Long-acting Porcine Sequence ACTH (Acton Prolongatum) Stimulation Test is a Reliable Alternative Test as Compared to the Gold Standard Insulin Tolerance Test for the Diagnosis of Adrenal Insufficiency

Sridevi Atluri, Vijaya Sarathi, Amit Goel\textsuperscript{2}, Shivaprasad Channabasappa\textsuperscript{2}, Shailaja Alapaty\textsuperscript{1}, Melkunte S. Dhananjaya, Ramdas Barure, Gautam Kolla

Departments of Endocrinology and \textsuperscript{1}Biochemistry, Vydehi Institute of Medical Sciences and Research Center, \textsuperscript{2}Department of Endocrinology, Sapthagiri Institute of Medical Sciences and Research Center, Bengaluru, Karnataka, \textsuperscript{3}A. G. Center For Diabetes, Thyroid, and Endocrine, Secunderabad, Telangana, India

Abstract

Context: As synacthen use is not licensed in India and there are concerns about the safety of the insulin tolerance test (ITT), an alternative dynamic test to diagnose adrenal insufficiency (AI) is required. Objective: The study aimed to evaluate the diagnostic performance of the Acton Prolongatum stimulation test (APST) with a standard ITT for the diagnosis of AI. Design: Prospective study comparing two diagnostic tests. Participants: Six healthy volunteers and 53 suspected or known AI patients. Measurements: Serum cortisol response to ITT and APST. Results: The median (95% confidence interval [CI]) peak cortisol levels among healthy volunteers in ITT and APST were 17 (14.58–19.08) and 30.5 (22.57–34.5) \( \mu \text{g/dL} \). Of the 53 patients (age: 39.6 ± 9.38 years; females: 38 [71.1%]), 34 had AI (peak ITT serum cortisol < 14.5 \( \mu \text{g/dL} \)) whereas 19 had a normal hypothalamic-pituitary-adrenocortical (HPA) axis. In the receiver operator characteristic curve analysis, 60-min APST cortisol had an area under the curve of 0.984 (95% CI: 0.904–1.00, \( P < 0.0001 \)). The best accuracy was obtained at a cut-off of 16.42 \( \mu \text{g/dL} \) (sensitivity: 97.7% [95% CI: 87.7–99.9%]; specificity: 100% [69.2–100%]). Forty-three of the 53 patients with suspected AI had hypoglycemic symptoms during ITT and two of them required intravenous dextrose, whereas, none had adverse events during APST. The ITT was incomplete in two patients whereas all completed APST. Conclusions: APST is a simple, safe, and reliable alternative to ITT for the diagnosis of AI; 60-min serum cortisol of 16.42 \( \mu \text{g/dL} \) in APST best distinguishes the AI patients from those with adequate cortisol response.

Keywords: Acton Prolongatum stimulation test (APST), adrenocortical insufficiency, hypocortisolism, hypothalamic-pituitary-adrenocortical (HPA) axis, insulin tolerance test (ITT)

