Liquid Chromatography and Radioimmunoassay Compared for Determination of Cortisol and Corticosterone in Plasma after a Dexamethasone Suppression Test

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We developed sensitive, specific "high-performance" liquid chromatography (HPLC) for determining suppressed cortisol and corticosterone in human plasma and compared its efficacy with that of conventional radioimmunoassay (RIA) at concentrations in the nanogram per liter range. Steroids from a 0.5-mL aliquot of plasma were extracted by rapid-flow fractionation, with diethyl ether as mobile-phase solvent, diatomaceous earth granules as stationary-support material. Analytical recovery of the steroids approached 100%. Concentrations in plasma were determined from peak-height ratio calibration (dexamethasone internal standard). The analytical column contained silica gel and the solvent system was water/methanol/dichloromethane/n-hexane (0.1/3/30/66.9 by vol). We could measure the steroids before and 20 h after oral administration of 0.5 mg of dexamethasone. The detection limit was 300 ng per liter of plasma for corticosterone, 500 ng/L for cortisol, with CVs of <4%. Determining corticosterone after administration of dexamethasone, in four of 20 such samples we could determine concentrations >300 ng/L; the others contained corticosterone between 100 and 300 ng/L, but these values could not be certified analytically. Mean concentrations of these hormones as determined by RIA substantially exceeded those by HPLC. Some cross reactions in RIA could not be considered negligible in spite of pre-column treatment of the extracts.

Additional Keyphrases: RIA compared · clinical psychiatric diagnosis · kidney transplant

Glucocorticoid assay is widely applied in clinical practice and in laboratory investigation of endocrine abnormalities such as Cushing's disease (1,2) and depressive psychosis (1, 3, 4). The dexamethasone suppression test (DST) (2, 5) is a useful clinical means for clarifying the functions of the hypothalamus–pituitary–adrenal axis. In the DST, owing to their low concentration, glucocorticoids must be determined by radioimmunoassay (RIA) (6) or competitive protein-binding techniques (7). In long-term glucocorticoid therapy of autoimmune diseases or organ transplantations, the patients have decreased adrenal cortical function (8). Under these clinical conditions, one needs to determine unusually low concentrations of cortisol and corticosterone in plasma, on the order of hundreds of picograms per milliliter. RIA and competitive protein-binding techniques have been applied to clinical uses, but the results obtained are attended with considerable error. The specificity of RIA, the most popular technique, varies with antisera and commercially available kits (10, 11). In the case of antisera, there is considerable cross reactivity with steroids such as hydroxy- pregnanes that are present in human plasma. Although more nearly accurate analytical results may possibly be obtained by "high-performance" liquid chromatography (HPLC) (12, 13), no comparative study between RIA and HPLC has so far been reported. Here we present a sensitive, specific HPLC method, which provides analytical results of greater accuracy than obtained by our original HPLC method (13).

Materials and Methods

Materials

Our HPLC system consisted of a reciprocal BIP-1 (Jasco, Hachiogi, Tokyo) solvent-delivery system with a Model 7125 (Rheodyne, Cotati, CA) syringe-loading injector and a UV-100-6 VI (Jasco) ultraviolet absorbance detector. It was also provided with a solvent degasser ERC-3310 (Erma, Chiyoda, Tokyo) and a high-pressure damper (Jasco). The chromatogram was recorded with a RC-150 (Jasco) pen recorder. The detector wavelength was set at 245 nm. The analytical accuracy of the system was confirmed at a sensitivity of 0.001 A full scale. A 4.0 mm (i.d.) × 250 mm stainless-steel column packed with 5-μm LiChrosorb Si-60 silica gel particles (Cica-Merck, Chuo, Tokyo) was prewashed with 200 μL of dilute (10 mL/L) sulfuric acid, then with distilled water until the eluent became neutral. Solvents for the mobile phase were obtained from Wako Chemical Co., Higashi, Osaka, Japan. The analytical standards of dexamethasone, cortisol, and corticosterone were purchased from Sigma Chemical Co., St. Louis, MO. Rapid-flow fractionation apparatus was obtained from Kusano Scientific Co., Sumida, Tokyo.

