Comparison of Adrenocorticotropin (ACTH) Stimulation Tests and Insulin Hypoglycemia in Normal Humans: Low Dose, Standard High Dose, and 8-Hour ACTH-(1–24) Infusion Tests*

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ABSTRACT

The efficacy of the standard high dose ACTH stimulation test (HDT), using a pharmacological 250-μg dose of synthetic ACTH-(1–24), in the diagnosis of central hypoadrenalism is controversial. The insulin hypoglycemia test is widely regarded as the gold standard dynamic stimulation test of the hypothalamo-pituitary-adrenal (HPA) axis that provides the most reliable assessment of HPA axis integrity and reserve. Alternatively, a prolonged infusion of ACTH causes a continuing rise in plasma cortisol levels that may predict the adrenals’ capacity to respond to severe ongoing stress.

In nine normal subjects, we compared plasma ACTH and cortisol levels produced by three iv bolus low doses of ACTH-(1–24) (0.1, 0.5, and 1.0 μg/1.73 m²; LDTs) with those stimulated by hypoglycemia (0.15 U/kg insulin) and with the cortisol response to a standard 250-μg dose of ACTH-(1–24). The normal cortisol response to an 8-h ACTH-(1–24) infusion (250 μg at a constant rate over 8 h) was determined using three modern cortisol assays: a high pressure liquid chromatography method (HPLC), a fluorescence polarization immunoassay (FPIA), and a standard RIA.

In the LDTs, stepwise increases in mean peak plasma ACTH were observed (124 ± 2.0, 48.2 ± 7.2, 120.2 ± 15.5 pmol/L for the 0.1-, 0.5-, and 1.0-μg LDTs, respectively; P values all < 0.0022 when comparing peak values between tests). The peak plasma ACTH level after insulin-induced hypoglycemia was significantly lower than that produced in the 1.0-μg LDT (69.6 ± 9.3 vs. 120.2 ± 15.5 pmol/L; P < 0.0002), but was higher than that obtained during the 0.5-μg LDT (69.6 ± 9.3 vs. 48.2 ± 7.2 pmol/L; P < 0.02). In the LDTs, statistically different, dose-dependent increases in peak cortisol concentration occurred (355 ± 16, 432 ± 13, and 482 ± 23 nmol/L; greatest P value is 0.0283 for comparisons between all tests). The peak cortisol levels achieved during the LDTs were very different from those during the HDT (mean peak cortisol, 580 ± 27 nmol/L; all P values < 0.00009).

However, the mean 30 min response in the 1.0-μg LDT did not differ from that in the HDT (471 ± 22 vs. 492 ± 22 nmol/L; P = 0.2). In the 8-h ACTH infusion test, plasma cortisol concentrations progressively increased, reaching peak levels much higher than those in the HDT [995 ± 50 vs. 580 ± 27 nmol/L (HPLC) and 1326 ± 100 vs 759 ± 31 nmol/L (FPIA)]. Significant differences in the basal, 1 h, and peak cortisol levels as determined by the three different assay methods (HPLC, FPIA, and RIA) were observed in the 8-h infusion tests. Similarly, in the HDTs there were significant differences in the mean 30 and 60 min cortisol levels as measured by HPLC compared with those determined by FPIA.

We conclude that up to 30 min postinjection, 1.0 μg/1.73 m² ACTH-(1–24) stimulates maximal adrenocortical secretion. Similar lower normal limits at 30 min may be applied in the 1.0-μg LDT and the HDT, but not when lower doses of ACTH-(1–24) are administered. The peak plasma ACTH level produced in the 1.0-μg LDT is higher than that in the insulin hypoglycemia test, but is of the same order of magnitude. The peak cortisol concentration obtained during an 8-h synthetic ACTH-(1–24) infusion is considerably higher than that stimulated by a standard bolus 250-μg dose, potentially providing a means of evaluating the adrenocortical capacity to maintain maximal cortisol secretion. Appropriate interpretation of any of these tests of HPA axis function relies on the accurate determination of normal response ranges, which may vary significantly depending on the cortisol assay used. (J Clin Endocrinol Metab 84: 3648–3655, 1999)

