Defining the normal cortisol response to the short Synacthen test: implications for the investigation of hypothalamic-pituitary disorders

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Summary

OBJECTIVE To define the normal cortisol response to the Short Synacthen Test using four different cortisol immunoassays and to assess the implications for the investigation of hypothalamic-pituitary disorders.

DESIGN AND PATIENTS The cortisol response to 250 μg im $ACTH_{1-24}$ (Synacthen, Ciba Geigy) in 100 healthy volunteers using four different cortisol immunoassays has been measured. In 44 newly diagnosed and untreated patients with pituitary disease, basal and 30 minute post-ACTH cortisol results were also determined using the four immunoassays.

RESULTS The distribution of cortisol results at all time points and for all methods were non-Gaussian and significant differences in the absolute values of the 5th – 95th percentiles were found between methods ($P<0{\cdot}01$). At 30 min post-Synacthen in normals the 5th percentile of the cortisol response ranged from 510 to 626 nmol/l with the different methods. Similarly the relationship between assay results differed at different time points. No effect of age on the cortisol response was found but for stimulated cortisol values and the incremental responses females showed significantly higher responses than males ($P<0{\cdot}05$) for most methods. Although there was a significant positive linear correlation ($P<0{\cdot}001$) between stimulated and basal cortisol values for all methods, no significant relationship was found between the incremental response and basal cortisol values. In the pituitary disease patients basal and 30 minute post-ACTH cortisol results were significantly lower ($P<0{\cdot}05$ and $<0{\cdot}001$) than the control group using the same cortisol assay. When the results were compared to the 5th percentile of the gender and assay specific control group 33·3% of male and 17·4% of female patients failed the Synacthen test at 30 min.

CONCLUSIONS The definition of the 'normal' response to Synacthen should be both gender and method related at all time points. The data suggest that up to one-third of untreated patients with pituitary disease may have subtle defects in the hypothalamic-pituitary-adrenal axis.

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The short Synacthen test (SST) was introduced first in the 1960s as a means of rapidly assessing adrenal function (Wood *et al*., 1965). Based on the premise that the adrenal gland will respond to an exogenous bolus of synthetic ACTH when there is endogenous ACTH reserve, the SST has also been used to assess the integrity of the hypothalamo-pituitary-adrenal (HPA) axis in patients with suspected or established pituitary disease (Lindholm & Kehlet, 1987; Stewart *et al*., 1988) and in patients treated chronically with corticosteroids (Kehlet & Binder, 1973; Kane *et al*., 1995). In both instances the cortisol response to the SST compares favourably with the response to the 'gold standard' of the insulin tolerance test (ITT). As a result many endocrinologists now use the SST as the first line test in documenting function of the HPA axis (Stewart *et al*., 1988; Grinspoon & Biller, 1994; Clayton, 1996), reserving the ITT for patients who fail the SST, or for those who also require an assessment of growth hormone reserve. However, based on the use of pass/fail cut-off values for the cortisol response to the SST and ITT, discrepancies have been reported between the two tests (Borst *et al*., 1982; Ammari *et al*., 1996; Soule *et al*., 1996; Streeton *et al*., 1996), leading some endocrinologists to question the predictive value of the SST. The definition of a 'pass' for the SST is not consistent in clinical practice, at least amongst British endocrinologists (Stewart *et al*., 1988). This may reflect the fact that the definition of a 'pass' response in the literature is derived from studies using the fluorimetric methods for the measurement of free 11-hydroxycorticoids in human

Table 1 Serum cortisol response to Synacthen in healthy volunteers. Results are expressed as median [5th-95th percentile] in nmol/l

Time	0 min	30 mins	60 mins	30–0 min	60–0 min
Method					
TDX	349 [164–870]	811 [626–1431]	972 [766–1655]	488 [289–776]	645 [433–1036]
ACS	352 [195–650]	741 [569–1078]	885 [661–1263]	399 [208–593]	520 [307–724]
Delfia	309 [163–620]	707 [510–1088]	849 [619–1291]	409 [222–641]	544 [329–810]
DPC	391 [200–904]	866 [590–1548]	1047 [722–1830]	494 [222–762]	650 [344–1037]

plasma (Mattingly, 1962; Wood *et al*., 1965) which is likely to give significantly higher results than immunoassays because of the measurement of both cortisol and corticosterone in the fluorimetric assays particularly at high concentrations. Furthermore there has been a proliferation of cortisol immunoassays and significant deviations from isotope dilution gas chromatography-mass spectrometry (GC-MS) results have been described for some methods, differences which are not reflected in different quoted reference ranges (De Brabandere *et al*., 1995). These deviations may reflect differences in assay specificity and calibration.

