

A One-Tube Micromethod for Radioimmunoassay of Plasma Cortisol

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A micro-scale method is presented for measurement of cortisol. Serum or plasma, 2 μ l, is diluted with buffer and the solution heated at 60 °C in the presence of high-affinity antibody and [³H]cortisol. Corticosteroid-binding globulin in the serum is denatured, releasing cortisol and making it available to the antibody, which is stable during the incubation. After cooling, ammonium sulfate solution is added, followed by a toluene-based scintillant that does not dissolve in the aqueous phase. The vial is shaken to extract the free cortisol into the scintillant. Antibody-bound cortisol remains in the aqueous phase and does not produce any scintillation. The amount of [³H]cortisol that is free at the end of the incubation, and which therefore gives rise to the measured counts, is related to the concentration of cortisol in the original serum, and may be calculated from a standard curve. The assay is sensitive and precise. Ranges are presented for normal and pathologic subjects.

Additional Keyphrases: *heat denaturation of cortisol-binding globulin · [³H]cortisol · solvent partition · liquid scintillation · normal values · pediatric chemistry*

Radioimmunoassay (RIA) methods for plasma cortisol differ principally in the means of releasing cortisol from its binding proteins, in the type of isotope used, and in the separation technique. Solvent extraction (1), acid pH (2), enzyme treatment (3), or heating (4, 5) have all been used to release cortisol from its binding proteins, principally corticosteroid-binding globulin (CBG) and albumin. Heating is a simple procedure, and if the cortisol antibody is stable under the conditions required to denature CBG, then release of cortisol and antibody incubation may be undertaken concurrently. Steroids labeled with γ -emitters—⁷⁵Se (6) or ¹²⁵I (7)—may be counted more readily than those labeled with ³H, but they are less stable. The substitution on cortisol of an ¹²⁵I-containing side chain may produce a steroid that has binding properties that are quite different from cortisol, and this fact may sometimes be used to advantage (8). Tritiated cortisol derivatives not only are more stable but also, because liquid scintillation is necessary for their detection and quantitation, it is possible to use solvent partition to separate bound and free steroid and thereby avoid the misclassification errors introduced by variable decanting of supernatant fluid from pellet or adsorbent.

Here, we present an assay in which the above factors are used to advantage. Serum is first placed directly into a scintillation "mini" vial, which also serves as incubation tube. Addition of further reagents, incubation, and solvent partition follow, after which the vial is counted in a liquid scintillation counter. This simplified protocol eliminates the errors and delay caused by transfers and decanting of incubation mixture and results in an efficient and precise microassay.

Materials and Methods

Reagents

Tris/HCl buffer, 50 mmol/liter, pH 7.0, containing albumin and γ -globulin. Add about 800 ml of water to 6 g of Trizma base (Sigma cat. no. T-1503). Adjust the pH_{20 °C} to 7.0 with 0.5 mol/liter HCl, and the volume to 1 liter. Add 1 g of bovine serum albumin (Sigma cat. no. A-4378) and 1 g of bovine serum globulin fraction II (Sigma cat. no. BG-II). When all have dissolved, mix briefly before storing. Stored frozen in 40- to 50-ml amounts in polyethylene bottles, the mixture is stable for at least six months.

Radioactive cortisol. [1,2,6,7-³H]Cortisol, approximately 100 kCi/mol (Amersham cat. no. TRK 407), is used. Dilute the contents of the ampule with ethanol to give a solution 25 μ Ci/ml (250 nmol/liter). Stored at 4 °C, this is stable for at least three months.

Standard cortisol. Dissolve 20.0 mg of cortisol (hydrocortisone; Sigma cat. no. H-4001) in 100 ml of ethanol (it dissolves slowly). Dilute 10 ml of this solution to 100 ml of ethanol (i.e., 20 mg/liter). This solution may be stored at 4 °C for up to one year. Dilute 2 ml of this solution to 100 ml with buffer (i.e., 400 μ g/liter). Serially dilute this solution with buffer to give 200, 100, 50, 25, and 0 μ g/liter. Mix equal volumes of the 400 and 200 μ g/liter standards to give 300 μ g/liter. Store the standards in 0.2- to 0.4-ml aliquots at -20 °C. They are stable for at least six months.

