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Gas chromatographic–mass spectrometric method for measuring stable isotope enrichments of underivatized cortisol from small plasma volumes

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ABSTRACT

Isotope dilution methods utilizing quantification of stable isotope enrichment by mass spectrometry has been used recently to determine cortisol production rates in humans. Studies of steroid production and utilization rates require frequent blood sampling, and while stable isotopes are safe for pediatric and perinatal use, the required blood volumes may be problematic using published methodologies. We have developed methods to isolate and quantify the plasma enrichment of cortisol with its stable isotope, [9,12,12-$^2H_3$]cortisol using a simple four-step isolation procedure and gas chromatography–mass spectrometry. Isolation is semi-quantitative, but reproducible (mean recovery: 51.5 ± 5.4% coefficient of variation) and the method does not require derivatization. In human studies, this method can determine plasma enrichments between 1 and 10 mol% at plasma concentrations of 2 μg/dl or more; only 1 ml of plasma is required. The concentrations of labelled cortisol added to plasma are low (0.2–0.5 μg/dl) and are not expected to interfere with the sensitive hypothalamic–pituitary–adrenal feedback system. We conclude that reproducible quantification of stable cortisol isotope enrichment can be achieved from small plasma volumes.

INTRODUCTION

Cortisol is the predominant glucocorticoid secreted by the human adrenal. At physiological concentrations, cortisol plays critical roles in diverse systems: these include adaptation to fasting [1,2]; the renal capacity to clear excess body water [3,4]; maintenance of myocardial contractility and vasomotor response to, and production of, catecholamines [5–7], and the modulation of lymphocyte activities by alteration of cytokine and antibody production [8,9]. The dynamics of cortisol homeostasis have been studied by timed urine collections [10,11], frequent blood sampling [12–14], and by isotope dilution techniques [15]. However, the concentration of hormone in the systemic blood only reflects the balance between hormone production and utilization. Hormone concentration may not be the only important parameter since coordination of biological response may not be uniquely concentration-dependent, but may also rely on oscillations in hormone production and utilization as a result of stimulation or inhibition by substrates or other secretagogues [16–18].

Although cortisol secretion has been approached in the past by deconvolution of plasma concentration patterns [14] and by radioactive isotope techniques [15], the previously described methods are cumbersome and required relatively large volumes of blood. We describe a new method to measure stable isotope enrichment of plasma cortisol by gas chromatography–mass spectrometry (GC–MS) from small (1 ml) volumes of

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plasma. Reduction in plasma volume requirements is indeed critical to frequent sampling and therefore to ascertainment of rapid homeostatic changes.

EXPERIMENTAL

Chemicals

Unlabeled reference standard grade cortisol was obtained from Sigma (St. Louis, MO, USA) (hydrocortisone: 4-Pregnen-11β,17α,21-triol-3,20-dione; Sigma H-5885, Lot 41H5950) and was used without further purification. [9,12,12-$^2H_3$]Cortisol was obtained from Cambridge Isotope Laboratories, Woburn, MA, USA (DLM-2057, Lot BK 1135). All solvents were analytical grade (acetone, petroleum ether and diethyl ether) or HPLC grade (methylene chloride, denatured ethanol). Tritiated cortisol (1,2-^{3}H-(N)-cortisol, 49.1 C_i/mmol) was obtained from NEN Research Products (Boston, MA, USA).

Plasma cortisol concentrations were assayed in duplicate by a fluorescence polarization immunoassay (TDX System, Abbott Diagnostics Division, Irvine, TX, USA). Inter-assay coefficients of variance for the cortisol immunoassay at concentrations of 4,15 and 40 μg/dl were 7.6, 6.5 and 6.0% respectively. The cortisol and deuterated cortisol concentration of stock solutions and infusates were assessed by triplicate immunoassay of serial dilutions of each solution. Stock solutions were stored at -70°C and the concentration was re-assessed every two weeks.

