INTRODUCTION

The 250 μg cosyntropin test has been conventionally used as a test for impaired adrenal reserve. The Cosyntropin that is commercially available is a 250 μg preparation; it contains the 1-24 amino acids of Adrenocorticotropic hormone (ACTH). Earliest studies with cosyntropin showed that responses to cosyntropin were similar to surgical stress or trauma.[1] In a study comparing 250 μg, 0.5 μg, 1 μg cosyntropin with insulin tolerance test (ITT) in healthy volunteers, The 1 μg test was found to produce plasma 1-24ACTH levels of 120 pmol/l, approximately twice as high than that of the ITT but of the same order of magnitude. The cortisol responses at 30 minute were comparable to that of the 250 μg synacthen test.[2] The peak level of 1-24ACTH produced during 250 μg test is about 22000 pmol/l nearly 220 times that produced during Insulin tolerance test (ITT) or 1 μg synacthen. Moreover 1 μg synacthen test has been used in studies looking into milder degrees of HPA axis suppression, such as patients with asthma using Inhaled corticosteroids (ICS).[3,4] Receiver operator curve analyses which are classically used to assess the utility of clinical screening tests suggest that 1 μg test is superior to 250 μg test.[5,6] There has been some criticism of the 1 μg test because the stability of reconstituted cosyntropin has been questioned and there has been one publication involving 8 normal individuals that suggests that cosyntropin if administered in
small quantities produces false positive results presumably because it tends to adhere to plastic tubing used to administer
the drug.\textsuperscript{[7]} Conflicting this report there is evidence in
literature for the stability of reconstituted cosyntropin in
saline for up to four months.\textsuperscript{[8]} Not withstanding these
controversies 1 μg cosyntropin test has been used widely
in research protocols to identify impaired adrenal reserve
both internationally\textsuperscript{[9,10]} and in India.\textsuperscript{[11]} The cut offs for a
clear pass response for the 1 and 250 μg tests have been
classically derived as a response above the 5th percentile
response in a group of normal individuals for each test.\textsuperscript{[12-14]}

Studies have pointed out the need for determining
normative responses for the cortisol assay because of
variability between different assay methods.\textsuperscript{[15,16]} Previous
metanalyses have suggested a cut-off of 16 μg/dl for the
30 min value while using the 1 μg test when the test is used
in patients with suspected adrenal insufficiency.\textsuperscript{[8]} Despite
this authors continue to use the empirical 18 μg/dl cut
off in India.\textsuperscript{[11]}

We performed a pilot study using the 1 μg cosyntropin test
in 49 normal individuals in order to obtain the 5th percentile
response for the test using an Immuno chemiluminometric
assay. We also simultaneously tested the hypothesis that
storage of synacthen in saline solution for up to 60 days
does not affect cortisol responses.

**MATERIALS AND METHODS**

Forty nine non-pregnant volunteer normal adults were
tested after Institutional ethical review board clearance and
informed consent. Following were the exclusion criteria:

- Use of any oral/topical/inhaled glucocorticoid in the
  past 3 years for any duration
- History of hypothalamo pituitary adrenal disease
- History of head injury in the past
- History of snake bite
- History of Disseminated intravascular coagulation in
  the past
- Any history of diseases known to cause adrenal
  insufficiency.

Reconstitution and storage: Synthetic ACTH1–24 1ml of
solution 250 μg/ml (Synacthen®, Novartis, Manufactured
by Alliance Pharmaceutical Ltd Chippenham, Wiltshire U.K)
was diluted in 499 ml of 0.9% saline in plastic Intravenous
fluid container. After reconstitution the solution was stored
in refrigerator 2-8° for up to 60 days.

Performance of the test: An i.v access with 21 gauge scalp
vein set was obtained. Blood was taken at 08:00 hrs for basal
cortisol, Two ml of the reconstituted solution containing
1 μg co-syntropin was administered intravenously through
the same cannula. The scalp vein set was flushed with 10 ml
of normal saline following administration of cosyntropin.

Cortisol assay was done using the automated Immuno
chemiluminometric Access2 assay system (Beckmann- Coulter
Gallaway, Ireland). Cumulative Coefficient of variation was
3.68% at 16.9 μg/dl and 9.77% at 2.77 μg/dl. Internal
Quality Control (IQC) material Lypocheck Immuno assay
Plus control lot no. 40240 from Biorad laboratories with
known cortisol concentrations were analyzed on the days
of sample run. Internal Quality Control values were within
cut off limits established by the laboratory.

**RESULTS**

The 1 μg cosyntropin test was performed in 49 subjects,
38 male (77.6%). The mean age of the subjects was
37.2 years, range (19-84 years). Majority of the subjects
were young with 59.2% being 36 years or younger, 14.3%
of subjects were 55 years or older[Figure 1].

