Immunoassay of Estradiol: Unanticipated Suppression by Unconjugated Estriol

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Background: Accurate measurement of estradiol is important in clinical settings. The quality of laboratory estimations of estradiol may be assessed through external quality-assurance surveys.

Methods: Estradiol was measured by microparticle enzyme immunoassay (MEIA) and other immunoassays. Proficiency testing of medical laboratories was conducted using samples prepared from normal male human serum supplemented with exogenous estradiol and other steroid and nonsteroid hormones, and participant laboratories measured estradiol by a variety of commonly used immunoassay techniques.

Results: The imprecision (CV) for measurement of estradiol [100–300 ng/L (367–1102 pmol/L)] was ≤22% for most analytical techniques. Greater imprecision, as high as 40% for the same concentration range, was observed for the (AxSYM) MEIA method in the proficiency testing event of September 2001. Results from this method were bimodal in distribution. We found that unconjugated estriol at concentrations >1.5 μg/L (>5.2 nmol/L) interfered with the MEIA method, leading to decreased recovery of added estradiol by up to 50%. This suppression in estradiol measurement was prevented by dilution of the specimen before measurement. Addition of unconjugated estriol gave a positive bias in some other immunoassay methods for estradiol. Poor comparability among the immunoassay methods for measurement of estradiol at clinically relevant concentrations [~60 ng/L (220 pmol/L)] was revealed.

Conclusions: A negative interference of unconjugated estriol with the MEIA method is a source of error for estradiol measurement. Lack of specificity and lack of comparability among immunoassay methods for estradiol may have detrimental effects on medical practice.

The steroid hormone estradiol is the most potent among the estrogens. Accurate determination of estradiol concentrations in human serum and plasma is important in many clinical settings (1–4). In most clinical laboratories, estradiol is measured by immunoassay methods in conjunction with sensitive detection technologies and automated instrumentation. Such measurements are usually robust, economical, and precise (5). Estradiol is classified as a “type A” analyte by the WHO because it is a chemically well-defined compound (6). Although its measurement should be method-independent, performance quality for estradiol assay has been questioned for more than a decade (7–11). Difficulties in measurement of estradiol may include interfering substances, low endogenous concentration in most patient samples, a lack of analytical specificity, errors in calibration, and lack of traceability.

In this study, we demonstrate that the presence of unconjugated estriol interferes with the measurement of estradiol in the AxSYM microparticle enzyme immunoassay (MEIA) method; we also study the nature of the interference, determine whether such interference occurs in other immunoassay methods, and examine the comparability among methods used in clinical practice for estradiol measurement.

Materials and Methods
Blood specimens were collected, without additives, from patients and from a healthy volunteer, and serum was prepared by leaving the specimens at room temperature for 30 min, followed by centrifugation at 2000g for 20 min. Serum was either used immediately or stored for <14 days at or below ~40 °C. This study was approved by the New York State Department of Health Institutional Re-
view Board. Processed male human serum used for the preparation of proficiency testing samples was from Bioresource Technology, Inc. All steroids, from Sigma Aldrich Co., were dissolved in methanol before addition to serum. The immunoassay instruments and associated reagents that were used for measurements in our laboratories were as follows: AxSYM and IMx (Abbott Laboratories), and Elecsys 2010 (Roche Diagnostics Co.). Other instruments and methods involved in the study included Architect (Abbott Laboratories); Immuno 1, ACS:180, and ADVIA Centaur Estradiol-6 assay (Bayer Corp.); Coat-A-Count, Immulite, and Immulite 2000 (Diagnostic Products Corp.); Access (Beckman Coulter Inc.); Vitros ECI (Ortho Clinical Diagnostics); and VIDAS (bioMérieux, Inc.).

The AxSYM method is a heterogeneous immunoassay in which estradiol from the specimen binds to rabbit polyclonal anti-estradiol antibodies that are linked to microparticles. On removal of unbound materials, estradiol–alkaline phosphatase conjugate is added and binds to available sites. After washing, 4-methylumbelliferyl phosphate is added, and the fluorescent product is measured, from the original 50% down to 1:5 automated dilution. (Abbott Laboratories has instructed users of the AxSYM instrument to perform a 1:5 automatic dilution of samples for some specimens.) Of the steroids tested, only unconjugated estriol showed an inhibitory effect on estradiol measurements, in the undiluted specimen. The magnitude of this effect was substantially reduced when the assay was performed with the 1:5 automated dilution protocol.