INTRODUCTION

Adrenal insufficiency (AI) is one of the commonly encountered endocrine diseases in clinical practice. Serum 8.00 am cortisol is the most commonly used initial test to diagnose adrenal insufficiency.\textsuperscript{1} It is a simple and less expensive test but fails to accurately distinguish AI from normal cortisol response in one-third to half of the patients evaluated for AI.\textsuperscript{2,3} Basal cortisol greater than 14–18 \( \mu \text{g/dL} \) is suggestive of an intact hypothalamic-pituitary-adrenal (HPA) axis whereas less than 3 \( \mu \text{g/dL} \) is specific for AI.\textsuperscript{2,4} In patients with suspected AI with a borderline serum basal cortisol (3–14 \( \mu \text{g/dL} \)), the confirmation or exclusion of AI requires dynamic testing of the HPA axis. Several dynamic tests like the insulin tolerance test (ITT),\textsuperscript{5,6} short synacthen test (SST),\textsuperscript{6,7} low-dose short synacthen test (LDDSST),\textsuperscript{7} corticotropin stimulating hormone (CRH) stimulation test,\textsuperscript{8} and metyrapone challenge test,\textsuperscript{9} have been evaluated with varied pros and cons. Of these, ITT is considered
as the gold standard test but it is a cumbersome procedure requiring expertise and is associated with complications like loss of consciousness and convulsions due to hypoglycemia. It is contraindicated in the elderly and patients with ischemic heart disease and epilepsy. The SST, used for the assessment of cortisol response to 250 µg of synacthen, is a safe and standard test for the diagnosis of AI.\(^{[10]}\) Synacthen is not licensed for use in India. So, the Acton Prolongatum\(^\text{®}\) (AP), a porcine sequence sterile solution of the adrenocorticotropic hormone (ACTH) as a carboxymethylcellulose complex in water for injection in either subcutaneous or intramuscular use, has been evaluated as an alternative to synacthen.\(^{[11‑13]}\) It is available as a 5-mL multi-dose vial with a concentration of 60 IU per mL and needs to be stored at 2–10°C. It is used for the treatment of various diseases such as infantile spasms and is easily available in India. The performance of Acton Prolongatum stimulation test (APST) in the evaluation of patients with suspected AI has been tested. A recent study from South India compared the APST with SST and reported high diagnostic accuracy of the former in the evaluation of AI.\(^{[15]}\) However, its diagnostic performance has not been evaluated against the gold standard test, ITT. There is limited data regarding cortisol response at multiple time points to stimulation with AP. Obtaining these data may enhance the diagnostic performance of APST. Hence, we compared the cortisol responses in APST and ITT among healthy volunteers and have tested the diagnostic accuracy of APST in the evaluation of AI.

**Methods**

**Participants**

This study was conducted at a tertiary care hospital, India, from January 2018 to February 2020. The study was approved by the Vydehi Institutional Ethics Committee (VIEC: ECR/747/Inst/KA/2015) and written informed consent was obtained from all the participants. The participants included healthy volunteers and patients evaluated for suspected AI or recovery of proven AI in the Department of Endocrinology. The patients with a history of coronary heart disease, epilepsy, cerebrovascular disease, chronic kidney disease, chronic liver disease, pregnancy or lactation, untreated thyroid dysfunction, and on estrogen-containing drugs, antiandrogens, ketoconazole, metyrapone, mifepristone, and ACTH were excluded.

A detailed history was taken and a thorough clinical examination was done. Laboratory evaluation for renal and liver function tests was performed in all the study participants (healthy volunteers and patients evaluated for AI). The etiological diagnosis of AI was based on clinical features, measurement of plasma 8:00 am ACTH, and imaging of adrenal and/or pituitary as appropriate. The patients with abnormal renal and liver function tests were excluded. In the patients on replacement doses of glucocorticoids, the glucocorticoids were withheld for 24 h and the replacement dose on the day of testing was postponed till the completion of the test.

All the healthy volunteers and patients evaluated for AI underwent both ITT and APST. A baseline fasting basal serum cortisol was measured at 8:00 am and those with levels less than 14.5 µg/dL underwent ITT and APST. All the participants were initially tested with ITT and APST was performed after a wash-out period of ≥ 48 h. For the patients who had a diagnosis AI in the ITT, glucocorticoid ± fludrocortisone replacement was initiated and continued till 24 h before the APST.

**Test protocols**

**Insulin tolerance test**

After an overnight fast of 8 h, intravenous access was secured at 30 min (insulin administration time is taken as 0 min) between 8:00–8:30 am. The baseline capillary glucose level was assessed at 0 min; 0.15 and 0.1 U/kg of regular insulin were administered as an intravenous bolus for healthy volunteers and cases, respectively. Capillary blood glucose levels were checked every 5 min till hypoglycemia was recorded. Hypoglycemia was defined as capillary blood glucose level < 40 mg/dL. A blood sample was collected in a fluoride vial at the time of the lowest documented blood glucose level in which plasma glucose was measured. The participants were given an oral carbohydrate-rich diet or intravenous dextrose as appropriate to terminate the hypoglycemia. Samples were drawn at 30, 45, 60, 90, and 120 min to measure the serum cortisol levels. If hypoglycemia did not occur with 0.1 U/kg of insulin by 40 min in the patients evaluated for AI, a repeat insulin dose of 0.15 U/kg was administered and the above-mentioned procedure was repeated. The lower limit of the 95% confidence interval (CI) of the peak serum cortisol level among healthy volunteers in ITT was used to diagnose AI and the corresponding APST cut-off was derived to diagnose AI using APST.

