Serum Estradiol Quantified by Isotope Dilution–Gas Chromatography/Mass Spectrometry, Huiqin Wu, Carol Ramsay, Panfilo Ozaeta, Lin Liu, and Hoda Aboleneen* (Abbott Laboratories, Diagnostics Division, 100 Abbott Park Rd., Abbott Park, IL 60064; * address correspondence to this author at: Abbott Laboratories, Department 90T, Bldg. AP20, 100 Abbott Park Rd., Abbott Park, IL 60064-3500; fax 847-938-7550, e-mail Hoda.aboleneen@abbott.com)

Estradiol measurements are important in evaluations of ovarian function, infertility, and menopause (1). The assays are challenging because physiologic concentrations of estradiol are typically <100 ng/L in plasma of adult men and postmenopausal women and in both sexes during infancy and childhood (1). Immunoassays are widely used, and they provide high sensitivity and short analysis times (2). Other methods are needed to determine the accuracy of immunoassays. Our aim was to develop a practical gas chromatography–mass spectrometry (GC/MS) method to quantify serum estradiol and use it to evaluate immunoassay accuracy.

Isotope dilution–GC/MS is widely regarded as the most accurate technique for organic analytes of interest in clinical chemistry (3). Several GC/MS methods have been reported for the quantification of estradiol in biological sources (4–14). The complexity of samples requires isolation and purification of estradiol before derivatization for GC/MS analysis.

The most common separation methods for estradiol involve solvent extraction followed by further purification with Sephadex LH-20 (4–5), strong ionic exchangers (6–9), weak ionic exchanger (9–11), HPLC (12–13), affinity chromatography (14), or their combinations. These methods, however, have limitations. The HPLC method for estradiol isolation cannot handle multiple samples simultaneously. The affinity column (14) is not commercially available and is very expensive to make. Other methods generally cannot sufficiently remove serum matrix materials to ensure rugged performance.

Here we report a GC/MS method for the quantification of serum estradiol in which estradiol is separated from the ether extract of serum samples by fractionation with a polystyrene divinylbenzene resin that has strong anion-exchange and adsorption properties. The method eliminates the use of HPLC in sample preparation and makes it suitable for routine analysis.

Samples were prepared by liquid–liquid extraction and solid-phase extraction followed by derivatization. Briefly, 1 mL of 1.0 mol/L acetate buffer (pH 5.0) and 100 µL of 10 µg/L estradiol-d3 were added to 2 mL of serum (sample, calibrator, or control) in a 15-mL polypropylene screw-cap centrifuge tube (Corning). The mixture was vortex-mixed for 30 s, equilibrated for 15 min, and saturated with ~20 mg of NaCl; 11 mL of ethyl ether was then added. The tube was then capped, and the estradiol and estradiol-d3 were extracted from the mixture by rocking the tube for 15 min on an aliquot mixer. After centrifugation at 1470g for 7 min, the ether layer was transferred to a 15-mL glass centrifuge tube. The solvent was evaporated under a gentle stream of nitrogen in a 50 °C water bath.

The ether extraction residue was reconstituted in 1.0 mL of 2-propanol–methanol (1:1 by volume) and 2.5 mL of 2.0 mol/L NaOH. After brief vortex-mixing, the solution was applied to a solid-phase XTRX type AXS extraction column (Creative Technology Systems, Inc.) and pulled through by reduced pressure (5–15 mmHg). The column was then washed with 4.0 mL of methanol, 1.5 mL of H2O, 3.0 mL of 50 mL/L acetic acid, and 2.0 mL of methanol–H2O (20:80 by volume). Each wash solution was pulled through the column by reduced pressure (5–15 mmHg). Estradiol and estradiol-d3 were eluted with 4.0 mL of methanol into a 13 × 100 mm culture tube. The methanol was evaporated under a gentle stream of nitrogen in a 60 °C water bath.

We added 100 µL of anhydrous acetonitrile and 30 µL of heptfluorobutyric anhydride to the final residue from the solid-phase extraction. After brief vortex-mixing, the tubes were capped and kept on a heating block at 65 °C for 45 min. We then added 1.0 mL of 1.0 mol/L phosphate buffer (pH 6.0) and 2.0 mL of hexane to the cooled reaction mixture. The tubes were vortex-mixed and centrifuged at 1470g for 4 min. The upper layer was transferred to a 12 × 75 mm culture tube and dried under a gentle stream of nitrogen in a 60 °C water bath. The residue was reconstituted in 20 µL of isoctane.

We injected 2 µL of the reconstituted solution into a gas chromatograph (Model 5890 Series II; Hewlett Packard) equipped with an MSD 5970B mass spectrometer (Hewlett Packard). Data acquisition and processing were carried out with the Hewlett Packard UX ChemStation Data System.