HYPOTHALAMO-pituitary-adrenal (HPA) axis dysfunction is a potentially life-threatening condition. It is of paramount importance that safe, reliable diagnostic tests be available to identify patients at risk for adrenal insufficiency. ACTH stimulation testing was originally developed using purified hormone extracted from animal pituitaries (1). Before reliable ACTH assays were available, performing 8-h infusions of ACTH on 3 consecutive days with measurement of urinary steroid metabolites (2) or plasma 17-hydroxycorticosteroids (3) was established as the best method of differentiating between primary and secondary adrenal insufficiency. More rapid tests were quickly introduced after the development of plasma cortisol assay methods (4) and the synthesis of ACTH-(1–24) (5, 6). The short ACTH stimulation test, using the standard dose of 250 μg ACTH-(1–24) but with varying routes of administration and sampling times, has been widely recommended as a convenient and reliable screening test for the diagnosis of primary or secondary hypoadrenalism (7–13). Quantifying the adrenal response to an 8-h infusion of synthetic ACTH, however, remains a valu-
able test of adrenocortical reserve that may more accurately reflect the degree of cortisol secretion required in response to severe stress (14, 15).

Several recent studies and a review of the performance characteristics of the high dose (250 μg) ACTH stimulation test (HDT) question its efficacy in the setting of secondary adrenal insufficiency (16–20). The accepted gold standard test of HPA axis function is the insulin hypoglycemia test (IHT) (21). Inducing hypoglycemia provides a potent stimulus for cortisol secretion via hypothalamic and pituitary activation (22), mimicking the effects of a major physical or psychological stress. Significant numbers of patients have been described who had a normal cortisol response to a bolus 250–μg dose of ACTH-(1–24) and yet had clearly subnormal responses to either the IHT (16, 17) or other tests designed to assess the integrity of the entire HPA axis, such as the overnight metyrapone test (18) or the glucagon stimulation test (19).

Peak plasma ACTH concentrations exceed 22,000 pmol/L after the administration of 250 μg synthetic ACTH-(1–24) (23), levels at least 2 orders of magnitude greater than those measured during major physical stresses such as severe sepsis or multiple trauma or during insulin-induced hypoglycemia (24). Graybeal and Fang (25) showed that very much lower doses of exogenous ACTH were sufficient to cause ACTH and cortisol levels similar to those stimulated by insulin hypoglycemia and suggested that administration of more physiological ACTH doses may improve diagnostic accuracy. Maximal short term cortisol secretion can be achieved with ACTH-(1–24) doses much lower than 250 μg; however, the dose suggested has varied in different studies (26–31). Low dose ACTH-(1–24) testing (using 1 μg) may be more sensitive than the standard 250-μg test in the detection of HPA axis dysfunction in patients with pituitary disease (31, 32) or using inhaled steroids (26, 30).

The purpose of this study was to determine the lowest ACTH-(1–24) dose that would stimulate a maximal acute cortisol response in normal subjects by performing three different low dose tests and comparing these with the standard 250-μg test. ACTH concentrations produced by the low dose tests (LDT) were measured using a sensitive RIA method to enable correlation with predicted ACTH levels and comparison with the ACTH response to insulin-induced hypoglycemia. In addition, we aimed to define the normal adrenal response to an 8-h ACTH-(1–24) infusion using three different cortisol assay techniques, i.e. a high pressure liquid chromatography (HPLC) method, a RIA performed in-house, and a fluorescence polarization immunoassay (FPIA) performed by a large commercial laboratory.

Subjects and Methods

Subjects

Nine healthy subjects, seven males and two females, aged 28–51 yr (mean, 38 yr), participated in this study. The subjects all had normal medical histories and physical examinations, were nonsmokers, and had never received any type of glucocorticoid therapy. No subject had any contraindications to the performance of an IHT, such as a history of cardiovascular or cerebrovascular disease or epilepsy. The oldest subject (51 yr) completed a standard exercise stress test to ensure satisfactory cardiovascular status. Serum chemistries, full blood counts, erythrocyte sedimentation rate, static anterior pituitary function tests, and electrocardiograms were all within normal limits. Mean body surface area (calculated using a height-weight nomogram) was 1.99 m² (range, 1.83–2.15 m²). Each subject underwent six tests. The LDTs were performed a minimum of 48 h apart, the 8-h infusions and IHTs were separated from all other tests by a minimum of 5 days.

