Enzyme Immunoassay for Specific Determination of the Synthetic Estrogen, Ethynyl Estradiol, in Plasma

A. Turkes, J. Dyas, G. F. Read,¹ and D. Riad-Fahmy

In this enzyme immunoassay for ethynyl estradiol, a conjugate of the 3-(O-carboxymethyl)ether with horse radish peroxidase is used as the label and a conjugate of the 6-(O-carboxymethyl)oxime with bovine serum albumin as the immunogen. A solid-phase antibody procedure is used in separating antibody-bound and free steroid. The lower limit of sensitivity of the assay is 2 pg per assay tube. Interference by synthetic progestagens was minimized by extracting the sample with an anti-estrogen serum before assay. Results obtained with a comparison radioimmunoassay and this procedure agreed well ($r > 0.98$).

Additional Keyphrases: oral contraceptives · steroids · drug assay · methods for the small laboratory and for field studies

Ethynyl estradiol (EE₂) is one of the most commonly used synthetic estrogens in combined oral-contraceptive formulations. Adverse clinical side effects associated with early formulations (1) led to the introduction of “low-dose” estrogen preparations, for which the acceptance rate in the U.S. two years later was 70% (2). In developing countries, only about 15% of women of reproductive age practice family planning, and of these some 70% discontinue it within two years (3). There is therefore a recognized need for formulations having greater acceptability in developing countries. Because physical status and dietary differences may influence the efficacy of orally active contraceptive formulations, it is advisable that field studies be done in the target country. In developing countries beta- and gamma-counting facilities are restricted, not only by the high capital cost of such equipment but also by difficulties in servicing it. Enzyme immunoassay (EIA), with a colorimetric endpoint readily determined with a simple spectrophotometer, represents an attractive alternative. Such assays also feature stable reagents, which helps minimize logistic difficulties, and thus have the high sample-throughput needed to process the many samples assayed in field studies.

The present paper describes an enzyme immunoassay for EE₂. An immunosorbent extraction and a suitable choice of antigen give the assay good specificity. The assay incorporates use of a horseradish peroxidase (EC 1.11.1.7) label with use of a solid-phase separation technique. Results obtained by this technique are compared with those of a conventional radioimmunoassay procedure.

Materials and Methods

Antiserum

Antiserum to ethynyl estradiol. The immunogen, ethynyl estradiol-6-(O-carboxymethyl)oxime coupled to bovine serum albumin, was prepared by standard procedures (4). Four New Zealand White rabbits were immunized with a suspension of 0.5 mg of the immunogen in an emulsion (7/3 by vol) of Freund’s complete adjuvant and isotonic saline, totaling 10 mL; a total of 2 mL of this emulsion was injected at 25–30 intradermal sites, at four- to five-week intervals, into each rabbit. Bordetella pertussis (Haemophilus pertussis) antigen, 0.5 mL, was given at the first injection only, to enhance immune response (5). All rabbits had produced antisera with adequate titer by the third injection.

Antiserum to estradiol-17β. This was raised against an estradiol 17-hemisuccinate conjugate in goats.

Absorption of antisera with bovine serum albumin. All antisera used in EIA procedures were absorbed with albumin (6). A precipitin curve, plotted as $A_{290}$ vs albumin concentration, showed the “zone of equivalence,” i.e., the concentration of albumin with which antibody precipitation was greatest (7). Antibodies to albumin were then precipitated from 5-mL volumes of antisera by adding albumin to give the “equivalence concentration.” After centrifugation, the supernate was decanted and stored in 0.5-mL aliquots at −20°C until required.

Coupling of antisera to cellulose. Microcrystalline cellulose (E. Merck AG, Darmstadt, F.R.G.; cat. no. 2330) was activated with cyanogen bromide (Aldrich Chemical Co., Dorset, U.K.) by the procedure of Wide (8). Anti-estradiol and anti-ethynyl estradiol sera were then coupled to activated cellulose according to Seth and Brown (9). The coupled antisera were diluted 100-fold in assay buffer and extracted with ethanol before use, to decrease the endogenous estrogen concentrations, as follows. Take a 10-mL aliquot of an antisemur suspension (100-fold dilution), centrifuge, and discard the buffer. Add 5 mL of ethanol, vortex-mix for 50 s, centrifuge, then aspirate the ethanol. Repeat this ethanol wash a further four times. Wash the pellet with 5 mL of buffer, and resuspend the coupled antiserum in 10 mL of assay buffer.

Coupled antisera when stored diluted 100-fold in assay buffer were stable at 4°C for at least six months.

