Establishment of reference values for standard dose short synacthen test (250 μg), low dose short synacthen test (1 μg) and insulin tolerance test for assessment of the hypothalamo–pituitary–adrenal axis in normal subjects

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Summary

OBJECTIVE To assess the integrity of the hypothalamo–pituitary–adrenal(HPA) axis, many authors have proposed the short synacthen test ($ACTH_{1-24}$, Tetracosactrin) as a replacement for the insulin tolerance test (ITT). The aim of this study was to compare the plasma cortisol response obtained with both short synacthen tests (high dose (HDT, 250 μg) and low dose (LDT, 1 μg)) with the peak reached during the ITT in healthy volunteers, and to establish the plasma cortisol cut-off level in each test.

SUBJECTS AND METHODS Thirty healthy subjects (16 F, 14 M), mean age 34 years, underwent both short synacthen tests. Twenty healthy subjects, 15 of whom (11 F, nine M) belonged to the above group, mean age 30 years, underwent an ITT. Plasma cortisol was measured using a chemiluminiscence immunoassay.

RESULTS There were no differences between plasma cortisol 30 minutes after both short synacthen tests (HDT: 684 ± 123, LDT: 669 ± 119 nmol/l) and the peaks reached with the LDT (691 ± 123 nmol/l) and the ITT (673 ± 99 nmol/l).The only difference ($P < 0{\cdot}001$) was found in the comparison of plasma cortisol peak reached with the HDT (802 ± 142 nmol/l) with the other tests. Plasma cortisol levels obtained in the 5th percentile in each test were: at + 30 minutes: (HDT: 537, LDT: 489 nmol/l), peak: (HDT 649, LDT 498, ITT: 539 nmol/l).

CONCLUSIONS Comparison of the plasma cortisol response at + 30 minutes with both short ACTH tests and the peak in the insulin tolerance test did not reveal differences. Each test, for each time point and for each biochemical method, requires its own minimum threshold of normality to assess the hypothalamo–pituitary–adrenal axis.

The insulin tolerance test (ITT) is considered the standard reference test for assessing the integrity of the hypothalamo–pituitary–adrenal (HPA) axis (Plumpton & Besser, 1969; Grinspoon & Biller, 1994). Plasma cortisol responses to hypoglycaemia correlate well with response to surgical stress (Plumpton & Besser, 1969). However, it is well known that this test is unpleasant for the patient, potentially hazardous in patients with coronary heart disease or with a history of seizures, and is not advisable in children and the elderly. In addition, close medical supervision is required, and the test is relatively expensive.

As a result, many authors have examined other tests, including short synacthen ($ACTH_{1-24}$) tests – standard, conventional or high dose (HDT, 250 μg) or low dose (LDT, 1 μg) – metyrapone, naloxone, and the CRH test (Lindholm *et al*., 1978; Lindhom & Kehlet, 1987; Crowley *et al*., 1991; Broide *et al*., 1995; Kane *et al*., 1995; Tordjman *et al*., 1995; Ammari *et al*., 1996; Hurel *et al*., 1996; Orme *et al*., 1996; Wang *et al*., 1996; Mukherjee *et al*., 1997). In most of these tests adequate maximum plasma cortisol response usually varied between 500 to 600 nmol/l for each test, though many of the studies did not include a control group.

Since Lindhom & Kehlet (1987) found a highly significant correlation between the plasma cortisol reached at + 30 minutes during the HDT and the plasma cortisol peak reached during the ITT, the HDT has increasingly gained acceptance as a good substitute for the ITT. However, the test result only reflects the presence or absence of adrenocortical atrophy secondary to corticotrophin insufficiency (Hjortrup *et al*., 1983). Furthermore,

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several researchers have considered that the HDT uses a supraphysiological dose and is not sensitive enough to detect mild degrees of secondary adrenal insufficiency. They report that the HDT underdiagnoses clinically significant adrenal insufficiency, with potentially serious consequences (Borst *et al*., 1982; Cunningham *et al*., 1983; Tsatsoulis *et al*., 1988; Ammari *et al*., 1996; Soule *et al*., 1996; Streeten *et al*., 1996).