Procedures

Extraction procedure. We used the rapid-flow fractionation technique to extract glucocorticoids from human plasma, following the general procedure described in our previous paper (13). A 0.5-mL aliquot of plasma was supplemented with 20 ng of dexamethasone (as internal standard) and injected onto a diatomaceous-earth column with a 3.5-mL inner volume. The column was connected to another one (0.8
mL inner volume) previously injected with 50 µL of a 50 g/L sodium hydroxide solution. The column system thus obtained was eluted with 7 mL of diethyl ether under nitrogen gas at a pressure of 2 kg/cm². The eluent, collected in a 10-mL glass test tube, was evaporated under a gentle stream of dried air at 30 °C. The neutral extracts in the residue were concentrated at the bottom of the tube by washing with 0.3 mL of methanol/dichloromethane (1/99 by vol), which also was evaporated.

**Extraction recovery.** Plasma specimens from patients who had received high doses of prednisolone for over two months were used in determining the extraction recovery of cortisol and corticosterone. No peaks for the samples were observed to correspond to cortisol or corticosterone at a sensitivity of 0.005 A. Trace quantities of both hormones were added to the specimens and extraction by rapid-flow fractionation was conducted without dexamethasone as the internal standard. The extracts were supplemented with 20 ng of dexamethasone before the solvent was evaporated. The residue remaining was dissolved in methanol/dichloromethane (1/99 by vol) and chromatographed. Recovery was calculated from peak-height ratio calibration.

**Chromatography.** Dried extracts were redissolved in approximately 20 µL of the methanol/dichloromethane mixture for HPLC. The mobile phase was a mixture of distilled water/methanol/dichloromethane/n-hexane (0.1/3/30/66.9 by vol). The flow rate was 1 mL/min. The concentration of each steroid was determined from a calibration curve, made on the basis of the ratio of the peak-height for the steroid to that for 20 ng of dexamethasone. The amount of cortisol standard ranged from 0.5 to 50 ng and that of corticosterone from 0.3 to 20 ng.

**Radioimmunoassay.** For cortisol and corticosterone determinations by RIA we used a kit (Teikoku-zoki Co., Kawasaki, Kanagawa, Japan) containing rabbit antiserum against cortisol-21-succinate–bovine serum albumin and corticosterone-3-oxime–bovine serum albumin. The diethyl ether extract of plasma was pre-treated on a Sephadex LH-20 micro column and the corresponding fraction was analyzed by RIA.

**Human Subjects**

Twenty healthy women, ages 23 to 61 y (mean 45.1, SD 12.1), participated in the test. Peripheral blood samples were collected in the afternoon at about 16:00 h. Plasma specimens obtained by centrifugation (2500 × g, 10 min) were used for HPLC and (or) RIA. To obtain plasma specimens with lower concentrations of the steroids, we performed the DST on volunteers administered 0.5 mg of dexamethasone just before bedtime, collecting blood specimens 20 h later, in the afternoon of the following day. These specimens were stored at -20 °C until analyzed.

**Results**

Table 1 shows that analytical recovery of cortisol and corticosterone from plasma by rapid-flow fractionation was approximately 100% in each case, essentially the same as that reported by the usual column technique (14). Other chromatographic approximations for the assay of the steroids are given in the following.

Figure 1 depicts a typical chromatogram of a plasma sample after administration of dexamethasone. The capacity ratio (k’ value) of the cortisol peak was 24.88, and it appeared at 42.20 min. The dexamethasone peak, corresponding to the internal standard added before the extraction, appeared at 38.05 min. The peak interval between cortisol and dexamethasone was wide enough to allow baseline separation with a separation factor (α) of 1.11. No endogenous dexamethasone in the plasma specimen could be detected on the chromatogram 20 h after its oral administration. The calibration of the cortisol on the basis of the ratio of the peak-height for cortisol to that for dexamethasone agreed reasonably; the CV was <3%. The regression equation was $y = 56.9966x - 0.5023$ ($r = 0.9998$), where $y$ is cortisol concentration (µg/L) and $x$ the peak-height ratio.

The peak for cortisol appeared at 15.18 min and its k’ value was 8.31. The separation factors for two adjacent peaks were 1.06 and 1.05, both these values being sufficient to permit baseline separation when the actual theoretical plate number was 11 000 per column. It is thus evident that the corticosterone/dexamethasone peak-height ratio can be accurately determined with a CV of <4%. The regression equation was $y = 8.9291x - 0.1834$ ($r = 0.9999$), where $y$ is corticosterone concentration (µg/L) and $x$ is the peak-height ratio of corticosterone to dexamethasone.