The Mean cortisol values at three time points are depicted in
Table 1. The cortisol values at 30 min were signiﬁcantly higher
than the 0 min and 60 min value \(P<0.001\) for both. Figure 2
represents the same data graphically. The lowest and highest
cortisol values at each time point and the fifth percentile

![Figure 1: The age distribution of studied subjects](image-url)
cortisol value at each time point are depicted in Table 1. The lowest four responses at 30 min were 13.86 μg/dl, 16.07 μg/dl, 16.60 μg/dl and 17.47 μg/dl. All the remaining cortisol values were above 17.5 μg/dl. The 5<sup>th</sup> percentile response was 16.33 μg/dl-rounded off to 16.5 μg/dl.

The incremental cortisol response was 8.53 ± 2.56 μg/dl (mean ± SD), ranging from 2.61-16.92 μg/dl. There was excellent correlation between the 30 min cortisol value and the incremental cortisol response Pearson’s R = 0.405. Table 2.

**DISCUSSION**

In our study we found that the 30 min cortisol response was higher than the 60 min cortisol response. The value of cortisol corresponding to 5<sup>th</sup> percentile cortisol response in our study population was 16.5 μg/dl. We also found that there was no association between duration of refrigeration of reconstituted synacthen and cortisol response for up to 60 days.

Classical comparative studies showed similar 30 min responses to cosyntropin for the 1 and 250 μg tests in the same individuals. In the 1 μg test the cortisol values quickly reduce and the 60 min value is always lower than the 30 min value. The obvious clinical implication for this will be that while using the 1 μg LDSST test one needs to ensure that the 30 min sampling not be delayed by a few minutes as one can obtain a falsely low value for the cortisol response.

Classically the cut – off for adrenal insufficiency have been derived by investigators as a 5<sup>th</sup> percentile response in normal individuals. But this method has been disputed by some investigators who feel that the cut point for cortisol value in a stimulation test has to be derived by comparison with a gold standard – usually the ITT and doing a ROC analysis. Unfortunately in the most common clinical situation where stimulation testing is necessary- Basal cortisol levels are equivocal with no clinical evidence of adrenal insufficiency and ITT is not feasible- there is no gold standard for diagnosis of adrenal insufficiency. Also in many cases the defect in HPA function could be transient and function may change over time. Some centers follow the protocol of using a response above the 5<sup>th</sup> percentile of normal response as a cut-off to rule out HPA axis suppression, They also use the 2.5<sup>th</sup> to 5<sup>th</sup> percentile values as abnormal requiring steroid supplementation as required. The practice of using 5<sup>th</sup> percentile responses and using them clinically is continuing with the understanding that the cut points so derived are only a guide to clinical decision making.

In a recent study Klose <sup>et al</sup>, showed that the 2.5<sup>th</sup> percentile response varied from 17.2 μg/dl (475 nmol/l) to 18.9 μg/dl (523 nmol/l) in three different contemporary assays and the stimulated cortisol values varied up to 110 nmol/l (3.98 μg/dl) in the same sample when assayed by different methods. The tendency for a downward shift in cortisol values has been attributed to higher specificity for cortisol and lesser cross reactivity with intermediaries of cortisol metabolism. The use of 550 nmol/l (20 μg/dl) or 500 nmol/l (18 μg/dl) as a cut off would lead to substantial number of false positives. This means that separate cut offs are needed for different cortisol assays.
Our study we found that the 5th percentile cut-off was 16.5 μg/dl this value is substantially lower than 18 μg/dl being used clinically. This figure is similar to 16 μg/dl identified by ROC curve analysis of patient level data of different published studies—while using the 1 μg cosyntropin test.[6]

The contribution of our study is that we have demonstrated that stimulated cortisol cut-offs lower than the currently used cut point is probably warranted with our assay. In order to arrive at a clinically useful cut off we need to study cortisol values at 30 min in patients with clinically suspected cortisol insufficiency with a longitudinal follow-up. We suggest that any cortisol value below 16.5 μg/dl at 30 min while using the 1 μg cosyntropin test will be suspicious of adrenal insufficiency based on our data alone.

One of the main criticisms against the 1 μg cosyntropin test is that storage of prepared cosyntropin solution is not possible and it has to be used immediately upon reconstitution. A previous study has shown stability of cosyntropin after storage for four months at 4°C.[6] Our study also had similar findings when we used the 30 min cortisol value as an indirect bioassay of the 1-24 ACTH levels, we found no difference in cortisol levels on storage up to 30 days or even longer. There was no linear relationship between number of days stored and cortisol response. This finding is of importance in a resource constrained country like ours where reconstituted cosyntropin can be used stored for up to two months and used in multiple patients. Measurement of 1-24 cosyntropin in the reconstituted solution and/or retesting in the same individuals after duration of storage could have better supported our hypothesis and these are shortcomings in our study.

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