The aim of this study was to define the normal cortisol response to the SST using four widely available immunoassays. Using the generated 'normal' reference range, the use of the SST in the investigation of the hypothalamic-pituitary-adrenal axis in newly diagnosed pituitary patients was also studied.

Materials and methods

Patients and controls

One hundred normal healthy volunteers on no therapy (67 females, aged 19–63 years, median 25 years) were studied. All subjects rested for 30 min prior to the test. A short Synacthen test was performed between 0900 and 1200 hours by the administration of $ACTH_{1-24}$ im (250 μg, Ciba Geigy). Blood was collected basally and at 30 and 60 min, into plain tubes. The samples were allowed to clot and serum separated and stored at − 20°C, prior to analysis.

In addition, 44 patients (23 females, aged 21–77 years, median 43 years) with newly diagnosed pituitary disease were investigated prior to any form of treatment. Thirteen patients had acromegaly, 18 prolactinomas, 9 nonfunctioning tumours and 4 idiopathic hypopituitarism. Twenty-eight patients had macroadenomas. A short Synacthen test was performed as described above (0900–1200 h) with blood samples collected basally and at 30 min.

The study had the approval of the local ethical committee and all patients and controls gave informed written consent.

Cortisol assays

Serum cortisol was measured by four different commercially available immunoassays. The assays used were chosen to include those widely used by laboratories, both automated and manual assays and those with isotopic and nonisotopic labels. The assays used were: TDX (Abbott Diagnostics, Maidenhead, UK), ACS 180 (Chiron Diagnostics, Halstead, UK), Delfia (Pharmacia Wallac, Milton Keynes, UK), Coat-a-Count (Diagnostic Products Corp DPC, Llanberis, UK). The following interassay imprecision was achieved, given as the coefficient of variation over the stated concentration range:

TDX: less than 8% (106–1099 nmol/l), ACS less than 10%

Table 2 Differences in the distribution of cortisol results and the incremental values for different methods

Methods	Time	*P*	Time	*P*	Time	*P*	Time	*P*	Time	*P*
TDX *vs* DPC	0	NS	30 mins	NS	60 mins	NS	30–0	NS	60–0	NS
TDX *vs* Delfia	0	NS	30 mins	< 0·001	60 mins	< 0·001	30–0	< 0·01	60–0	< 0·01
TDX *vs* ACS	0	NS	30 mins	NS	60 mins	< 0·05	30–0	< 0·01	60–0	< 0·001
DPC *vs* Delfia	0	0·05	30 mins	< 0·001	60 mins	< 0·001	30–0	< 0·001	60–0	< 0·01
DPC *vs* ACS	0	NS	30 mins	< 0·01	60 mins	< 0·001	30–0	< 0·001	60–0	< 0·001
Delfia *vs* ACS	0	NS	30 mins	NS	60 mins	NS	30–0	NS	60–0	NS

Kolmogorov Smirnov test, NS, not significant.

Table 3 Bias ratio of the cortisol assays compared to the ACS method

Method	Bias ratio (mean)	Significance of difference (P) 0 *vs* 30 min	0 *vs* 60 min	30 *vs* 60 min
TDX *vs* ACS				
0 min	1·053	< 0·01		
30 min	1·152		< 0·01	
60 min	1·185			NS
DPC *vs* ACS				
0 min	1·181	NS		
30 min	1·207		NS	
60 min	1·244			NS
Delfia *vs* ACS				
0 min	0·899	< 0·05		
30 min	0·964		< 0·05	
60 min	0·983			NS

(NS, not significant).