In recent years several authors have reduced the dose, demonstrating that the plasma cortisol response to 1 μg is equivalent to that obtained with 250 μg in normal subjects (Oelkers *et al*., 1988; Crowley *et al*., 1991; Daidoh *et al*., 1995; Rasmuson *et al*., 1996; Talwar *et al*., 1998) and that the test is thus able to detect mild adrenal insufficiency. Without modifying the reference test's threshold of stimulated plasma cortisol, Tordjman *et al*. (1995) and Dickstein *et al*. (1991) found that the LDT increased sensitivity for assessment of secondary adrenal insufficiency. However, Mayenknecht *et al*. (1998) established that the diagnostic sensitivity of the tests is the same, provided different thresholds of normality are applied for each test. The lower normal limit of plasma cortisol response varies widely from study to study and this is one cause of discrepancies in sensitivity and specificity. To address this issue we attempted to compare the response of plasma cortisol achieved with both short synacthen tests (HDT and LDT) and with the ITT in healthy volunteers and for each test, to establish the normal cut-off level at which the stimulated plasma cortisol excludes impairment of the HPA axis.

Subjects and methods

Control subjects

Thirty-five healthy subjects – mostly Hospital personnel or patients' family members – were recruited to establish the normal range of plasma cortisol responses to HDT, LDT and ITT. None were taking any medication known to affect the HPA axis. Thirty of them (16 females, 14 males, aged 21–64 years, mean 34 years, body mass index (BMI): 24·8 ± 4·8 kg/m^2), underwent both short synacthen tests in random order. Twenty healthy volunteers, 15 of whom belonged to the above group (11 females, nine males, aged 21–56 years, mean 30 years, BMI 23·1 ± 3·7 kg/m^2) underwent an ITT. All tests were performed in the morning between 0800 and 0900 hours after a fast. The minimum and maximum intervals between tests were 1 week and 1 month, respectively. The study was approved by the hospital's ethical committee; each subject was fully informed and gave written consent.

Test protocol

The synacthen tests were performed in subjects who had been quietly seated in armchairs for 30 minutes An indwelling venous cannula was placed in one forearm and 250 μg Tetracosactrin ($ACTH_{1-24}$) (Synacthen, Novartis, Basel, Switzerland) was injected as a bolus after a basal blood sample had been taken to measure plasma cortisol. Additional blood samples were obtained 30 and 60 minutes after injection. The LDT was also performed in these subjects, at a dose of 1 μg. One ml of the content of the Tetracosactrin ampule (250 μg) was diluted in 49 ml of saline serum; later, 1 ml of this solution was diluted again with 49 ml more of saline serum. Ten ml of the solution thus contained 1 μg of Tetracosactrin. This freshly prepared solution was also injected as a bolus, and blood samples for plasma cortisol measurement were withdrawn at baseline and 30 and 60 minutes postinjection. No adverse effects were reported with either the HDT or the LDT.

The insulin tolerance test was performed in the morning between 0800 and 0900 h. The insulin dose was 0·1–0·15 IU/kg body weight, with sampling at -15, $0, +30$, 45, 60 and 90 minutes The results were accepted if blood glucose levels had dropped below 2·2 mmol/l (40 mg/dl). During the test the capillary glucose was monitored with the glucose–oxidase method (glucocard, Menarini Diagnostics, Kyoto, Japan). All subjects recovered normoglycaemia quickly and spontaneously.

Laboratory methods

For all tests blood samples were immediately centrifuged at 2000 ***g*** at 4 °C for 15 minutes and the resulting plasma was stored at − 20 °C until plasma cortisol measurement.

Plasma cortisol was measured using a direct chemiluminiscence immunoassay (ACS 180, Chiron Diagnostics, Halstead, UK). The intra-assay and interassay coefficients of variation were 5·1–7·0% and 6·4–9·7%, respectively.

