Polarization Fluoroimmunoassay of 11-Deoxycortisol in Serum and Saliva

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We developed polarization fluoroimmunoassays for 11-deoxycortisol in serum and saliva. To avoid interfering factors, the steroid was initially extracted from the biological fluids with dichloromethane. Assays could then be completed without any further separation procedures or need to correct for blank signals. The serum assay was suitable for following the response to the metyrapone test and results correlated acceptably with those by an established, specific, direct 125I-radiiodimmunoassay. The method is not sufficiently sensitive to detect 11-deoxycortisol in normal saliva, but greatly increased concentrations were found in post-metyrapone saliva and results agreed well with those by the radioimmunoassay as modified for salivary assay. Salivary 11-deoxycortisol assay would provide a convenient means of monitoring results of the metyrapone test.

Additional Keyphrases: metyrapone test · steroids

Response to administration of metyrapone, which blocks the conversion of 11-deoxycortisol to cortisol, is a useful indicator of pituitary–adrenal reserve (1). Relatively nonspecific colorimetry of urinary 17-oxo steroids (2) has commonly been used to monitor the test, but more recently radioimmunoassay (RIA) or "high-performance" liquid-chromatographic methods have been applied to determine 11-deoxycortisol in serum or plasma (3–6). Although test protocols vary, a normal response typically sees circulating 11-deoxycortisol increase from <50 nmol/L to values in the region of 250 to 750 nmol/L (3–6).

Measurement of steroids in saliva has many potential advantages (2, 7, 8). Salivary values generally reflect those of the unbound steroid fraction in the circulation, which is believed to relate directly to physiological effects and may show a more marked response than the total steroid concentration in dynamic function tests (6). Specimen collection is easy and noninvasive, which facilitates studies on children (8) and tests requiring serial sampling (2).

We developed and validated polarization fluoroimmunoassay (PIA) methods for 11-deoxycortisol in serum and saliva and compared results for metyrapone test specimens with those obtained by a previously described direct 125I-RIA (4).

Materials and Methods

Reagents

11-Deoxycortisol and other steroids were obtained from Sigma, Poole, Dorset, U.K.; metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propanone] from Ciba, Horsham, West Sussex, U.K.; dichloromethane ("Analar" grade, used without further purification) and gelatin from BDH Chemicals, Poole, Dorset, U.K.; activated charcoal from Hopkin and Williams, Chadwell Heath, Essex, U.K.; pooled normal human serum from ILS, Newbury St., London EC1, U.K.; and 11-deoxycortisol-[2-3H] from New England Nuclear, Dreieich, F.R.G.

Glass centrifuge tubes, 110 × 17 mm, were obtained from Corning, Stone, Staffordshire, U.K.; glass test tubes, 76 × 16 mm, from Hoslab, Ilford, Essex, U.K.; and polystyrene test tubes type LP3, 63.5 × 9.5 mm, from Luckham, Burgess Hill, Sussex, U.K.

Fluorescein-labeled 11-deoxycortisol. Following procedures previously described for cortisol (9), we prepared 11-deoxycortisol-3-(O-carboxymethyl)oxime and conjugated it to fluoresceino-carbamyl ethylenediamine. The product was purified by thin-layer chromatography (Rf = 0.75), and its concentration estimated spectrophotometrically, as before (9).

Anti-11-deoxycortisol serum. We immunized three mature Border–Leicester cross ewes with 11-deoxycortisol-3-(O-carboxymethyl)oxime conjugated to bovine albumin (4) and antiserum from the animal with the highest-titer response.

Serum standards. Prepare sterile-human serum by charcoal stripping. Mix pooled normal human serum with activated charcoal (100 g/L) by gentle rotation for 18 h at 37 °C, then centrifuge for 45 min at 4 °C (10 000 rpm). Remove charcoal fines from the supernate by pressure filtration (we used equipment from Sartorius, Belmont, Surrey, U.K.) through membranes with successively smaller nominal pore sizes from 2 to 0.22 µm. The product contained no detectable 11-deoxycortisol by RIA (4).

Prepare standards by adding 11-deoxycortisol dissolved in methanol (1 mmol/L) to steroid-free human serum.

