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

Youichi FUJII,*,[a] Masanori TERANISHI,[a] Keiji NAKADA,[a] Mitsuru YAMAZAKI,[a] Shigeru KISHIDA,[b] and Susumu MIYABO[b]

School of Pharmacy, Hokuriku University,[a] 3 Ho, Kanagawa-machi, Kanazawa 920–11, Japan and Fukui Medical School,[b] Shimoaizuki, Matsuoka-cho, Yoshida-gun, Fukui 910–11, Japan. *Received July 9, 1991*

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 extraction of the plasma with ethyl acetate and purification on a Sephadex LH-20 column. In order to obtain the immunogen for 2-methoxyestriol, 6-oxo-2-methoxyestriol was converted to its 6-(*O*-carboxymethyl)oxime derivative. The derivative was then coupled to bovine serum albumin by the mixed anhydride method, and rabbits were immunized with this conjugate. The antiserum 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).[1] 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-$MeOE_3$) *via* 2-hydroxyestriol.[3] In recent years, Kono *et al.*[4] have shown that 2-$MeOE_3$ has a hypocholesterolemic effect in rats and that the mechanism of this action involves no estrogen receptors.

The determination of 2-$MeOE_3$ 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-$MeOE_3$ in plasma because of its low concentration and the complicated pretreatment. A highly sensitive and specific radioimmunoassay (RIA) method for 2-$MeOE_3$ should provide the solution 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-$MeOE_3$.

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-$MeOE_3$ antiserum by immunization with hapten–bovine serum albumin (BSA) conjugate and the quantitation of plasma 2-$MeOE_3$ in pregnant women using the RIA method.[9]

Experimental

Materials [6,7-^{3}H]Estrone (60.0 Ci/mmol), [6,7-^{3}H]estradiol (53.0 Ci/mmol), [2,4,6,7-^{3}H]estriol (94.0 Ci/mmol), and the scintillation solution (Riafluor) were purchased from New England Nuclear (Boston, MA). Estriol, mushroom tyrosinase, *S*-adenosyl-L-methionine, COMT, and BSA (fraction V) were supplied by Sigma Chemical (St. Louis, MO). Sephadex LH-20, aminohexyl (AH)-Sepharose 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. (Elkhart, IN), silica gel pre-coated 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-$MeOE_3$ 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] [^{3}H]2-Methoxyestrone and [^{3}H]2-methoxyestradiol 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 guaiacol 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 ethylenediaminetetraacetic acid disodium salt (EDTA) (7.45 g) in H_2O (500 ml); the resultant 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 spectral (MS) measurements were run on a JEOL JMS-DX 300 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 (^{1}H-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-3,16α,17β-trihydroxy-1,3,5(10)-estratrien-6-one Triacetate (3) A solution of CrO_3 (81 mg, 0.81 mmol) in H_2O (0.2 ml)–AcOH (1.6 ml) was added dropwise to a solution of 2-methoxy-1,3,5(10)-estratriene-3,16α,17β-triol triacetate (**2**) (111 mg, 0.25 mmol) in AcOH (3 ml), and the mixture was stirred at room temperature for 200 min. The excess reagent was then reduced with EtOH (2 ml). After dilution with H_2O, the reaction mixture was extracted with AcOEt and the extract was washed with cold 2% $NaHCO_3$ solution, then dried over anhydrous Na_2SO_4. 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 (*Rf* 0.57) corresponding to the product with AcOEt gave **3** (90 mg, 79%) as colorless oil. MS *m/z*: 458 $[M]^+$, 416 $[M-COCH_3+H]^+$, 314 $[M-3\times COCH_3-CH_3]^+$. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, s, 18-$CH_3$), 2.06 and 2.10 (3H, s, 16α- or 17β-$OCOCH_3$), 2.31 (3H, s, 3-$OCOCH_3$), 3.90 (3H, s, 2-OCH_3), 4.99 (1H, d, J = 5.6 Hz, 17α-H), 5.20 (1H, m, 16β-H), 6.91 (1H, s, 1-H), 7.73 (1H, s, 4-H).