Testing protocols

All tests were performed in a random order and single blinded. The subjects were free of medication for at least 4 weeks and abstained from alcohol and caffeine for at least 24 h before each test. The LDTs and HDTs were performed in the early to late afternoon, a time of low basal secretory activity for ACTH and cortisol. The subjects were fasted for 3 h before the insertion of two forearm venous cannulas (at least 45 min before injection), one for drug administration and the other with a three-way tap to allow blood sampling and volume replacement with isotonic saline. An ACTH-(1–24) solution was prepared freshly for each LDT by injecting 250 μg ACTH-(1–24) (Synacthen, Ciba-Geigy, Basel, Switzerland) into a 1000-mL bag of 5% dextrose, resulting in a concentration of 0.25 μg/mL. The appropriate dose (standardized for a body surface area of 1.73 m²) was withdrawn shortly before iv injection. Each subject underwent three separate tests with the following doses: 0.1, 0.5, and 1.0. Blood samples for plasma ACTH and cortisol measurements were collected at −15, 0, 2 (ACTH only), 5, 10, 15, 20, 30, 45, and 60 min. The HDT was performed using a standard protocol; the dose was injected iv at 0 min, blood sampling was performed at each time point (0, 20, 30, 45, and 60 min) for cortisol measurement by HPLC, separate samples were collected at 0, 30, and 60 min for cortisol measurement by FPIA.

The IHTs were commenced at 0800 h after an overnight fast with bilateral cannulas insertion as described above. Blood samples for cortisol and ACTH determination were collected at 15-min intervals from 15 min before to 120 min after injection of 0.15 U/kg soluble insulin (Actrapid, Novo-Nordisk, Copenhagen, Denmark). Blood glucose was measured at 5-min intervals from 0 min to ensure nadir detection. All of the tests were satisfactory according to standard criteria, with all subjects experiencing hypoglycemic symptoms and achieving blood glucose levels below 1.5 mmol/L (range, 0.4–1.4 mmol/L; mean nadir, 0.9 mmol/L). No test complications occurred.

The ACTH-(1–24) infusion test was also commenced at 0800 h, but no fasting before or during the test was required. ACTH-(1–24) (250 μg Synacthen, Ciba-Geigy) was injected into a 500-mL bag of normal saline and infused at a constant rate over 8 h. Blood sampling was performed hourly for cortisol measurement by HPLC, RIA, and FPIA (n = 6), with additional samples at 0, 1, 7, and 8 h for plasma ACTH measurements.

Blood pressure and heart rate were recorded at each blood sampling time point in every test by a Dinamap (Critikon, Tampa, FL) automatic monitor. An observer, who noted the subject’s condition and the occurrence of any side-effects, was in attendance throughout each test, and all IHTs were also directly supervised by a medical practitioner. The study protocols were approved by the University of Queensland human ethics committee and the Greenslopes Private Hospital ethics committee.

Hormone assays

ACTH was measured in unextracted plasma by RIA, using a polyclonal anticorticotropin serum, IgG-ACTH1–41 (IgG Corp., Nashville, TN), which is directed at the ACTH-(5–18) sequence (33). All assays were performed in duplicate. The routine detectable concentration of ACTH was 1.1 pmol/L, and the inter- and intraassay coefficients of variation were 7.8% and 3.7%, respectively, at 7.7 pmol/L (n = 7).

Cortisol was measured in extracted plasma by a previously described HPLC method (34), using prednisolone as the internal standard and extraction with ether-dichloromethane (60:40). All assays were performed in duplicate. Recovery was 95–100% for cortisol and prednisolone. The limit of detection of the assay was 30 nmol/L. The inter- and intraassay coefficients of variation at 165 nmol/L were 6.2% and 4.5%, respectively (n = 7).

Additional samples were collected at each time point in the 8-h infusion tests (n = 8) and the 0, 30, and 60 points in the HDT for cortisol measurement by a commercial laboratory in unextracted plasma by a FPIA (TDX, Abbott Laboratories, North Chicago, IL) using a polyclonal
antibody and fluorescein tracer. Interassay coefficients of variation were 10%, 4.5%, and 3.6%, at 98, 600, and 980 nmol/L, respectively (n = 200).

In the 8-h infusion tests, cortisol was also measured in unextracted plasma using a RIA kit assay (Amerlex, Ortho-Clinical Diagnostics, UK) performed in-house. The intraassay coefficients of variation were 5.7%, 4.3%, and 5.8% at 55, 225, and 707 nmol/L, respectively. The interassay coefficients of variation were 8.9%, 7.7%, and 8.0% at 61, 229, and 742 nmol/L, respectively.

**Statistical methods**

Results are expressed as the mean ± se. Statistical significance was taken as P < 0.05. Comparisons of mean hormone levels between and within tests were performed by between- and within-subject ANOVA with repeated measures. Derived parameters [mean peak change and area under the curve (AUC)] were compared by two-way ANOVA with planned comparisons. Comparisons of mean basal and peak cortisol levels obtained using the different assay methods were performed by paired t tests (dependent variables). All statistical analyses were performed using the software package Statistica (Statsoft, Tulsa, OK).