(53–993 nmol/l), Delfia less than 8% (76–925 nmol/l), DPC less than 10% (80–1033 nmol/l), respectively. The quoted cross-reactivity of these assays with corticosterone is 6·3, 2·8, 27·7 and 0·9%, respectively. The manufacturers recommended procedures were followed. The TDX assay was used to assay the samples from the pituitary patients.

Statistical methods

The distribution of cortisol results in the healthy volunteers for each time point, analysed by each method showed a non-Gaussian distribution. As a result, cortisol data were expressed as medians and the 5th and 95th percentiles.

The distribution of cortisol results at each time point and the distribution of the differences in cortisol results between time points, i.e. 30 min minus basal and 60 min minus basal, were compared for each assay using the Kolmogorov-Smirnov test with a Bonferroni correction for multiple comparisons.

The relative bias of the methods was studied by arbitrarily choosing one method (ACS) against which to compare the others, thus for each sample a result by a given method was expressed as a ratio to the matched ACS result (bias ratio) and this was calculated for each time point. Comparison of the bias ratio at different time points was performed using the Student's t-test. The Mann–Whitney U-test with correction for ties was used to investigate the effect of age and gender on the cortisol response and to compare the cortisol responses of the newly diagnosed pituitary patients and normal volunteers. The relationship between the 30 and 60 minute responses to the basal value were examined by least squares linear regression analysis.

Results

The distribution of serum cortisol results and the cortisol increments in the control subjects obtained with the four different immunoassays are shown (Table 1). Significant differences in these distributions ($P < 0·01$) were found at each time point between methods (Table 2).

The bias ratios were found to be significantly different between methods and within a method at different time points (Table 3).

Comparing the results for each time point and increment for each method from the healthy volunteers aged less than 40 years with those aged greater than 40 years, revealed no significant differences. Significant differences, however, were

Table 4 Serum cortisol response to Synacthen in healthy volunteers according to gender

Time	Gender	Method TDX	ACS	Delfia	DPC
0 min	M	313 [166–527]	326 [186–578]	309 [164–475]	356 [203–573]
	F	368 [150–884]	352 [194–691]	309 [162–632]	424 [200–935]
30 min	M	750 [585–909]	705 [554–876]	689 [501–900]	786 [605–1040]
	F	871*** [629–1456]	786** [543–1193]	729 [510–1383]	920** [586–1571]
60 min	M	888 [676–1077]	830 [624–998]	814 [553–956]	990 [632–1282]
	F	1066*** [800–1879]	927** [662–1354]	897** [631–1317]	1057** [778–1834]
30–0 min	M	404 [238–552]	350 [120–509]	376 [218–533]	423 [219–698]
	F	509*** [295–808]	406* [193–607]	417 [216–673]	511 [206–773]
60–0 min	M	540 [344–716]	468 [254–660]	481 [221–657]	547 [255–864]
	F	681*** [488–1037]	539** [351–742]	582** [337–830]	676* [375–1037]

Values are given as median, [5th – 95th percentiles], nmol/l. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ *vs* males.

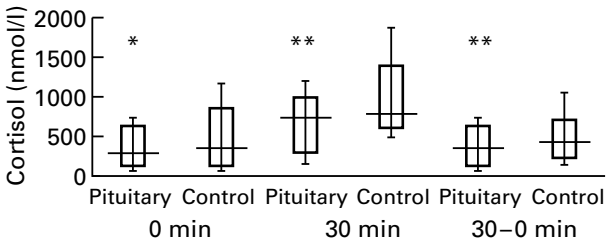

Fig. 1 Distribution of cortisol results in patients with pituitary disease at diagnosis and controls. Results are expressed as median, [5th – 95th percentile] in nmol/l and were obtained using the TDX assay. *$P<0{\cdot}05$, **$P<0{\cdot}01$ compared to the control group.

found for stimulated and incremental values (but not basal values) for all assays between male and female volunteers (Table 4).

