.53 ± 0.84	—	1.25 ± 0.08
		2	Yes	10	0	8.12 ± 1.33		
		2	Yes	10	1	8.73 ± 2.18	—	No[d]
		2	Yes	10	10	9.54 ± 1.80		
		1	Yes	0	10	2.13	—	0.21
		7	Yes	0	0	9.11 ± 2.96		
		6	Yes	0	10	10.91 ± 2.32	—	No[e]
		8	Yes	0	0	10.75 ± 1.98		
		7	Yes	0	10	13.85 ± 1.97	—	0.31[f]
	Plasma[c]	4	Yes	0	0	12.03 ± 2.04		
		4	Yes	0	10	10.54 ± 1.67	—	No[g]
		3	Yes	0	0	11.94 ± 2.36		
		2	Yes	0	5	16.82 ± 3.10	—	No[h]
1% Human plasma, ^{3}H-corticosterone, 8.4 mg. Florisil	Ethanol[b] solution of steroid	2	No	10	0	—	7.27 ± 0.45	0.73 ± 0.04
1% Human plasma, ^{3}H-corticosterone, 59.1 mg. fuller's earth	Ethanol[b] solution of steroid	2	No	5	0	—	4.97 ± 0.35	1.00 ± 0.06
5% Human plasma, ^{3}H-cortisol, 59.1 mg. fuller's earth	Plasma[b]	8	Yes	0	0	—	10.96 ± 3.86	No[i]
5% Human plasma, ^{3}H-cortisol, 59.1 mg. fuller's earth	Plasma[b]	4	Yes	10	0	—	11.97 ± 5.87	

[a] Interference determined by subtracting the known concentration (mcg./100 ml.) of desired steroid from "found" concentration (mcg./100 ml.) of steroid and dividing the difference so obtained by the concentration of interfering steroid known to be in the system. [b] Recovery correction by yield determination. [c] Recovery correction by internal standard method. [d] $F < 1$; interference not significant. [e] $t = 1.20$; interference not significant, where t is Student's t. [f] $t = 3.30$; interference significant ($p < 0.01$). [g] $t = 1.13$; interference not significant. [h] $t = 2.03$; interference not significant. [i] $t = 0.36$; interference not significant.

Table V—Separation of Corticosterone and Cortisol by Carbon Tetrachloride/Water Partitioning[a]

Replicate	Water Phase		Carbon Tetrachloride Phase	
	Cortisol Recovery	Corticosterone Crossover	Corticosterone Recovery	Cortisol Crossover
1	74.1 ± 2.5[c]	15.6 ± 1.2[b]	48.5 ± 4.3[c]	6.3 ± 0.6[c]
2	74.1 ± 1.0[c]	6.9 ± 0.3[c]	76.2 ± 2.4[c]	7.5 ± 0.4[c]
3	—	8.6 ± 0.3[c]	62.0 ± 1.8[c]	—
4	—	12.6 ± 1.2[c]	35.4 ± 2.4[c]	—

[a] Results expressed as average percent ± standard deviation. [b] Number of samples equals 3. [c] Number of samples equals 4.

assayed using System 4, but negative values were still obtained. Therefore, crossover of sample protein to the assay step did not explain the failure of the assay. Evaporation of the carbon tetrachloride in a stream of nitrogen rather than air also did not improve the results. Systems 6 and 9, when applied to the carbon tetrachloride residue, gave positive values. System 9 gave adequate specificity for corticosterone after carbon tetrachloride/water partitioning and assay of the carbon tetrachloride residue. Specificity using System 6 was not confirmed.

Recovery—Recovery of cortisol and corticosterone from plasma after ethanol precipitation was checked using ^{3}H-steroids. The recovery was determined as 69.2 ± 11.8% (n = 12) for cortisol and 89.2 ± 7.3% (n = 12) for corticosterone. Recovery was reproducible on two different days.

Recovery of ^{3}H-cortisol and ^{3}H-corticosterone from aqueous solutions was determined after carbon tetrachloride/water partitioning (Table V). Replicates were performed on different days. The recovery of cortisol was reproducible from day to day [74.1 ± 1.8% (n = 8)], and crossover of cortisol into carbon tetrachloride was 6.9 ± 0.8% (n = 8). The recovery of corticosterone was reproducible on any given day but varied from day to day. For this reason, daily recovery indicators were needed in the total assay procedure. Two approaches were employed.

