Daily Cortisol Production Rate in Man Determined by Stable Isotope Dilution/Mass Spectrometry

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ABSTRACT. Growth retardation as well as the development of Cushingoid features in adrenally insufficient patients treated with the currently accepted replacement dose of cortisol (35-41 μmol/day·m²; 12-15 mg/m²-day) prompted us to reevaluate the cortisol production rate (FPR) in normal subjects and patients with Cushing's syndrome, using a recently developed thermospray liquid chromatography-mass spectrometry method. The stable isotope [9,12,12-2H3]cortisol was infused continuously for 31 h at about 5% of the anticipated FPR. Blood samples were obtained at 20-min intervals for 24 h, spun, and pooled in 4-h groups. Tracer dilution in plasma was determined by liquid chromatography/mass spectrometry. The method was validated with controlled infusions in 6 patients with adrenal insufficiency. Results from 12 normal volunteers revealed a FPR of 27.3 ± 7.5 μmol/day (9.9 ± 2.7 mg/day) or 15.7 μmol/day·m²; 5.7 mg/m²-day). A previously unreported circadian variation in FPR was observed. Patients with Cushing's syndrome demonstrated unequivocal elevation of FPR (84.7 ± 25.7 μmol/day) and loss of circadian rhythm. FPR and cortisol concentration correlated with the currently accepted replacement dose of cortisol (33-41 μmol/day·m²; 12-15 mg/m²-day) prompted us to reevaluate the cortisol production rate (FPR) in normal subjects and patients with Cushing's syndrome, using a recently developed thermospray liquid chromatography-mass spectrometry method. The stable isotope [9,12,12-2H3]cortisol was infused continuously for 31 h at about 5% of the anticipated FPR. Blood samples were obtained at 20-min intervals for 24 h, spun, and pooled in 4-h groups. Tracer dilution in plasma was determined by liquid chromatography/mass spectrometry. The method was validated with controlled infusions in 6 patients with adrenal insufficiency. Results from 12 normal volunteers revealed a FPR of 27.3 ± 7.5 μmol/day (9.9 ± 2.7 mg/day) or 15.7 μmol/day·m²; 5.7 mg/m²-day). A previously unreported circadian variation in FPR was observed. Patients with Cushing's syndrome demonstrated unequivocal elevation of FPR (84.7 ± 25.7 μmol/day) and loss of circadian rhythm. FPR and cortisol concentration correlated during each sample period in normal volunteers, indicating that cortisol secretion, rather than metabolism, is mainly responsible for changes in plasma cortisol. Our data suggest that the FPR in normal subjects may be lower than previously believed. (J Clin Endocrinol Metab 71: 39-45, 1991)

The daily cortisol production rate (FPR) in man has been estimated to be approximately 33-41 μmol/day·m² (12-15 mg/m²-day) (1-3). These doses of cortisol administered to patients with adrenal insufficiency, panhypopituitarism, or congenital adrenal hyperplasia, however, often cause weight gain and development of Cushingoid features as well as growth retardation in children. This observation suggested that some of the earlier studies may have overestimated the FPR. Four methods have been employed widely for this measurement: 1) urinary production rate by isotope dilution (4), 2) urinary production rate from endogenous metabolite excretion (5), 3) plasma production rate based on the metabolic clearance (MCR) of a single injection (6) or a constant infusion (7) of radioactive cortisol, and 4) plasma production rate by summation of incremental secretion (8). A comparison of the four methods in a large group of patients revealed considerable variation (8). The limitations in FPR determinations by radiotracers have been reviewed previously and are thought to involve: 1) incomplete sample recovery, 2) inability to take into account the diurnal variations of cortisol, 3) the assumption that a unique metabolite of cortisol has been analyzed, and 4) relative lack of specificity in the quantitative analysis of plasma cortisol and its urinary metabolites (e.g. fluorometry) (2, 8-10). It is noteworthy that some investigators have reported lower values of FPR using radiotracers (11-13).

We have developed an alternate analytical procedure using stable isotope dilution thermospray liquid chromatography/mass spectrometry (LC/MS) methodology that enables a more direct measurement of plasma FPR in man than previously possible using radioactive isotopes (14, 15). The method offers several technical and clinical advantages over radiotracer techniques. First, the production rate determination depends exclusively upon dilution of the tracer by the natural cortisol. Second, stable isotopes can be infused for long periods of time, such as a 24-h period, allowing inclusion of the effects of the circadian variation in the production rate measurements. Third, studies can be performed safely in children and pregnant women (16).
In the present investigation we have applied this methodology to the study of FPR in normal subjects and patients with Cushing’s syndrome, validating the technique using different cortisol infusion schedules in patients with adrenal insufficiency.