**Acton Prolongatum stimulation test**

The first study on APST and a few subsequent studies have recommended using 25 units of AP (equivalent to 250 µg of ACTH 1–24) by drawing AP till mark 16 or 40 in 40 or 100 IU insulin syringe, respectively.\(^{[11,14]}\) However, when calculated accurately, this corresponds to 24 units.\(^{[12]}\) Acton Prolongatum\(^\text{®}\) was drawn into a 1 mL syringe (100 units) with a detachable needle till mark 40 (24 units); the needle of the insulin syringe was replaced with a 24G needle, and the drug was administered intramuscularly into a deltoid muscle.\(^{[11,14]}\) Samples were drawn at 30, 60, 90, and 120 min for assessment of cortisol levels in healthy volunteers whereas in patients evaluated for AI, a single sample, at the earliest time point, at which all healthy volunteers, had attained serum cortisol of 18 µg/dL.

**Laboratory methods**

For cortisol measurement, 2 mL of blood sample was collected into gel tubes and centrifuged for separation of the serum and were stored at −20°C till assay was performed. Cortisol assay was performed by the chemiluminescence method with Beckman Coulter, Dxc-860i Auto-Analyzer (Beckman Coulter, CA, USA) with REF 33600 kit with a reference range of 5–23 µg/dL. The inter-assay coefficient of variance (CV) was 6.0–7.9% and the intra-assay CV was 4.4–6.7%.
**Statistical analysis**
The data were analyzed using the MedCalc Statistical Software, Version 19.2.6 (MedCalc Software Ltd, Ostend, Belgium). Assuming an alpha error of 5%, a beta error of 20% (power: 0.8), rank correlation coefficients of 0.8 in both positive and negative cases, a ratio of negative cases to positive cases of 0.6 (as previously diagnosed AI cases were also included), and an area under the curve of 1 for the gold standard test (ITT) and 0.923 for the APST, a total sample size of 48 was estimated (30 positive cases and 18 negative cases). The continuous variables were expressed as mean ± SD or median (95% CI) as appropriate whereas the categorical variables were expressed as absolute numbers and percentages. The receiver-operating characteristic curves (ROC) were derived with sensitivity on the Y-axis and 1-specificity on the X-axis and the cut-offs with the best diagnostic accuracy were noted for APST and basal cortisol. A two-tailed $P$ value < 0.05 was considered statistically significant.

**RESULTS**

**Healthy volunteers**

ITT and APST were conducted on six healthy volunteers. This group included two females and four males with the median (95% CI) age of 33 (26.96–38.51) years. During ITT, all the healthy volunteers developed symptomatic hypoglycemia. ITT was conducted between 8 am and 12 pm, with the starting time between 8.00 and 9.00 am and the samples were drawn depending on the time of insulin injection and hypoglycemia development. APST was done between 8 and 11 am. Their baseline serum cortisol and cortisol responses to ITT and APST are depicted in Figure 1. All the volunteers achieved peak serum cortisol response at 45–60 min in ITT. The median (95% CI) peak cortisol in ITT was 17 (14.12–19.08) µg/dL. The lower limit of the 95% CI (14.5 µg/dL) was used to define adequate cortisol response in ITT.

In APST, the serum cortisol demonstrated a continuous rise from 0 to 120 min. The median peak serum cortisol at 120 min was 30.52 (22.57–34.6) µg/dL. In APST, five of the six and all six healthy volunteers achieved the conventional cut-off of ≥ 18 µg/dL at 30 min and 60 min, respectively. Hence, serum cortisol was measured in a single sample at 60 min in the patients evaluated for AI.

