Radioimmunoassay for the Determination of 2-Methoxyestriol Concentration in Plasma of Pregnant Women

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A specific assay method has been developed for the determination of 2-methoxyestriol in plasma of pregnant women. The quantitation was achieved by radioimmunoassay after the extraction of the plasma with butyl acetate and purification on a Sephadex LH-20 column. In order to obtain the immunoagent for 2-methoxyestriol, 6-oxo-2-methoxyestriol was converted to its 6-(O-carboxymethyl)oxime derivative. This derivative was then coupled to bovine serum albumin by the mixed anhydride method, and rabbits were immunized with this conjugate. The antisemur obtained was partially purified by affinity chromatography on estrone 17-(O-carboxymethyl)oxime–aminohexyl Sepharose conjugate to eliminate the cross-reactive antibodies. Plasma 2-methoxyestriol concentrations in pregnancy were estimated to be mean values of 41.1 pg/ml (12th–14th week), 85.3 pg/ml (27th–29th week), and 97.5 pg/ml (37th–41st week).

Keywords 2-methoxyestriol; radioimmunoassay; plasma; pregnant woman; 2-methoxyestriol 6-(O-carboxymethyl)oxime; anti-2-methoxyestriol antiserum; 2-methoxyestriol–bovine serum albumin conjugate; cross-reactivity; Sephadex LH-20; affinity chromatography

It is well known that catechol estrogens, formed by the ring A hydroxylation of primary estrogens at either the C-2 or C-4 position, are converted to the corresponding monomethyl ethers by the enzyme catechol-O-methyltransferase (COMT). 11 Considerable attention has been focused on the physiological significance of catechol estrogens and their methyl ethers. 2 Thus, it is extremely important to establish an assay method for the quantitative determination of these compounds in plasma.

Estriol, one of the most reliable indices of fetal well-being in late pregnancy, is metabolized to 2-methoxyestriol (1, 2-MeOE3) via 2-hydroxyestriol. 3 In recent years, Kono et al. 4 have shown that 2-MeOE3 has a hypcholesterolemic effect in rats and that the mechanism of this action involves no estrogen receptors.

The determination of 2-MeOE3 in pregnancy urine by capillary gas chromatography and mass spectrometry has been proposed by Fotsis et al. 5 and Gerhardt et al. 6 However, it seems that this method cannot be readily applied to the measurement of 2-MeOE3 in plasma because of its low concentration and the complicated pretreatment. A highly sensitive and specific radioimmunoassay (RIA) method for 2-MeOE3 should provide the solutions to these problems. Although several attempts have been made to develop RIA of 2-methoxyestrone and 2-methoxyestradiol, 7 there have been no reports on RIA of 2-MeOE3.

We previously developed a specific RIA for 2-hydroxyestriol and applied it to the measurement of this substance in plasma of pregnant women. 8 The present paper describes the preparation of anti-2-MeOE3 antisemur by immunization with hapten–bovine serum albumin (BSA) conjugate and the quantitation of plasma 2-MeOE3 in pregnant women using the RIA method. 9

Experimental

Materials [6,7-3H]Estriol (60.0 Ci/mmol), [6,7-3H]estradiol (53.0 Ci/mmol), [2,4,6,7-3H]estriol (90.4 Ci/mmol), and the scintillation solution (Riafluor) were purchased from New England Nuclear (Boston, MA). Estriol, muscle tyrosinate, S-adenosyl-l-methionine, COMT, and BSA (fraction V) were supplied by Sigma Chemical (St. Louis, MO). Sephadex LH-20, aminohexyl (AH)-Sephose 4B, cellulose powder, and dextran T-70 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), Amberlite XAD-2 resin from Rohm and Haas (Philadelphia, PA), Freund’s complete adjuvant from Difco Lab. (Detroit, MI), bovine gamma-globulin from Miles Lab. (Elk hart, IN), silica gel precoated on aluminum sheets for thin-layer chromatography (TLC) from E. Merck (Darmstadt, Germany), and other general reagents from Wako Pure Chemical Industries (Osaka, Japan), 2-MeOE3 was synthesized by the method of Watanabe and Menzies. 10 Catechol estrogens and their methyl ethers used for cross-reaction studies were prepared in these laboratories by the known methods. 11 [3H]2-Methoxyestrone and [3H]2-methoxyestriadiol were prepared enzymatically from labeled estrone and estradiol, respectively, by treatment with mushroom tyrosinase 12 and subsequent methylation with COMT using S-adenosyl-l-methionine. 13 The resulting guaicol estrogens were purified by paper chromatography. 14 RIA was performed in ascorbic acid buffer (pH 7.4) containing ascorbic acid (2.2 g), bovine gamma-globulin (1.0 g), and ethylendiaminetetraacetic acid disodium salt (EDTA) (7.45 g) in H2O (500 ml); the result pH was adjusted with 1 N NaOH.