Enzyme Label

Synthesis of EE₂-3(O-carboxymethyl)ether. Reflux 1 g of ethynyl estradiol for 10 min in 35 mL of ethanol containing 0.32 g of sodium metal. Add 0.41 g of chloroacetic acid and reflux the solution for a further 2 h. Remove the ethanol under reduced pressure, dissolve the residue in 50 mL of water, and wash the solution twice with 50-mL portions of ethyl acetate. Acidify to pH 2 with 4 mol/L hydrochloric acid and extract the solution with two 50-mL portions of ethyl acetate. Dry the organic solution over sodium sulfate, remove the solvent under reduced pressure. Dissolve the residue in hot acetic acid, add hexane dropwise until the solution is opalescent, and allow it to cool. Filter off the crystalline product (0.14 g; mp 161–165°C).

Synthesis of EE₂ enzyme label. Couple EE₂-3-(O-carboxymethyl)ether to horseradish peroxidase by a modified mixed-anhydride procedure (10), and purify the label by gel exclusion chromatography on Sephadex G-25.

Solvent and Reagents

Ethynyl estradiol, bovine serum albumin, Norit-A charcoal, gelatin (B.P. grade), and horseradish peroxidase (Reinheit's

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Received Nov. 28, 1980; accepted March 17, 1980.
Zahl, the ratio of absorbances at 280 and 403 nm, = 3.0) were all from Sigma Ltd., London, U.K.

[6,7-3H]Ethynyl estradiol (specific activity 58 kCi/mol) was from NEN Chemicals GmbH, Dreieich, G.F.R. All scintillation-grade chemicals were from Koch-Light Laboratories, Colnbrook, Bucks, U.K. The liquid scintillant was prepared by dissolving 5 g of 2,5-diphenyloxazole in a mixture of 500 mL of Triton X-100 surfactant and 1 L of toluene.

Ethynyl estradiol solutions. A stock standard solution (100 mg of EE2 per litre of ethanol) was stored at 4 °C. Working standards (10, 20, 40, 60, 80, and 100 pg/100 μL of ethanol) for use in constructing the dose–response curve were prepared by dilution when required. Tritiated EE2 was stored in benzene/ethanol (9/1 by vol) at 4 °C. A 50-μL aliquot was dried under nitrogen, and the residue was redissolved in 30 mL of assay buffer just before use.

Assay reagents. In the comparison radioimmunoassay (11) we used the reagents previously described (12) for a testosterone radioimmunoassay procedure: phosphate-buffered isotonic saline, pH 7.4; assay buffer (phosphate-buffered saline containing 1 g of gelatin per litre); and dextran-coated charcoal suspension.

In the EIA we used the reagents previously described in a testosterone assay (19): phosphate-buffered isotonic saline containing 1 g of bovine serum albumin per litre; substrate sodium phosphate buffer (0.2 mol/L., pH 6.0); and the substrate (α-phenylenediamine, 2.8 mmol/L, and hydrogen peroxide, 2 mmol/L).

Sample-extraction procedure. This procedure is common both to the EIA and radioimmunoassay. In labeled 12 × 75 mm glass tubes, place 300 μL of plasma and 300 μL of solid-phase anti-estradiol serum, suitably diluted (1/300) in assay buffer. Mix briefly and incubate overnight at 4 °C. Add 4 mL of phosphate-buffered saline, centrifuge (1300 × g, 10 min), and discard the supernate. Repeat this procedure twice. Add 300 μL of ethanol and extract the antibody-bound ethynyl estradiol in the pellet on a multivortex-type shaker for 10 min. Centrifuge and transfer 200 μL of the ethanolic phase to similarly labeled assay tubes.

Determine the ethynyl estradiol content of extracts either by the comparison radioimmunoassay, according to the technique of Dyson et al. (11) or by EIA.

Radioimmunoassay procedure. Evaporate ethanol extracts under a stream of nitrogen at 30 °C, add 100 μL of liquid-phase anti-estradiol serum, mix briefly, allow to stand at room temperature for 30 min, and add 100 μL of tritiated ethynyl estradiol. Incubate for a further 1 h at 30 °C, and separate antibody-bound and free steroid by treatment with 0.5 mL of dextran-coated charcoal. Centrifuge, decant the supernate into plastic scintillation vials, and count the radioactivity associated with the antibody-bound fraction in a β-scintillation counter.