Procedures

Polarization fluorometer. We used a Model 4000 polarization fluorometer (SLM Instruments, Urbana, IL 61801) as described in detail elsewhere (10).

Specimen collection. We collected unstimulated whole mixed saliva as described previously (8). Salivary specimens were frozen (−20 °C) until required, then thawed, centrifuged (10 min, 2000 rpm) to sediment any debris or aggregated material, and the supernates taken for assay.

PIA diluent buffer. Prepare all working assay reagents in sodium phosphate buffer (100 mmol/L, pH 8.0) containing, per liter, 1 g of gelatin and 1 g of sodium azide.

PIA of serum 11-deoxycortisol. Pipet 500 µL of serum specimens or standards into glass centrifuge tubes and vortex-mix thoroughly with 4.5 mL of dichloromethane for 2 min. Allow the tubes to stand for 60 min, then centrifuge (10 min, 2000 rpm). Transfer 3-mL aliquots of the organic (lower) layers to glass test tubes, evaporate the solvent under reduced pressure (Searle Vortex Evaporator; Buchler Instruments, Fort Lee, NJ 07024), and reconstitute the residues in 300 µL of diluent buffer. Pipet the reconstituted serum extracts (100 µL, in duplicate) into polystyrene test tubes and add 100 µL of a 50 mmol/L solution of fluorescein-labeled 11-deoxycortisol, followed by 100 µL of a 250-fold dilution of antiserum. Incubate for 30 min at room tempera-

Received Mar. 28, 1983; accepted June 7, 1983.
ture, add 1.2 mL of diluent buffer, and measure the fluorescence polarization.

**PIA of salivary 11-deoxycortisol.** Pipet 1.5 mL of salivary specimens or standards prepared in diluent buffer into glass centrifuge tubes and extract with 6.5 mL of dichloromethane as above. Evaporate 5.2-mL aliquots of the organic layers after centrifugation and reconstitute the residues in 1.2 mL of diluent buffer. Pipet the reconstituted salivary or buffer extracts (500 μL in duplicate) into polystyrene test tubes and add 100 μL of 50 nmol/L fluorescein-labeled 11-deoxycortisol, followed by 100 μL of 500-fold diluted antiserum. Incubate this mixture for 30 min at room temperature, add 800 μL of diluent buffer, and measure the fluorescence polarization.

**RIA of serum 11-deoxycortisol.** We performed 125I-RIA using an established method (4) with 125I-histamine-labeled 11-deoxycortisol-3-(O-carboxymethyl)oxime and a rabbit antiserum. Direct (nonextraction) assay was possible because of the high specificity of the antiserum and the use throughout of a pH 4 buffer to obviate serum protein matrix effects.

**RIA of salivary 11-deoxycortisol.** We modified the serum RIA method for direct assay of salivary 11-deoxycortisol. To 100 μL of salivary specimen or standard prepared in buffer add 100 μL of a 33 pmol/L solution of 125I-labeled 11-deoxycortisol, followed by 100 μL of antiserum (diluted 25 000-fold). Incubate for 90 min at room temperature, add 500 μL of dextran-coated charcoal suspension (4), centrifuge, aspirate the supernates, and count the radioactivity in the charcoal pellets for 150 s.

**Results**

**Assay optimization.** We equilibrated a trace amount of tritiated steroid with specimens of human serum or saliva, or diluent buffer, for at least 4 h at room temperature. Analytical recovery after dichloromethane extraction from serum was 93 ± 8% (mean ± SD) and from either saliva or buffer 98 ± 5%. The serum 11-deoxycortisol PIA was accordingly calibrated with standards prepared in steroid-free human serum and the salivary assay with standards prepared in diluent buffer.

Although binding of tracer by antiserum was complete within 5 min in both assay systems, we used an incubation time of 30 min for routine convenience.

**PIA of serum 11-deoxycortisol.** Figure 1 shows a typical standard curve. From the SD of the polarization signal for 20 replicates of the zero-concentration standard the minimal detectable concentration at the 95% confidence level was estimated (11) as 3.6 nmol of 11-deoxycortisol per liter of serum.