Cortisol antibody Type M 14417276. The preparation and characterization of this antiserum, raised against cortisol-21-monosuccinate-bovine serum albumin conjugate, has been described previously (5). The antiserum is diluted 20-fold in the buffer and 200- μ l aliquots are transferred to polyethylene microtubes and stored at -20 °C until required. They are stable for at least six months.

Antibody/[³H]cortisol mixture. Prepare freshly on the day the mixture is required. For up to 30 sera (86 vials), place 100 μ l of [³H]cortisol (25 μ Ci/ml concentration) in a suitable disposable glass container—e.g., a 20-ml glass scintillation vial. Carefully evaporate the alcohol under air or nitrogen. Add 10 ml of buffer to the residue and mix. Transfer the contents of a tube of dilute antibody (200 μ l) to the solution with a Pasteur pipette and rinse out the microtube several times with a little of the buffer/[³H]cortisol mixture. Mix and allow to equilibrate for longer than 15 min before use.

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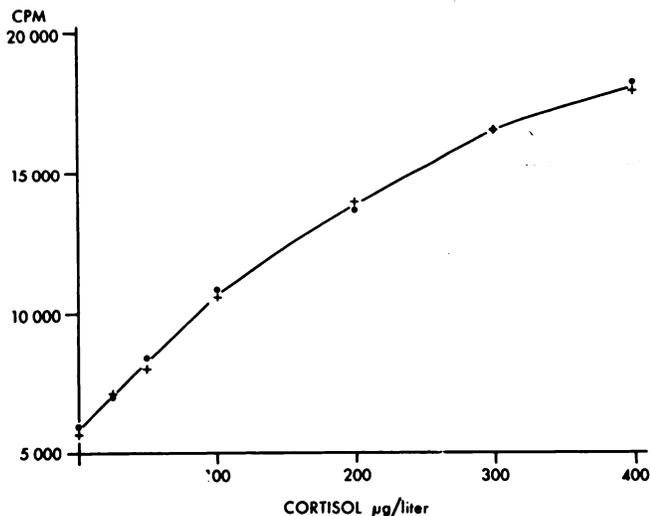


Fig. 1. Typical standard curve obtained during a routine assay
Total counts, 24 900 cpm

Ammonium sulfate solution (saturated). This contains more than 540 g/liter of solution (ample time for saturation should be allowed; the solution should *not* be prepared immediately before use). Filter the stock solution through a coarse filter into a suitable 0.5-ml pipettor (e.g., Oxford Model R). It is stable indefinitely at room temperature, but avoid storage in a part of the laboratory subject to wide temperature variation.

Scintillant. Dissolve 5 g of PPO (2,5-diphenyloxazole; Sigma cat. no. D-4630) and 0.5 g of dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]benzene; Sigma cat. no. P-3879) in 1 liter of scintillation grade toluene.

Scintillation vials. Glass "vialettes," 7-ml capacity (Amersham cat. no. 196280). Polypropylene mini-vials of the same capacity may be used, but they are opaque, preventing viewing of the reaction contents, they are less efficient conductors of heat, and they are more prone to float out of the rack during incubation.

Procedure

Prepare antibody/[³H]cortisol mixture as above. Using a suitable diluter, such as that of Micromedic Systems Inc., dilute standard or sample 10-fold with buffer and add 20 µl into each of two 7-ml glass vialettes. (Because of the small sample size, it is important to remove all air bubbles from the syringes before starting, to be very careful about wiping the sample probe tip, to avoid the introduction of any air bubbles during sampling, and to beware of very small clots, which can easily block the sample probe.) Add 100 µl of antibody/[³H]-cortisol mixture with use of (e.g.) a Hamilton repeating dispenser, mix gently, and transfer the vialette to a suitable rack. To two vialettes labeled "total counts," add 100 µl of [³H]-cortisol/antibody mixture and 200 µl of 0.5 mol/liter HCl to disrupt antibody binding. Place all vialettes in a water bath, set at 60 ± 0.5 °C and containing sufficient water to go 1.5 to 2 cm up the side of the vial. Do not cover the bath. Leave them for 30 ± 2 min, then remove them from the bath and allow to cool to room temperature (5–10 min). Add 500 µl of saturated ammonium sulfate solution to each vial, and mix gently. Add 4 ml of scintillant with (e.g.) an Oxford pipettor, Model SA. Cap the vials tightly and transfer them to vial holders (Amersham, cat. no. 196301) in a rack. Shake the rack vigorously, manually or mechanically, with the vials horizontal, for 2 min. Transfer them to a refrigerated scintillation counter, and allow to equilibrate for 30–60 min before starting counting. Count the radioactivity for 1 min, using a ³H-setting without external