EIA procedure. Evaporate 200-μL aliquots of ethanolic plasma extracts, or 200 μL of working standards, under a stream of nitrogen at 30 °C. Redissolve the steroid-containing residue with 100 μL of assay buffer, add 100 μL of solid-phase anti-estradiol serum, mix briefly, and allow to stand at room temperature for 30 min. Add 100 μL of enzyme label and incubate overnight at 4 °C. Add 4 mL of substrate buffer, centrifuge, discard the supernate, then repeat this procedure once. Add 2 mL of substrate solution (α-phenylenediamine/hydrogen peroxide), and allow the tubes to stand for 1 h at room temperature in the dark. Terminate the reaction by adding 0.6 mL of 2 mol/L sulfuric acid and measure the absorbance of the resulting solution at 492 nm (we used a Gilford Stasar II rapid-sampling spectrometer).

Calculate the concentration of ethynyl estradiol in plasma samples from the dose–response curve. We used a PDP 11/34 computer and the four-parameter model of Rodbard and Hutt.

Fig. 1. Composite of standard curves for 12 successive assays: The CV is indicated at each point (left). Data linearized by log-logit plot are shown on the right.

(13), but interpolation from a manually charted plot is acceptable.

Results

Analytical Variables

The dose–response curve obtained in the EIA procedure is shown in Figure 1. Twelve replicate assays established that the precision of the EIA was satisfactory at all points and closely approximated that of the comparison method.

Sensitivity of the standard curve, defined here according to the criteria of Kaiser and Specker (14) as the least amount distinguishable from zero at the 95% confidence level, was nearly the same for the two analytical techniques: 2 pg per assay tube for the EIA, 1 pg per assay tube for the radioimmunoassay.

Sensitivity of the assay. Sensitivity in previously published procedures (15, 16, 19) has been limited by difficulties associated with plasma "blanks," i.e., significant EE2 concentrations being observed in samples provided by volunteers who were taking no synthetic steroids. In the present assay, samples extracted with diethyl ether were associated with high blank values (11.5 ± 1.6 pg) equivalent to 30 pmol/L. This problem was eliminated by the solid-phase immunosorbent technique, blank values indistinguishable from zero (0 ± 0.1 pg per assay tube) being observed.

Specificity. Table 1 shows the cross reactivity of the anti-EE2 serum with structurally related steroids and others that may be co-administered. Significant cross reactions were observed with lynestrenol (8.45%), norethisterone (8.45%), and norgestrel (1.17%). Interference by these progestogenic ste-
plasmas were allowed to stand overnight at 4 °C. Recovery for the solid-phase immunoabsorption technique was excellent (Table 4), always exceeding 94%.

As a further check on recovery, various amounts of unlabeled EE2 were added to a plasma sample provided by a healthy woman who was taking no synthetic steroids, the samples were allowed to equilibrate overnight at 4 °C, then assayed. The data (Table 5) indicate close agreement between observed and calculated concentrations, confirming the efficiency of extraction.

The addition of tracer to monitor recovery was therefore considered unnecessary in routine practice.

Results by EIA and Radioimmunoassay Compared

EE2 concentrations in 25 samples from subjects taking a contraceptive formulation containing EE2 (35 µg) and norethisterone (500 µg) were determined by the EIA and the comparison radioimmunoassay (Figure 2). The agreement is satisfactory and regression analysis shows that the slope does not differ significantly from one (m = 0.978), or the intercept from zero (c = 10.97 pmol/L).

The assay has been shown to conform to accepted validation criteria and has subsequently been used to determine EE2 concentrations in plasma samples from subjects undergoing pharmacokinetic and bioavailability studies.

![Graph showing linear relationship between EIA and RIA concentrations.](image)

*Fig. 2. Results compared for plasma ethynyl estradiol as measured by radioimmunoassay and by EIA.*

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### Table 2. Effect of Protein Content, Lipemia, and Hemolysis

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Blank plasma</th>
<th>Buffer</th>
<th>Lipemic plasma</th>
<th>Hemolyzed plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1248</td>
<td>1248</td>
<td>1248</td>
<td>1248</td>
</tr>
<tr>
<td>1/2</td>
<td>1332</td>
<td>1256</td>
<td>1188</td>
<td>1164</td>
</tr>
<tr>
<td>1/4</td>
<td>1308</td>
<td>1284</td>
<td>1248</td>
<td>1208</td>
</tr>
<tr>
<td>1/8</td>
<td>1296</td>
<td>1040</td>
<td>1080</td>
<td>784</td>
</tr>
<tr>
<td>1/16</td>
<td>1248</td>
<td>1184</td>
<td>1216</td>
<td>928</td>
</tr>
</tbody>
</table>

