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Short Communication

Corticotropin hormone assay interference: A case series

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ABSTRACT

Measuring the plasma corticotropin (ACTH) concentration is an important step in determining the underlying cause in patients with hypercortisolemia. Interfering substances in immunoassays can lead to erroneous results impacting clinical management. We describe a case series of 12 patients, the majority of whom were being investigated for possible Cushing's syndrome and in whom inconsistencies between the clinical picture and biochemical testing raised concerns of assay interference. ACTH assay interference resulted in falsely elevated ACTH concentrations using the Siemens Immulite assay and consequently led to additional unnecessary testing. Communication between physician and laboratory as well as appropriate investigation (including sample dilution, use of blocking antibodies and testing on an alternate platform) resulted in assay interference identification. Recognition of biochemical results which are clinically discrepant remains an essential step in patient assessment.

1. Introduction

Corticotropin (ACTH) is a polypeptide hormone produced and secreted by the anterior pituitary corticotroph cells. Plasma ACTH quantitation plays an essential role in the evaluation of adrenal dysfunction, particularly in determining the level of abnormality (adrenal or hypophyseal), as this can then direct appropriate treatment [1]. Excess endogenous cortisol production in Cushing syndrome can be ACTH-dependent (pituitary or ectopic) or ACTH-independent (adrenal); whereas deficient cortisol production can be due to primary adrenal insufficiency (characterized by increased serum ACTH concentrations) or secondary insufficiency (low serum ACTH concentrations). Thus, precise and accurate ACTH measurements are critical.

Currently, ACTH is measured by two-site immunometric “sandwich” assays. Analytical interferences in these assays, although rare, could lead to misdiagnosis and result in adverse consequences [2,3]. Various analytical sources of immunoassay interference have been described including antibodies (heterophile, auto or anti- animal antibodies) and substances such as medications or their metabolites. As a consequence, several techniques have been developed to overcome and troubleshoot these interferences, but recognition of potential artefactual results remains the crucial step. We describe 12 cases where ACTH assay interference could have negatively impacted clinical management and outline an assessment strategy to be used when the clinical and biochemical pictures are discordant.

2. Methods

A case series of 12 patients with clinically discrepant plasma ACTH concentrations using Siemens Immulite (Los Angeles, CA) were identified after approval by the Mayo Clinic Institutional Review Board. Eligible cases were identified as those patients necessitating further ACTH measurement. This was prompted by the primary clinician who had been in contact with the clinical laboratory to express concerns of clinically discrepant results. ACTH assay interference in the plasma samples was investigated by: a) repeat testing; b) serial dilutions (ranging from 2- to 10-fold and using the manufacturer's ACTH sample diluent); c) measurement using a different analytical platform (Roche Cobas, IN); and, d) heterophile blocking reagents (HBT, Scantibodies laboratory Inc., CA) assessment using the sample with the reported suspicious result. Troubleshoot testing was performed in parallel. For heterophile investigations, HBT (Scantibodies catalog # 3IX762) were used. The reagent is in the form of a lyophilized pellet. Patient sample (500ul) is added to the tube, mixed and incubated for 1 h at room

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Table 1
Summary of clinical presentation, investigations performed and outcomes among those found to have ACTH assay interference.