Cortisol extraction

Human blood was collected in evacuated EDTA tubes (Beckton-Dickinson, Rutherford, NJ, USA) and kept at 4°C until separation by centrifugation (1100 *g*, 4°C) and storage at -70°C. Protein precipitation of 1-ml aliquots of plasma was performed with 2 ml of cold acetone (15 min, at 4°C). Two aliquots of 5 ml of petroleum ether–diethyl ether–acetic acid (90:10:1, v/v/v) were added to the aqueous acetone layer obtained after centrifugation (1100 *g*, 4°C). The resultant aqueous acetone layer was then extracted with two 5-ml volumes of methylene chloride. The methylene chloride layer was dried under nitrogen at 60°C and resuspended in 1 ml of HPLC-grade methylene chloride. Silica columns (500 mg silica, 2.8-ml column, Alltech Associates Deerfield, IL, USA) were sequentially rinsed with 3 ml of ethanol and 3–5 ml of methylene chloride. The 1-ml methylene chloride fractions were poured on the silica columns and allowed to drain by gravity. The columns were rinsed with 3 ml of methylene chloride and the steroids subsequently eluted with 5 ml of ethanol. The ethanol fractions were dried under nitrogen and resuspended in 20–50 μl of ethanol in conical-bottom autosampler microvials (Kimble, 60750-1232, Vineland, NJ, USA). Quantitative steroid recoveries were assessed by adding 1,2-^{3}H(N)-cortisol to the 1-ml plasma volumes prior to the extraction procedure.

The purity of steroid containing fractions was initially monitored by thin layer chromatography. Solvent fractions from 1–2 ml of plasma were dried under nitrogen, resuspended in 10–20 μl of that solvent and applied to 20 × 20 cm Silica G analytical plates (Fisher Scientific, Norcross, GA, USA). Neutral lipids were run in petroleum ether–diethyl ether–acetic acid (90:10:1, v/v/v) and polar lipids in chloroform–methanol–water (60:30:4.5, v/v/v) [19]. Iodine vapor was used for visualization.

Gas chromatograph–mass spectrometry (GC–MS)

The GC–MS system consisted of a Hewlett-Packard 5890 Series 2 gas chromatograph and a 5970 Series Hewlett-Packard quadrupole mass selective detector with HP Unix data retrieval and analysis operations systems. Samples were injected by an automated 7673 sampler. Splitless injections were made into a DB-17 10 m × 0.25 mm I.D. column (0.15 μm film thickness, J and W Scientific, Folsom, CA, USA). Injector temperature was 290°C. The column was held at 40°C during injection, ramped to 280°C over 6.0 min, and held at 280°C for 4 min with ultrapure helium at a linear velocity of 25–30 cm/s as carrier. Cortisol typically eluted at 7.88 ± 0.08 min. The electron impact ionization was at 70 eV. The

detector was autotuned with perfluorotributylamine prior to use. Selected-ion monitoring at m/z 302 and 305 was used for natural and D3-cortisol, respectively. The fragment at 302.2 has been identified by the NBS54.K1 library as 11-β-hydroxyandrost-4-ene-3,17-dione (M_r = 302.19). Isotopic purity of the cortisol and D3-cortisol was assessed by ion spectral analysis of ethanolic solutions of pure compound at their respective GC retention times [20]. Cortisol contained 0.19% trilabeled (m + 3) species; the deuterated cortisol contained 2.5% unlabeled (m) species. The biochemical purities of cortisol and its deuterated isotope as assessed by linear regression of immunoassay results of multiple serial dilutions were 96.5 and 86.9% ($r^2 > 0.98$ for both). The biochemical purities assessed by HPLC [$<C_{18}$ column, mobile phase methanol–water (70:30, v/v)] were 98.4 and 85.6 for cortisol and deuterated cortisol espectively. The isotopic and biochemical purities from HPLC were used to correct the weighed amounts of standards used to establish the standard curve.

Patient study

Deuterated cortisol enrichments were measured in plasma obtained during isotopic infusions performed on three healthy males, ages 24–29. Written informed consent was obtained according to protocols approved by the Institutional Review Board of Baptist Medical Center (Jacksonville, FL, USA). Endogenous steroid production was suppressed by oral administration of 2 mg dexamethasone every 6 h during the 12 h before the study. Baseline plasma (08:00 h) was analyzed for cortisol concentration and enrichment. Infusions of unlabeled cortisol (350–810 $\mu g/m^2/h$) enriched with [9,12,12-$^2H_3$]cortisol (25–43 $\mu g/m^2/h$) were then started and continued for 6 h. After a 3-h equilibration, 1-ml samples of plasma were drawn every 30 min and analyzed for plasma cortisol concentration and enrichment. All infusates were sterile-filtered (0.22 μm) and had been tested negatively for endotoxin prior to use (E-Toxate, Sigma).