2-Methoxy-3,16α,17β-trihydroxy-1,3,5(10)-estratrien-6-one (4) To a solution of **3** (88 mg, 0.19 mmol) in MeOH (5.3 ml), 10% KOH (0.3 ml) was added and the mixture was allowed to stand at 50 °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 H_2O and dried over anhydrous Na_2SO_4. The aqueous layer was percolated through an Amberlite XAD-2 column (10×0.6cm i.d.). The column was washed with H_2O, 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 AcOEt 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 **4** (45mg, 71%) as colorless needles, mp 155—158°C, $[\alpha]_D^{20}$ +0.91° (*c*=0.20, MeOH). *Anal.* Calcd for $C_{19}H_{24}O_5 \cdot 1/3H_2O$: C, 67.44; H, 7.35. Found: C, 67.34; H, 7.34. MS *m/z*: 332 $[M]^+$, 314 $[M-H_2O]^+$. UV λ_{max}^{MeOH} nm (ε): 278 (10500), 322 (6900). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1650 (C=O), 1605 (aromatic). ^{1}H-NMR [$CDCl_3$–CD_3OD (1:1, v/v)] δ: 0.81 (3H, s, 18-CH_3), 3.52 (1H, d, *J*=5.6Hz, 17α-H), 3.96 (3H, s, 2-OCH_3), 4.10 (1H, m, 16β-H), 6.88 (1H, s, 1-H), 7.45 (1H, s, 4-H).

2-Methoxy-3,16α,17β-trihydroxy-1,3,5(10)-estratrien-6-one 6-(*O*-Carboxymethyl)oxime (5) A solution of (*O*-carboxymethyl)hydroxylamine·1/2HCl (171mg, 1.56mmol) and AcONa (165mg) in H_2O (1.95ml) was added to a solution of **4** (40mg, 0.12mmol) in EtOH (20ml), and the mixture was refluxed for 4h. After addition of H_2O (5ml) to the reaction mixture, EtOH 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 Na_2SO_4. The aqueous layer was percolated through an Amberlite XAD-2 column (10×0.6cm i.d.). The column was washed with H_2O, 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 a Sephadex LH-20 column (60×2cm i.d.) using benzene–MeOH (4:1, v/v). The eluted product was recrystallized from CH_2Cl_2–EtOH to give **5** (20mg, 41%) as colorless needles, mp 209—212°C (dec.), $[\alpha]_D^{19}$ −15.9° (*c*=0.23, MeOH). *Anal.* Calcd for $C_{21}H_{27}NO_7$: C, 62.21; H, 6.71; N, 3.45. Found: C, 61.93; H, 6.85; N, 3.42. MS *m/z*: 331 $[M-OCH_2COOH+H]^+$. UV λ_{max}^{MeOH} nm (ε): 270 (13800), 310 (8200). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH, COOH), 1730 (COOH), 1600 (C=N). ^{1}H-NMR [$CDCl_3$–CD_3OD (1:1, v/v)] δ: 0.76 (3H, s, 18-CH_3), 3.50 (1H, d, *J*=5.8Hz, 17α-H), 3.87 (3H, s, 2-OCH_3), 4.09 (1H, m, 16β-H), 4.66 (2H, s, -OCH_2CO-), 6.77 (1H, s, 1-H), 7.39 (1H, s, 4-H).

2-Methoxy-3,16α,17β-trihydroxy-1,3,5(10)-estratrien-6-one 6-(*O*-Carboxymethyl)oxime–BSA Conjugate (6) Tributylamine (30μl) and isobutyl chlorocarbonate (15μl) were added to a solution of **5** (22mg, 0.054mmol) in dry dimethylformamide (0.64ml) under ice-cooling, and the mixture was stirred for 15min. The reaction mixture was added to a solution of BSA (79mg) in H_2O (3.9ml)–dimethylformamide (2.4ml) containing 0.025 N NaOH (0.4ml) under ice-cooling. After being stirred for 1h, the resulting solution was dialyzed overnight at 4°C against a constant flow of cold 0.2ppm ascorbic acid solution (20l). Lyophilization of the solution afforded **6** (96mg) as a fluffy powder.