Patient	Age	Sex	Reason for referral	Symptoms	Testing	Imaging	Outcome
1	43	F	Possible Ectopic CS	Weight gain, abdominal striae, bruising, hypertension, metacarpal stress fracture	(+) 1 mg ODST, elevated MN salivary cortisol CRH Test: suggestive of ectopic ACTH **ACTH (S):** 56 pg/mL, **ACTH (R):** ACTH < 1 pg/mL	indium-111-pentreotide scintigraphy: negative FDG-PET-CT: negative CT Chest: 30 mm right adrenal adenoma	Right adrenalectomy With resolution of symptoms
2	48	F	Possible CS	Thin skin, fatigue, easy bruising, acne and decreased muscle strength. CT abdomen for pancreatitis - > right adrenal nodule identified	(+) 1 mg DST, Elevated 24-h UFC **ACTH (S):** 19 pg/mL **ACTH (R):** 1.76 pg/mL	CT abdomen: revealed 38 mm right adrenal adenoma	Right adrenalectomy with resolution of symptoms
3	59	M	Possible CS	Cold intolerance, hypertension, palpitations, low libido	Normal cortisol and 24-h UFC **ACTH (S):** 142 pg/mL **ACTH (R):** 20 pg/mL	MRI Brain: no pituitary abnormality	No evidence of CD, false elevation of ACTH
4	26	F	Possible CS	Weight gain, abdominal striae, hypertension	Elevated 24-h UFC **ACTH (S):** 98.5 pg/mL **ACTH (R):** < 5 pg/mL	MRI Brain: no pituitary abnormality CT abdomen: 42 mm left adrenal adenoma	Left adrenalectomy with resolution of symptoms
5	46	F	Possible CS	Weight gain, plethora, abdominal striae, hirsutism, secondary amenorrhea, and muscle weakness	(+) 1 mg ODST, Elevated MN salivary cortisol and 24-h UFC **ACTH (S):** 14 pg/mL **ACTH (R):** < 1 pg/mL	IPSS: no central peripheral gradient CT Abdomen: 33 mm right adrenal adenoma	Right adrenalectomy with resolution of symptoms
6	39	F	Possible CS	Weight gain, acne, hirsutism excess sweating. Exogenous steroid use, stopped for testing	Normal 1 mg ODST, UFC. 2 day LDDST + CRH: cortisol < 1, but ACTH remained elevated after suppression test **ACTH (S):** 21 pg/mL **ACTH (R):** 1.3 pg/mL	MRI Brain: no pituitary abnormality	No evidence of endogenous cortisol excess, false elevation of ACTH
7	42	F	Possible CS with bilateral hyper-aldosteronism	History of hypertension AVS: bilateral hyperaldosteronism Work up included ACTH which was elevated	Normal: MN salivary and am cortisol **ACTH (S):** 137 pg/mL **ACTH (R):** 48 pg/mL	CT abdomen: No adrenal adenoma	No evidence of hypercortisolism treated medically for primary hyperaldosteronism.
8	33	M	Possible persistent CS	TSS for CD due to 9 mm pituitary adenoma	Normal cortisol **ACTH (S):** 95 pg/mL **ACTH (R):** 24 pg/mL	MRI Brain: no residual/ recurrent adenoma	No evidence of recurrent CD, false elevation of ACTH
9	77	F	Possible CS	History of TSS for Rathke's cleft cyst	Normal Cortisol **ACTH (S):** 138 pg/mL **ACTH (R):** 8.9 pg/mL	MRI Brain: no new abnormality	No evidence of CD, false elevation of ACTH
10	27	F	Panhypo-pituitarism	Craniotomy for craniopharyngioma with anterior pituitary insufficiency and diabetes insipidus on levothyroxine, hydrocortisone, estrogen and ddAVP replacement. No clinical signs of cortisol excess	IGF-1 < 10, Free thyroxine normal (on replacement) **ACTH (S):** 261 pg/mL **ACTH (R):** < 5 pg/mL	MRI Brain: empty sella no residual tumor	No change to medication, false elevation of ACTH
11	46	M	Possible AI	Muscle pain, spasms, intermittent weakness	Cosyntropin stimulation test: normal response **ACTH (S):** 115 pg/mL **ACTH (R):** 22 pg/mL	NA	No evidence of adrenal insufficiency, false elevation of ACTH
12	46	M	Possible AI	Graves' disease treated with radioactive iodine, type 1 diabetes and ankylosing spondylitis	Cosyntropin stimulation test: normal response 21-hydroxylase antibody negative **ACTH (S):** 77 pg/mL **ACTH (R):** 5.6 pg/mL	NA	No evidence of adrenal insufficiency, false elevation of ACTH