To confirm the results described above and to eliminate the possibility of a matrix effect in the pooled human serum, we prepared two series of specimens by adding various amounts of unconjugated estriol and a constant concentration of estradiol (200 ng/L) to fresh human serum and to the proficiency serum matrix. The concentrations for unconjugated estriol corresponded to the range of concentrations found in nonpregnant and pregnant women (first 28 weeks), and the estradiol concentration of 200 ng/L was characteristic of late follicular and mid-luteal phases (13). Estradiol was measured by the AxSYM method in both an undiluted sample and in a 1:5 automatic dilution. The measured estradiol concentrations in the specimens of both series were substantially decreased, by up to 50%, in the presence of unconjugated estriol at 20 µg/L (Fig. 1, solid lines). However, the inhibitory effect of unconjugated estriol corresponded to the range of concentrations found in nonpregnant and pregnant women (first 28 weeks), and the estradiol concentration of 200 ng/L was characteristic of late follicular and mid-luteal phases (13).

We also examined the effect of unconjugated estriol on the measurement of estradiol with the IMx, an instrument that also uses the MEIA technique, at four different concentrations of estradiol (Fig. 2). At a serum estradiol concentration of 750 ng/L, unconjugated estriol at 20 µg/L suppressed the measured result by ~20%, and the effect on the IMx assay (Fig. 2) was generally similar to that found for the AxSYM (Fig. 1).

To determine whether an increase in the estradiol concentration could reduce or eliminate the inhibitory effect of unconjugated estriol, we prepared two sets of

<table>
<thead>
<tr>
<th>Steroid added</th>
<th>Measured estradiol, ng/L</th>
<th>Relative recovery (undiluted/1:5 dilution),%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconjugated estriol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 µg/L</td>
<td>729</td>
<td>918</td>
</tr>
<tr>
<td>10.0 µg/L</td>
<td>547</td>
<td>873</td>
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<tr>
<td>Cortisol</td>
<td></td>
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<tr>
<td>108 µg/L</td>
<td>993</td>
<td>1033</td>
</tr>
<tr>
<td>308 µg/L</td>
<td>&gt;1000</td>
<td>1047</td>
</tr>
<tr>
<td>Estriol 3-sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 µg/L</td>
<td>984</td>
<td>1112</td>
</tr>
<tr>
<td>10.0 µg/L</td>
<td>&gt;1000</td>
<td>1084</td>
</tr>
<tr>
<td>Testosterone</td>
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<tr>
<td>4.2 µg/L</td>
<td>&gt;1000</td>
<td>1038</td>
</tr>
<tr>
<td>5.2 µg/L</td>
<td>&gt;1000</td>
<td>1198</td>
</tr>
<tr>
<td>Progesterone</td>
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<td></td>
</tr>
<tr>
<td>7.4 µg/L</td>
<td>946</td>
<td>967</td>
</tr>
<tr>
<td>15.4 µg/L</td>
<td>919</td>
<td>780</td>
</tr>
</tbody>
</table>

* A range of 86–96% was obtained with use of three fresh patient sera without addition of exogenous estradiol and unconjugated estriol.


diagram

Table 1. Effect of other steroids on measurement of estradiol by the AxSYM (MEIA) method.

4 The conversion factors to molar units are: estradiol, ng/L × 3.67 = pmol/L; unconjugated estriol, µg/L × 3.47 = nmol/L.

5 Details regarding this proficiency test, as well as others, are available at http://www.wadsworth.org/chemheme.
specimens using both fresh human serum and proficiency serum matrix. To each set of samples, unconjugated estriol was added at a concentration of 10 \( \mu \text{g/L} \) and estradiol was added at concentrations ranging from 100 to 1000 ng/L. Estradiol was then measured by the AxSYM method without dilution. Increasing estradiol concentrations did not increase the percentage recovery of estradiol, nor did they reduce the inhibitory effect of unconjugated estriol (Fig. 3).