Immunization Procedure The hapten–BSA conjugate (**6**) (6mg) was dissolved in sterile isotonic saline (2.4ml) and emulsified with complete Freund's adjuvant (3.6ml). The emulsion was injected into multiple subcutaneous sites along both sides of the back of six domestic strain male albino rabbits. The procedure was repeated at intervals of two weeks for three months. After confirmation of the increase of the antibody titer, the blood was collected by bleeding from the marginal ear veins. The sera were separated by centrifugation and stored at −18°C in small aliquots.

Preparation of Estrone 17-(*O*-Carboxymethyl)oxime–AH-Sepharose Conjugate A solution of estrone 17-(*O*-carboxymethyl)oxime (30mg, 0.087mmol)[15] in dimethylformamide (3ml) was added to a suspension of AH-Sepharose 4B (150mg) in H_2O (1.4ml), and the whole was adjusted to pH 5 with 0.1 N HCl. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (120mg, 0.63mmol) was added to the suspension, and the reaction mixture was stirred at room temperature for 72h. After filtration of the mixture, the precipitate was washed with water and MeOH several times, and dried.

Elimination of Cross-Reactive Antibodies by Affinity Chromatography Estrone 17-(*O*-carboxymethyl)oxime–AH-Sepharose (30mg) was added to the 1:50 diluted antiserum (50ml) in 0.01 M phosphate buffer (pH 7.4), and the whole was stirred at 4°C for 3h. After filtration of the mixture, the filtrate was used for RIA at a final dilution of 1:7000.

Preparation of [^{3}H]2-MeOE$_3$ [2,4,6,7-^{3}H]Estriol (340μCi) was converted enzymatically into [^{3}H]2-hydroxyestriol by mushroom tyrosinase.[12] This labeled compound was purified by a Sephadex LH-20 column (14×0.6cm i.d.) using benzene–MeOH (9:1, v/v) saturated with ascorbic acid. To a solution of [^{3}H]2-hydroxyestriol (180μCi) in 0.1 M Sörensen phosphate buffer (pH 6.0) (3ml), COMT (0.29mg), *S*-adenosyl-L-methionine (1.5mg), and $MgCl_2 \cdot 6H_2O$ (1.0mg) were added, and the mixture was incubated at 37°C for 5h under a nitrogen stream. The reaction mixture was extracted with AcOEt and washed with H_2O. After evaporation of the solvent, the resultant product was subjected to a Sephadex LH-20 column (14×0.6cm i.d.) using benzene–MeOH (95:5, v/v) saturated with ascorbic acid. The monomethyl ether fraction obtained was then submitted to a cellulose column (30×0.4cm i.d.) using cyclohexane–AcOEt–formamide (86:14:0.1, v/v), in order to separate [^{3}H]2-MeOE$_3$ from the isomeric by-product ([^{3}H]2-hydroxyestriol 3-methyl ether). After evaporation of the eluate, the resulting [^{3}H]2-MeOE$_3$ (18μCi) was stored in benzene–EtOH (1:2, v/v) containing 0.1% ascorbic acid and 0.05% AcOH at −18°C. The specific activity was determined to be approximately 55Ci/mmol using the RIA system.[16] The radiochemical purity was checked by TLC using $CHCl_3$–MeOH–AcOH (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 ascorbic 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 (250mg) and dextran T-70 (25mg) in cold ascorbic acid buffer (40ml) for 15min prior to use.

[^{3}H]2-MeOE$_3$ (*ca.* 8000cpm) in assay buffer (0.1ml) and diluted antiserum (0.5ml) were added to test tubes containing standard or unknown amounts of 2-MeOE$_3$ in assay buffer (0.1ml). All tubes were shaken in a vortex mixer and incubated at 4°C overnight. Dextran-coated charcoal suspension (0.3ml) was added to each tube, vortexed, incubated for 10min in an ice water bath, and centrifuged at 1700×*g* for 10min at 4°C. The supernatant (0.5ml) was withdrawn into counting vials and a scintillation solution (5ml) 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 1000pg.

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-MeOE$_3$ and test compounds necessary for 50% displacement of the antibody-bound labeled 2-MeOE$_3$.

Determination of 2-MeOE$_3$ in Plasma Blood was collected into vacutainer tubes (5ml) containing EDTA (10mg) and ascorbic acid (15mg), and then centrifuged at 4°C. The plasma obtained was promptly frozen at −18°C until assayed.