Materials and Methods

Subjects

Studies of FPR were carried out in normal subjects (n = 12) and patients with Cushing’s disease secondary to pituitary adenoma (six studies; n = 5; in whom the diagnosis was established by conventional tests). The clinical data are presented in Table 1. Three patients with adrenal insufficiency were given cortisol as a continuous iv infusion. Three additional patients received cortisol as a series of iv boluses designed to simulate the normal pulsatile secretion of cortisol.

Materials

\[9,12,12-\text{H}_3\]Cortisol (d₃-cortisol; 98 atom % \text{H}; KOR Isotopes, Inc., Cambridge, MA) was used without further purification. High pressure liquid chromatography solvents were the same as described previously (14).

Human studies

The infusion studies were approved by the Human Clinical Research Subpanel of the NICHD, and written informed consent was obtained from each subject. An iv catheter was inserted into one arm at 0800 h (Fig. 1). A second iv catheter was inserted in the other arm for the administration of the d₃-cortisol tracer. The tracer dose [83-165 nmol/h d₃-cortisol; \~5\% of the anticipated daily FPR, as determined by Kenny et al. (1)] was prepared by the Pharmaceutical Development Branch in 1 mL sterile pyrogen-free 95\% ethanol at 2.76 nmol/L and diluted in 1 L normal saline before iv administration. Tracer infusion was started at 0100 h on the second day of admission. The rate of infusion was controlled by a pump, the accuracy of which was checked before each study. At 0800 h, after 7 h of continuous iv infusion of tracer (equivalent to 4–6 times the half-life of cortisol), 3-mL blood samples were obtained through the heparinized iv catheter at 20-min intervals for 24 h. At the end of each study the isotope concentration was determined by LC/MS. The total volume of the infusate actually received by each patient was verified by measurement of the initial volume and the volume of the infusate remaining at the end of the study.

Analysis of samples

Samples of cortisol were centrifuged, and 1-mL aliquots of plasma were grouped into six 4-h pools. One aliquot (1 mL) of each pooled plasma sample was combined in a 24-h pool; another aliquot (1 mL) was kept for plasma cortisol quantification by LC/MS. The pooled plasma samples were extracted twice with equal volumes of methylene chloride-ethyl ether (3:1). The combined organic phases were evaporated to dryness under a stream of argon at 24 C, reconstituted in 100 \mu L methanol, and filtered (0.45 \mu m). Sample recovery was more than 95\%. LC conditions used for the analysis of cortisol were chosen to separate cortisol from other steroids normally present in serum (14). The thermospray interface has been described previously (17). The MS analysis was performed using a modified Finnigan model 4000 Quadrupole Mass Spectrometer (San Jose, CA) controlled by a Teknivent Data System (Teknivent, St. Louis, MO), as described previously (14).

Isotope dilution mass spectrometry

Calibration of the cortisol IDMS method was carried out by using d₃-cortisol (98 atom % \text{H}) as internal standard and monitoring ions at mass 303 and 363, and 306 and 366, corresponding to unlabeled and labeled material, respectively. Standard curves were obtained by plotting relative peak area abundances between labeled and unlabeled cortisol ions vs. the expected ratio in the range of ratios 0.01:1 to 0.2:1. Intra- and interassay variations for labeled/unlabeled cortisol ratios of 0.05:1 were less than 6\%. Similar precision was observed when duplicates of biological samples were analyzed.

Calculations

The peak area ratio (labeled/unlabeled cortisol) was used to determine plasma enrichment (PE). FPR was calculated from the product of the known infusion rate (IR) and the ratio of tracer infusedate enrichment (IE) to tracer dilution in plasma: FPR = IR (IE/PE).

Duplicate determinations were averaged for each sample and expressed as the mean ± SD. Correlation between parameters was carried out with use of standard linear regression analysis. The significance of all parameter differences was determined by Student’s t test.

Results

The daily FPR was determined on the basis of the 6-4-h pooled samples from each subject. The results from

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<th>Table 1. Physical characteristics and clinical data of normal volunteers (NV) and patients with Cushing’s syndrome (CS)</th>
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UFC, Urinary cortisol; 17OH-S, 17-hydroxysteroids; Cr, creatinine.
12 normal volunteers revealed an FPR of 27.3 ± 7.5 μmol/day (9.9 ± 2.7 mg/day), equivalent to 15.7 μmol/day·m² (5.7 mg/m²·day; Table 1). The circadian pattern of cortisol secretion observed in this group during continuous iv infusion of d₃-cortisol is illustrated in Fig. 2. Each bar represents the estimated instantaneous FPR from a single pooled sample obtained from 12 serum samples drawn at 20-min intervals over a 4-h period. The range of the circadian pattern of cortisol production varied by more than 3-fold, with a nadir of 1.94 ± 0.7 μmol/h (0.70 ± 0.25 mg/h) at 2000-2400 h and a peak of 7.31 ± 2.94 μmol/h (2.65 ± 1.07 mg/h) at 0400-0800 h.