Statistical methods

Using the Kolmogorov–Smirnov test, the distribution of plasma cortisol results in the healthy volunteers for each time point was found to be normal. The results are expressed as mean ± standard deviation (m ± SD). However, as their distribution was non-Gaussian, plasma cortisol data were also expressed as medians and percentiles. Statistical analysis was performed by one-way ANOVA. Differences between two tests in subjects were analysed with the paired two-tailed Students' test. Correlations were examined by computing Pearson's correlation coefficients. The highest value of plasma cortisol at + 30 or + 60 minutes in each test was regarded as peak. The plasma cortisol increment was obtained by determining the difference between the peak and the basal plasma cortisol concentration. A *P*-value less than 0·05 was considered significant.

Table 1 Plasma cortisol response at + 30 minutes and peak cortisol with low dose test (LDT), standard dose test (HDT) and peak cortisol with insulin tolerance test (ITT). Results are expressed as 5th, 10th and 50th percentiles

Percentile	Peak ITT	+ 30 minutes HDT	+ 30 minutes LDT	Peak HDT	Peak LDT
5th	539	537	489	649	498
10th	562	564	534	654	534
50th	654	632	649	806	657

Results

There were no differences between the mean basal plasma cortisol levels on any of the three tests. There were no differences between the mean plasma cortisol level reached at + 30 minutes after both short synachten tests and the peaks reached with the LDT and the ITT (at + 30 minutes with HDT: 684 ± 123 nmol/l and LDT: 669 ± 119 nmol/l; peak with LDT: 691 ± 123 nmol/l and ITT: 673 ± 99 nmol/l. $P > 0{\cdot}87$). The only difference ($P < 0{\cdot}001$) found was between the peak plasma cortisol with the HDT (802 ± 142 nmol/l) and the other tests. Likewise, the plasma cortisol mean increment after the HDT (439 ± 127 nmol/l) was significantly higher ($P < 0{\cdot}001$) than the increment after the other two tests (LDT: 311 ± 117; ITT: 316 ± 131 nmol/l).

The plasma cortisol peak with the HDT was obtained in 100% of cases at + 60 minutes In contrast, with the LDT the peak was reached at + 30 minutes in 23 subjects, and at + 60 minutes in only seven.

The correlation coefficients between plasma cortisol levels were as follows: between + 30 minutes with HDT and the peak with ITT $r = 0{\cdot}51$ $P < 0{\cdot}004$; between + 30 minutes with LDT and the peak with ITT $r = 0{\cdot}11$ $P = 0{\cdot}65$; between + 30 minutes with both short synachten tests $r = 0{\cdot}49$ $P < 0{\cdot}006$.

There was no statistically significant correlation regarding sex and age at any point in any of the tests (data not shown). The only significant correlation was between plasma cortisol response and the weight of healthy volunteers at certain points with the LDT (at + 30 minutes, $r = -0{\cdot}37$ $P = 0{\cdot}039$; at 60 min, $r = -0{\cdot}53$, $P = 0{\cdot}002$; peak, $r = -0{\cdot}42$, $P = 0{\cdot}02$). There were no correlations between the other tests at any point and the basal plasma cortisol with the LDT.

The plasma cortisol responses, expressed as median and 10th and 5th percentiles, are shown in Table 1. The plasma cortisol level values in the 10th percentile with LDT at + 30 minutes and the values in the 5th percentile at the peak during ITT and at + 30 minutes in HDT were very similar. Figure 1 shows the distribution of plasma cortisol results in our healthy subjects expressed as median and different percentiles.

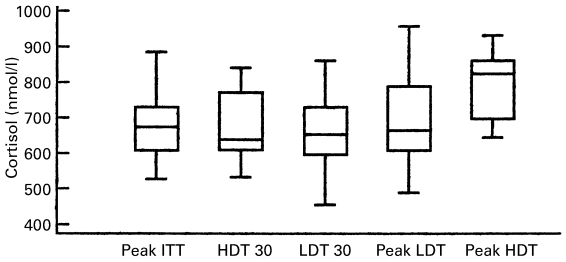

Fig. 1 Box plots showing median values, together with the 5th, 25th, 50th, 75th and 95th percentiles of plasma cortisol level for peak with insulin tolerance test (PEAK ITT), at + 30 minutes with standard and low dose synacthen test (+ 30 MIN HDT, + 30 MIN LDT), and peak with standard and low dose synacthen test (PEAK HDT and PEAK LDT).