The first was recovery correction by isotope yield determination. With each batch of samples, two yield determination standards were included. One contained a measured amount of cortisol-1,2-^{3}H in 0.2 ml. of plasma, while the other contained corticosterone-1,2-^{3}H in the same volume of plasma. These standards were taken through the procedure of protein precipitation and partitioning in parallel with the other samples. The standards were processed no further except for counting of radioactivity. Each standard yielded two counting samples, one for recovery and one for crossover. The fractional recovery and crossover were calculated by comparison to the initial radioactivity added. Because the normal concentration of

Table VI—Comparison of Fluorometric, Double-Isotope Analysis, and Competitive Protein-Binding Radioassay Methods

Reference	SD[a]	Precision Range[a]	n	Normal Values, mcg./100 ml.[b] (n) Cortisol	Corticosterone	Comments
				Fluorometric		
16	—	—	—	9.8	0.2	—
17	1.7	10–20	24	21.96 ± 4.76[c]	—	8–9 a.m.
18	0.55	10–20	6	11 ± 4.4 (36)	—	10–12 a.m.
19	—	—	—	10.9 ± 0.55 (20)	3 ± 0.2 (20)	—
20	—	—	—	6–15	0.7–2.9	—
21	—	—	—	11.71 ± 4.31 (48)	—	Male, age 16–78
	—	—	—	13.01 ± 4.27 (33)	—	Female, age 12–69
	—	—	—	12.24 ± 4.34 (81)	—	Both sexes, all values 7:30 a.m.
22	—	—	—	9.2 ± 1.5 (30)	1.2 (9)	8–9 a.m.
23	—	—	—	11 ± 1	2 ± 0.5	—
24	0.80	10–19.9	39	9.6 ± 3.0 (33)	—	8–9:30 a.m.
	0.21	0–0.9	36	—	0.6 ± 0.6 (19)	8–9:30 a.m.
25	0.1–0.2	—	—	—	—	—
26	0.2	10–20	33	21.4 ± 5.6[c] (18)	—	7 a.m.
	—	—	—	13.0 ± 3.3[c] (18)	—	9 a.m.
27	—	—	—	15 (20)	1.1 ± 0.3 (20)	—
28	—	—	—	18 ± 4.3 (30)	—	—
29	—	—	—	19.1 ± 3.1[c] (18)	—	—
30	0.082	15–35	44	—	—	—
31	2.12	10–20	9	9.6 ± 2.7 (27)	—	9:30 a.m.
	0.34	1–2	9	—	0.4 ± 0.2 (27)	9:30 a.m.
32	1	—	—	—	—	—
33	2.1–2.2	—	16	13.8	5.2	—
34	1.2	10–20	75	14.6 ± 3.7 (50)	—	8 a.m.
				Double-Isotope Analysis		
35	—	—	—	10.5 (159)	2.5 (19)	8–10 a.m.
36	—	—	—	10.27 ± 3.1 (29)	0.56 ± 0.21 (28)	a.m.
	—	—	—	6.77 ± 3.13 (14)	0.34 ± 0.1 (13)	p.m.
37	—	—	—	11.8 ± 4.3 (25)	0.34 ± 0.25 (25)	9–10 a.m.
38	0–19	—	10	9.8 (17)	—	—
	0.03	—	11	—	0.66 (29)	—
				Competitive Protein-Binding Radioassay		
8	4.9	0–100	167	—	—	—
14	—	—	—	15.7 ± 4.6 (26)	—	8 a.m.
	—	—	—	8.2 ± 2.3 (25)	—	2 p.m.
	—	—	—	6.4 ± 2.7 (12)	—	8 p.m.
1	2.0	10–20	18	15.2 ± 5.1[c] (12)	—	Male, 9 a.m.
	—	—	—	17.6 ± 4.3[c] (12)	—	Female, 9 a.m.
3	1.2	10–20	20	—	—	—
4	2.0	10–20	20	8–24[c]	—	—
	—	—	—	6–18	4	9 a.m.
10	1.2	10–20	12	15[c]	—	8 a.m.
Present study	2.65	9–22	39	11.71 ± 1.50 (3)	—	a.m.
Present study	2.52	4–8	13	—	1.84 (1)	a.m.