*All results in pmol/L (100 pmol/L = 29.8 pg/mL).*

### Table 3. Intra- and Inter-assay Variance in High-, Medium-, and Low-concentration Quality-control Samples

<table>
<thead>
<tr>
<th>Dilution</th>
<th>High Ethynyl estradiol, pmol/L</th>
<th>Medium Ethynyl estradiol, pmol/L</th>
<th>Low Ethynyl estradiol, pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td>Mean: 951</td>
<td>594</td>
<td>277</td>
</tr>
<tr>
<td></td>
<td>SD: 62.4</td>
<td>42.1</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>CV, %: 6.6</td>
<td>7.1</td>
<td>9.3</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>Mean: 972</td>
<td>569</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>SD: 38.8</td>
<td>51.8</td>
<td>31.9</td>
</tr>
<tr>
<td></td>
<td>CV, %: 4</td>
<td>9.1</td>
<td>9.8</td>
</tr>
</tbody>
</table>

### Table 4. Analytical Recovery of [3H]Ethynyl Estradiol from Plasma

<table>
<thead>
<tr>
<th>Ethynyl estradiol (pg/assay tube)</th>
<th>Recovery, % (and SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99.6 (0.9)</td>
</tr>
<tr>
<td>10</td>
<td>97.7 (1.3)</td>
</tr>
<tr>
<td>50</td>
<td>97.6 (1.8)</td>
</tr>
<tr>
<td>100</td>
<td>97.2 (1.4)</td>
</tr>
<tr>
<td>200</td>
<td>97.6 (1.6)</td>
</tr>
<tr>
<td>400</td>
<td>96.6 (1.6)</td>
</tr>
<tr>
<td>800</td>
<td>94.1 (2.1)</td>
</tr>
</tbody>
</table>

Results were obtained using EIA or radioimmunoassay.

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Norethisterone was decreased to <0.15% by a solid-phase immunoabsorption technique (Table 1). After this pre-assay purification, no compound appears to cross react greatly enough to interfere significantly with assay of clinical samples.

**Linearity.** A high-titre plasma sample provided by a healthy woman taking an EE2-containing contraceptive formulation (EE2 34 µg; norethisterone 500 µg) was serially diluted with buffer and normal, grossly hemolyzed, or hyperlipemic plasma provided by subjects not receiving synthetic steroids. These data (Table 2) indicate that the assay is not sensitive to changes in protein concentration, and that moderate hemolysis and high lipid concentration in the samples causes no significant interference in the assay.

**Precision.** Three plasma pools of high, medium, and low titres were established. Twenty-four aliquots of each pool, titrated in one assay, gave the intra-assay variance shown in Table 3. These pools were subsequently split into aliquots stored at −20 °C, and used as quality controls for subsequent routine assays, giving the inter-assay variance (Table 3).

**Analytical recovery.** Tritium-labeled EE2 was added to plasmas containing known amounts of added EE2 and the
Table 6. Ethinyl Estradiol Concentrations in Plasma of Subjects Taking Combined and Norethisterone-Only Contraceptive Formulations

<table>
<thead>
<tr>
<th>Day of treatment cycle</th>
<th>Concn, pmol/L (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–7</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Combined</td>
<td>607 (83)</td>
</tr>
<tr>
<td>NET only</td>
<td>104 (7.2)</td>
</tr>
<tr>
<td>14–16</td>
<td>429 (72)</td>
</tr>
<tr>
<td></td>
<td>107 (5.8)</td>
</tr>
<tr>
<td>23–25</td>
<td>421 (41)</td>
</tr>
<tr>
<td></td>
<td>107 (4.8)</td>
</tr>
</tbody>
</table>

Plasma EE₂ concentrations in three volunteers increased rapidly after oral administration of a preparation containing EE₂ (35 μg) and norethisterone (500 μg), reaching peak values in 30 to 90 min (Figure 3), and declined rapidly thereafter. The maximum concentration of EE₂ in plasma varied slightly from subject to subject, the mean peak values observed being 525 pmol/L (range, 500–752 pmol/L). EE₂ was still detectable at 24 h in the plasma of two subjects, but not in the third, whose concentration was <20 pmol/L.

Plasma EE₂ concentrations in women taking a norethisterone-only formulation (500 μg) were also determined, to check its in vivo conversion to EE₂. Participants in this study (n = 10) took the preparation for 21 days, starting on the fifth day after menstruation, and samples were collected within 2 h after administration. EE₂ concentrations in the samples collected at the beginning (five to seven days), middle (14–16 days), and end (23–25 days) of their treatment cycles were determined by EIA. The data (Table 6) indicate that the norethisterone-only preparation to circulating EE₂ concentrations is about 100 pmol/L (range, 47 to 199 pmol/L) throughout the treatment cycle. It is noteworthy that EE₂ concentrations in plasma samples obtained from the same subjects in the pre-treatment cycle in which they receive no synthetic steroids were always indistinguishable from zero. These data suggest that the values observed in the treatment cycle were not due to plasma “blank” effects.