**Results**

**LDTs vs the HDT and IHT**

The results from these five tests are shown in Table 1. Mean basal ACTH levels were similar in all of the tests (P = 0.58). Basal cortisol levels were higher before the IHT, a test performed in the morning. In the LDTs, stepwise increases in mean peak plasma ACTH occurred as the dose of administered ACTH-(1–24) increased. The mean peak plasma ACTH concentrations following each dose were significantly different compared with those at all other doses (P < 0.0023 for all). Statistical analyses using the derived parameters of increase from basal (delta) and the AUC were very similar, with statistically significant differences for all comparisons between the different LDTs: P < 0.0022 for all Δ values, and P < 0.00018 for all AUCs (data not shown). Direct comparison with the ACTH levels achieved after hypoglycemia is shown in Fig. 1. As shown in Table 1, the mean peak ACTH concentration after a hypoglycemic stimulus was significantly higher than that during the 0.5-μg LDT (P < 0.02), but lower than that during the 1.0-μg LDT (P < 0.0002). As expected, the duration of the increase in plasma ACTH during the IHT was much greater than that in any of the low dose tests due to the ongoing stimulation of ACTH release. Measured peak ACTH levels in the LDTs correlated closely with predicted plasma ACTH levels calculated using approximated plasma volumes (Fig. 2).

All four synthetic ACTH doses achieved significantly different peak cortisol concentrations (P < 0.02 for all). The cortisol responses to the low doses were of shorter duration, with levels declining at 60 min, whereas in the HDT a continuing rise occurred (Fig. 3). Peak cortisol levels measured by HPLC during the HDT and IHT were very similar (58 ± 27 vs. 576 ± 33 nmol/L; P = 0.9). The mean cortisol level at 30 min during the 1.0-μg LDT was not statistically different from the mean 30 min cortisol level in the HDT (471 ± 22 vs. 492 ± 22 nmol/L; P = 0.2). The ranges of cortisol levels measured at 30 min were also similar in these two tests (370–580 vs. 399–602 nmol/L for the 1.0-μg LDT and the HDT, respectively). The mean cortisol levels at 30 min in the 0.1- and 0.5-μg LDT were significantly different from those in the HDT (279 ± 19 nmol/L; P = 0.00033 and 436 ± 18 nmol/L; P = 0.0254, respectively). The ranges of cortisol responses at 30 min in these two tests were also much lower

![Fig. 1. Plasma ACTH levels after iv administration of low dose ACTH (1–24) (0.1, 0.5, and 1.0 μg/1.73 m²) and insulin-induced hypoglycemia.](https://academic.oup.com/jcem/article-abstract/84/10/3648/2660644/1)

**TABLE 1.** Plasma ACTH concentrations and cortisol (measured by HPLC) responses during three different low dose ACTH-(1-24) stimulation tests (all doses per 1.73 m²), the standard 250-μg high dose test (HDT), and the insulin hypoglycemia test (IHT)

<table>
<thead>
<tr>
<th>Test</th>
<th>ACTH (pmol/L) Basal</th>
<th>Cortisol (nmol/L) Basal</th>
<th>ACTH (pmol/L) Peak</th>
<th>Cortisol (nmol/L) Peak</th>
<th>30 min cortisol level (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μg</td>
<td>3.0 ± 0.4</td>
<td>138 ± 15</td>
<td>12.4 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>355 ± 16&lt;sup&gt;a&lt;/sup&gt; (263–410)</td>
<td>279 ± 19 (171–393)</td>
</tr>
<tr>
<td>0.5 μg</td>
<td>2.6 ± 0.5</td>
<td>120 ± 12</td>
<td>48.2 ± 7.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>432 ± 13&lt;sup&gt;b&lt;/sup&gt; (359–473)</td>
<td>435 ± 17 (320–493)</td>
</tr>
<tr>
<td>1.0 μg</td>
<td>2.8 ± 0.5</td>
<td>155 ± 17</td>
<td>120.2 ± 15.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>482 ± 23 (370–580)</td>
<td>471 ± 22 (370–580)</td>
</tr>
<tr>
<td>HDT</td>
<td>Not measured</td>
<td>186 ± 22</td>
<td>Not measured</td>
<td>580 ± 27 (490–706)</td>
<td>492 ± 22 (399–602)</td>
</tr>
<tr>
<td>IHT</td>
<td>2.5 ± 0.6</td>
<td>205 ± 20</td>
<td>69.6 ± 9.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>576 ± 32 (449–746)</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM. Response ranges are contained in parentheses.

