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Spuriously Elevated Total Estrogens Due to Immunoassay Interference in a Young Man With Normal Estrogen Fractions

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Hal Farkas [1], Ellen Leschek [2]

1. Medical School, Nova Southeastern University Dr. Kiran C. Patel College of Osteopathic Medicine, Fort Lauderdale, USA 2. Pediatric Endocrinology, Potomac Pediatrics, Rockville, USA

Corresponding author: Ellen Leschek, eleschek@potomacpediatrics.com

Abstract

During a routine wellness visit, a 19-year-old male complained of fatigue and mood fluctuations and requested hormone testing. Testosterone was normal, but total estrogens by immunoassay were 5987 pmol/L (1631 pg/mL; reference range 206-782 pmol/L (56-213 pg/mL)), approximately eight times the upper limit of normal. He had no signs of excess estrogen, and ultrasounds of the testes and adrenal glands were normal. Total estrogens remained elevated on multiple occasions at the same laboratory over six months. Estrone, estradiol, and estriol, the components of total estrogens (measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS)), were normal. Estradiol, alpha-fetoprotein, and human chorionic gonadotropin, as measured by immunoassay, were normal, localizing the issue to a single immunoassay. The patient received rabies vaccinations and equine rabies immunoglobulin following a dog bite three to four weeks before the first abnormal total estrogen immunoassay result. Both interventions are a recognized source of antibodies that can falsely elevate hormone measurements obtained by immunoassay. The spurious total estrogen elevation in this individual is very likely attributable to antibody-mediated immunoassay interference. When a total analyte and its components are measurable, gross discordance between them and a lack of clinical correlation are suggestive of assay interference.

Categories: Endocrinology/Diabetes/Metabolism
Keywords: anti-animal antibodies, heterophile antibodies, hyperestrogenism, immunoassay interference, liquid chromatography-tandem mass spectrometry, total estrogens

Introduction

Immunoassays are a mainstay of endocrine testing but are susceptible to interference from antibodies (endogenous and exogenous), biotin, paraproteins, and other cross-reacting compounds. Reported frequencies range from 0.4% to 4.0% [1], and in reviews of documented interference affecting thyroid function and cardiac biomarker immunoassays, roughly one-half led to misdiagnosis and/or inappropriate treatment [1,2]. Large analytes, such as thyrotropin, are measured by immunometric (“sandwich”) assays, in which two antibodies bind separate sites on the molecule and signal increases by raising concentration. Estradiol is too small for conventional two-site immunometric assays and is therefore routinely measured by competitive immunoassay. Estradiol from the patient sample competes with a fixed quantity of labeled estradiol for a limited number of antibody binding sites, causing signal to fall as concentration rises. Any interference that suppresses signal, such as an antibody that blocks the reagent antibody, binds the labeled tracer, or disrupts detection, results in false elevation [3]. Falsely elevated estradiol from this type of interference has been reported in 15 patients, with serious consequences resulting, such as unnecessary oophorectomy [4-6], diagnostic laparoscopy [7], cancelled in vitro fertilization cycles [8,9], and inappropriate aromatase inhibitor therapy [10,11]. Most reported cases involved estradiol immunoassay, and all but one occurred in women. There is no prior report of interference affecting a total estrogens immunoassay. We describe a young man with spuriously elevated total estrogens as measured by immunoassay. This assay lacks specificity and is prone to cross-reactivity from endogenous and exogenous antibodies, exogenous supplements, and structurally similar steroid hormones. Estrogen concentrations are normally low in young adult males, so the extremely high total estrogens in this individual prompted a six-month investigation demonstrating a gross discordance between total estrogens and its individually measured components, measured by liquid chromatography-mass spectrometry and mass spectrometry, which proved highly informative.