To determine the relationship between the concentrations of estradiol and unconjugated estriol in association with the observed suppression, we prepared a series of 24 samples, using six fresh patient sera, with various concentrations of endogenous and/or added estradiol (20–980 ng/L). These sera were aliquoted into four subsamples, and unconjugated estriol (up to 30 \( \mu \text{g/L} \)) was added. Estradiol was measured on the AxSYM instrument without dilution. We observed three different types of effect depending on the amount of estradiol in the specimens: a positive interference for estradiol concentrations of <100 ng/L; no apparent interference for estradiol at ~100 ng/L; and a negative interference for estradiol >150 ng/L (Fig. 4).

We examined the effect of unconjugated estriol on other immunoassay methods for estradiol by preparing two proficiency samples: one containing unconjugated estriol (at 4 \( \mu \text{g/L} \)), and the other with no such addition. These samples were analyzed by participant laboratories using various immunoassay methods in the May 2001 and January 2002 proficiency testing events conducted by our laboratory. The results, summarized in Table 2, show a positive interference of unconjugated estriol in all of these methods, with one exception; however, the effect seen in this latter method was inconclusive because of the low number of participants and relatively high imprecision. The increase in the measured estradiol concentration attributable to addition of unconjugated estriol ranged from 21% to 389%. We repeated this study using patient serum, rather than proficiency serum matrix, and similar results were obtained (data not shown).
Further examination of Table 2 shows a high variability among the methods used to measure estradiol in the absence of unconjugated estriol. The overall method mean was 40.4 ng/L with a range of 26.6–64.6 ng/L; the relative difference for each method is presented in Fig. 5. To rule out the possibility of a matrix effect, we also performed this experiment with serum from a nonpregnant female and obtained results similar to those shown in Fig. 5 (data not shown).

Discussion

Although estriol is the most abundant of the estrogens, it is less potent than estradiol. In pregnancy, the concentration of estriol can be increased several hundredfold. Interference attributable to unconjugated estriol in immunoassays has been described and is usually the result of positive cross-reactivity with the antibodies used for estradiol measurement (14). Unconjugated estriol is usually present at concentrations far exceeding the concentration of estradiol (15). However, a negative interference of unconjugated estriol in estradiol immunoassays has not been reported previously. We found that unconjugated estriol lowers the recovery of estradiol in the AxSYM (MEIA) method at several concentrations of the two steroids and in both patient specimens containing exogenous estradiol and estradiol in proficiency testing specimens.

Our study revealed three types of effects of unconjugated estriol on estradiol measurements; the type of effect varied according to the concentration of estradiol in the specimen (Fig. 4). At estradiol concentrations <100 ng/L, the effect of estriol resembled that of a cross-reacting interferent. At estradiol concentrations >200 ng/L, the effect of estriol was to suppress the measured concentration of estradiol. In the range of ~100–200 ng/L estradiol, there was little apparent effect, presumably because of a cancellation effect between the two phenomena. Although we did not investigate the mechanism of these interferences, a suggestion put forth by Valdes and Jortani (12) sheds light on the possible mechanism of the negative type of effect. They proposed that interfering substances bind to antibodies during the initial reaction and then dissociate during the wash step. As a consequence, relatively more binding sites become available for phosphatase-labeled conjugate to occupy, giving an increased amount of fluorescent product and a decreased analytical result. Negative interference with the MEIA technique, similar to the one we present here, has been reported for the measurement of digoxin (16–20). Further investigations would be required to confirm whether this may be a general problem of the assay design in MEIA technology.

The accuracy of measurement of specimens containing estradiol >200 ng/L and an increased concentration of estradiol was improved by dilution. Abbott Laboratories, through the AxSYM estradiol package insert, currently has explicit instructions to users that "patient specimens with an estradiol assay value greater than or equal to 250 pg/mL (ng/L) must be diluted, retested, and the diluted..."
result reported”. However, at various time points this concentration was either 150 or 250 ng/L. This procedure was to minimize “issues associated with depressed results for fertility management patients”. The cause of interference was not known, and we here identify unconjugated estriol as the likely source of error. Results from our proficiency surveys indicate that laboratories using the AxSYM method do not uniformly follow the manufacturer’s guidance. We therefore observed a bimodal distribution of results from this method, with apparent centers of ∼120 and 240 ng/L. Participant laboratories in the former group reported results from 99 to 140 ng/L and likely failed to dilute the specimen. Laboratories in the latter group reported results from 201 to 275 ng/L and likely did perform the dilution that minimized the interference of estriol. No laboratories reported results in the range 141–200 ng/L. The use of differing protocols among participant laboratories was likely the origin of the large CV obtained for the AxSYM method in the proficiency surveys.