Apparatus All melting points were determined with a Yanagimoto micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. Electron impact mass spectrometry (MS) measurements were run on a JEOL JMS-50 instrument. Ultraviolet (UV) spectra were obtained on a Hitachi 323 recording spectrophotometer and infrared (IR) spectra on a JASCO IRA-2 spectrophotometer. Proton nuclear magnetic resonance (1H-NMR) spectra were recorded using tetramethylsilane as an internal standard on a JEOL FX-100 spectrometer at 100 MHz. Abbreviations used: s = singlet, d = doublet, and m = multiplet.

2-Methoxy-16a,17β-trihydroxy-1,3,5(10)-estratrien-6-one Triacetate (3) A solution of CrO3 (81 mg, 0.81 mmol) in H2O (0.2 ml) AcOH (1.6 ml) was added dropwise to a solution of 2-methoxy-1,3,5(10)-estrenatrie-16a,17β-triolic triacetate (2) (111 mg, 0.25 mmol) in AcOCH (3 ml), and the mixture was stirred at room temperature for 200 min. The excess reagent was then reduced with Et3O (2 ml). After dilution with H2O, the reaction mixture was extracted with AcOEt and the extract was washed with cold 2% Na2CO3 solution, then dried over anhydrous Na2SO4. Removal of AcOEt gave an oily residue, which was submitted to preparative TLC using hexane:AcOEt (2:1, v/v) as a developing solvent. Elution of the zone (Rr 0.57) corresponding to the product with AcOEt gave 3 (90 mg, 79%) as colorless oil. MS m/z: 458 [M+H]+, 416 [M–COCH3+H]+, 314 [M–3 COCH3+H]+. 1H-NMR (CDCl3) δ: 0.85 (3H, s, 18-CH3), 2.06 and 2.10 (3H, s, 16a–20-CH3), 2.51 (3H, s, 3-OCOC6H5), 3.90 (3H, s, 2-OCOC6H5), 4.99 (1H, d, J = 5.8 Hz, 17H, 5.20 (1H, m, 16H), 6.91 (1H, s, 1H, 7.73 (1H, s, 4-H).

2-Methoxy-16a,17β-trihydroxy-1,3,5(10)-estratrien-6-one (4) To a solution of 3 (88 mg, 0.19 mmol) in MeOH (3.5 ml) 10% KOH (0.3 ml) was added and the mixture was allowed to stand at 30°C for 1 h. The reaction mixture was acidified by 1 N HCl, then MeOH was removed under reduced pressure. The resulting solution was extracted with AcOEt, and the organic layer was washed with H2O and dried over