Table 1. Precision of the Method

	Manu- facturer's stated value	n	Mean µg/liter	SD	CV, %
<i>Within batch</i>					
Serum A	—	10	20.70	5.76	27.8
Serum B	—	10	79.20	5.87	7.4
Serum C	—	10	247.80	7.50	3.0
<i>Between batch</i>					
Serum pool	—	48	189.02	8.93	4.7
Lederle RIA Control 1, Lot 2945-405	124.6 ^a	45	119.78	8.06	6.7
Hyland Q Pak					
Radioreceptor controls, Lot 3272NOOIA)					
Control A	100.9 ^b	25	103.10	7.06	6.9
Control B	233.2 ^b	25	238.50	7.98	3.3

^a Mean value calculated from the results reported for nine kits.

^b Mean value calculated from the results reported for eight of the nine kits.

standardization. Draw a standard curve of counts/min vs. cortisol concentration of the standards. Read off the values of the unknown sera from this (Figure 1).

Results

Incubation Conditions

These are similar to those of Morris (5), but the antibody concentration has been adjusted so as to retain adequate sensitivity over the range of serum cortisol concentration 0–400 µg/liter at the higher incubation temperature. We studied the effect of duration of incubation at 60 °C on release of cortisol from serum and on stability of the antibody by preparing standard curves and analyzing nine serum samples with incubations of 5, 10, 20, 30, 60, and 90 min, followed in each case by a 10–15 min cooling period at room temperature before ammonium sulfate was added. Release of cortisol was incomplete at 5 min, but the cortisol measured in the various sera changed little as the duration of incubation at 60 °C was increased from 10 to 90 min. There was no evidence of antibody inactivation over that period.

The potential interference of serum in the assay was investigated by omitting antibody from the assay system. The effects of 2, 10, and 20 µl of each of four sera were investigated with incubation at room temperature for 30 min or for 10 or 30 min at 60 °C. The mean counts, expressed as per cent of counts in the absence of serum were 62.8, 38.0, and 28.7% in the presence of 2, 10, or 20 µl of serum after incubation at room temperature (CBG not denatured). Corresponding figures after a 10-min incubation at 60 °C were 98.7, 89.5, and 81.1% and those after 30-min at 60 °C were 98.0, 90.8, and 84.1%. Similar results to those obtained at 60 °C after a 30-min incubation were obtained when serum was heated for as long as 60 min at 60 °C or at 70 °C for 10 to 45 min. We conclude that when 2 µl of serum is used in the assay, residual binding is negligible after incubation at 60 °C for 30 min, and these conditions were selected for the recommended procedure.

Separation of Bound and Free [³H]Cortisol

We found it necessary, because of the geometry of our scintillation counter, to increase the volume of scintillant from the 2 ml recommended by Morris (5) to 4 ml, to avoid the er-

rors incurred when the smaller volume was used. The amount of saturated ammonium sulfate added to 0.3 ml of incubation mixture was varied between 0.2 and 0.8 ml. Sensitivity was improved when we used 0.5 ml (final ammonium sulfate concentration, 2.56 mol/liter).

Standard curves were run and assays performed with use of either sodium chloride at final concentrations of 1.22–3.06 mol/liter or polyethylene glycol (av M_r 6000 to 7500) at final concentrations of 62.5 to 156 g/liter in place of the ammonium sulfate solution. These standard curves were much less sensitive, and the measured counts at 2 °C drifted much more during 25 h than those of the ammonium sulfate system.