Abbreviations used: ACTH, corticotropin; AI, Adrenal insufficiency; AVS, Adrenal vein sampling; CS, Cushing syndrome; CRH, corticotropin releasing hormone; FDG, 18F-fluorodeoxyglucose; IPSS, inferior petrosal sinus sampling; MN salivary cortisol, midnight salivary cortisol; NA, not applicable; 1 mg ODST, 1 mg overnight dexamethasone suppression test; PET, positron emission tomography;(R) Roche assay - reference range 7.2–63.3 pg/mL; (S) Siemens assay - reference range 10–60 pg/mL; TSS, transsphenoidal surgery; 24-h UFC, 24-h urinary free cortisol.

temperature. The sample mix is then tested in the ACTH immunoassay.

Two assays were used for ACTH measurements: Siemens Immulite and Roche Elecsys ACTH assays. The inter-assay precision for the Siemens Immulite assay is < 5% CV at concentrations of 30 and 400 pg/mL. The inter-assay precision for the Roche Immulite assay is < 3% CV at concentrations of 38 and 900 pg/mL. Comparison between the Siemens (x-axis) and Roche (y-axis) assays ($n = 54$) showed an R^2 of 0.91, slope of 0.94 and intercept of −1.71 by Passing-Bablok regression fit.

Medical records were also reviewed for relevant medical history or potential medications that may lead to assay interference.

3. Results

Among the 12 patients with clinically discrepant plasma ACTH concentrations, the mean (± SD) age was 43 ± 14 years and 8 were female (Table 1). The reasons for referral were hypercortisolism or investigation for the cause of hypercortisolism in 9 patients, whereas adrenal insufficiency was the reason for referral in 3 patients. Review of the medical records did not reveal a prescribed or an over the counter medication that was common to all patients. Additionally, only one patient (patient #12) had evidence of an autoimmune dysfunction with a history of type 1 diabetes, Graves' disease, and ankylosing spondylitis. Rheumatoid factor was not measured.

In all cases, assay interference led to falsely elevated plasma ACTH concentrations when using the Siemens Immulite assay (Table 2). Repeat measurement (except in patient #4 and #10 in who the measurement was not repeated) using the same assay confirmed the original result. Serial dilutions in all patient samples resulted in a nonlinear dose response suggesting the presence of an analytical interference and also led to a notable rise in concentration with further dilution. In plasma samples from 8 patients, incubation with HBT did not remove the analytical interference and concentrations remained falsely elevated. Evaluation on a different analytic platform (Roche Cobas) resulted in a lower plasma ACTH concentration with the largest discrepancy seen in patient #10 who had panhypopituitarism following transcranial surgery for resection of a craniopharyngioma.

In several cases, unnecessary laboratory testing or radiological imaging was performed as a consequence of ACTH assay interference and resultant aberrant ACTH measurements. Prior to referral to our institution, patient #5, presumed to have ACTH-dependent endogenous hypercortisolemia, had inferior petrosal sinus sampling (IPSS) because the plasma ACTH concentration was not suppressed, suggesting ACTH-dependent disease. Consequently, IPSS was performed elsewhere to determine if the source was pituitary or ectopic. IPSS did not reveal a pituitary-to- peripheral gradient and CT scans of the chest and abdomen were obtained in an attempt to identify an ectopic source of ACTH production. The only abnormality identified was a 3.3 cm adrenal adenoma. Given the clinical and biochemical discordance, assay interference was suspected and confirmed when the ACTH measurement was repeated on a different platform (plasma ACTH concentration < 1 pg/mL). The patient underwent resection of the adrenal adenoma and was cured. In patient #1 who presented with Cushing syndrome, the ACTH concentration was 56 pg/mL and did not increase in response to corticotropin-releasing hormone administration (suggesting ectopic ACTH syndrome) and led to 3 additional imaging procedures for tumor localization (indium-111-pentreotide scintigraphy, 18F-fluorodeoxyglucose (FDG) positron emission tomography CT scan, and chest CT scan). Although an adrenal adenoma (3 cm) was identified on imaging, no ectopic source was seen and the patient was referred for further assessment. Again, concerns regarding assay interference were raised. By simply re-measuring plasma ACTH on a different platform it was found to be undetectable and consistent with ACTH-independent Cushing syndrome. The patient was cured by resecting the cortisol-secreting adrenal adenoma identified. Additional unnecessary imaging with pituitary MRI was performed in patients #3, 4, 6, 9, 10, 11 who did not have evidence of endogenous hypercortisolemia and were prompted by persistently elevated plasma ACTH measurements.