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anhydrous Na₂SO₄. The aqueous layer was percolated through an Amberlite XAD-2 column (10 x 0.6 cm i.d.). The column was washed with H₂O, then the desired material, which could not be extracted with AcOEt, was eluted with MeOH. The AcOEt extract and the MeOH eluate were combined and submitted to preparative TLC using n-hexane-ethyl acetate (95:5, v/v) as a developing solvent. The zone (Rf 0.30) corresponding to the product was eluted with AcOEt. The eluted product was recrystallized from AcOEt to give 45 mg, 71% as colorless needles, mp 155–158 °C, [α]D 1° +0.91° (c = 0.20, MeOH). Anal. Calc. For C₁₁H₂O₃O₃: C, 62.05; H, 6.71; N, 3.45. Found: C, 62.03; H, 6.71; N, 3.45. MS m/z: 331 [M - H]⁺; 279 [M - COOH]⁺; 174 [M - COOH - H⁺]⁺; 113 [M - H - O]⁺. UV (λ max nm) (c): 278 (10500), 322 (6900). IR (KBr): 3433 (OH), 3039 (CH), 2924 (CH), 1717 (C = O), 1635 (C = N), 1542 (C = C), 1451 (C - O - C), 1265 (C - N), 1112 (C - O - C), 834 (C - H). 

2-Methodology 3.16,17-trihydroxy-1,3,5(10)-estratrien-6-one 6 (4-O-Carboxybenzyl)oxime (5) A solution of (4-carboxybenzyl)hydroxylamine·HCl (171 mg, 1.56 mmol) and AcONa (165 mg in H₂O (1.95 ml) was added to a solution of 4 (40 mg, 0.12 mmol) in EtOH (20 ml), and the mixture was refluxed for 4 h. After addition of H₂O (5 ml) to the reaction mixture, EOH was removed under reduced pressure. The resulting solution was extracted with AcOEt, and the organic layer was washed with a saturated NaCl solution and dried over anhydrous Na₂SO₄. The aqueous layer was percolated through an Amberlite XAD-2 column (10 x 0.6 cm i.d.). The column was washed with H₂O, then the desired material, which could not be extracted with AcOEt, was eluted with MeOH (10 ml). The MeOH eluate and the MeOH eluate were combined and submitted to a Sephadex LH-20 column (14 x 0.6 cm i.d.) using benzene-MeOH (95:5, v/v) saturated with acetic acid. The monomethyl ether fraction obtained was then submitted to a cellulose column (30 x 0.4 cm i.d.) using cyclohexane-ethyl acetate-formamide (86:14:1, v/v), in order to separate [H₂]-MeOE₃ from the isomeric by-product ([H₂]-hydroxystroel 3-methyl ether). After evaporation of the eluate, the resulting [H₂]-MeOE₃ (18 mc) was stored in benzene-EtOH (1:2, v/v) containing 0.1% acetic acid and 0.05% AcOH at –18 °C. The specific activity was determined to be approximately 55 Ci/mmol using the RIA system. The radiochemical purity was confirmed by TLC using CHCl₃-MeOH- 

AcCl (90:10:0.8, v/v) as a developing solvent.

RIA Procedure All dilutions of the standard or sample, tracer, and antiserum were performed in acetic acid buffer, and the antiserum was used in the assay at a final dilution of 1:7000. Dextran-coated charcoal suspension was prepared by continuously stirring Norit A (250 mg) and dextran T-70 (25 mg) in cold acetic acid buffer (40 ml) for 15 min prior to use.

[3H]-2-MeOE₃ (ca. 8000 cpnm in assay buffer (0.1 ml) and diluted antiserum (0.5 ml) were added to test tubes containing standard or unknown amounts of 2-MeOE₃ in assay buffer (0.1 ml). All tubes were shaken in a vortex mixer and incubated at 4°C overnight. Dextran-coated charcoal suspension (0.3 ml) was added to each tube, vortexed, incubated for 10 min, centrifuged at 1700 x g for 10 min at 4°C. The supernatant (0.5 ml) was withdrawn into counting vials. A scintillation solution (5 ml) was added. The radioactivity of tritium was measured in a Beckman LS-9000 liquid scintillation spectrometer. The radioactivity bound to the antibody was calculated after correction for the blank value of the assay buffer. The standard curve was constructed in duplicate with doses ranging from 10 to 1000 pg.

Specificity of Antiserum The specificity of antibody was tested by calculating the percentage of cross-reaction with other steroids. Cross-reactivity was determined by the above-mentioned assay procedure, by comparing the concentrations of non-labeled 2-MeOE₃ and test compounds necessary for 50% displacement of the antibody-bound labeled 2-MeOE₃.