<sup>a</sup> P < 0.0023 for all comparisons between low dose tests.
<sup>b</sup> P < 0.00002 vs. 0.5 μg; P < 0.00009 vs. 1.0 μg.
<sup>c</sup> P < 0.0283 vs. 1.0 μg.
<sup>d</sup> P < 0.02 vs. 0.5 μg; P < 0.0002 vs. 1.0 μg.
LOW DOSE, HIGH DOSE, AND 8-h ACTH TESTS

Fig. 2. Correlation between measured (peak) and predicted ACTH levels after the administration of three low doses of ACTH-(1–24). Predicted levels were calculated by assuming that 0.1–1.0 μg ACTH-(1–24) was distributed in 3 L plasma (allowing for a mol wt of 2853.5 for ACTH-(1–24) compared with a mol wt of 4541.4 for ACTH (1–39)).

than those in the HDT (171–393 and 320–493 nmol/L for the 0.1 and 0.5 μg LDTs, respectively).

**HDT and 8-h infusion**

Mean basal ACTH before the 8-h infusion tests was 2.8 ± 0.4 pmol/L, a level similar to that measured before every other test (P = 0.78). Mean plasma ACTH concentrations at 1, 7, and 8 h were 289.2 ± 20.7, 234.6 ± 16.1, and 243.8 ± 20.6 pmol/L, respectively (n = 8).

The mean basal cortisol level in the 8-h infusion tests as measured by HPLC was significantly lower than that determined by either RIA or FPIA: 200 ± 15 vs. 240 ± 17 (P < 0.017) and 222 ± 14 (P < 0.024) nmol/L, respectively. There was no difference between the mean basal cortisol levels as measured by RIA and FPIA (P = 0.75). At the 1 h point after commencing the infusion, the mean cortisol level as measured by HPLC was similar to that measured by RIA (620 ± 24 vs. 646 ± 24 nmol/L; P = 0.48), but was significantly lower than when measured by FPIA (620 ± 24 vs. 767 ± 34 nmol/L; P < 0.0001). The 1-h cortisol measurement by RIA was significantly lower than by FPIA, P < 0.04. The mean peak cortisol level as determined by HPLC was lower than when measured by RIA (995 ± 50 vs. 1122 ± 48 nmol/L), although it did not reach statistical significance (P = 0.07). It was significantly lower than when measured by FPIA (995 ± 50 vs. 1377 ± 87 nmol/L; P < 0.001). The mean peak cortisol level measured by RIA was significantly lower than that determined by FPIA (P = 0.031). The lowest individual peak cortisol levels (different subjects for each assay method) were 804 (HPLC), 897 (RIA), and 1129 (FPIA) nmol/L (Fig. 4).

In the HDTs, mean basal cortisol levels measured by HPLC or FPIA were similar: 186 ± 22 vs. 203 ± 25 nmol/L (P = 0.65). At each subsequent time point, however, mean cortisol levels determined by HPLC were significantly lower than those measured by FPIA: at 30 min, 493 ± 22 vs. 609 ± 30 nmol/L (P < 0.004); and at 60 min (mean peak level), 580 ± 22 vs. 759 ± 31 nmol/L (P < 0.0001). The lowest individual peak cortisol levels (different subjects for each assay method) were 490 (HPLC) and 618 (FPIA) nmol/L.

**Discussion**

Confirmation of the diagnosis of central adrenocortical insufficiency is essential, both in patients who present with suggestive symptoms as well as in those known to be at risk because of concurrent disease, such as pituitary tumor. The IHT is widely regarded as the gold standard dynamic test of HPA axis function and is considered to simulate physiological stresses (14, 21). Unfortunately, this test is costly, unpleasant, and contraindicated in certain patients (e.g., those with cerebrovascular or cardiovascular disease), thus limiting clinical use (21). The metyrapone test, which relies on intact pituitary feedback mechanisms, is also a sensitive test of HPA axis function (18, 31, 35–37), but it requires access to an 11-deoxycortisol assay and may precipitate acute adrenal insufficiency (15, 21). ACTH stimulation tests provide an indirect assessment of hypothalamic and pituitary function, relying on the detection of adrenocortical atrophy secondary to ACTH deficiency. Despite this, the potential advantages of safety and convenience continue to make ACTH testing an attractive method for screening patients with suspected secondary hypoadrenalism.