[^{3}H]2-MeOE$_3$ (*ca.* 700cpm) was added to plasma (2ml) in order to estimate the recovery during the procedure. After addition of AcOEt (15ml), the mixture was shaken on a vortex mixer for 1min and centrifuged. The organic layer was evaporated at 40°C under a nitrogen gas. The residue was applied to a Sephadex LH-20 column (14×0.6cm i.d.) using cyclohexane–EtOH (87:13, v/v). The eluate corresponding to 2-MeOE$_3$ fraction (32—46ml) was dried under reduced pressure and then dissolved in EtOH (0.5ml). An aliquot (0.1ml) of this solution was measured for radioactivity to determine the recovery, and another aliquot (0.3ml) was evaporated to dryness under a nitrogen gas and subjected to RIA as described above.

Recovery Test Aliquots (2ml) of pooled normal male plasma were added to each of the centrifuge tubes containing non-labeled 2-MeOE$_3$ (48.8, 97.6, and 195.0pg/ml) and [^{3}H]2-MeOE$_3$ (*ca.* 700cpm). The sample preparation and assay were then carried out in the manner described above.

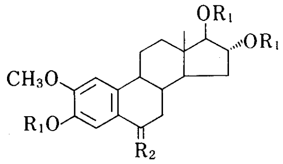
Results and Discussion

An initial project was directed towards the synthesis of 6-oxo-2-MeOE$_3$ 6-(*O*-carboxymethyl)oxime–BSA conjugate (**6**) for the production of anti-2-MeOE$_3$ antiserum. 2-MeOE$_3$ triacetate (**2**), prepared from estriol according to the methods[10,17] 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*-benzylic position of the steroid nucleus.[18] Subsequent alkaline hydrolysis of **3** yielded 6-oxo-2-

MeOE$_3$ (**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[15] 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 immunoadsorption on affinity chromatography media[19] 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 [^{3}H]2-MeOE$_3$), 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-MeOE$_3$ showed a linear relationship over the range of 10 to 1000 pg.

The assay system for 2-MeOE$_3$ in plasma of pregnant women was then developed using anti-2-MeOE$_3$ antiserum. The extent of cross-reactivities of 2-methoxyestradiol and 2-methoxyestrone makes overestimation inevitable in the direct assay. Moreover, primary estrogens that occur in plasma of pregnant women at high concentrations exert significant influence on the determination of 2-MeOE$_3$, 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-MeOE$_3$ 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-MeOE$_3$ fraction (32—46 ml), [^{3}H]estriol was chromatographed on the Sephadex LH-20 column. The result indicated that 4.7% (range 3.9—5.5%, n=6) of



1 : R$_1$=H, R$_2$=H$_2$
2 : R$_1$=Ac, R$_2$=H$_2$
3 : R$_1$=Ac, R$_2$=O
4 : R$_1$=H, R$_2$=O
5 : R$_1$=H, R$_2$=NOCH$_2$COOH
6 : R$_1$=H, R$_2$=NOCH$_2$CONH–BSA

Chart 1

Table I. Per Cent Cross-Reactions of Crude and Purified Anti-2-MeOE$_3$ Antisera with Selected Steroids

Compound	% cross-reactivity (50%)	
	Crude antiserum	Purified antiserum
2-MeOE$_3$	100	100
2-Methoxyestradiol	42	51
2-Methoxyestrone	3.8	2.2
2-Hydroxyestriol	0.20	0.46
2-Hydroxyestradiol	0.05	0.15
2-Hydroxyestrone	0.02	0.01
4-Methoxyestriol	0.14	0.18
4-Methoxyestradiol	<0.01	<0.01
4-Methoxyestrone	<0.01	<0.01
4-Hydroxyestriol	0.24	0.15
4-Hydroxyestradiol	0.05	0.06
4-Hydroxyestrone	<0.01	<0.01
2-Methoxyestriol 3-methyl ether	0.30	0.42
2-Hydroxyestriol 3-methyl ether	0.28	0.39
4-Hydroxyestriol 3-methyl ether	0.06	0.06
Estriol	0.52	0.13
Estradiol	0.28	0.06
Estrone	0.02	<0.01
Androsterone	<0.01	<0.01
Dehydroepiandrosterone	<0.01	<0.01
Etiocholanolone	<0.01	<0.01
Testosterone	<0.01	<0.01
Corticosterone	<0.01	<0.01
Cortisone	<0.01	<0.01
Hydrocortisone	<0.01	<0.01
Pregnanediol	<0.01	<0.01
Progesterone	<0.01	<0.01
Cholesterol	<0.01	<0.01