Determination of 2-MeOE₃ in Plasma Blood was collected into vacutainer tubes (5 ml) containing EDTA (10 mg) and acetic acid (15 mg), and then centrifuged at 4°C. The plasma obtained was then frozen at –18°C until assayed.

[3H]-2-MeOE₃ (ca. 700 cpnm) was added to plasma (2 ml) in order to estimate the recovery during the procedure. After addition of AcOE₃ (15 ml), the mixture was shaken on a vortex mixer for 1 min and centrifuged. The organic layer was evaporated at 40°C under a nitrogen gas. The residue was applied to a Sephadex LH-20 column (14 x 0.6 cm i.d.) using cyclohexane-EtOH (87:13, v/v). The eluate corresponding to 2-MeOE₃ fraction (32–46 ml) was dried under reduced pressure and then dissolved in EtOH (0.5 ml). The aliquot (0.1 ml) of this solution was measured for radioactivity to determine the recovery, and another aliquot (0.5 ml) was evaporated to dryness under a nitrogen gas and subjected to RIA as described above.

Recovery Test Aliquots (2 ml) of pooled normal male plasma were added to each of the centrifuge tubes containing non-labeled 2-MeOE₃ (48.8, 97.6, and 195.0 pg/ml) and [3H]-2-MeOE₃ (ca. 700 cpnm). The sample preparation and assay were then carried out in the manner described above.

Results and Discussion

An initial project was directed towards the synthesis of 6-oxo-2-MeOE₃, 6-(4-carboxybenzyl)oxime-BSA conjugate (6) for the production of anti-2-MeOE₃ antiserum. 2-MeOE₃ triacetate (2), prepared from estrol according to the methods previously reported, was oxidized with chromium trioxide in acetic acid to provide the 6-oxo derivative (3) in 79% yield. The high yield of 3 is due to the activating effect of the electron donating 2-methoxy group on the p-phenolic position of the steroid nucleus. The subsequent alkaline hydrolysis of 3 yielded 6-oxo-2-
MeOE3 (4). Condensation of 4 with (O-carboxymethyl)hydroxylamine gave the 6-(O-carboxymethyl)oxime (5). The oxime derivative was then linked covalently to BSA by the mixed anhydride method to yield the hapten–BSA conjugate (6). The number of steroid residues incorporated per molecule of BSA, as determined spectrophotometrically at 270 nm, was 41.

The immunogen thus obtained was administered to six rabbits for producing the antibody. Among the antisera elicited in the rabbits, the most specific antiserum discriminating the ring A structure of estrogen, especially primary estrogens, was selected for further experiment. This antiserum was partially purified by immunosorption on affinity chromatography media using estrone 17-(O-carboxymethyl)oxime-Sepharose conjugate, in order to eliminate antibodies cross-reactive with classical monophenolic estrogens. Although the titer of the antiserum decreased to 1:7000 dilution from 1:28000 (binding 50% of ca. 8000 cpm of [3H]2-MeOE3), the specificity towards classical estrogens was improved by purification (Table I). The antiserum exhibited 51% cross-reaction with 2-methoxyestradiol, 2.2% with 2-methoxyestrone, and a very low or negligible value with all other compounds tested. The standard curve obtained with the antiserum is presented in Fig. 1. The plot of logit percent bound radioactivity vs. logarithm of the amount of non-labeled 2-MeOE3 showed a linear relationship over the range of 10 to 1000 pg.

The assay system for 2-MeOE3 in plasma of pregnant women was then developed using anti-2-MeOE3 antiserum. The extent of cross-reactivities of 2-methoxyestradiol and 2-methoxyestrone makes overestimation inevitable in the direct assay. Moreover, primary estrogens which occur in plasma of pregnant women at high concentrations exert significant influence on the determination of 2-MeOE3, even if their cross-reactivities are extremely low. It is therefore necessary to introduce a purification step by Sephadex LH-20 chromatography before RIA, to separate 2-MeOE3 from these interfering compounds. Figure 2 shows the elution pattern of a mixture of tritiated primary estrogens and 2-methoxyestrogens on a Sephadex LH-20 column using cyclohexane–EtOH (87:13, v/v) as the eluent. In order to evaluate the contamination with estriol in the 2-MeOE3 fraction (32–46 ml), [3H]estriol was chromatographed on the Sephadex LH-20 column. The result indicated that 4.7% (range 3.9–5.5%, n=6) of