[a] In mcg./100 ml.; if standard deviation given for several concentration ranges, the standard deviation for normal range is quoted. [b] ± Standard deviation. [c] Normal values apply to corticosteroids, not cortisol only.

cortisol in human plasma is much higher than that of corticosterone, crossover is less important in the determination of cortisol. In the case of corticosterone, correction for crossover is important to avoid overestimating the corticosterone concentration of the sample. To do this, the value of cortisol (corrected for recovery) is multiplied by the fraction crossover of cortisol into carbon tetrachloride. This figure is the contribution of cortisol to the estimate of corticosterone, so it is subtracted from the recovery-corrected amount of corticosterone to give the corticosterone (corrected) concentration. In physiological states where the ratio of cortisol to corticosterone is reversed, this procedure would be used to find the cortisol (corrected) concentration in the sample.

The second approach to recovery correction was the internal standard method. In this procedure, a sample containing a known amount of added cortisol (usually 10 ng./0.1 ml.) and another containing a known amount of added corticosterone are prepared, as well as a sample of the plasma containing no added steroid. These three samples are processed in parallel with the other samples. From the difference between the amount of steroid detected in the plasma-only sample and the sample with added steroid, the recovery can be determined.

In both cases, the recovery-corrected amount of steroid was calculated by dividing the uncorrected weight of detected steroid by the fractional recovery factor. The results are expressed in ng./0.1 ml. of plasma and are numerically equal to the concentration in mcg./100 ml. of plasma.

Precision—The precision of the total method was estimated by calculation of the standard deviation of replicate determination on aliquots of several plasma samples. System 9 was used to assay both cortisol and corticosterone. For cortisol in the range 9–22 mcg./100 ml., the standard deviation was 2.65 (n = 39). For corticosterone in the range 4–8 mcg./100 ml., the standard deviation was 2.53 (n = 13).

Accuracy—The accuracy of the method was determined using aliquots of four different plasma samples. The concentration of cortisol in each sample was determined; then 10 mcg./100 ml. of cortisol was added, and the concentration was again determined. Several replicate determinations were made in each case. When the internal standard method of recovery correction was used, the additional increments detected were 8.24, 9.12, 11.83, and 12.45 mcg./100 ml. compared to 8.40, 9.02, 15.94, and 4.57 mcg./100 ml. using the yield determination method of recovery correction. It was judged that the internal standard method gave more accurate results.

Normal Values—Several morning (10:00 a.m. to 12:30 noon) blood samples were drawn from a normal male subject to determine normal estimates of corticoids by the total method. Cortisol concentration, determined using System 9, was 11.71 ± 1.50 mcg./100 ml. (n = 3, recovery correction by internal standard method). When the method was applied to pooled human serum, the cortisol concentration was found to be 13.79 mcg./100 ml. (recovery correction by yield determination method). Using System 9, corticosterone concentration (12:30 noon) was determined to be 4.96 mcg./100 ml. (recovery correction by yield determination method). With System 5, the corticosterone concentration (10:15 a.m.) was determined as 1.84 mcg./100 ml. In this case, no recovery correction was needed since all samples and standards were processed in parallel through all the steps of protein precipitation, partitioning, and assay.

DISCUSSION

The results of the present study did not confirm the observation of Murphy (4) that the use of fuller's earth as an adsorbent for separation of bound and free steroid produced a system with increased specificity for cortisol. Using fuller's earth as the adsorbent, corticosterone was detected to an equal or greater extent than cortisol. When Florisil was the adsorbent, corticosterone was detected to a greater extent than cortisol. It seems reasonable that this effect would be observed since the measurement of steroid is dependent only on the decrease in activity observed. The adsorbent role is to remove free steroid after the equilibration of the sample steroid with transcortin. The effect of the adsorbent on the assay is dependent only on the form of radioactive steroid used, since adsorbent removal of other steroids goes undetected. The source of the protein used determines specificity. Since human transcortin has approximately equal affinity for cortisol and corticosterone (4), both are detected and there is no specificity for one or the other. ^{3}H-Cortisol on the transcortin is displaced by any steroid with equal or greater affinity for the transcortin, and the free ^{3}H-cortisol is removed from the solution by adsorption, depending on the affinity of the adsorbent for cortisol. The different adsorbents affect the slope of the standard curves and thus the sensitivity and precision of the assay, but they do not affect the specificity of the assay.