In the early studies that examined the effects of exogenous
ACTH in humans, multiple ACTH infusions lasting several hours or continuous infusions over a number of days were the protocols of choice (2, 3, 38). This enabled the adrenal response to be quantified by the measurement of increased levels of either urinary or plasma cortisol metabolites (2, 3). Administration of ACTH as a prolonged infusion was recognized as a means of evaluating the adrenal glands’ capacity to achieve a sustained response after exhaustion of immediately available hormone stores (3) as well as differentiating between primary and secondary hypocortisolism (2). The synthesis of an equally bioactive form of ACTH (39) and the development of methods for determining plasma cortisol levels (4) simplified the assessment of adrenocortical function. The short ACTH-(1–24) test was first introduced by Wood et al. (5) and Greig et al. (6), who measured plasma cortisol 30 min after a single im 250-μg dose of ACTH-(1–24) in patients with Addison’s disease and receiving long term glucocorticoid therapy. Speckert et al. (7) then proposed that a short ACTH test was also an accurate screening test in patients with pituitary disease, although in their protocol 250 μg ACTH-(1–24) were given iv, and cortisol was measured 60 min postinjection. A similar conclusion was reached by Kehlet and colleagues, who showed that cortisol concentrations 30 min after an iv injection of 250 μg ACTH-(1–24) were similar to those achieved by insulin-induced hypoglycemia (9, 10) and to cortisol levels measured 1 h after skin incision during major surgical procedures (8). Despite the significant methodological differences in studies assessing the utility of the 250-μg ACTH test (HDT) (5–10), including the route of ACTH-(1–24) administration, the timing of blood samples postinjection, and the control populations tested to establish a reference range, it has been widely accepted as a safe, reliable test and suggested as a potential replacement for the IHT in the investigation of suspected secondary hypocortisolism (13).

However, a normal immediate cortisol response to a bolus 250-μg dose of synthetic ACTH-(1–24) does not necessarily predict the patient’s ability to maintain a continuing response to severe stress (14). Almost 30 yr ago, Plumpton and Besser (40) demonstrated in patients undergoing major surgery that peak cortisol levels (measured using a fluorometric technique) ranged from 607–2069 nmol/L intraoperatively, a response that was significantly greater and more prolonged than that to hypoglycemia. Furthermore, elevated plasma cortisol concentrations and absence of the normal circadian rhythm persist for at least 72 h postoperatively (41). After myocardial infarction, plasma cortisol concentrations are maintained at almost 1000 nmol/L for 12 h and then gradually decreased over a 72-h period (42). In this study, we showed that administration of 250 μg ACTH-(1–24) as a continuous infusion maintains plasma ACTH concentrations of greater than 230 pmol/L. This is higher than ACTH levels measured during severe physical stresses, such as major sepsis or multiple trauma (24), therefore achieving ongoing maximal adrenocortical stimulation. As expected, plasma cortisol levels progressively increased during the infusion, resulting in peak concentrations that were much higher than those at 60 min in the HDT, i.e. mean peak cortisol 995 vs. 580

![Graph](image-url)
nmol/L (HPLC) or 1377 vs. 759 nmol/L (FPIA). The peak cortisol responses in the 8-h infusion test ranged from 804-1245 nmol/L (HPLC), 896-1306 nmol/L (RIA), and 1129–1920 nmol/L (FPIA). The normal peak response during an 8-h infusion test has been previously defined as greater than 1062 nmol/L (cortisol measured by RIA) (14). Using a similar normal criterion (>1080 nmol/L), Streeten et al. (15) reported two patients with convincingly normal responses to a standard 250 µg ACTH-(1–24) test (cortisol at 60 min, 756 and 728 nmol/L) whose responses to an 8-h infusion were subnormal (peak cortisol, 690 and 1008 nmol/L, respectively). Neither patient underwent an IHT, but both subsequently had abnormal metyrapone tests and showed significant clinical improvement with steroid replacement (15). In patients with suspected secondary hypoadrenalism, the 8-h ACTH infusion test remains an indirect method of assessing pituitary or hypothalamic function. However, it provides a valuable estimate of the adrenal capacity for sustained cortisol secretion that may more accurately reflect the cortisol levels required in response to severe stress. This sustained response may be impaired in patients with partial adrenal atrophy who are still able to respond acutely to a high bolus dose of ACTH-(1–24).