A significant positive linear relationship ($P<0{\cdot}001$) was obtained between the results at 30 and 60 min and with the basal cortisol concentrations for all methods (30 mins *vs* 0 min, range for all cortisol assays: slope = 0·836–1·049, intercept = 407–485 nmol/l, $r = 0{\cdot}7113–0{\cdot}8396$, 60 minute *vs* 0 minute, range for all cortisol assays: slope = 0·777–1·162, intercept = 524–662 nmol/l, $r = 0{\cdot}6473–0{\cdot}8050$). No significant correlation was obtained between the incremental values (30–0 min and 60–0 min and the basal cortisol concentration. In the newly diagnosed pituitary patients both basal cortisol (290 [113–650] nmol/l) (median [5^{th}-95^{th} percentile]) and 30 minute cortisol (748 [302–1000] nmol/l) were significantly lower than the control group ($P<0{\cdot}05$ and $P<0{\cdot}001$, respectively, Mann Whitney *U*-test) and this was independent of tumour type and size (Fig. 1). Indeed, compared to their gender matched controls (Table 5) the pituitary patients gave significantly lower results at all time points for females and for the 30 minute post-Synacthen value for males.

Fourteen of the 44 patients (32%) had a 30 minute serum cortisol concentration less than the 5th percentile of the control group with the appropriate assay (< 626 nmol/l). When the results were compared to the 5th percentile of the gender specific control group, 7 of the male patients (33·3%) and 4 of the female patients (17·4%) had a 30 minute cortisol less than the gender and method specific ranges.

Discussion

The use of the short Synacthen test for the investigation of disorders of the hypothalamic-pituitary-adrenal axis has come under increasing scrutiny as comparisons of its diagnostic efficiency with the insulin tolerance test have shown a number of discrepancies (Borst *et al*., 1982; Ammari *et al*., 1996; Soule *et al*., 1996; Streeton *et al*., 1996). It has been recognized that a number of factors may contribute to this situation. Thus, for example, there has been much debate as to whether the peak cortisol value or the cortisol increment should be used, whether the route of administration should be iv or im, the dose of ACTH that should be used and whether the diurnal variation in ACTH/cortisol secretion is of importance. Whilst these issues have been addressed and can be taken into consideration (Grinspoon & Biller, 1994), discrepancies between the SST and ITT may still be found, in patients with Cushing's disease and immediately following a pituitary insult such as surgery or apoplexy when the adrenal glands can still respond to ACTH (Hjortrup *et al*., 1983). This aside, the greatest clinical concern relates to patients who 'pass' the SST but who 'fail' the ITT (Borst *et al*., 1982; Ammari *et al*., 1996; Soule *et al*., 1996; Streeton *et al*., 1996). Central to these studies is the definition of a 'normal' response to Synacthen (and also to the ITT). Early studies using a fluorimetric assay for 11-hydroxycorticoids (Mattingly, 1962) are likely to have overestimated the cortisol

Table 5 Range of cortisol results in patient with pituitary disease at diagnosis

Group	Gender	0 min	30 min	30–0 min
Pituitary	M	290 [110–511]	660* [300–995]	420 [114–720]
Control	M	313 [166–527]	750 [585–909]	404 [238–552]
Pituitary	F	290* [69·5–688]	780** [219–995]	364* [110–608]
Control	F	368 [150–884]	871 [629–1456]	509 [295–808]
Pituitary	M & F	290* [113–650]	748** [302–1000]	373** [112–667]
Control	M & F	349 [164–870]	811 [626–1431]	488 [289–776]
Pituitary				
Micro adenoma	M & F	274 [108–715]	720 [291–1073]	400 [112–575]
Macro adenoma	M & F	290 [85–572]	760 [224–963]	370 [105–713]

Results are expressed as median [5th – 95th percentile] in nmol/l and were obtained using the TDX assay. *$P<0{\cdot}05$, ** $P<0{\cdot}01$ compared with control group.

response to Synacthen because of the nonspecificity of the assays. Despite the development of immunoassays for the measurement of serum cortisol significant method-related variations in the measurement of serum cortisol have been reported. Indeed during 1984, 65% of all laboratories would have more than a 20% positive bias from GC-MS targets, the then reference method (Moore *et al*., 1985). There remain significant method-related differences in measured serum cortisol concentrations despite improvements in calibration and specificity of immunoassays (De Brabandere *et al*., 1995). These differences might well be reflected in different reference ranges for basal cortisol and for the cortisol response to Synacthen and also the ITT.