RESULTS

Cortisol extraction

Thin-layer chromatography (TLC) was used to monitor the partitioning of nonprotein substances from plasma to the final analyte. After separation, 100× concentrates of 2-ml plasma extracts showed only cortisol and other polar constituents remaining at the origin of non-polar TLC plates. Reproducibility and yield at the various steps was monitored by spiking the initial plasma sample with tritiated cortisol. Over eight assays, the final ethanol eluate from the silica column contained 51.5 ± 5.4% (C.V.) of the initial amount of tritated cortisol (Fig. 1). Selected ion monitoring analyses of cortisol extracted from plasma (Fig. 2) show virtually no interference from contaminants and are indistinguishable from analysis of reference cortisol.

A standard curve is used to relate measured peak-area ratios (305/302) to the expected molar ratios (m + 3/m) calculated from molecular masses and purities. Over six assays, the overall relationship between expected and calculated molar ratios was 0.971 x + 0.255, r^2 = 0.992. The coefficient of variation of the slope was 3.15%.

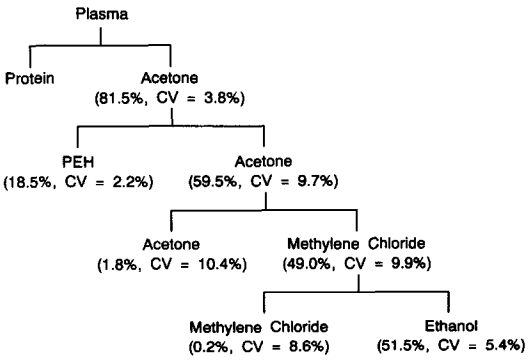

Fig. 1. Extraction of [$^3H_2$]cortisol from 1 ml of plasma. Plasma aliquots (1 ml) were spiked with 5 nCi [1,2-$^3H_2$-(N)]cortisol and processed as described in Experimental. The four branch points are: protein precipitation in cold acetone; back extraction of the aqueous acetone with petroleum ether-diethyl ether–acetic acid (PEH); extraction of the aqueous acetone with methylene chloride; silicagel G column chromatography of the methylene chloride fraction with final ethanol elution. The numbers in parentheses refer to percentage of original counts remaining and the coefficient of variation from eight assays.

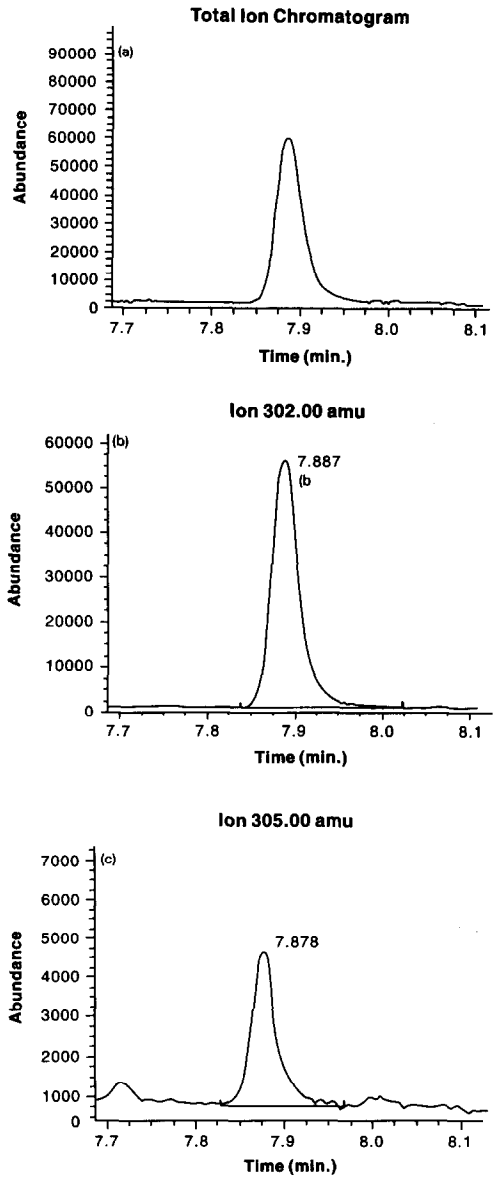

Fig. 2. GC–MS analysis of cortisol and [9,12,12-$^2H_3$]cortisol extracted from 1 ml human plasma. (a) Total ion current at 7.88 min retention time, (b) mass detection of cortisol fragment ion at *m/z* 302, (c) mass detection of [9,12,12-$^2H_3$]cortisol at *m/z* 305.