4. Discussion

These cases illustrate the importance of clinical vigilance and laboratory communication when results are incompatible with the clinical picture—a step than can avoid unnecessary costly investigations. Interference in immunoassays by endogenous antibodies including heterophile antibodies, human anti-animal antibodies, and rheumatoid factor or other interfering agents (e.g. biotin) have been described but often under-recognized [4]. Such interferences could lead to erroneous results impacting clinical decision making and lead to additional unnecessary or incorrect testing as demonstrated in this case series [3,5,6].

Compared to bio- and radio- immunoassays, the development of two-site immunometric assays heralded advancements in specificity and ease of use. However, pre-analytical and analytical variables as well as interfering substances still need to be considered as potential sources of error. Pre-analytical practices were not assessed in this case series. However, inappropriate handling of specimens, including maintenance samples at room temperature, delay in centrifugation and separation of plasma, hemolysis and excess EDTA, can lead to a false decrease in plasma ACTH concentrations [7–9]. This was not observed in our series. Regarding potential analytical confounders, there is no international ACTH reference material available. This means that each assay developer must manufacture its own reference material making the comparison of assay results challenging [10]. Moreover, despite the

Table 2
Laboratory assessment performed in those patients with ACTH assay interference.

	Siemens ACTH (Reference range, 10–60 pg/mL)				HBT (pg/mL)	Roche ACTH (Reference range, 7.2–63.3 pg/mL)
Patient	Original ACTH	Repeat ACTH	X 2 dilution	X 4 dilution		
1	56	34.8	50.8	100.4	27.5	< 1
2	19	13.7	25.6	42.4	20.3	1.76
3	142	145	51*	82*	NP	20
4	98.5	NP	550	730	292	1.9
5	14	15	24.6	66.4	7.2	< 1
6	21	22	30	56	154	1.3
7	137	127	97.2	72.4	NP	48.44
8	95	93	77.6	160	NP	24
9	138	98	290	632	NP	8.9
10	261	NP	NP	238	930 (1.89 Cobas)	1.74
11	60	49	80	88	67	22
12	77	70	140	172	98	5.6

Abbreviations used: ACTH, corticotropin; HBT, heterophile blocking tube, NP, not performed.
* Dilution performed was 5- and 10-fold.

fact that most immunoassays are fully automated, minimizing potential human variability, Giraldi et al. noted significant intra- and inter-assay variability of ACTH results measured by 35 different Italian laboratories [11]. In our case series, all except for one sample, were re-analyzed and results were found to be consistent, excluding assay variability as a source of error.