GC separation was performed on a J&W DB-1 column (15 m × 0.25 mm i.d.; 0.25-µm film thickness) in splitless mode with helium as the carrier gas. The injection port temperature was 240 °C. The transfer line and detector temperature were at 260 °C. The oven temperature was ramped linearly from 80 to 240 °C at 15 °C/min and held at 240 °C for 5 min. The mass spectrometer was tuned with perfluorotributylamine and used in selected-ion monitoring mode. The two target ions were m/z 664.4 for estradiol and m/z 667.4 for estradiol-d3. The dwell time was 80 ms for both m/z 664.4 and 667.4, which corresponds to 20 cycles (data points) under each peak. The peak-area ratio of m/z 664.4 to m/z 667.4 was obtained for each calibrator, sample, and control.

For the removal of neutral, acidic, and basic compounds in the ethyl ether extract of serum, ion-exchange chromatography has been widely used (4–11). We used a poly(styrene divinylbenzene copolymer resin linked to a quaternary amine anion-exchange moiety to remove, from ether extract, nonacidic species and those showing weak adsorption to polystyrene. The AXS resin used in the procedure offers both anionic and adsorption properties, which have been used for selective extraction of synthetic growth promotants from animal tissues (15–16). Estradiol in the phenolate anion form was first exchanged to the resin at pH 11. Organic bases and neutral lipid and...
steroids were then removed during the methanol and H2O washes. The washes with 50 mL/L acetic acid were used to displace the estradiol from the charged portion of the resin; estradiol was then adsorbed to the polystyrene matrix in a reversed-phase fashion. This step allowed the elution of a large quantity of interfering anionic substances previously bound to the resin. The subsequent wash with methanol-H2O (20:80 by volume) removed 95% of estradiol and additional matrix materials. Finally, estradiol was released from the resin by elution with 4 mL of methanol. Under these experimental conditions, estradiol and estradiol-d3 had virtually identical retention times (~11.6 min), and they were monitored on two channels at m/z 664.4 and m/z 667.4, respectively.

Two calibration curves were constructed by plotting the peak-area ratio of estradiol to the internal standard (E2/E2-d3) against the estradiol concentration, [E2]. For the low curve, estradiol was added at 50–1000 ng/L (50, 100, 200, 500, 800, and 1000 ng/L); for the high curve, it was added at 400–3000 ng/L (400, 500, 1000, 2000, and 3000 ng/L). Linear regression analyses of the two curves were then performed. For the high curve, the equation was: \( \frac{E_2}{E_2-d_3} = 0.00211[E_2] + 0.00784 \) \( (r^2 = 0.999) \). The SDs of the slope and y-intercept (n = 6) were 0.00005 and 0.01, respectively. For the low curve, the equation for the line was: \( \frac{E_2}{E_2-d_3} = 0.00207[E_2] + 0.00386 \) \( (r^2 = 0.999) \). The SDs of the slope and y-intercept (n = 6) were 0.00005 and 0.01, respectively. The use of two calibration curves produced a better fit to points at the low end of the concentration range than could be achieved by the use of a weighting factor (1/x, where x equals the estradiol concentration) for linear regression analysis of a single curve. The limit of detection was 15 ng/L [signal-to-noise (S/N) ratio = 3].

The limit of quantification was 30 ng/L (S/N ratio = 10). The S/N ratios for the calibrators at 50, 100, 200, 400 ng/L were 65, 100, 240, and 390, respectively. The use of silanized glassware did not improve the detection limit.

Accuracy and precision of the method were assessed by preparing two independent sets of estradiol calibrators and controls in charcoal-stripped normal human serum. For the determination of accuracy, all estradiol concentrations in the two sets were analyzed in triplicate by two different analysts on different days (n = 6). The calibration curve was constructed with the data obtained from one set and was used to determine the concentrations of the second set. The mean deviation from the target concentrations was < 7.1%, and the CV was < 6.1% across the entire range. The three controls (75, 200, and 800 ng/L) of the second set were analyzed in triplicate by three different analysts on different days (n = 9). The CV was < 6.1% for all concentrations in both low and high curves. The overall extraction recovery of estradiol from serum was 52.7%. For a 100 ng/L sample, 10.5 pg of estradiol was actually injected onto the column, and the corresponding S/N ratio was ~100.

The specificity, accuracy, and precision of the method were examined by analyzing Certified Reference Materials (CRMs): CRMs 576, 577, and 578 from the Community Bureau of Reference of the European Commission. Six replicates of each CRM were analyzed (Table 1). The measured values were within 4.2% of stated values, and CVs were ≤ 5.2%. The S/N ratios for the three CRMs were 16, 140, and 480, respectively.