Case Presentation

History

During a routine wellness visit, a 19-year-old male requested hormone testing for fatigue and mood fluctuations. Total testosterone (Roche electrochemiluminescence immunoassay (ECLIA)) was 20.2 nmol/L (581 ng/dL; reference range 5.2-27.2 nmol/L (150-785 ng/dL)). Total estrogens (enzyme-linked immunosorbent assay (ELISA)) was 5987 pmol/L (1631 pg/mL; reference range 206-782 pmol/L (56-213

pg/mL)), and this result was verified. Two months later, total estrogens at the same laboratory were 2004 pmol/L (546 pg/mL), and testosterone was 16.0 nmol/L (461 ng/dL). A complete blood count showed mild erythrocytosis with low red cell indices. Complete metabolic panel, lipid panel, and thyroid function studies were normal. 25-Hydroxyvitamin D was 44 nmol/L (17.6 ng/mL; reference range 75-250 nmol/L (30-100 ng/mL)), and supplementation was initiated.

He described puberty as occurring on the late side of normal, with pubertal gynecomastia beginning at about age 12. He said that the gynecomastia decreased but never completely resolved. He denied exogenous testosterone or estrogen exposure, including oral and topical (direct or indirect) sources. He denied the use of alcohol, cannabis, tea tree oil, and/or lavender-containing products. He reported occasional use of creatine and whey protein supplements. He denied muscle-enhancing or any other hormone supplements. He trained at the gym intensively and regularly with weights. Family history was notable for maternal hypothyroidism. His fatigue and mood symptoms resolved by the time of endocrine evaluation.

About three weeks prior to the initial elevated total estrogen result, he was bitten by a stray dog while visiting family in a rural area abroad. He received equine rabies immunoglobulin and a rabies vaccine series.

Physical examination

Examination showed a well-developed, muscular male with full facial and axillary hair, a normal adult male phallus, 25 mL testes bilaterally, and Tanner stage 5 pubic hair. Very minimal diffuse breast tissue was palpable bilaterally and not visible when supine or seated. Nipples did not appear stimulated by estrogen. There were no other signs of estrogen excess.

Diagnostic assessment

Estrogen laboratory results are summarized in Table *1*.

Interval From First Result	Analyte	Laboratory A						Laboratory B			
		ELISA	ELISA Normal Range	ICMA	ICMA Normal Range	Roche ECLIA	Roche ECLIA Normal Range	Immunoassay	Immunoassay Normal Range	LC-MS/MS	LC-MS/MS Normal Range
Day 0	Total Estrogens	5987 pmol/L (1631 pg/mL)[a,b]	206–782 pmol/L (56–213 pg/mL)	—	—	—	—	—	—	—	—
Day 70	Total Estrogens	2004 pmol/L (546 pg/mL)	206–782 pmol/L (56–213 pg/mL)	—	—	—	—	—	—	—	—
Day 102	Total Estrogens	3076 pmol/L (838 pg/mL)[b]	206–782 pmol/L (56–213 pg/mL)	—	—	—	—	—	—	—	—
	Estradiol	—	—	—	—	75 pmol/L (20.5 pg/mL)	28–156 pmol/L (7.6–42.6 pg/mL)	—	—	—	—
	Estriol	—	—	0.7 nmol/L (0.2 ng/mL)	not established	—	—	—	—	—	—
Day 114	Estrone	—	—	—	—	—	—	—	—	93 pmol/L (25 pg/mL)[c]	≤252 pmol/L (≤68 pg/mL)
Day 115	Total Estrogens	—	—	—	—	—	—	1674 pmol/L (456 pg/mL)	≤1483 pmol/L (≤404 pg/mL)	—	—
Day 137	Total Estrogens	—	—	—	—	—	—	1061 pmol/L (289 pg/mL)	≤1483 pmol/L (≤404 pg/mL)	—	—
	Estradiol	—	—	—	—	—	—	<110 pmol/L (<30 pg/mL)	≤143 pmol/L (≤39 pg/mL)	59 pmol/L (16 pg/mL)	≤106 pmol/L (≤29 pg/mL)
	Estrone	—	—	—	—	—	—	—	—	78 pmol/L (21 pg/mL)	≤252 pmol/L (≤68 pg/mL)
	Estriol	—	—	—	—	—	—	—	—	<0.35 nmol/L (<0.10 ng/mL)	≤0.62 nmol/L (≤0.18 ng/mL)
Day 165	Total Estrogens	1949 pmol/L (531 pg/mL)[b]	206-782 pmol/L (56-213 pg/mL)	—	—	—	—	1149 pmol/L (313 pg/mL)	≤1483 pmol/L (≤404 pg/mL)	—	—