**Patients**

A total of 55 patients evaluated for suspected or recovery of AI were included, but two patients were excluded in the final analysis for the reasons mentioned below. The final diagnoses of the patients evaluated for AI are summarized in Table 1. A patient who did not achieve adequate hypoglycemia during ITT and another whose 60- and 90-min ITT blood samples were hemolyzed and did not consent for repeat ITT were excluded from the analysis.

The mean age of the study population $n = 53$) was 39.6 ± 9.38 years. There were 38 (71.1%) females and 15 (28.3%) males. Five participants had diabetes and two were prediabetics. The insulin dose used for ITT was

![Figure 1: Serum mean cortisol response to Acton Prolongatum stimulation test and insulin tolerance test in healthy volunteers ($n = 6$)](image)

![Figure 2: Receiver-operating characteristic curve to diagnose adrenal insufficiency using 60-min Acton Prolongatum stimulation test serum cortisol](image)

**Table 1: The final diagnoses in patients evaluated for adrenal insufficiency ($n = 55$)**

| Diagnosis                                      | Count |
|-----------------------------------------------|-------|
| Exogenous glucocorticoid use                  | 24    |
| Sheehan syndrome                              | 5     |
| Hypophysitis                                   | 3     |
| Viperine snake bite                           | 5     |
| Partial empty sella (incidentally detected)   | 4     |
| Postoperative sellar tumors                   | 7     |
| Postoperative ectopic ACTH secreting thymic NET | 1    |
| Postoperative corticotropinoma                | 1     |
| Autoimmune polyglandular syndrome 2           | 3     |
| Primary adrenal insufficiency                 | 2     |
0.1 ± 0.02 U/kg. The baseline capillary blood glucose level was 91.0 ± 20.02 mg/dL. All the 53 achieved hypoglycemia and the time to hypoglycemia from insulin administration was 30.36 ± 15.75 min. The lowest blood glucose documented during ITT was 34.19 ± 3.46 mg/dL. Forty-three of the 53 participants had hypoglycemic symptoms during ITT, two of whom required 25% dextrose to terminate hypoglycemia due to their inability to consume an oral diet. Neither healthy volunteers nor patients had any adverse effects during APST. The baseline serum cortisol and cortisol response to ITT and APST are summarized in Table 2.

Out of the 53 patients analyzed, 34 had AI (peak ITT cortisol < 14.5 µg/dL) whereas 19 had a normal HPA axis. In the ROC curve analysis, 60-min APST stimulated cortisol had an AUC of 0.984 (95% CI: 0.904–1.00, P < 0.0001) [Figure 2]. The best accuracy was obtained at a cut-off of 16.42 µg/dL.

Table 2: Response to insulin tolerance test and Acton Prolongatum stimulation test in patients evaluated for adrenal insufficiency

| Parameter                                      | Mean cortisol ± SD |
|------------------------------------------------|--------------------|
| Baseline serum cortisol (µg/dL)                | 3.86 ± 2.69 (n = 53) |
| Insulin tolerance test                         |                    |
| 30 min serum cortisol (µg/dL)                  | 5.57 ± 4.48 (n = 50) |
| 45 min serum cortisol (µg/dL)                  | 5.99 ± 4.87 (n = 51) |
| 60 min serum cortisol (µg/dL)                  | 7.84 ± 5.43 (n = 51) |
| 90 min serum cortisol (µg/dL)                  | 6.36 ± 4.74 (n = 51) |
| 120 min serum cortisol (µg/dL)                 | 5.06 ± 3.9 (n = 48)  |
| Acton Prolongatum stimulation test             |                    |
| 60 min serum cortisol (µg/dl)                  | 11.53 ± 6.05 (n = 53) |

Twelve‑two patients had basal cortisol of less than 3µg/dL. When these were excluded and repeat ROC was performed, 60-min APST had an AUC of 0.970 (0.836–0.999%) [Figure 3]. The best accuracy was obtained at a cut-off of 16.42 µg/dL with a sensitivity of 95.45% (95% CI: 77.2–99.9%) and specificity of 100% (95% CI: 84.6–100%). A sensitivity of 100% (95% CI: 91.8–100%) was obtained at a cut-off of 21.17 µg/dL but with a marked reduction in specificity to 30% (95% CI: 6.7–65.2%).