Precision

Table 1 shows the within- and between-batch precision.

Analytical Recovery

Cortisol standards in alcohol were evaporated in glass test tubes and pooled serum from hypopituitary patients was added to the residue. After 2 h of incubation at room temperature, the original and supplemented samples were analyzed. Recoveries ($n = 10$) were 95% on addition of 100 μg of cortisol per liter of serum and 87% when 200 μg /liter was added.

Comparison of Results by the Present Method with Those of Other Laboratories

During the 12-month period that the method has been used routinely, nine samples have been analyzed as part of the College of American Pathologists Quality Control Scheme. Results obtained by the present method were on average 0.225 standard deviation intervals [SDI = (our value – consensus mean)/consensus SD] higher than mean values reported for the 70 laboratories using RIA's with [^3H]steroid tracer (range, –0.380 SDI to 0.663 SDI), while our results were on average 0.632 SDI lower than those obtained by 450 laboratories using [^{125}I]steroid tracer (range, –0.226 to –1.293).

As shown in Table 1, control sera analyzed by the present method with each routine batch of analyses gave mean results close to the mean reported by the manufacturer.

Correlation with Existing Methods

Thirty-two samples were analyzed by the present method and by the competitive protein binding assay of Murphy (9) with use of horse serum. (This assay was imprecise in our hands.) A correlation coefficient of 0.961 was obtained, with a regression equation: result by present assay = 0.967 (result by method of Murphy) + 12.6 μg /liter. We assayed 37 sera by the present method and that of Morris (5). The correlation coefficient was 0.961 and the regression equation: result by present method = 1.06 (result by method of Morris) – 7.1.

Time Dependence of the Counting

On three occasions, we prepared standard curves and assayed six sera by the recommended procedure. After preparation, the scintillation vials were placed in a scintillation counter thermostated to 2, 9, or 20 °C. After a 1-h equilibration, the vials were each counted for 1 min, then the counting of the whole batch was repeated several times during the next 14 h. The cortisol concentrations of the sera were calculated from the appropriate standard curves drawn after various times and temperatures of incubation within the counter. The values obtained for patients' samples were independent of the time or temperature at which measurement was made. However, because dissociation of the [^3H]cortisol–antibody complex was much more marked at room temperature than at 2 °C, the standard curves became progressively much less

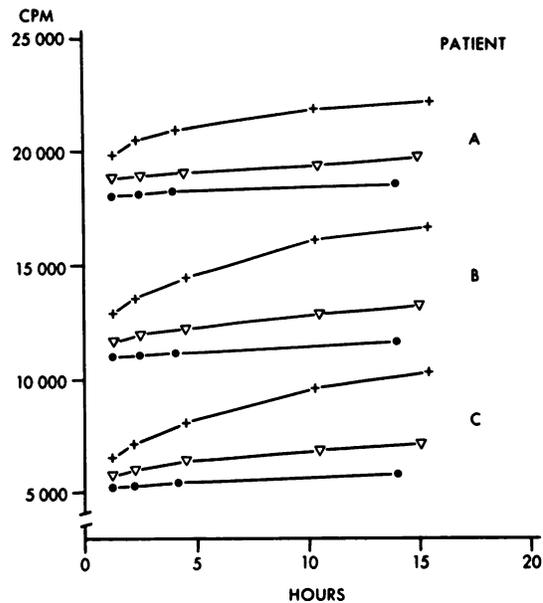


Fig. 2. Stability of the measured counts at various temperatures

Results are shown for vials prepared from three patients' samples (A, B, and C) with the scintillation counter maintained at: ●, 2 °C; ▽, 9 °C; and +, 20 °C

sensitive at room temperature. Figure 2 shows the effect of time and temperature on the measured counts obtained from three patients' samples.

Cortisol in Serum of Normal and Pathologic Subjects

Cortisol (capillary blood) was measured in specimens collected at 0800 to 0900 h from 33 healthy laboratory staff (11 men, 22 women), ages 18 to 60 years, none of whom was taking oral contraceptives. The mean cortisol value was 130.8 μg /liter (SD, 31.4 μg /liter) with an observed range of 58 to 220 μg /liter. A cumulative probability plot gave values of 70 and 190 μg /liter at the 2.5 and 97.5th percentiles.