The discrepancy between the results obtained in the 8-h infusion test when using three different cortisol assay methods deserves specific comment. Definitions of normal response criteria are critical in the interpretation of dynamic tests of the HPA axis. There has been extensive debate during the last decade, for example, regarding the appropriate cut-off for a normal response in the standard short ACTH stimulation test (13, 16, 20, 43–45). The normal response values quoted in the literature are frequently derived from studies using methods such as the fluorometric assay (4) that include measurements of both corticosterone and cortisol. Fluorometric cortisol assays have been reported as showing a 20–30% positive bias compared with various RIAs (46) or a mean positive bias of 96 nmol/L (19). Accordingly, in some studies measuring cortisol by RIA (19, 32) cut-off values were adjusted, although new normal response criteria specific for the RIA used were not determined. It is not always appreciated that cortisol measurements vary significantly among the many different immunoassays now available. De Brabandere et al. (47) compared three routinely used cortisol immunoassay kits (a fluorometric enzyme immunoassay and two RIAs) with results obtained by isotope dilution gas chromatography-mass spectrometry (ID GC-MS) [a recognized reference method for cortisol measurement (46)] in a panel of 15 patients’ sera that encompassed a wide range of cortisol concentrations. All three immunoassays gave varying results that deviated significantly from those obtained by ID GC-MS (47). Across a wide range of cortisol concentrations, the mean positive bias ranged from 9.7% (RIA 1) to 49% (RIA 2), with positive bias at low cortisol concentrations as high as 91% (47). In a recent study by Clark et al. (48), 100 normal subjects underwent a standard ACTH stimulation test [250 µg ACTH-(1–24), im] with cortisol measurements performed using 4 different commercially available immunoassays, including the FPIA (TDX, Abbott Laboratories) used in our study. In keeping with our results, significant differences were observed between the cortisol levels obtained using the different immunoassay methods, with less variation being evident in the baseline measurements (48). In this study, the cortisol concentrations measured by FPIA after ACTH-(1–24) stimulation were consistently higher (about 10% and 15%) than those with two of the other immunoassays used. Similarly, we showed that the mean 1 h and peak cortisol levels in the 8-h infusion test as determined by FPIA were significantly higher (18–23%) than when measured by the RIA we used. These method and time-related variations are potentially explained by the release of other adrenocortical steroids in response to ACTH-(1–24) (49, 50) that may affect to a varying extent the results obtained by different immunoassays, either by direct cross-reactivity or by an effect on cortisol release from cortisol-binding globulin (48).

Although more labor-intensive than immunoassays, liquid chromatography methods enable accurate separation and quantification of plasma cortisol even when other steroids and their metabolites are present that may cross-react in an immunoassay (51). Canalis et al. (52) directly compared HPLC with two different RIAs (one performed in-house, the other at a commercial laboratory) in patients undergoing assessment of HPA axis function, including morning cortisol measurements and dynamic tests such as the short ACTH-(1–24) stimulation test, the IHT, and the metyrapone test (52). Cortisol values (baseline or stimulated) obtained by HPLC correlated with those determined by RIA, but were generally lower (between 15–50%); this discrepancy was more pronounced with the RIA performed in the commercial laboratory. The difference between cortisol values obtained by HPLC and RIA became more marked after metyrapone, presumably due to the high circulating levels of 11-deoxycortisol, resulting in increased cross-reactivity in the RIAs (52). In our study, cortisol levels as measured by HPLC were also generally lower than when measured by either RIA or FPIA, with a larger discrepancy being observed between HPLC and FPIA. In both the HDT and the 8-h infusion test the difference between cortisol measurements performed using the various assay methods increased at later time points after ACTH stimulation. This observation is consistent with the hypothesis proposed by Clark et al. (48) that the release of other adrenocortical steroids in response to ACTH-(1–24) may affect the cortisol measurements obtained by different immunoassays, in this case the FPIA more than the RIA. As others have emphasized (53), the interpretation of any test of HPA axis function is highly dependent on an accurate determination of the lower limit of normality. Our study clearly illustrates that this determination is highly assay method dependent and that reference to previously published normal response ranges is not always appropriate.

As early as 1964, while investigating the dose-response characteristics of the newly available synthetic ACTH, Landon and colleagues (54) demonstrated that 3 and 100 µg ACTH-(1–24) infused over 1 h produced similar peak cortisol concentrations at 60 min. In later studies, 250 ng (35) and 400 ng ACTH-(1–24) (55) bolus iv doses stimulated similar acute adrenocortical responses to the 250-µg dose. It was clearly recognized that the standard HDT employed a pharmacological dose of synthetic ACTH, but in these early studies this was not believed to reduce the value of the test (56). In 1985, Graybeal and Fang (25) reintroduced the concept of physi-
them. The mean cortisol levels at 30 min in the 1.0-
gave a similar cortisol response equivalent to that of the 250-μg dose was achieved by 5 μg, but not 1.0 μg, ACTH-(1–24).
Dickstein et al. (26) studied 2 groups of 10 normal volunteers and showed that at 30 min, there was no difference in cortisol levels after bolus injections of 250, 5, or 1.0 μg ACTH-(1–24). Weinroth et al. (57) obtained similar results in children using 1.0 μg/1.73 m² synthetic ACTH.