![Chart 1](image)

**Table I. Per Cent Cross-Reactions of Crude and Purified Anti-2-MeOE3 Antiserum with Selected Steroids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>% cross-reactivity (50%)</th>
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<tr>
<td></td>
<td>Crude antiserum</td>
</tr>
<tr>
<td>2-MeOE3</td>
<td>100</td>
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<tr>
<td>2-Methoxyestradiol</td>
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<td>2-Methoxyestrone</td>
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</tr>
<tr>
<td>4-Methoxyestrone</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4-Hydroxyestradiol</td>
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</tr>
<tr>
<td>4-Hydroxyestrone</td>
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</tr>
<tr>
<td>2-Hydroxyestradiol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2-Methoxyestradiol 3-methyl ether</td>
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</tr>
<tr>
<td>2-Hydroxyestradiol 3-methyl ether</td>
<td>0.28</td>
</tr>
<tr>
<td>4-Hydroxyestradiol 3-methyl ether</td>
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</tr>
<tr>
<td>Estriol</td>
<td>0.52</td>
</tr>
<tr>
<td>Estradiol</td>
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</tr>
<tr>
<td>Estrone</td>
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</tr>
<tr>
<td>Androsterone</td>
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</tr>
<tr>
<td>Dehydroepiandrosterone</td>
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<td>Etiocladenolone</td>
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<tr>
<td>Cortisone</td>
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<tr>
<td>Progesterone</td>
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<tr>
<td>Cholesterol</td>
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![Fig. 1](image)

**Fig. 1. Standard Curve for Radioimmunoassay of 2-MeOE3 Using Anti-2-MeOE3 Antiserum (1:7000 Dilution)**

![Fig. 2](image)

**Fig. 2. Elution Pattern of Primary Estrogens and 2-Methoxyestrogens from a Sephadex LH-20 Column (14 x 0.6 cm i.d.) Using Cyclohexane–EtOH (87:13, v/v)**

estriol applied to the column was eluted in the 2-MeOES fraction. The plasma was extracted with ethyl acetate and purified on the Sephadex LH-20 column. The eluate corresponding to the 2-MeOES fraction was dried and then used for RIA. Observed values obtained by RIA were corrected on the basis of the recovery rate (mean 57.8%, range 49.7–64.2%) of [3H]-2-MeOES added to each plasma sample.

The accuracy of the assay method was examined by the addition of known amounts of 2-MeOES to pooled male plasma. Regression analysis gave the linear relationship of \(Y = 0.95X + 17.1\) with a correlation coefficient of 0.97, as depicted in Fig. 3. The mean recovery was 105.5% with a range of 90.1–115.8%. Intra- and inter-assay coefficients of variation at a plasma level of 98 pg/ml were 8.6% (n = 12) and 10.5% (n = 8), respectively.

The measurement of 2-MeOES in plasma samples obtained from normal pregnant women was carried out by this assay procedure, and plasma concentrations are summarized in Table II. Plasma levels during pregnancy were found to be mean values of 41.1 pg/ml (12th–14th week), 85.3 pg/ml (27th–29th week), and 97.5 pg/ml (37th–41st week). It thus seems that the plasma concentration of 2-MeOES, as well as estriol and 2-hydroxyestriol, tends to increase in the third trimester of pregnancy.

The determination of plasma 2-MeOES in pregnant women was achieved by RIA using anti-2-MeOES antiserum after purification of the samples by Sephadex LH-20 chromatography. This assay method has been demonstrated to be reliable and sensitive for the measurement of 2-MeOES in plasma, and it is hoped that it can be adopted for the assay of conjugated 2-MeOES in plasma and urine of pregnant women with prior deconjugation.

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