We assessed precision by measuring each of three pools of selected sera 10 times in one assay, giving mean results of 15, 135, and 625 nmol of 11-deoxycortisol per liter, with within-assay CVs of 8.5, 9.2, and 10.2%, respectively. Measurement of the same pools on each of 10 different days gave between-assay CVs of 10.0, 9.8, and 11.0%, respectively.

11-Deoxycortisol was added at final concentrations of 45, 75, 90, 120, and 190 nmol/L to a serum pool that contained 10 nmol of endogenous steroid per liter. Mean analytical recovery of the added steroid by PIA was 100% (SD 2%).

Cross reactivities (12) of other steroids in the PIA were: for cortisol 0.5%, 17α-hydroxyprogesterone 7%, progesterone 2%, 11-deoxycorticosterone 7%, corticosterone 0.5%, and prednisolone 0.6%. Cortisone, dehydroepiandrosterone, aldosterone, metyrapone, and dexamethasone all showed less than 0.01% cross reaction.

Normal serum specimens, and sera from normal subjects or patients given metyrapone, were assayed by PIA and by RIA. For 68 specimens that had RIA results between 9 and 705 nmol/L, the values by PIA (γ) were related to those by RIA (x) by the least-squares regression equation y = x − 15, with a correlation coefficient (r) of 0.97.

**PIA of salivary 11-deoxycortisol.** We made the salivary assay about fourfold more sensitive, relative to the serum PIA, by halving the amount of antiserum and taking a greater volume of the biological specimen. Even so, we could not detect 11-deoxycortisol in normal saliva.

Two post-metyrapone salivary specimens measured 16 times each in one assay gave mean results for 11-deoxycortisol of 7 and 15 nmol/L, with within-assay CVs of 6.7 and 7.1%, respectively. The same specimens gave between-assay CVs of 9.2 and 9.8%, respectively, for assays performed on 10 different days.

11-Deoxycortisol was added at final concentrations of 35 or 50 nmol/L to four saliva specimens that had endogenous steroid concentrations between 3 and 20 nmol/L. Mean analytical recovery of the added steroid by PIA was 101% (SD 4%).

For 17 post-metyrapone saliva specimens from normal subjects or patients whose RIA results were between 18 and 172 nmol/L, PIA (γ) and RIA (x) values were related by y = 1.03x − 1, with r = 0.998.

**Discussion**

PIA is one of the simplest immunoassay techniques (13); and instrumentation for the precise and convenient measurement of fluorescence polarization is now being introduced (10, 14–16). Fluorescent and other components of biological fluids may potentially interfere in PIA (13), however, and because of the comparatively low concentrations of 11-deoxycortisol in serum and saliva we extracted the steroid before assay. The PIAs could then be completed without any further separation procedures or need to measure and correct for individual sample blank signals (17, 18).

Our antiserum had specificity similar to that used in the direct RIA (4) and there was no need for chromatographic separation of extracted steroids as in a recently reported solid-phase fluorimunoassay for serum 11-deoxycortisol (19).

The serum PIA covered a working range suitable for monitoring of the metyrapone test. Sensitivity was just sufficient to cover the normal range (3, 4) as well. Precision was adequate for the proposed application, in which gross changes in steroid concentrations are followed. PIA results
for normal and post-metyrapone sera correlated acceptably with those by the established RIA.

Because binding of 11-deoxycortisol by plasma protein is similar to that of cortisol (20), normal concentrations of it in saliva are expected to be much lower than those in serum, as is true for cortisol (7, 8). We were unable to detect 11-deoxycortisol in normal saliva by PIA, indicating that concentrations are 1 nmol/L or less. The precision of the salivary 11-deoxycortisol PIA was noticeably superior to that of the serum assay; this may be attributable to the greater ease of extraction of the steroid from saliva with its much lower protein content. Although a relatively large sample volume was required, most subjects, including children (8), have no difficulty in providing adequate amounts of saliva. 11-Deoxycortisol concentrations were greatly increased after metyrapone administration, and results by the PIA correlated well with those by the modified RIA procedure. These studies suggest that salivary 11-deoxycortisol assay would provide a convenient means of monitoring the metyrapone test.

A. A. K. Al-Ansari gratefully acknowledges support by the Ministry of Health, Kuwait.

References