Results for serum or plasma samples submitted from hospitalized patients for cortisol determinations were examined, together with the patients' records. Results for patients who were being treated with steroids or who had hypopituitarism, adrenal disease, brain tumors, hydrocephalus, or hypertension were eliminated. The mean cortisol values (μg /liter) for the remaining 47 patients (ages 1 to 17 years, mean 10.3 years) were 168 (SD = 55) at 0800 to 0900 h (95% of results were in the range 69 to 275) and 51 (SD = 30) for samples collected at 2000 h (95% of results were in the range less than 10 to 110). The cortisol in the specimen collected at 2000 h was less than 50% of that of the 0800-h specimen in 88% of cases. Only one person, a 13-year-old girl, has presented with Cushing's disease in the past year. Pre-treatment cortisol values for her ranged between 190 and 295 μg /liter, with notable absence of diurnal variation. Of the eight patients with hypopituitarism, all had values at 0800 h of less than 32 μg /liter.

Discussion

We have presented a micro-scale radioimmunoassay for determination of plasma cortisol. Because the antibody has good specificity and affinity and is stable at 60 °C, serum may be assayed directly without prior extraction, by using heat to denature its CBG. Other workers have previously advocated this technique (4, 5, 10). The likelihood of the steroid being nonspecifically adsorbed onto the walls of the incubation vial or on pipette tips is greatly decreased in the present method,

because the CBG is denatured in the presence of antibody in the vial in which it is to be counted. Because the initial dilution of the serum is made into a vial and the incubation and the final extraction into scintillant take place in the same vial, and because transfers of reagents or decanting procedures are not required, the assay is labor saving and has good precision.

The technique of separating bound and free steroid by extracting the free steroid into a scintillant was first used by Castanier and Scholler (11) in the determination of estrone and estradiol. These authors flash froze the aqueous phase and decanted the toluene layer for scintillation counting. It was necessary to cool the reaction mixture and toluene to 4 °C to reduce the rate of dissociation of bound estradiol and to repeat the extraction with scintillant to ensure completeness. Refrigeration of incubation mixture and addition of cold scintillant was also necessary in the aldosterone assay of Jowett et al. (12), and the cortisol method of Keane et al. (13). The addition of ammonium sulfate solution to the aqueous phase before addition of scintillant, as recommended by Edwards (14) and subsequently used by Morris (5), helps stabilize the antibody-steroid complex so that no refrigeration is required during handling before scintillation counting. The precise role of ammonium sulfate is not understood; in addition to its role in the stabilization of the antibody-steroid complex, it also helps salt out the free steroid into the organic phase. It precipitates the proteins in the buffer, but does not denature the antibody, because standard curves are unaffected if ammonium sulfate is added to incubation mixtures containing standards before antibody-³H]cortisol mixture. (This protocol is not recommended routinely because precision is adversely affected.) The ammonium sulfate could not be replaced satisfactorily by sodium chloride or polyethylene glycol.

As shown in Figure 2, the measured counts vary considerably over a period of time as antibody-bound ³H]cortisol slowly dissociates and is trapped by the scintillant. The effects of this potential source of interference may be minimized by placing standards at the beginning and end of a batch and (or) by reversing the order of the second set of sample duplicates. Dissociation may be minimized by using a refrigerated scintillation counter at 2–4 °C and keeping counting times to 1 min. In practice, drift is not a major problem, and batches of 60–80 vials have been handled routinely.

During the past year the method has produced clinically meaningful results. The normal range obtained for adults is similar to that observed by others (4, 5, 7). Our correlation with a competitive binding technique is good, like that of Demers and Derk (15) and Jiang et al. (16), and the large differences between competitive protein binding and RIA methods reported by Farmer and Pierce (17) were not observed, possibly because these authors used dog serum as source of CBG, a less specific source than the horse serum we used.

The principles of the assay presented should prove useful to laboratories that are dissatisfied with their existing cortisol

methods and that require a micro capability for samples from pediatric or geriatric populations.

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