	Estradiol	—	—	—	—	—	—	—	—	128 pmol/L (35 pg/mL)	≤106 pmol/L (≤29 pg/mL)
	Estrone	—	—	—	—	—	—	—	—	111 pmol/L (30 pg/mL)	≤252 pmol/L (≤68 pg/mL)
	Estriol	—	—	—	—	—	—	—	—	<0.35 nmol/L (<0.10 ng/mL)	≤0.62 nmol/L (≤0.18 ng/mL)
Day 179	Total Estrogens	1839 pmol/L (501 pg/mL)	—	—	—	—	—	—	—	—	—
	Estradiol	—	—	—	—	57 pmol/L (15.4 pg/mL)	28–156 pmol/L (7.6–42.6 pg/mL)	323 pmol/L (88 pg/mL)	≤143 pmol/L (≤39 pg/mL)	59 pmol/L (16 pg/mL)	≤106 pmol/L (≤29 pg/mL)
	Estrone	—	—	—	—	—	—	—	—	74 pmol/L (20 pg/mL)	≤252 pmol/L (≤68 pg/mL)
	Estriol	—	—	—	—	—	—	—	—	<0.69 nmol/L (<0.20 ng/mL)	≤0.62 nmol/L (≤0.18 ng/mL)

TABLE 1: Estrogen measurements by method and laboratory over six months of evaluation.

ELISA: enzyme-linked immunosorbent assay. This assay detects and measures an antigen or antibody. Specific antibody–antigen binding occurs on a solid surface. Then an enzyme attached to an antibody converts a substrate into a colored, luminescent, or fluorescent product. The strength of that signal tracks how much of the target is in the sample. The enzyme amplifies the signal, making the test sensitive, and antibody binding makes it specific.

ICMA: immunochemiluminometric assay. This is a two-site ("sandwich") immunometric assay. A capture antibody holds the analyte, and a second antibody, tagged with a chemiluminescent label, measures it.

ECLIA: electrochemiluminescence immunoassay. This assay involves antibody–antigen binding that is detected by light emitted from a label when voltage is applied at an electrode. It has high sensitivity, low background, and a wide dynamic range.

LC-MS/MS: liquid chromatography–tandem mass spectrometry. This is a two-stage assay. First, liquid chromatography physically separates molecules. Next, a tandem mass spectrometer identifies and measures the molecules according to their mass-to-charge ratio before and after fragmentation. Specific parent mass and fragment mass give this assay very high selectivity.

a: Total estrogens is a mixed analyte; conversion to pmol/L uses the molecular weight of estradiol (factor 3.671), its principal component, by convention. b: Reported by the laboratory as “results verified by repeat testing.” c: Performed by laboratory A’s mass spectrometry reference division.

At the initial endocrine evaluation (day 102), total estrogens (ELISA, laboratory A) were 3076 pmol/L (838 pg/mL). Each estrogen component was subsequently measured separately. Estradiol (Roche ECLIA, laboratory A) was 75 pmol/L (20.5 pg/mL; reference range 28-156 pmol/L (7.6-42.6 pg/mL)) and estriol (immunochemiluminometric (ICMA) assay, laboratory A) was 0.7 nmol/L (0.2 ng/mL; no reference range established). Estrone, measured twelve days later by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at laboratory A, was 93 pmol/L (25 pg/mL; reference range 37-185 pmol/L (10-50 pg/mL)). On day 102, alpha-fetoprotein (<1.8 µg/L; reference range ⩽5.7) and beta human chorionic gonadotropin (<1 IU/L; reference range ⩽3), both measured by Roche ECLIA at laboratory A, were normal. Total estrogens measured by laboratory B’s immunoassay (ELISA) on day 115 were 1674 pmol/L (456 pg/mL; reference range ⩽1483 pmol/L (⩽404 pg/mL)).