Table 2 shows that the proportion of falsely subnormal HPA responses varies according to the test and time point used. The percentage of falsely subnormal responses at the cut off level of 500 nmol/l with the LDT at both times (peak, + 30 minutes), was very similar to the percentage of falsely subnormal responses using a cut off of 550 nmol/l with the ITT and at + 30 minutes in the HDT.

Discussion

The comparisons of the mean plasma cortisol levels obtained by each test in our healthy volunteers are in agreement with those of other researchers in patients with pituitary disease without HPA impairment. Lindholm & Kehlet (1987) carried out a

Table 2 Percentage of falsely subnormal responses calculated for our series of healthy subjects at the different cut-off levels (500, 550, 600 nmol/l) habitually used in the literature at + 30 minutes and peak with low dose test (LDT), standard dose test (HDT) and peak to insulin tolerance test (ITT)

Cortisol	Peak ITT	+ 30 minutes HDT	+ 30 minutes LDT	Peak HDT	Peak LDT
≥ 500	0%	0%	7%	0%	7%
≥ 550	5%	7%	13%	0%	13%
≥ 600	10%	17%	30%	0%	23%

study in a group of patients with pituitary diseases in whom, in most cases, the HPA axis was preserved. In those patients, the authors found no differences between the mean plasma cortisol level at + 30 minutes with the HDT and peak plasma cortisol with the ITT. In another study carried out in children with profound adrenal insufficiency, Weintrob *et al*. (1998) found no differences between the mean plasma cortisol level at + 30 minutes with both short synacthen tests and the plasma cortisol peak during the ITT.

The large differences between the short synacthen tests and the ITT reported in the literature are probably due to the fact that the three tests were compared in patients with mild adrenal gland atrophy (Borst *et al*., 1982; Cunningham *et al*., 1983; Jones *et al*., 1994; Kane *et al*., 1995; Tordjman *et al*., 1995; Ammari *et al*., 1996; Hurel *et al*., 1996; Orme *et al*., 1996; Rasmuson *et al*., 1996; Soule *et al*., 1996; Streeten *et al*., 1996; Mukherjee *et al*., 1997; Bangar & Clayton, 1998 Mayenknecht *et al*., 1998). Mayenknecht *et al*. (1998) consider that this state of partial or mild secondary adrenal insufficiency occurs when the peak plasma cortisol during the ITT is between 500 and 550 nmol/l.

In order to reduce the problems associated with the reported lack of sensitivity of the short synacthen test, the LDT has been proposed as a substitute for the ITT. Tordjman *et al*. (1995) evaluated only the plasma cortisol peak, and arbitrarily applied the same normality threshold (500 nmol/l) for three different $ACTH_{1-24}$ stimuli (1, 5 and 250 μg). Other authors showed that the LDT (1 μg) is the most sensitive test for detecting states of partial adrenal insufficiency in patients with pituitary disease (Dickstein *et al*., 1991; Broide *et al*., 1995; Rasmuson *et al*., 1996; Dickstein *et al*., 1997; Talwar *et al*., 1998; Weintrob *et al*., 1998), though the increased sensitivity of the test is only due to the use of the same threshold for this dose.

However, Mayenknecht *et al*. (1998) found no differences in the plasma cortisol stimulated by the two short synacthen tests. They found the two tests to be equally valid if different normality thresholds were established for each, and observed that both tests identify patients with moderate or severe adrenal insufficiency but not mild degrees of insufficiency.