Serum baseline cortisol had an AUC of 0.819 (95% CI: 0.689–0.911%) to predict AI. The best accuracy was obtained at a cut-off of 5.13 µg/dL with a sensitivity of 76.7% (95% CI: 61.4–88.2%) and specificity of 90% (95% CI: 55.5–99.7%) [Figure 4]. Serum cortisol of 3.05 µg/dL had 100% specificity for the diagnosis of AI but the sensitivity was 51.16%.

Discussion

The present study is the first study to compare the diagnostic performance of APST in the evaluation of AI with ITT, the gold standard test for the diagnosis of AI. Sixty-minute APST serum cortisol of 16.42 µg/dL provided the best accuracy for the diagnosis of AI. The study also compares the cortisol responses to ITT and APST in healthy volunteers, albeit in a smaller sample size.
To assess the serum cortisol response to AP, it was compared with ITT in six healthy volunteers. Acton Prolongatum led to incremental responses in the serum cortisol levels from 0 to 120-min with a maximal increment from 0 to 30-min. Synacthen Depot, a long-acting form of ACTH, led to a similar incremental response in serum cortisol beyond 5 h after administration (Synacthen Depot product monograph). This suggests that serum cortisol response after stimulation with AP is time-dependent and cut-offs for serum cortisol to diagnose AI by APST need to be time-specific. A trend for increasing serum cortisol with time after stimulation with AP was also observed in the only previous study that evaluated cortisol response at more than one time points (30 and 60 min) among healthy volunteers.[12] However, all participants achieved serum cortisol of ≥ 18 µg/dL at 30 min in this study, whereas in our study, one participant did not do so. Hence, we decided to measure serum cortisol at 60 min after stimulation with AP when all achieved serum cortisol of ≥ 18 µg/dL, instead of 30 min.

In the first study on APST by Gundagarthi et al.,[11] the 60-min serum cortisol level among the healthy volunteers (n = 24) was 29.62 ± 5.86 µg/dL (radioimmunoassay, Immunotech, Beckman Coulter), whereas in a subsequent study by Wagmode et al.,[12] it was 31.59 ± 6.40 µg/dL (chemiluminescence immunoassay, ADVIA Centaur XP, Siemens). In contrast, the 60-min cortisol level in APST in our study was relatively lower (25 ± 4.96 µg/dL). However, the 60-min cortisol level in APST in our study was comparable to that (22.57 ± 5.19 µg/dL) in another study by Nair et al.[13] in which serum cortisol was measured by a second-generation assay (Roche cortisol II). These observations suggest an impact of the assay used on 60-min serum cortisol attained in APST and the need for assay-specific cut-offs. Even a study by Emilia Sbardella et al.[16] also showed that cortisol cut-offs should be assay-specific.

The cortisol levels measured by the second-generation assays are significantly lower (~20–30%) than those measured by the previous assays.[17–19] Notably, the second-generation cortisol assays use monoclonal antibodies and are as specific as as liquid chromatography-tandem mass spectrometry (LC-MS/MS) with comparable cortisol levels measured by the two methods.[18–20] Such findings have not only been reported for Elecsys® Cortisol II in Cobas (Roche) and Architect Cortisol in i2000 Architect (Abbott) but also for Access Cortisol in Unicel Dxi800 (Beckman Coulter), the assay used in our study.[16,17,19] Several recent studies have suggested lowering (12.6–15 µg/dL) the serum cortisol cut-offs in diagnosing AI with SST while using the second-generation cortisol assays to reduce its overdiagnosis.[17–19,21] The need for lowering the serum cortisol cut-off (14–18 µg/dL) to define adequate response was also recognized by the Endocrine Society Guidelines on Congenital Adrenal Hyperplasia.[22] Similarly, the peak cortisol levels in ITT among healthy volunteers in our study were lower (14.12–20.96 µg/dL) with only one-third (2/6) meeting the conventional criteria ≥ 18 µg/dL for adequate cortisol response. The lower limit of the 95% CI for peak cortisol in our small cohort of healthy volunteers was 14.5 µg/dL which was used as a cut-off to define an adequate response with ITT, and then, the corresponding APST cut-off was derived using the AUC.