Three weeks later, at laboratory B, total estrogens (ELISA) was 1061 pmol/L (289 pg/mL), estradiol by immunoassay was <110 pmol/L (<30 pg/mL; reference range ⩽143 pmol/L (⩽39 pg/mL)), and LC-MS/MS estrogen fractions were normal: estradiol 59 pmol/L (16 pg/mL; reference range ⩽106 pmol/L (⩽29 pg/mL)), estrone 78 pmol/L (21 pg/mL; reference range ⩽252 pmol/L (⩽68 pg/mL)), and estriol <0.35 nmol/L (<0.10 ng/mL; reference range ⩽0.62 nmol/L (⩽0.18 ng/mL)). Sex hormone-binding globulin was 10 nmol/L (reference range 10-50 nmol/L).

Approximately one month later, specimens were simultaneously assayed at both laboratories. At laboratory A, total estrogens (ELISA) were 1949 pmol/L (531 pg/mL; verified). At laboratory B, total estrogens (ELISA) were 1149 pmol/L (313 pg/mL), and by LC-MS/MS estradiol was 128 pmol/L (35 pg/mL), marginally above the reference range, with estrone 111 pmol/L (30 pg/mL) and estriol <0.35 nmol/L (<0.10 ng/mL).

Two weeks later, specimens were again drawn simultaneously. At laboratory A, total estrogens (ELISA) were 1839 pmol/L (501 pg/mL), and estradiol (Roche ECLIA) was 57 pmol/L (15.4 pg/mL). At laboratory B, estradiol by immunoassay was 323 pmol/L (88 pg/mL), whereas by LC-MS/MS estradiol was 59 pmol/L (16 pg/mL), estrone 74 pmol/L (20 pg/mL), and estriol <0.69 nmol/L (<0.20 ng/mL).

Total testosterone was normal on four occasions (12.4-22.2 nmol/L (356-640 ng/dL)), and free testosterone (direct analog enzyme immunoassay) on three occasions (108-122 pmol/L (31.1-35.1 pg/mL)). Dehydroepiandrosterone (LC-MS/MS) was 18.6 nmol/L (536 ng/dL; reference range 1.4-17.0 nmol/L (40-491 ng/dL) for ages 17-19, 1.1-24.3 nmol/L (31-701 ng/dL) for ages 20-50). Dehydroepiandrosterone sulfate (ECLIA), androstenedione (LC-MS/MS), and 17-hydroxyprogesterone (LC-MS/MS) were normal. Total protein, albumin, and globulin were normal.

Ultrasonography of the testes and adrenal glands performed following initial endocrine assessment was normal. A consultant steroid biochemist with expertise in hormone assay methodology concurred that there was no clinical or laboratory evidence of estrogen excess.

Treatment

Creatine and whey protein supplements were discontinued following the initial endocrine evaluation. Total estrogens remained elevated on subsequent assessments. No pharmacologic or surgical treatment was administered.

Outcome and follow-up

Total estrogens by immunoassay at laboratory A were elevated on five determinations over six months. Apart from one marginally elevated estradiol, LC-MS/MS estrogen fractions were normal on three occasions. The patient remained well throughout with no clinical evidence of estrogen excess.

Discussion

Evidence demonstrated that the elevated total estrogens did not reflect clinical estrogen status. Sustained total estrogens at this high level in a young adult male would likely produce clinical effects, and none were present. Further, total estrogens measurement was not consistent with measured estrogen fractions. Estrone, estradiol, and estriol by LC-MS/MS were normal on three occasions, while total estrogens were approximately eight times the upper limit of the normal range. Therefore, the discrepancy between total estrogens and estrogen fractions was likely due to immunoassay detection of interfering substances. To date, most reports of falsely elevated estrogen measurements involve a single estrogen analyte (estradiol) with no components to fractionate. One report described use of an insufficient reagent dose in a prepubertal girl whose falsely elevated estradiol did not reproduce on repeat assay [12]. Interference with the total estrogens immunoassay has not previously been described. LC-MS/MS is essentially unaffected by antibody interference [13], and use of this assay to measure estrogen analytes generated normal results throughout the evaluation and follow-up of this patient. The total estrogen issue for this patient was assay-specific rather than specimen- or laboratory-specific. Estradiol, alpha-fetoprotein, and human chorionic gonadotropin measured by Roche ECLIA on the same serum at the same laboratory were normal. Prior reports established method specificity by comparing laboratories [4,5,7,10,11]. Within-laboratory, within-specimen dissociation is a tighter control. Elevated total estrogens in this patient were reproduced in the same laboratory on multiple occasions across six months. Standardization of the approach to detecting and addressing assay interference could alleviate issues associated with unexpected and/or inappropriate abnormalities in laboratory results.