After carbon tetrachloride/water partitioning, specificity was improved so that cortisol could be determined in the presence of 5–10 times the normal concentration of corticosterone. The interference of corticosterone detected in some cases probably was due to the variable recovery of corticosterone into carbon tetrachloride. Corticosterone could be determined in the carbon tetrachloride phase without interference from cortisol. The carbon tetrachloride phase contains other steroids than corticosterone. It takes up 11-deoxycortisol just as avidly as corticosterone (partition coefficient water/carbon tetrachloride: corticosterone, 0.75; 11-deoxycortisol, 0.74) (7). Bowman and DeLuna (8) reported that 80% of 11-deoxycortisol is removed by extraction of plasma with 10 volumes of carbon tetrachloride. The normal levels of 11-deoxycortisol are low (0–2 mcg./100 ml.) (9) but may be significant when compared to the low levels of corticosterone in plasma (1 mcg./100 ml.). 11-Deoxycortisol is detected quite strongly in a competitive protein-binding type of assay (4, 10); therefore, the "corticosterone" value would be inflated by the presence of 11-deoxycortisol. This would be especially important in cases of pharmacological blockage of 11-hydroxylation with metyrapone[5], where levels of 11-deoxycortisol rise sharply.

Rudd and Black (11) showed that pregnanetriol and pregnanetriolone are present in the carbon tetrachloride phase. The blood levels of these two steroids are normally low, but they may be elevated in adrenal hyperplasia where the 21-hydroxylation is reduced. These two compounds would not likely be detected by competitive protein binding (10). Other steroids that would favor the carbon tetrachloride phase, judged from partition coefficient data (7), are testosterone, progesterone, and androstenedione. The normal levels of these steroids are 0.5 mcg./100 ml. for testosterone, 0.17 mcg./100 ml. for androstenedione, and less than 0.8 mcg./100 ml. for progesterone in males and less than 2 mcg./100 ml. in nonpregnant females (4, 12, 13). These levels are significant relative to corticosterone concentration. The two androgens would not interfere in a competitive protein-binding assay; however, progesterone would inflate the values of corticosterone estimated from the carbon tetrachloride phase (4). Recently, Kolanowski and Pizarro (14) published data showing that testosterone is strongly detected in a competitive protein-binding assay, so it may pose an interference problem.

The standard deviation of replicate determinations of cortisol in plasma samples where the concentration of cortisol was in the range 9–22 mcg./100 ml. was 2.65. This is comparable to precision of other assays reported in the literature (Table VI).

Normal morning concentrations of cortisol were found to be 11.71 mcg./100 ml. For corticosterone, using System 9, the morning concentration was found to be 4.96 mcg./100 ml.; using System 5, the level of corticosterone was determined as 1.84 mcg./100 ml. For cortisol, the value compares favorably with values from more specific double-isotope dilution methods and also values determined by fluorometric and competitive protein-binding methods (Table VI). The value for corticosterone using System 9 seems high compared to other reported values, although Van der Vies (33), using a a fluorometric method, reported a similar value. Both the present method and the Van der Vies method employed carbon tetrachloride/water partitioning to separate cortisol and corticosterone. Possibly some common interference affected both values. The high value of corticosterone detected with System 9 may be related to the better detection of corticosterone noted in the specificity study. Corticosterone may have greater affinity for the protein binding sites under these conditions. This is in keeping with the "polarity rule" (15). Two different sites might be involved in the binding of cortisol and corticosterone.

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Removal of Ferrous Ions by *N,N'*-Ethylenediaminetetraacetic Acid in Microbiological Assay of Pyridoxine

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Abstract □ The application of a general microbiological method for the determination of pyridoxine was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A modification was made to the method by using *N,N'*-ethylenediaminetetraacetic acid to sequester Fe^{+2} before acid hydrolysis of the preparations. By using this modification, 100% of the pyridoxine can be detected compared with 75 or 77% for 20:1 or 12:1 ratios of Fe^{+2}–pyridoxine, respectively.

Keyphrases □ Pyridoxine, multivitamin combinations—bioassay, ferrous-ion removal with *N,N'*-ethylenediaminetetraacetic acid □ Multivitamin combinations—pyridoxine bioassay □ *N,N'*-Ethylenediaminetetraacetic acid—ferrous-ion complexing, pyridoxine bioassay

In 1943, Atkin *et al.* (1) reported a microbiological method which employed a yeast, *Saccharomyces carlsbergensis*, to determine pyridoxine. Other studies (2–5) further supported this method. In natural products and pharmaceutical preparations, pyridoxine exists partly as the combined form which must be hydrolyzed prior to assay. The method was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A combination of Fe^{+2} with multivitamins is generally recommended for the treatment of iron-deficiency anemia (6). The interference of Fe^{+2} might be prevented by the addition of a compound such as *N,N'*-