Results from six studies in five patients with Cushing's syndrome revealed an FPR of 84.7 ± 25.7 μmol/day (30.7 ± 9.3 mg/day) or 49.9 μmol/day·m² (18.1 mg/m²·day). The circadian rhythm was blunted (P < 0.001), with a nadir to peak ratio that varied between 1.2-2.4 (Fig. 3). The analysis of the 24-h pooled sample differed by less
than 7% ($P = NS$) from the sum of the instantaneous FPR calculated after analysis of the six 4-h pooled samples. The results obtained from normal volunteers and patients with Cushing's syndrome are summarized in Fig. 4. The daily FPR of one of the patients was almost within the normal range, but that was not the case when samples from either 2000-2400 h or 2400-0400 h were compared.

Plasma cortisol concentrations were also measured by LC/MS in each of the 4-h pooled samples from seven subjects studied. Mean daily cortisol concentration obtained from the averaged values of the six pooled samples from each subject was remarkably constant (165.5 ± 24.8 nmol/L). These results did not differ from those obtained by a conventional RIA method (168.3 ± 35.9 nmol/L; $n = 10; P = NS$). The positive correlation between mean FPR and mean cortisol concentration at each pooled sample period in normal volunteers is shown in Fig. 5. In comparison, mean daily cortisol concentration measured in patients with Cushing's syndrome was 422.1 ± 99.3 nmol/L ($n = 3$), more than twice the normal mean ($P < 0.001$).

**Validation studies**

The sensitivity, specificity, and accuracy of the methodology were evaluated as follows. Three subjects with
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adrenal insufficiency were infused continuously for 31 h with unlabeled cortisol at rates of 12.7, 42.5, and 74.5 μmol/day, respectively. The d₃-cortisol was administered at tracer/tracee ratios of 5.07, 5.4, and 6.15 mol %, respectively. Seven hours after the initiation of the iv infusion, blood samples were obtained every 20 min, as described above. Figure 6 shows the FPR (mean ± SD) of the values obtained from the analysis of each of the 4-h samples in the three patients studied. Daily FPR determinations in the three patients studied differed from the expected value by less than 5% (P = NS).

Three additional studies were performed in adrenally insufficient patients to address the issue of accuracy of the method in nonsteady state conditions characterized by pulsatile secretion and circadian variation, such as those that exist in normal subjects. The tracer dose was infused continuously during 30 h, while cortisol was given in a series of iv boluses to simulate normal pulsatility and circadian variation. Pooled samples were analyzed every 2 h. The measurements of the apparent daily FPR in the three patients studied differed from the total cortisol infused over the same period by 7% (4.6–13.1%; P = NS; Table 2).

Discussion

The current mean daily FPR of 27.3 ± 7.5 μmol/day (9.9 ± 2.7 mg/day) or 15.7 μmol/day·m⁻² (5.7 mg/m²·day) determined in 12 normal volunteers is significantly lower than the FPR reported by previous investigators (1–8). However, this value is closer to that reported by other investigators (11–13). As expected, FPR varied during the day, with the peak production rate between 0400–0800 h, and the nadir between 2000–2400 h. The pattern of circadian variation in FPR in normal subjects has not been reported previously due to the limiting factor of radiation exposure. We observed that the mean FPR (as determined by analysis of the 4-h pooled samples) correlated positively with the cortisol concentration measured at the same 4-h period in normal volunteers, indicating that hormone secretion, rather than metabolism, was mainly responsible for changes in plasma cortisol concentration.

The IDMS method was chosen because of the high accuracy and specificity of LC/MS (14). This method overcomes several difficulties associated with radiotracer techniques. These include the following.

1) Inability to attain equilibrium. Because of the risk of radiation exposure, previous studies have been limited to a single injection or a constant infusion for a short period of time, which was then extrapolated to 24 h. In the face of constantly changing plasma cortisol concentrations, the metabolic fate of a pulse injection of labeled cortisol will be largely dependent upon the plasma cortisol concentration at the time of injection (10). Thus, the metabolism of the tracer may not mirror the cumulative transformation of all endogenous, episodically secreted cortisol. In the present study tracer was infused over 31 h, and FPR was calculated using the equation: IR(IE/PE).

While this expression is generally used for calculations...
of production rates in systems at steady state, the circadian rhythm of cortisol precludes the existence of a steady state. Rather, we use the expression in this case to determine the dilution of the tracer in a particular 4-h pooled sample, with the tracer being delivered at a fixed rate, as a measure of the instantaneous FPR. We believe that this approach enables the determination of production rates in systems that undergo circadian as well as pulsatile variation.