Endogenous and exogenous antibodies interfere with competitive immunoassays indirectly by binding the capture antibody, labeled analyte, or detection system. Each suppresses signal, which the assay reports as elevated analyte [3]. Wauthier et al distinguishes heterophile antibodies, which are low-affinity, polyspecific, and diagnosed only when there is no evidence of animal antigen exposure, from human anti-animal antibodies, which are monospecific and typically follow a known exposure [1]. Our patient had two documented animal antigen/antibody exposures: equine rabies immunoglobulin and a rabies vaccine series. Anti-animal antibody is the most likely etiology of this patient's spuriously elevated total estrogens. The temporal relationship is consistent, and the gradual decline in total estrogen levels with a months-long plateau rather than resolution favors an active endogenous antibody response rather than residual passive exogenous immunoglobulin. Biotin interference is unlikely, as there was no history of biotin supplementation, and total estrogens remained elevated after all dietary supplements (creatine and whey protein) were discontinued.

Limitations include the fact that heterophile blocking, polyethylene glycol precipitation, and serial dilution studies were not performed. Therefore, an antibody was inferred rather than directly measured. Method comparison alone does not discriminate between interference mechanisms [1]. Serum protein electrophoresis was not performed, although normal electrophoresis may not have excluded a monoclonal interferent. In one report, an immunoglobulin A lambda paraprotein bound 88% of the assay tracer despite electrophoresis showing no monoclonal peak [6]. No pre-exposure specimen exists, so the association with antibodies generated by rabies prophylaxis is suggestive but unverifiable. Laboratory B's total estrogens immunoassay was marginally elevated on one occasion, and its estradiol immunoassay was elevated on a day when laboratory A's estradiol (ECLIA) and laboratory B's estradiol (LC-MS/MS) were normal, consistent with an interferent affecting immunoassays to differing degrees depending on assay architecture [14]. Lastly, we cannot rule out the possibility that matrix components suppressed analyte ionization in our estrogen

component LC-MS/MS measurements. However, the lack of clinical evidence of estrogen excess makes this unlikely.

Increased utilization of assays with greater specificity, such as LC-MS/MS, could reduce the occurrence of inaccurate laboratory results. Improvement could also be accomplished with the design of next-generation reagents and better characterization of unknown cross-reactants. Automated interference-flagging algorithms and standardization of pre-treatment protocols could also decrease the generation of factitious laboratory results.

The evaluation of this patient spanned more than seven months, two reference laboratories, five estrogen assays, two imaging studies, and an external consultation. He was not exposed to any procedures posing risk throughout his evaluation, unlike multiple published cases [4-7]. Measurement of estrogens in a young adult male without clinical signs of estrogen excess set the reported cascade of events in motion.

Conclusions

The spurious elevation of total estrogens in this young adult male is very likely due to antibody-mediated immunoassay interference. The ability to measure total estrogens and its measurable components demonstrated gross discordance. This, coupled with a lack of clinical correlation and a history of anti-animal antibody exposure, is highly suggestive of assay interference. Comprehensive investigation avoided the performance of risk-laden testing and procedures.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Ellen Leschek, Hal Farkas

Acquisition, analysis, or interpretation of data: Ellen Leschek, Hal Farkas

Drafting of the manuscript: Ellen Leschek, Hal Farkas

Critical review of the manuscript for important intellectual content: Ellen Leschek, Hal Farkas

Supervision: Ellen Leschek

Disclosures

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