Adrenal Status Assessed by Direct Radioimmunoassay of Cortisol in Whole Saliva or Parotid Saliva

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We describe a direct radioimmunoassay for cortisol in 10-μl volumes of parotid saliva or whole saliva. Binding proteins are absent from these fluids, as demonstrated by the excellent correlation between results for samples assayed directly and by a comparison procedure involving extraction with 1,2-dichloroethane. The direct assay is specific, precise, and had a lower limit of sensitivity of 4 pg per assay tube. Comparison of cortisol concentrations in plasma, parotid saliva, and whole saliva in persons undergoing investigations for assessing adrenal function, including stimulation with cosyntropin (Synacthen) and suppression with dexamethasone, indicated that changes in plasma cortisol concentration were accurately and immediately reflected in saliva from either the parotid-gland or whole saliva. A marked circadian rhythm has also been demonstrated for cortisol in parotid-gland saliva and whole saliva. We had to modify the 1,2-dichloroethane extraction procedure for accurate determination of cortisol in parotid saliva and whole saliva of patients undergoing treatment with metyrapone.

Additional Keyphrases: steroids • normal values • effects of stimulation and suppression • circadian rhythm • elimination of interference by 11-deoxycortisol • assessing adrenal function • screening

Assessment of adrenal status by measuring cortisol in either whole saliva or that from the parotid gland only (parotid fluid) is an attractive alternative to assay of cortisol in plasma and urine. Such determinations in urine are complicated by collection difficulties, and the stress associated with the repeated venepuncture now required in stimulation and suppression tests may invalidate studies in which plasma is used. Collection of saliva and parotid fluid by non-invasive stress-free procedures obviates many of these problems.

Recent reports (1, 2) on concentrations of biologically active compounds in plasma and parotid fluid suggest that cortisol concentrations in saliva and parotid fluid reflect only the "free" (i.e., non-protein-bound) steroid in the blood-stream. Because cortisol in plasma is largely bound to transcortin, its concentration in saliva and parotid fluid is relatively low, and accurate determination of these low concentrations requires the sensitivity and specificity provided by radioimmunoassay (RIA) techniques. Early studies (3–5) in which the nonspecific Porter–Silber reaction was used to determine 17-hydroxycorticosteroids gave little indication of the potential value of parotid fluid assays for assessing adrenal function.

Parotid fluid reportedly contains no corticosteroid binding proteins (5), and this it would appear to be ideal for the direct clinical determination of cortisol without the need for tedious extraction.

This communication describes the development of a direct radioimmunoassay for cortisol in small volumes (10 μl) of saliva and parotid fluid. The direct assay was validated by comparing results with an RIA involving extraction with an organic solvent and thin-layer chromatographic purification before assay.

We investigated the possibility of using cortisol values in parotid fluid or saliva for assessing adrenal function by collecting matched specimens of plasma, parotid fluid, and whole saliva from apparently normal volunteers and from hospitalized patients undergoing commonly used tests of adrenal function.

The cortisol antiserum used in this study had a 10% cross reaction with 11-deoxycorticol. In most circumstances this was negligible. However when metyrapone, known to inhibit steroid 11b-monooxygenase (EC 11.14.15.4) activity, was administered, circulating 11-deoxycorticol increased significantly. Interference by this steroid in the assay system was eliminated by extracting the parotid fluid or saliva with petroleum ether/benzene (65/35 by vol).

Materials and Methods

Subjects

Seven normal, apparently healthy volunteers (five men and two women) provided matched saliva, parotid fluid, and plasma samples for our investigation of circadian rhythms, adrenal stimulation by cosyntropin (Synacthen), and suppression by dexamethasone. We investigated the effect of increased concentrations of circulating transcortin on cortisol concentrations in salivary secretions in three normal women who had been taking an estrogen-containing oral contraceptive for at least three months.

Samples from subjects with disturbances of the hypothalamic–pituitary–adrenal axis were kindly provided by Professor M. E. Besser, St. Bartholomews Hospital, London.

Collection of Samples

Previous experience indicated that painful venepuncture could increase cortisol concentrations in saliva and parotid fluid; therefore we always collected saliva samples before venepuncture. Subjects providing saliva rinsed their mouths with water and after 15 minutes spat directly into a glass tube. Parotid fluid was collected by fitting a Curby cup (6) over the duct of the parotid gland. Parotid secretion was stimulated by placing drops of a citric acid syrup on the tongue, so as to maintain a flow rate of about 0.5 ml/min. Blood was sampled by venepuncture into tubes containing lithium heparin anticoagulant. All samples were stored at −20 °C until assayed.
Solvents and Reagents

Cortisol, bovine serum albumin, activated-charcoal no. C-5260, and gelatin (B.P.) were from Sigma Ltd., London. [2,4,6,7,-³H]Cortisol (specific activity 82 kCi/mol) was from the Radiochemical Centre, Amersham, Bucks., U.K.