Antibodies such as heterophile, human anti-animal antibodies, or autoantibodies including rheumatoid factor may lead to interference in immmunoassays [3,5,6,12]. Although the prevalence of potential interfering agents is reported to be high, clinically relevant interference has been reported to occur in 0.5–3% of specimens [13,14]. Serial dilutions can be used to confirm the presence of analytic interference. In our case series, serial dilutions were performed and exhibited lack of linearity following dilution, which indicated the presence of an interfering substance. Interestingly, the measured concentration increased following dilutions which is contrary to what is typically seen in the setting of heterophile antibodies [15]. Current immunoassay contain blocking reagents significantly reducing the incidence of heterophile antibodies interferences however these reagents have been unable to completely eliminate the problem. Blocking agents may be specific such as heterophile blocking reagents which capture human immunoglubulins or nonspecific such as non-immune globulins typically from sera of the species used to develop the assay antibodies [4]. For troubleshooting purposes laboratories can use commercially available heterophile antibodies blocking reagents. If antibodies are present, use of such blocking agents may eliminate the interfering antibody. In our case series, use of HBT did not eliminate the interfering substance and resulted in variable response (some of which were increased, similar or decreased) compared to the original measured ACTH. Given these inconsistences more emphasis was given to the nonlinearity of dilutions and the result of the Roche ACTH assay to determine if interference was present. Additionally, precipitation of interfering antibodies using polyethylene glycol (PEG) can be performed. Polyethylene glycol can be used to precipitate high molecular weight proteins such as immunoglobulins which may interfere with the assay. This was not performed at our institution, but a subset of samples [5 samples from 4 patients were sent, selected solely on the basis of residual sample volume availability (patients #3, 4, 8 and 9, with two samples collected 1 month apart from patient #8)] were sent to the Immulite ACTH assay manufacturer (Siemens Healthcare Diagnostic) for investigation. PEG precipitation and PolyMak block testing (a polymeric monoclonal antibody against the Fc and Fab of IgG1 to eliminate mouse antibodies) were performed and concluded that the findings were most consistent with heterophile interference.

Lastly, assessment using a different analytical platform that uses different capture or detection antibodies may negate the effect of interfering substances as was seen in this case series. Testing using the Roche Cobas platform compared to the Siemens Immulite yielded different results; those measured on the Roche Cobas platform were concordant with the clinical presentation. Differences in reagent composition are proprietary, but the Siemens Immulite assay uses polyclonal antibodies, whereas the Roche Cobas uses monoclonal antibodies. More recently biotin supplementation has been recognized to cause assay interference [16]. Both the Roche and Siemens Immulite ACTH assays are sandwich immunoassays. In these assays the presence of biotin would result in a negative bias if biotin concentrations are significant. For the Siemens ACTH assay, biotin concentrations of up to 1500 ng/mL does not cause significant interference, whereas for the Roche ACTH assay, biotin concentrations of > 60 ng/mL may be associated with interference. All patients denied biotin use except patient #11 who had a remote history of biotin supplementation, but had not consumed biotin on the day of testing. Biotin interference was not suspected in these cases given that the Siemens result was considered false positive and the expected biotin interference, if any, would have resulted in a falsely low result.

Although several steps were taken to identify the interfering agent, the exact identity is not as important as the recognition that an interfering agent is present which may have clinical consequences highlighted here. Arguably, assay interference should always be part of any differential diagnosis and therefore part of the “assessment strategy”, but cannot be confirmed or supported without laboratory analysis. When discrepancies between the clinical scenario, imaging, and assay results are noted, repeating the plasma ACTH measurement under optimal circumstances (to exclude pre-analytical factors and analytical factors) in addition to investigative trouble shooting which may include serial dilutions, HBT pretreatment, and testing using a different platform should be considered. Although, not all laboratories may have the resources to offer all means for troubleshooting, based on our experience at least two troubleshooting steps should be performed. We found the greatest success demonstrating lack of linearity following serial dilutions and testing by a different assay.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research report.

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Author contribution

Donegan- Data collection, manuscript writing and revision, Hamidi- data collection, manuscript revision, Bancos- data collection and manuscript revision, Nippoldt- data collection and manuscript revision, Young- manuscript revision and expertise, Algeciras-Schimnich- data collection, manuscript revision and expertise, Erickson- manuscript revision and expertise.

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