The important role of proficiency testing in the unmasking of laboratory errors is apparent from this study. Those proficiency samples that were found to have associated with them an increased imprecision for measurement of estradiol contained unconjugated estriol at concentrations >3 µg/L. Concentrations far exceeding 3 µg/L may be found during pregnancy, and even some nonpregnant females have been shown to have serum concentrations up to 2.4 µg/L (15). Any concentration of estriol exceeding 1.5 µg/L was found to depress the measured concentration of estradiol when estradiol exceeded 200 ng/L. The concentration range of estradiol that we examined also mimicked concentrations characteristic of pregnancy (>200 ng/L) and of the early follicular phase (<100 ng/L). Using patient specimens with added estriol and estradiol, we were able to reproduce all of the effects found with pooled serum or proficiency test samples. We therefore believe that the samples used in this study were commutable (21), both for analyte concentration and for the matrix used. Definitive confirmation of our findings would require obtaining clinical specimens with endogenous concentrations of estradiol and estriol similar to those that we prepared by adding exogenous steroids.

Although the error attributable to the interference of estriol in the MEIA procedure may approach 50%, the clinical impact of this inhibitory effect may not be widespread because of the typically low concentration of unconjugated estriol in healthy nonpregnant women and because of the use of the recommended 1:5 dilution of patient samples by a proportion of laboratories. However, for certain medical conditions in which the unconjugated estradiol concentration increases, e.g., pregnancy and obesity (22), results of estradiol measurements using the MEIA method on an AxSYM or IMx instrument may need to be confirmed by another technology.

Poor agreement among methods was also revealed by proficiency testing (Table 2 and Fig. 5). We also found similar poor agreement when patient specimens were tested. This lack of agreement among methods may be related to issues of calibration and/or antibody specificity. A strategy for improving intermethod agreement has emerged from an experiment involving recalibration of all methods, using a standardized reference material (23). However, the continued existence of poor method agreement (Table 2 and Fig. 5) indicates that the latter model has yet to be fully adopted or that unstandardized reference methods are used by manufacturers within the in vitro diagnostics industry. The existing “reference” methods vary in their internal standards, derivatives, extraction procedures, and gas chromatography–mass spectrometry conditions (24–29). Any of these variables can potentially influence measurements (29). For example, two different derivatization techniques to determine estradiol produced significantly different results (26); perhaps, therefore, standardization of the so-called reference methods for estradiol measurements may be required.

Accurate measurement of estradiol is particularly important in an in vitro fertilization setting. Estradiol concentration is often used for dose adjustment during gonadotropin treatment and as an estimate of ovarian reserve, ovarian stimulation response, and pregnancy outcome (1–4). The discrepancies in the literature (30–32) regarding the recommendations for decisions based on serum estradiol concentration may well be attributable to the differential effect of estriol on different analytical methods or to the differences among the methods that we describe here (Table 2 and Figs. 1–5). For the specimen without exogenous estriol, the reported concentrations of estradiol ranged from 26.6 to 64.6 ng/L depending on the method used (Table 2); individual laboratory results ranged from <20 to 191 ng/L. For the specimen with exogenous estriol at 4 µg/L, the reported concentrations of estradiol ranged from 39 to 159 ng/L, depending on the
method used (Table 2); individual laboratory results ranged from 23 to 196 ng/L. These data show that the current state of practice for measurement of estradiol does not meet clinical needs, at least for certain applications (31). The presence of unconjugated estradiol in specimens further compromises reliability (Table 2).

In summary, an external quality-assurance/proficiency testing program revealed a source of error in the estimation of estradiol in serum. Deficiencies identified in this study occurred in three areas: (a) unconjugated estradiol interferes with the measurement of estradiol in the AxSYM (MEIA) method; (b) inconsistency of participant laboratories in compliance with the manufacturer-recommended dilution protocol for the AxSYM instrument leads to poor interlaboratory agreement; and (c) there is a lack of agreement in results among immunoassay techniques for measurement of estradiol.

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References