Common solvents and reagents (Analar grade) were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K., except for methanol and ethanol, which were supplied by James Burroughs Ltd., London, and petroleum ether (30–60 °C) from Eastman Kodak, Rochester, N.Y. 14650. The latter was purified by washing first with concentrated sulfuric acid and then with distilled water, then dried over calcium chloride and refractionated.

All scintillation-grade reagents were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. The ligand scintillant was prepared by dissolving 5 g of 2,5-diphenyloxazole in a mixture of 500 ml of the surfactant Triton X-100 and 1 litre of toluene.

For thin-layer chromatography we used precoated silica gel 60 F254 plates (0.25 mm layer) and benzene (spectroscopic grade) from E. Merck, Darmstadt, W. Germany.

Cosyntropin (Synacthen, 0.25 mg/ml; tetracosactrin BP 1973, tetracosactrin I.N.N.) was purchased from Ciba Labs., Horsham, Sussex, U.K.

Dexamethasone, 0.5 mg, was from Organon Labs., Ltd., Morden, Surrey, U.K.

Citric acid syrup was prepared by the hospital pharmacy to contain citric acid (350 g), simple syrup (500 ml), quillia tincture (10 ml), Lemon Spirit (5 ml), concd chlorine water (25 ml), and Comp. Tartrazine, made up to 1 litre with water.

Rabbit antiserum to cortisol. This was raised against cortisol-3-((O-carboxymethyl)-oxime–bovine serum albumin as described by Fahmy et al. (7). We suitably diluted and stored 500-μl portions at −20 °C. Just before use, the antiserum was diluted in the appropriate volume of buffer (pH 7.4 or pH 4) to give 2500, 10 000-, and 7000-fold dilutions for use in the reference procedure and direct assay at pH 7.4 and pH 4.0, respectively.

Cortisol solutions. A stock standard solution (1 mg of cortisol per 10 ml of ethanol) was stored at 4 °C. Cortisol working standards were prepared by diluting the stock standard (100 μl) to 10 ml with either ethanol or the appropriate assay buffer. Further dilution of these working standards provided the working standards used to prepare the dose–response curve (12.5, 25, 50, 75, 100, 125, 175, and 250 pg/10 μl). Tritiated cortisol was stored in 10 ml of benzene/ethanol (9/1 by vol) at 4 °C. Aliquots were dried under nitrogen and redissolved in the appropriate volume of buffer (pH 7.4 or pH 4.0) to give solutions containing 54 500 dpm (reference procedure), 12 100 dpm direct assay (pH 7.4), and 16 700 dpm direct assay (pH 4.0) in 100 μl, giving a mass of 110, 24, and 33 pg per assay tube, respectively.

Assay reagents. In the comparison RIA we used reagents as previously described (8) for a testosterone assay: phosphate-buffered saline (pH 7.4); an assay buffer (pH 7.4) consisting of phosphate-buffered saline containing 1 g of gelatin per liter; dextran-coated charcoal, a homogenized suspension of dextran (0.25 g/litre) and charcoal (2.5 g/litre); and phosphate–citrate buffer (pH 4; Documenta Geigy Scientific Tables, 7th ed., p. 281).

Assay Procedures

Cortisol in plasma. Cortisol in plasma was determined by the method of Fahmy et al. (7).

Cortisol in saliva and parotid fluid (comparison procedure). Add 200-μl portions of saliva or parotid fluid to labeled 12 × 100 mm glass-stoppered assay tubes. Add 3 ml of 1,2-dichloroethane and vortex-mix for 10 min. Centrifuge for 10 min at 2500 rpm and 4 °C. Aspirate the extracted saliva and transfer duplicate 1-ml aliquots of the extract to labeled 75 × 12 mm glass tubes. Add 10-μl aliquots of ethanolic standard solutions to labeled tubes and then 1 ml of 1,2-dichloroethane. Evaporate the contents of all tubes under nitrogen at 30 °C, add 100 μl of antiserum, and allow the tables to stand for 30 min at room temperature. Add 100 μl of cortisol tracer to all tubes and incubate at 30 °C for 1 h. Transfer all tubes to an ice bath for 10 min and add 0.5 ml of ice-cold dextran-coated charcoal suspension to all tubes. Mix briefly, allow to stand for a further 17 min in ice, and centrifuge at 4 °C for 10 min. Transfer 0.5-ml aliquots of the supernates to labeled scintillation vials, add 6 ml of scintillation fluid, and count the radioactivity. Calculate cortisol concentrations in unknown samples from the dose–response curve obtained by similar treatment.

Cortisol in saliva and parotid fluid, direct assay. Add 10 μl of parotid fluid or saliva and 10-μl aliquots of standards in the appropriate assay buffer (pH 4 or pH 7.4) to labeled 75 × 12 mm glass tubes. Add 100 μl of antiserum to all tubes and continue the assay as described in the comparison procedure.

Cortisol in saliva and parotid fluid of patients taking metyrapone. Add 200 μl of saliva or parotid fluid and 3 ml of petroleum ether/benzene (65/35 by vol) to labeled 100 × 12 mm assay tubes. Vortex-mix for 10 min, centrifuge, and discard the extract. Re-extract with 3 ml of 1,2-dichloroethane and assay by the comparison procedure.