For six Abbott calibrators, GC/MS ratios were within 3% of the target concentrations (Table 1). In a correlation study, estradiol concentrations of 61 patient samples were measured by microparticle enzyme immunoassay (MEIA); Abbott Architect Estradiol assay) and by GC/MS (Fig. 1). The GC/MS and MEIA results were generated by two independent laboratories within Abbott and were submitted to an independent group for statistical analysis. A linear regression was obtained with the following equation: MEIA = 1.09 GC/MS + 44; \( r^2 = 0.997 \). The 95% confidence intervals of the slope and intercept

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Target</th>
<th>Found</th>
<th>SD, %</th>
<th>CV, % (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal 1</td>
<td>0</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cal 2</td>
<td>50</td>
<td>51.26</td>
<td>2.52</td>
<td>4.5</td>
</tr>
<tr>
<td>Cal 3</td>
<td>100</td>
<td>99.49</td>
<td>-0.51</td>
<td>4.1</td>
</tr>
<tr>
<td>Cal 4</td>
<td>200</td>
<td>202.9</td>
<td>1.45</td>
<td>3.2</td>
</tr>
<tr>
<td>Cal 5</td>
<td>500</td>
<td>495.8</td>
<td>-0.84</td>
<td>1.5</td>
</tr>
<tr>
<td>Cal 6</td>
<td>1000</td>
<td>1004</td>
<td>0.40</td>
<td>0.43</td>
</tr>
<tr>
<td>CRM 576</td>
<td>31.0</td>
<td>32.28</td>
<td>4.1</td>
<td>5.2</td>
</tr>
<tr>
<td>CRM 577</td>
<td>187.4</td>
<td>193.4</td>
<td>3.2</td>
<td>2.9</td>
</tr>
<tr>
<td>CRM 578</td>
<td>364.5</td>
<td>360.9</td>
<td>-1.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Cal, calibrator; ND, not detected; NA, not applicable.

![Fig. 1. Correlation of estradiol concentrations for 61 patients measured by GC/MS vs MEIA.](https://academic.oup.com/clinchem/article-abstract/48/2/364/5641575)
were (1.07–1.11) and (10–65), respectively. The SD of residuals (S_y|x) was 73 ng/L in the concentration range of 0–5000 ng/L and 22 ng/L in the range of 0–500 ng/L. The agreement between the GC/MS and MEIA assays indicated low interference from other estrogenic compounds and their metabolites in the latter assay.

In conclusion, we have developed a GC/MS method for the quantification of estradiol in patient serum samples and serum-based immunoassay calibrators; the method was validated by use of CRMs. The utility of the method was further demonstrated by the presented correlation between estradiol concentrations in patient samples measured by MEIA and GC/MS.

We thank Dr. Greg Williams and his team at Abbott Laboratories for providing the immunoassay data for the patient samples.

References

Rapid Quantification of CYP3A4 Expression in Human Leukocytes by Real-Time Reverse Transcription-PCR, Isabella Novakowski-Gashaw, 1 Przemyslaw M. Mrozikiewicz, 1 Ivor Roots 1, and Jürgen Brockmøller 1,2 1 Institute of Clinical Pharmacology, Charité University Medical Center, Humboldt University of Berlin, 10099 Berlin, Germany; 2 Department of Clinical Pharmacology, University Medical Center, Georg-August University of Göttingen, 37075 Göttingen, Germany; * address correspondence to this author at: Department of Clinical Pharmacology, University Medical Center, Georg-August-University of Göttingen, Robert-Koch-Strasse 40, 37075 Göttingen, Germany; fax 49-551-3912767, e-mail jürgen.brockmøller@med.uni-goettingen.de

Cytochrome P450 3A4 (CYP3A4) contributes to the metabolism of a wide variety of drugs and endogenous substrates, such as steroid hormones (1, 2). Variations in the catalytic activity of CYP3A4 are predominantly caused by enzyme induction mediated by transcriptional activation or by competitive substrate inhibition. Such variation may strongly influence the bioavailability of drugs and may modulate drug interactions. CYP3A4 is one of the predominant CYPs in the human liver, accounting for ~30% of the total hepatic cytochrome P450 protein (2, 3). Relatively high CYP3A4 concentrations have been found in the small intestinal epithelium (70% of total CYP protein) and in the kidney (2). There are conflicting results concerning the amount of CYP3A4 in human peripheral blood lymphocytes. Several authors could not detect any CYP3A4 mRNA or protein, whereas some studies reported poor CYP3A4 expression in the white cell fraction (4–6). Thus, we assumed that CYP3A4 is expressed in lymphocytes in very small amounts and that only a very sensitive method could detect them. We developed a sensitive quantitative real-time reverse transcription-PCR (RT-PCR) method that allows rapid and correct determination of CYP3A4 mRNA expression in leukocytes.

We investigated CYP3A4 mRNA expression in 31 human blood samples from healthy volunteers (20 males and 11 females; mean age, 29 years; range, 20–64 years) and in three human liver samples obtained from the International Institute for the Advancement of Medicine (Exton, PA). Before blood collection, all volunteers signed informed consents that were accepted by the Ethical Committee of the Charité. Leukocytes were separated from 8 mL of whole blood in a Vacutainer® opt cell preparation tube system (Becton Dickinson). Small liver fragments were disrupted with a homogenizer (Potter; Braun). Samples were stored at ~80°C. Total cellular RNA was extracted by the TRIzol® LS method according to the manufacturer’s protocol (Life Technologies). The procedure was modified by the addition of 10 ng of RNase-free glycogen (Roche) before homogenization. The RNA solution was digested with 10 U of RNase-free RQ1 DNase (Promega) for 15 min at 37°C to exclude genomic DNA from the preparation; extraction with TRIzol LS was then repeated. RNA concentration and purity were deter-