In the present study, the best accuracy to diagnose AI using APST was obtained at a serum cortisol level of 16.42 µg/dL (sensitivity: 97.7%; specificity: 100%). A higher cut-off in APST than ITT suggests stronger stimulation with ACTH (synacthen or AP) than insulin. Similar results with higher serum cortisol cut-offs to diagnose AI in SST and APST than ITT have been reported previously.[15–23] In a study by Nair et al.,[13] where APST was compared with SST (cut-off: 18 µg/dL), 60-min APST serum cortisol of 17 µg/dL provided the best diagnostic accuracy (95% sensitivity and 88% specificity). These observations suggest comparable cut-offs using APST and SST. Notably, 120-min serum cortisol provided better diagnostic accuracy at cut-off of 19.5 µg/dL (sensitivity of 100% and specificity of 88%) in the Nair et al.[13] study; however, 120-min APST serum cortisol was not measured in patients with suspected AI in our study. A sensitivity of 100% to diagnose AI was obtained at 21.17 µg/dL but with markedly lowered specificity (33.3%) in our study, whereas in the Nair et al. Study,[13] 100% sensitivity was obtained at a 60-min cortisol level of 20.3 µg/dL.

Various studies have attempted to diagnose AI using basal serum cortisol levels so that dynamic testing can be avoided. The basal serum cortisol cut-offs to diagnose AI varied from 3.0 to 5.2 µg/dL with widely variable specificity.[24–27] The latest Endocrine Society Clinical Practice Guidelines recommend a basal serum cortisol cut-off of 5 µg/dL to diagnose AI.[10] Nevertheless, the guidelines recognized that the lower limit of normal using the contemporary assays is lower (4.1–4.3 µg/dL). In our study, the basal serum cortisol of 5.13 µg/dL yielded a specificity of 90% and a sensitivity of 76.7% whereas the cut-off of 3.05 µg/dL provided 100% specificity but with 51.16% sensitivity. The use of basal serum cortisol of 3 µg/dL significantly increases the need for dynamic testing (~one-fourth of the patients evaluated for AI) but with a marginal increase in the specificity. A study by Perton et al.[28] had shown a reduction of dynamic testing by 12% if a cut-off of 5.2 µg/dL is used instead of a conventional cut-off of 3 µg/dL. So, a basal serum cortisol cut-off of 5 µg/dL may be adopted to diagnose AI in clinical practice.

The APST protocol used in our study is less expensive than ITT and SST but has comparable accuracy to ITT as demonstrated in our study, and to SST, as demonstrated in Nair et al.’s study.[13] Also, it is safer than ITT and more widely available in India than SST. The strengths of the study include a comparison of this emerging simple, safe, easily available, and cost-effective test against the gold standard, ITT, both among healthy volunteers and the participants evaluated for AI. The study also had a few limitations. First, the comparison of cortisol responses to APST and ITT was performed in only a small number of healthy volunteers. Second, serum cortisol
level was measured only at 60 min but not at 30 and 120 min after APST in the participants evaluated for AI; hence, the ideal time to measure serum cortisol that provides the best diagnostic accuracy could not be determined.

**Conclusions**

APST is a simple, safe, less expensive, and reliable alternative to ITT in the evaluation of AI. Serum cortisol of 16.42 µg/dL at 60 min in APST provides the best diagnostic accuracy to delineate AI patients from those with a diagnostic accuracy of cortisol response.

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**Conflicts of interest**

There are no conflicts of interest.

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