Patient study

The cortisol concentrations and deuterated cortisol enrichments during dexamethasone suppression and infusion of a mixture of unlabeled cortisol and deuterated cortisol are depicted in Fig. 3. During the last 3 h of the 6-h infusions, the

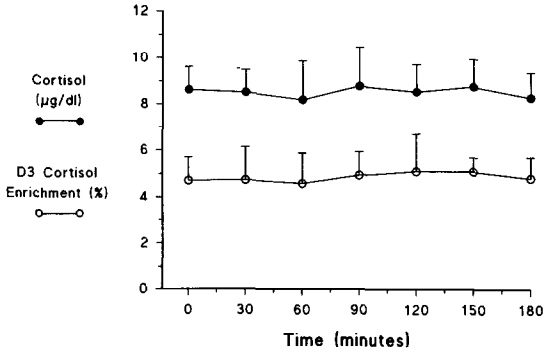

Fig. 3. Man cortisol concentrations (±S.D.) and [9,12,12 $^2H_3$]cortisol (D3) enrichments (±S.D.) in three subjects during combined cortisol and [9,12,12-$^2H_3$]cortisol infusions. The infusions commenced at time zero; *i.e.* 3 h before plasma sampling started.

cortisol concentration had reached an apparent plateau of 8.30 μg/dl; the coefficient of variation among time points was 8.9% (individual C.V. 6.1–11%) while the variation in cortisol replication by TDX was 4.1%. The mean infusate enrichment in [9,12,12-$^2H_3$]cortisol was 5.8 ± 2.2%. During the 180 min of sampling, the mean plasma enrichment was 4.9% with a coefficient of variation between time points of 4.9% (individual C.V. 8.9–15%) and a variation in enrichment replicates of 5.0%. Isotopic steady state was achieved as attested by (1) coefficients of variation less than 10%, and (2) absence of a significant slope when enrichments were plotted against sampling time. Pre-infusion cortisol determinations showed apparent complete suppression of endogenous cortisol concentration (<0.4 μg/dl). A difference was observed between infusate and plasma enrichments (5.83 *versus* 4.86%). An isotope effect of differential clearance between cortisol and deuterated cortisol is unlikely since no systematic increase or decrease in plasma enrichment is noted between 180–360 min: this difference is therefore thought to reflect low level residual endogenous cortisol production.

DISCUSSION

We sought an extraction procedure that would allow rapid, economical and reproducible

amounts of cortisol to be extracted from 1-ml samples of plasma. Assay precision and column performance required the purified sample to be substantially free of other steroid contaminants. A suitable balance between analytical yield and purity was found with initial protein precipitation with cold acetone, back extraction of non-polar lipids from the aqueous acetone with petroleum ether–diethyl ether–acetic acid and finally extraction of polar lipids into methylene chloride with subsequent preliminary fractionation on silica G columns.

The ability of a hormone to generate a physiological response is known to be concentration dependent, but more recent evidence suggests that oscillations in hormonal concentration may also be important [17–19]. Therefore, we sought to devise an assay system for stable isotopes of cortisol that would allow analyses from the small plasma volumes required by frequent or prolonged sampling protocols. The present cortisol isolation procedure requires four simple, inexpensive steps with an inter-assay variability in yield of approximately 5–6%. In practice, 30–40 samples may be extracted in one day. Although only 50% of cortisol is recovered, suitable levels of sensitivity and reproducibility were achieved in the low physiological range of cortisol concentrations (6–10 μg/dl) and with concomitant low level enrichments of deuterated cortisol (3–6%). Additionally, TLC of plasma concentrates and selected ion monitoring of recovered cortisol and deuterated cortisol show no appreciable contamination by any interfering compound. Thus, 1-ml samples of plasma may be used to generate reproducible measurements of isotope enrichments within the low physiological concentration range of cortisol. At isotope enrichments of 3–6% the calculated concentration of deuterated cortisol is subphysiological (0.2–0.5 μg/dl), no interference by the deuterated steroid on the sensitive hypothalamic–pituitary–adrenal feed-back system would be anticipated.

The present method employs electron-impact GC–MS (EI–GC–MS) and may find more widespread application than liquid chromatography or thermospray mass spectrometry since EI–GC–MS is more common in clinical use. The current scheme requires no derivatization step and therefore is less time consuming and more cost effective. The ability to use small plasma volumes makes frequent sampling feasible in adults and children. The use of stable isotopes will make the application of isotope dilution studies safe for use in children and in pregnant women.

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