In our study, peak and incremental cortisol were higher with the HDT than with the other tests (LDT, ITT). Our findings suggest that plasma cortisol at 60 minutes with the HDT should not be used or, at least, should be evaluated with another threshold. This conclusion would invalidate the many studies which consider the same threshold for peak and increment in all tests (Brost *et al*., 1982; Cunningham *et al*., 1983; Tsatsoulis *et al*., 1988; Jackson *et al*., 1994; Jones *et al*., 1994; Kane *et al*., 1995; Tordjman *et al*., 1995; Soule *et al*., 1996; Streeten *et al*., 1996; Ambrosi *et al*., 1998; Talwar *et al*., 1998; Weintrob *et al*., 1998).

In our study, the LDT and the ITT were not well correlated. This may be because the low dose was not adjusted for body weight, or because there may have been a large variability in the dose during handling. The insulin dose in all subjects was adjusted to reach the necessary glycaemic level; the standard dose was supraphysiological and did not require adjustment for body weight. In fact, we only found a relationship between plasma cortisol response and weight in LDT; there was no correlation with weight in the other tests.

In a large group of healthy volunteers, Clark *et al*. (1998) showed that with plasma cortisol stimulated with $ACTH_{1-24}$ administered via intramuscular injection the results varied widely according to the biochemical method used. They found that the value of plasma cortisol at + 30 minutes varied between 510 and 626 nmol/l. For their part, Mayenknecht *et al*. (1998) used the DPC (Diagnostic Products Corp DPC, Llanberis, UK), one of the methods that obtains the highest values of plasma cortisol. Applying the result of mean − 2SD as the normality threshold (although it presented a high standard deviation) they obtained a value around 535 nmol/l at + 30 minutes with the LDT and 618 and 725 nmol/l at minutes 30 and 60 with the HDT. In that study, reference levels were obtained in a group of healthy subjects and a group of hospitalized patients without adrenal disorders. For the ITT, a plasma cortisol peak of 550 nmol/l was applied as a cut-off point. However, the results of Oelkers (1998) for ITT in healthy volunteers showed a mean − 2SD value of 569 nmol/l, below the minimum normal level (599 nmol/l). As in our study, Oelkers obtained very similar minimum values at + 30 minutes with the HDT (596 nmol/l) and with the peak on the ITT. In addition, the lowest range at + 30 minutes with the LDT was lower (483 nmol/l) than with the other two tests. In that study the low dose was adjusted for body surface area. Using the same biochemical method, Ambrosi *et al*. (1998) would not have obtained a similar result with the LDT; their normality threshold (336 nmol/l) would be far below the lowest value obtained in the control group. This result would give an unacceptably low sensitivity. Hurel *et al*. (1996) used a different biochemical method, but in that study the normality threshold can be deduced to be around 390 nmol/l for the HDT. They considered a value of around 520 nmol/l as normal during the ITT when applying confidence intervals, and 600 nmol/l at + 30 minute with HDT, because with this value they obtained a false negative rate of only 10%.

Two recent studies (Bangar & Clayton, 1998; Abdu *et al*., 1999) propose raising the cut-off to 600 nmol/l in order to increase sensitivity with both synacthen tests. We found that this arbitrary value gives rise to a high proportion of falsely subnormal response (up to 30%) with both synacthen tests and therefore many patients with pituitary disease would be administered unnecessary chronic treatment with hydrocortisone.

We agree with Clark *et al*. (1998) that the plasma cortisol results obtained in healthy subjects with both short synacthen tests and with ITT do not have a Gaussian distribution.

Therefore, if we consider the mean − 2SD result as threshold, the plasma cortisol value considered as normal would be far below the lowest value obtained in our group of healthy volunteers. For this reason, we prefer to study the distribution of plasma cortisol results in percentiles, and consider the result obtained in the 5th percentile in each of the three tests and in each of the times as the plasma cortisol cut-off. The cut-off at + 30 minutes with the HDT and at the peak with the ITT would thus be the same (around 540 nmol/l). The cut-off at + 30 minute and at the peak on the LDT would be lower, around 500 nmol/l. The cut-off at the peak on the HDT would be around 650 nmol/l.

We conclude that different minimum normality thresholds should be established for each test, for each point, and for each biochemical method. These data should be applied to a group of patients with pituitary disease to establish the sensitivity and specificity of each test.

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