Other investigators have suggested that a dose of 0.5 μg (variously adjusted for body surface area) stimulates a similar peak cortisol level compared with 250 μg (29) or achieves equivalent levels at 15 min (27), 20 min (30), or 30 min (23) postinjection. We found that significantly lower peak cortisol levels occurred after all three low doses compared with the 250-μg dose. This reflects the sustained increase in plasma cortisol concentrations at 60 min during the HDT, whereas the cortisol responses to lower doses were of shorter duration. The mean cortisol levels at 30 min in the 1.0-μg LDT and the HDT were not significantly different, and the ranges of cortisol response measured at 30 min in these 2 tests were also similar. These results provide strong evidence that a dose of 1.0 μg/1.73 m² synthetic ACTH-(1–24) stimulates maximal adrenocortical secretion for 30 min postinjection. In the 0.1- and 0.5-μg LDTs, however, the mean 30 min cortisol levels and ranges were significantly lower. As others have emphasized (23), these results provide further evidence that different lower normal limits for the 30 min cortisol response must be applied when interpreting low dose ACTH stimulation tests if using a dose less than 1.0 μg/1.73 m² synthetic ACTH-(1–24).

Graybeal and Fang (25) evaluated the ACTH and cortisol responses to insulin hypoglycemia in a group of normal subjects and then infused three low doses of ACTH-(1–24) (1, 0.2, and 0.05 μg/kg) over 10 min on separate occasions in an attempt to mimic a physiological HPA axis response. Mean peak ACTH concentrations at the end of the 0.2 μg/kg infusion and 5 min later were higher than the mean peak ACTH levels during the IHTs (25). However, the integrated ACTH concentrations (AUC) and integrated cortisol responses were similar (25). In our study, lower doses of ACTH-(1–24) were administered as bolus injections. Although the tests produced peak ACTH levels on the order of 1.0 μg LDT > IHT > 0.5 μg LDT (120, 2, 69.6, and 48.2 pmol/L, respectively), the considerably greater duration of ACTH stimulation in the IHT resulted in a 7-fold greater integrated ACTH response in this test than in the 1.0-μg LDT (30008.8 vs. 416.3 pmol/minL). Administration of bolus doses of exogenous ACTH-(1–24), even in very low doses, does not produce a similar pattern of change in ACTH concentration to insulin hypoglycemia even though the peak plasma ACTH levels produced are of the same order of magnitude.

Concerns have been raised regarding the possibility of error when administering such low doses of ACTH-(1–24) (58); potential problems include incomplete injection and binding of ACTH to plastic surfaces. Currently, a two-stage procedure remains necessary, reflecting the availability of ACTH-(1–24) in 250-μg amounts only. Dickstein et al. (26) and Tordjman et al. (31) used similar protocols; a prediluted solution of ACTH-(1–24) was kept refrigerated, and then a further dilution was performed before each test. We prepared a fresh solution of 250 μg ACTH-(1–24) in 1000 mL 5% dextrose before each test, a protocol similar to that used by Crowley et al. (27, 28), and then withdrew the appropriate dose adjusted for body surface area shortly before injection. The incremental rises in measured ACTH levels with increasing doses of ACTH-(1–24) and the close correlation with predicted plasma levels confirm the robust accuracy of this technique.

In the past, the standard ACTH stimulation test using 250 μg ACTH-(1–24) has been accepted as a convenient and reliable test despite evidence to the contrary. This pharmacological dose of ACTH-(1–24) provides a grossly supra-physiological stimulus that may mask partial adrenocortical insufficiency. In agreement with other published data, our study confirms that the much lower dose of 1.0 μg/1.73 m² stimulates maximal adrenocortical secretion in normal subjects up to 30 min postinjection. The cortisol response ranges measured at 30 min (by HPLC) during the 1.0-μg LDT and the HDT are comparable, indicating that a similar lower normal limit may be applied in these two tests, but not when a lower dose of ACTH-(1–24) is used. The peak plasma ACTH levels produced in the LDTs are of the same order of magnitude as those in the IHTs (27, 28), and then withdrew the appropriate dose. The rise in plasma ACTH levels produced in the LDTs are of the same order of magnitude as those in the IHTs (27, 28), and then withdrew the appropriate dose.

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References