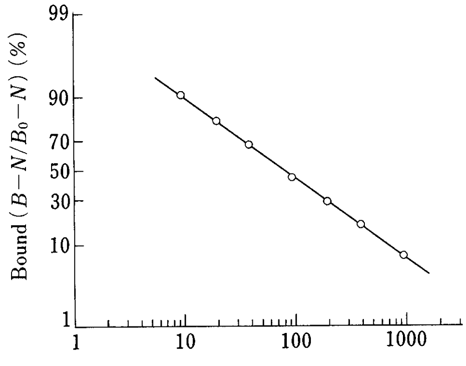


Fig. 1. Standard Curve for Radioimmunoassay of 2-MeOE$_3$ Using Anti-2-MeOE$_3$ Antiserum (1 : 7000 Dilution)

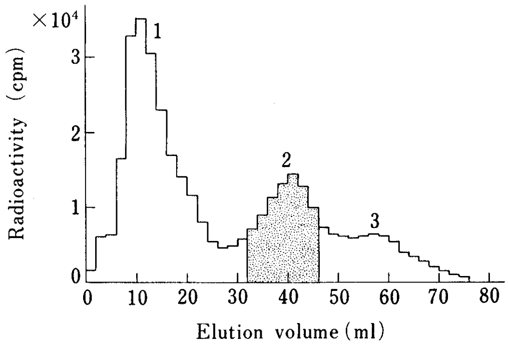


Fig. 2. Elution Pattern of Primary Estrogens and 2-Methoxyestrogens from a Sephadex LH-20 Column (14 × 0.6 cm i.d.) Using Cyclohexane–EtOH (87 : 13, v/v)

1, [^{3}H]2-methoxyestrone, [^{3}H]estrone, [^{3}H]2-methoxyestradiol, and [^{3}H]-estradiol; 2, [^{3}H]2-MeOE$_3$; 3, [^{3}H]estriol.

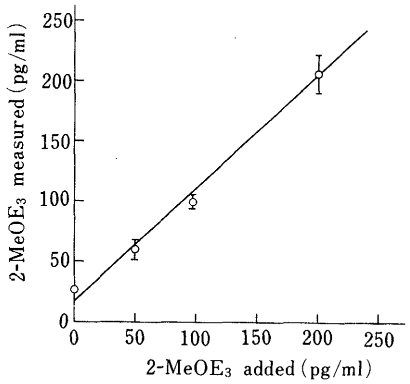


Fig. 3. Recovery Test for 2-$MeOE_3$ Added to Pooled Male Plasma

Each point and bar indicate the mean value and standard deviation, respectively, for four determinations. $Y=0.95X+17.1$.

Table II. Plasma Concentrations of 2-$MeOE_3$ in Normal Pregnant Women

Weeks of pregnancy	Number	Mean (pg/ml)	Range (pg/ml)
12—14	6	41.1	26.4- 56.9
27—29	7	85.3	71.1-106.1
37—41	7	97.5	66.1-180.0

estriol applied to the column was eluted in the 2-$MeOE_3$ fraction. The plasma was extracted with ethyl acetate and purified on the Sephadex LH-20 column. The eluate corresponding to the 2-$MeOE_3$ 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 [^{3}H]2-$MeOE_3$ added to each plasma sample.

The accuracy of the assay method was examined by the addition of known amounts of 2-$MeOE_3$ 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 the 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-$MeOE_3$ 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-$MeOE_3$, as well as estriol and 2-hydroxyestriol, tends to increase in the third trimester of pregnancy.

The determination of plasma 2-$MeOE_3$ in pregnant women was achieved by RIA using anti-2-$MeOE_3$ 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-$MeOE_3$ in plasma, and it is hoped that it can be adopted for the assay of conjugated 2-$MeOE_3$ in plasma and urine of pregnant women with prior deconjugation.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research (No. 61303018) from the Ministry of Education, Science, and Culture, Japan, which is gratefully acknowledged.

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