Discussion

Previously reported radioimmunoassays for EE₂ (15–17) were associated with unacceptably high “plasma blank” values and featured antisera having significant cross reactivity with progestogenic steroids. These progestogens, frequently co-administered in relatively high concentrations, may therefore cause serious interference in assays for EE₂, because highly specific anti-EE₂ sera are not currently available. Conventional pre-assay purification procedures involving thin-layer and column chromatography are unacceptable in routine practice; a solid-phase immunoabsorbant technique (18) was therefore incorporated into our procedure and conferred specificity on an assay in which a relatively nonspecific anti-EE₂ serum is used.

Radioimmunoassays featuring tritiated ligands are probably the least cost-effective of all immunoassay procedures. Running costs may be decreased by substituting enzyme immunoassays having the sensitivity, specificity, and precision of conventional radioimmunoassays (19–21). Although good sensitivity has been achieved in homologous EIA’s for naturally occurring steroids, a limited degree of heterology may facilitate development of a sensitive assay whilst retaining adequate specificity; such a system has been described for estradiol (22) involving 3/6 heterology. In the assay described here we therefore used an antisera raised against an EE₂-6-[(O-carboxymethyl)oxime/bovine serum albumin conjugate and a label prepared by conjugating peroxidase to EE₂-3-(O-carboxymethyl)ether. Similar 3-(O-carboxymethyl)ether derivatives have been used in the radioimmunoassay of naturally occurring estrogens (23). Increased throughput in the

Fig. 3. Plasma ethinyl estradiol concentrations in three normal women after oral administration of 35 μg of EE₂ and 500 μg of norethisterone

EIA of EE₂ was ensured by using a cellulose-coupled solid-phase antiserum, facilitating the separation of antibody-bound and free steroid. The time independence of this separation procedure allows many samples to be processed in one batch without misclassification errors (24).

Widely different EE₂ concentrations have been reported in previous publications, the highest concentrations of EE₂ being observed in women taking combined preparations with norethisterone or norgestrel. In the study of Pasqualini et al. (16) on volunteers receiving EE₂ (50 μg) norethisterone (1000 μg), EE₂ concentrations ranged from 405 to 3580 pmol/L and in the study of Elstein et al. (17) concentrations ranged from 2680 to 4610 pmol/L in women receiving EE₂ (30 μg) d-norgestrel (150 μg). These values are considerably higher than ours (Figure 3), where EE₂ plasma concentrations did not exceed 900 pmol/L in subjects given a combined preparation (EE₂ 35 μg; norethisterone 500 μg). These disparate results may reflect differences in assay specificity, but unfortunately neither of the previous publications report data relevant to this.

The study of Nilsson and Nygren (15) indicates that subjects receiving an EE₂-only formulation (EE₂ 30 μg and 50 μg) had the lowest reported EE₂ values (170–304 pmol/L). These authors claim that the significantly lower circulating EE₂ concentrations are ascribable to the absence of cross-reacting progestogens. However, these differences may arise by partial conversion of norethisterone to EE₂. Because evidence for this conversion is controversial (25), we determined EE₂ concentrations by the specific EIA in women who were taking a norethisterone-only formulation. Significant concentrations of EE₂ were found (Table 6) by EIA and confirmed by a gas-chromatographic/mass-spectrometric technique (26).

Women taking a norethisterone-only preparation are therefore not exposed to “unopposed” progestational effects, because interconversion provides EE₂ concentrations that are as high as 25% of those achieved on a combined preparation containing EE₂ (35 μg) norethisterone (500 μg).

The cost-effective EIA described in this paper allows at
least 40 samples to be processed in any one batch. It is ideally
suited for routine use in well-established laboratories and
could well be particularly useful for processing the samples
generated by field studies of new contraceptive formulations
in developing countries.

We would like to thank Dr. M. Morton for synthesis of ethynyl es-
tradiol-3-(O-carboxymethyl)ether and Dr. B. Furr of I.C.I. Pharma-
caceuticals Division, Macclesfield, Cheshire, for a generous gift of an-
tisera to estradiol-17/3. We are also grateful to Professor K. Griffiths
for helpful advice and the Tenovus Organisation for financial sup-
port.

One of us (A.T.) is grateful to the WHO for the provision of a Re-
search Training Grant.

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