In this study we have demonstrated that the response of serum cortisol to the SST in healthy volunteers shows a non-Gaussian distribution and significant method related differences. This has been shown for both the basal, stimulated values at both 30 and 60 min post-Synacthen and for the incremental values. The nature of the distribution of cortisol results may not have been noted in earlier studies either because of the small number of subjects studied or the imprecision of the assays used (Stewart *et al*., 1988; Hurel *et al*., 1996) but because of this inappropriate statistical techniques may have led to an incorrect definition of the 'normal' response. The data presented here shows significant differences in the cortisol distributions between methods, particularly at 30 and 60 min post-Synacthen.

In addition to the variation in the distribution of the results with different cortisol assays, the significant differences between the methods shown by the bias ratio do not show consistency at the different time points. This greater method-related difference in specimens taken after Synacthen compared to basal specimens might be explained by the release of steroids other than cortisol in response to Synacthen which affect some of the immunoassays more than others either by direct cross-reactivity or by their effect on the displacement reaction of cortisol from cortisol binding globulin. Synacthen is known to cause the release of other adrenal steroids including 17 α–hydroxyprogesterone, 17 α–hydroxy-pregnenolone, dehydroepiandrosterone, androstenedione, androstenediol and aldosterone (Grunwald *et al*., 1990; Lashansky *et al*., 1991). Whether such substances contribute significantly to the 'cortisol' result will depend on individual assay specificity and the absolute concentration of cortisol released. This difference in bias has important implications for the comparison of methods. It is clear that where a study has involved the use of more than one cortisol immunoassay comparison must be made on both basal and stimulated samples and full details published (Hurel *et al*., 1996; Orme *et al*., 1996). The use of quality control materials alone to compare assays is not likely to give data applicable to patient samples and comparison should be based on analysis of samples collected from patients basally and post-stimulation. Some indication of the relative bias of cortisol assays is given by the six month cumulative bias for the period of the study reported by UK NEQAS (Dr J Middle, personal communication): for TDX = 9·80%, DPC = 5·30%, ACS = – 1·75%, Delfia = – 11·0% which approximates the ranking of the methods as shown in Table 1. It should be emphasized that all analytical methods are subject to variation over a period of time, for example the subsequent recalibration of the DPC method and this should be taken account of when determining reference ranges.

There was no effect of age on the response to Synacthen in this adult population, but the effect of gender on the response to cortisol was significant with some variation between the methods. Gender differences in the cortisol response to the SST have not been noted in earlier studies using fluorimetric assays (Wood *et al*., 1965) though analysis of the data using parametric statistics combined with the imprecision and nonspecificity of the assay may have obscured any differences. If steroids other than cortisol do cross-react in the assays, it is possible these are released in response to Synacthen in higher concentrations in females *vs* males. Alternatively, studies of ACTH and cortisol pulsatility (Horrocks *et al*., 1990; Roelfsema *et al*., 1993) suggest that there may be a greater sensitivity of the adult female adrenal cortex to ACTH. The dose of ACTH used in this study, however, is supraphysiological (Oelkers, 1996) suggesting an analytical explanation for the gender differences may be the most likely.

Several studies have demonstrated a negative correlation between the incremental cortisol response and basal concentrations (Leisti & Perheentupa, 1978; Kukreja & Williams, 1981; May & Carey, 1985; Dickstein *et al*., 1991) though these studies have been limited by small numbers, the use of nonspecific fluorimetric cortisol assays or have studied an unselected, hospital population.Within the tightly defined conditions of this study no such relationship was demonstrated for either incremental value (30–0 min or 60–0 min).

Defining a 'pass' for the SST as a 30 minute cortisol response greater than the 5th percentile value for the assay used, we have demonstrated that 32% of newly diagnosed and untreated pituitary patients had some dysfunction of the HPA axis. When the appropriate gender-related 5th percentile value at + 30 min was used, this value was 33·3% for male patients and 17·4% for females. Rather surprisingly this was unrelated to the underlying endocrine status or size of the pituitary lesion, suggesting that any deficiency of the HPA axis may be secondary to neuroendocrine abnormalities rather than structural loss of pituitary corticotrophs.

We conclude that the definition of the 'normal' response to Synacthen should be both method and gender related at all time points. The statistical techniques used to compare data from different groups should also take into account that the distributions are non-Gaussian and that the relationships of the

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