The cortisol concentration determined by HPLC before administration of dexamethasone was 61.6 (SD 23.5) µg/L. This value decreased to 23.3 (SD 21.8) µg/L 20 h after the

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**Table 1. Efficiency of Extraction of Cortisol and Corticosterone from Plasma**

<table>
<thead>
<tr>
<th>Steroid concn. µg/L</th>
<th>Recovery, %, mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>Corticosterone</td>
</tr>
<tr>
<td>1</td>
<td>100.0 (4.3)</td>
</tr>
<tr>
<td>10</td>
<td>99.0 (2.5)</td>
</tr>
</tbody>
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administration. In the assay of corticosterone the average value before administration of dexamethasone was 0.88 (SD 0.61) μg/L, a value less than those obtained in the morning—3.91 (SD 1.61) μg/L—owing to circadian rhythm. However, with the present HPLC technique we could not quantitatively determine highly suppressed corticosterone after administration of dexamethasone. In only four of 20 treated specimens was it possible to determine concentrations exceeding 300 ng/L. Although other specimens contained corticosterone at concentrations between 100 to 300 ng/L, these values could not be certified analytically.

The major improvement in steroid HPLC demonstrated here is the enhanced analytical sensitivity, which allows more precise determination of corticosterone. As little as about 300 ng of this hormone per liter in the plasma can be determined at a signal-to-noise ratio of 3 and 0.001 A full scale, a sensitivity about 10-fold that possible by the original method of Rose and Jusco (12). Thus, liquid-chromatographic determination of corticosterone in human plasma with measurement by ultraviolet absorbance is now possible for the first time without any tedious preliminary chemical derivatizations. In contrast, only about 1 μg of cortisol per liter of plasma could be determined, owing to its larger k' value. However, when chromatography was carried out using a more polar solvent system such as one containing 60 mL of methanol per liter, as little as 500 ng/L could be detected.

In Figures 2 and 3, we compare cortisol and corticosterone concentrations as determined by HPLC and RIA. Note the significant statistical deviation between RIA and HPLC.

**Discussion**

Currently, RIA is the technique most widely used to determine glucocorticoids in human biofluids. However, in the case of low concentrations of hormones such as those of corticosterone and cortisol in DST and in long-term gluco-corticoid therapy, RIA cannot be used to obtain sufficiently accurate results owing to the occurrence of numerous cross reactions with other steroids present in the plasma. For instance, the cross reactivity of antiserum against corticosterone-3-oxime–bovine serum albumin with cortisol has been reported to be 2.20% and with progesterone, 5.47%.

These values show such cross reactivity to be sufficiently high as to interfere with accurate determination of low concentrations of corticosterone. In glucocorticoid therapy, cortisol determination becomes greatly inaccurate, owing to co-existing drugs such as prednisolone that cross react with antiserum significantly. Thus, at the present, the application of RIA should be preceded by more-accurate chromatographic pre-treatment of the extract to lessen as much as possible the influence of the cross reactions. This consequently renders RIA in glucocorticoid assay a somewhat tedious and time-consuming procedure. Still, the results obtained by the two techniques were found to differ considerably, the more reliable values being obtained by HPLC. The improved HPLC described in the present paper facilitates more accurate and selective determinations of low concentrations of steroids and is a simpler procedure. This is evident from the comparison of results shown in Figure 2 and 3, which demonstrate the more-accurate analytical determinations of very low amounts of glucocorticoids by the present method. From these results, recently reported diagnostic indices such as the corticosterone/cortisol ratio (15–19) appear measurable by the present highly sensitive HPLC. Although our method proved to be inadequate for measuring amounts of corticosterone <300 ng/L in plasma, this should pose no clinical problem in DST, where the diagnostic criteria of depression are at higher-than-normal values for glucocorticoids (19).

Our method has been tested through application to over 1000 samples from kidney-transplant patients who were taking prednisolone. Plasma cortisol concentrations have been determined, to monitor adrenal function during therapy. The endogenous cortisol of these patients was suppressed to as little as 1 to 5 μg/L in the plasma after the start of drug therapy (20).

Our improved method has also been carried out to determine plasma corticosterone/cortisol ratios, and the values obtained were found to be more nearly accurate than those by RIA (9). In the present experiment, the ratio obtained by HPLC, 0.0139 (SD 0.0046), was lower than that obtained by RIA, 0.0201 (SD 0.0089). The statistical significance of the differences between the two methods was P <0.02. Ratio increment due to either adrenal suppression or stimulation has recently been reported by workers using RIA (15, 19). However, we recommend that more precise values be ob-
tained by the present HPLC or more reliable RIA to eliminate outstanding inaccuracies in clinical diagnosis.

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