Results

Analytical Variables

The dose–response curve obtained by the direct assay (pH 7.4) is shown in Figure 1. Fifteen replicate assays established that precision in the assay was satisfactory.

Sensitivity, defined according to Kaiser and Specker (9) as the least amount distinguishable from zero at the 95%
confidence level, was 30 pg/assay tube in the comparison procedure and 4 pg/assay tube in the direct assays.

Specificity. The cross reactivity of cortisol and structurally related steroids has been presented elsewhere (7). The only known steroid that present in high concentrations could cause significant interference was 11-deoxycortisol. Extraction with petroleum ether/benzene removed 80% of the 11-deoxycortisol, with no significant loss of cortisol, thus eliminating this source of error.

Specificity of the direct assay (pH 7.4) was investigated by assaying 24 parotid fluid and 12 saliva samples by this procedure and by the comparison method. The excellent agreement in titres shown in Figure 3 confirms the specificity of the direct assay.

Precision of the direct assay (pH 7.4). Four pooled samples of parotid fluid having suitable cortisol concentrations were established. Aliquots (n = 12) of each pool were assayed in one assay and used to assess intra-assay variance (Table 1). These pools were apportioned, stored at −20 °C, and used as quality controls for subsequent assays, thus giving the inter-assay variance. Cortisol in parotid fluid and saliva samples is stable for at least six months when stored under these conditions.

Analytical recovery. Tritiated cortisol was added to 12 parotid fluid and 12 saliva samples known to have low and high cortisol titres. Recovery on 1,2-dichloroethane extraction exceeded 95% in all samples.

Assessment of Adrenal Function

Parotid fluid vs. plasma. The response, in normal volunteers, of parotid fluid cortisol to adrenal stimulation after administration of Synacthen (250 µg, i.m.) is shown in Figure 4. Cortisol reached peak values in both plasma and parotid fluid 60 min after Synacthen stimulation and returned to baseline values after 5.5 h. The eightfold increase in parotid fluid cortisol considerably exceeded the threefold increase observed in plasma. In a patient with secondary adrenal atrophy (Figure 4), cortisol concentrations remained low in parotid fluid and plasma after Synacthen administration, the values approximating those seen in dexamethasone-treated volunteers (Figure 5). Also shown in Figure 4 are the mean changes in cortisol concentrations in plasma and parotid fluid when Synacthen was administered to the three healthy volunteers on estrogen-containing oral contraceptive. Plasma cortisol values were increased above basal values and, on stimulation, the response considerably exceeded normal. The time taken to reach maximum values in plasma was 2 h. In marked contrast, basal values for cortisol in parotid fluid were normal and the increase in these values was also within the normal range. This anomaly could well be due to an increase in circulating globulin, considered to be induced by the estrogen component of the contraceptive preparation (10).

Parotid fluid vs. saliva. Ten-microliter samples of parotid fluid and saliva were assayed at pH 4 and pH 7.4. At pH 4, cortisol is known to be released from binding proteins (11). No significant difference in titres was observed, indicating that cortisol is bound neither to specific nor nonspecific proteins in these fluids. Addition to the tubes used in preparing the dose–response curve of 10 µl of parotid fluid or saliva, collected during dexamethasone suppression, caused no significant change in binding. Because there is no interference

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Fig. 3. Correlation of cortisol concentrations in parotid fluid (left) and whole saliva (right) assessed by the direct assay (pH 7.4) and by the 1,2-dichloroethane extraction procedure.
by binding proteins in either fluid, cortisol concentrations can be directly determined at pH 7.4. Four samples of saliva were collected with no stimulation during 5 min. This was immediately followed by collection of four parotid fluid samples, with dropwise administration of citric acid syrup to maintain maximal flow rate. This procedure for collecting matched samples was repeated three times during a 24-h period. Cortisol titres in the samples of parotid fluid (n = 16) and saliva (n = 16) agreed well (Figure 6), indicating that cortisol secretion is independent of flow rate. Thus the determination of cortisol concentrations in whole saliva, which is collected with relative ease, could well replace parotid saliva in the assessment of adrenal function.

Discussion

Our direct assay requires only 10 μl of fluid for determining cortisol in either parotid fluid or whole saliva. Changes in cortisol concentrations in the plasma of normal volunteers after Synacthen stimulation or after dexamethasone suppression of adrenal activity are accurately and immediately reflected by cortisol concentrations in both parotid fluid and whole saliva. Cortisol titres in parotid fluid and saliva are independent of flow rate, a finding in keeping with the studies of Paxton et al. (12) and Levy et al. (13). This non-invasive sampling technique, coupled with the small volumes required for analysis, facilitates serial clinical investigations. Assay of cortisol in either parotid fluid or saliva may therefore be of great value, not only as a screening procedure; it could become the method of choice for accurately assessing adrenal function.

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References