2) Incomplete recovery of the sample. Previous investigators have considered complete recovery of the sample when more than 60% of the radioactive dose was recovered, assuming identical metabolism of the isotope throughout the study. Determinations of FPR by stable tracer/MS are independent of any quantification of sample recovery, since only single measurements of isotope ratios are required.

3) Assumption that a unique metabolite of cortisol has being analyzed. None of the urinary metabolites of cortisol usually studied are known to be derived exclusively from plasma cortisol (9). Further complexity is added by the finding that isolated perfused rat kidneys or suspended tubular fragments generate metabolites from cortisol, making the interpretation of results obtained by urinary secretion rate methods difficult and questionable (18).

4) Variability in the methods used to quantify plasma cortisol for FPR determinations. Specific activity, the typical unit of radioactivity measurements, is expressed in terms of concentration; thus, studies of hormone production performed with radioactive tracers yield results in terms of clearance rates and depend upon independent measurements of the mass of material present in the sample. Consequently, production rates in these cases must be calculated from a knowledge of the volume of distribution and concentration. The quantification of cortisol by techniques less specific than high pressure liquid chromatography or GC/MS can result in significantly higher concentration values and can be a factor in the overestimation of FPR (19). In a typical selected ion-monitoring LC/MS method, the labeled and unlabeled substrates are measured simultaneously. Furthermore, since there is a single sample preparatory technique for the determination, and since the analysis conditions are identical for the labeled and unlabeled material, propagated errors are reduced, and the assay is frequently simpler and more precise than determination of radiotracer specific activity, where different techniques are generally used to measure isotope and substrate content. Furthermore, by judicious choice of LC/MS conditions, confidence in the specificity of the measurement is virtually assured. The results of determinations of production rates by stable tracer/MS are expressed directly in units of mass/time and independent of any determination of volume of distribution. Since the production rate, mass/time, is the goal of these studies, our methodology offers advantages over radioactive tracers in simplicity and reliability, because only single measurements of isotope ratios are required.

5) Inability to take into account the diurnal variations in cortisol. The pattern of circadian variation in FPR in normal subjects has not been reported previously due to the limiting factor of radiation exposure. The present study showed values that varied more than 3-fold. Although this finding is in agreement with that estimated by Weitzman et al. (20), we may have underestimated the magnitude of variation in FPR without affecting total FPR by pooling the serum samples into 4-h groups.

6) Stress-evoked cortisol responses. The iv lines were inserted 24 h before sampling, so that the stress-evoked cortisol rise associated with venipuncture (usually within 30 min) would not artifactually elevate the FPR. Furthermore, studies were performed on the second inpatient day to allow adaptation to the environment, since hospital admission has been shown to be associated with elevated plasma cortisol and urinary free cortisol levels (21).

We validated our methodology in three patients with adrenal insufficiency by administering unlabeled cortisol in a dose similar to what would be endogenously produced. We spanned the range of expected cortisol production rates as suggested by the literature (12.7, 42.5, and 74.5 μmol/day). These studies addressed the issues of reliability of the method through a wide range of secretion, and absence of a measurable in vivo isotope effect.

To address the issue of accuracy of the method during pulsatile and circadian secretion, three additional studies were performed in adrenally insufficient patients. Tracer was infused continuously, as described above, while natural cortisol was given in a series of iv boluses to simulate normal pulsatility and circadian variation. Although there were differences at each sample point, probably due to the mixing process, the areas under the curve were almost identical. Daily FPR determinations did not differ significantly from the total cortisol amount infused over the same period.

Mean daily cortisol concentration, as determined by LC/MS, was remarkably constant in normal volunteers. These results were very similar to those obtained by a conventional RIA method and did not differ from the mean daily cortisol concentration reported previously in healthy men, determined by the same RIA method (22).

Since there was a positive correlation between FPR and plasma cortisol concentration during each pooled
the hypothalamic-pituitary-adrenal axis. Despite the unlikely that a negative feedback resulted in measuring mass of the infused tracer, mean daily cortisol concentration significantly decreased if there were a negative feedback on cortisol concentration and FPR would have been significantly lower in normal subjects. We hypothesize that our finding that the variation in FPR in normal subjects and its absence in adrenalectomized adults to assess the question of whether the dose of glucocorticoid administered needs to be adjusted when the route of administration is oral rather than parenteral. The optimal replacement dose of glucocorticoid may need to be accounted for the bioavailability and/or first pass effect of an oral dose (23). We are currently conducting studies in adrenalectomized adults to assess the rate of appearance of hydrocortisone after several schedules of oral administration designed to simulate the normal pattern of cortisol secretion. Further studies are needed to determine the appropriate glucocorticoid oral replacement dose.

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References