RADIOIMMUNOASSAY FOR
2-METHOXYOESTRONE IN HUMAN PLASMA

By
Günter Emons, Peter Ball,
Gertrud v. Postel and Rudolf Knuppen

ABSTRACT

A bovine serum albumin conjugate of 2-methoxyoestrone was used for the preparation of highly specific antibodies in rabbits. Cross-reactivity for catecholoeestrogens and monophenolic steroids was below 0.3 %. Only 2-methoxyoestradiol cross-reacted with 44 %. An assay procedure for the determination of unconjugated and conjugated 2-methoxyoestrone in human plasma is described. The following mean plasma concentrations (pg/ml) were found (unconjugated/conjugated): children 61/1130, young men 74/1320, elderly men 109/1260, cycling women 131/1040, post-menopausal women 102/1420, and pregnant women 3980/5850.

Abbreviations and trivial names:
2-OHOe = 2-hydroxyoestrone = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one;
2-OHOe2 = 2-hydroxyoestradiol = 1,3,5(10)oestratriene-2,3,17β-triol;
2-OHOe3 = 2-hydroxyoestriol = 1,3,5(10)-oestratriene-2,3,16α,17β-tetrol;
2-OHOe 2-Me = 2-methoxyoestrone = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one 2-methyl ether;
2-OHOe2 2-Me = 2-methoxyoestradiol = 1,3,5(10)-oestratriene-2,3,17β-triol 2-methyl ether;
2-OHOe3 3-Me = 2-hydroxyoestrone 3-methyl ether = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one 3-methyl ether;
2-OHOe2 3-Me = 2-hydroxyoestradiol 3-methyl ether = 1,3,5(10)-oestratriene-2,3,17β-triol 3-methyl ether;
4-OHOe = 4-hydroxyoestrone = 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one;
4-OHOe2 = 4-hydroxyoestradiol = 1,3,5(10)-oestratriene-3,4,17β-triol;
4-OHOe3 = 4-hydroxyoestriol = 1,3,5(10)-oestratriene-3,4,16α,17β-tetrol.
It is now well established that the 2-hydroxyoestrogens (catecholoestrogens) play an important role in the metabolism of oestradiol, 2-OHOe being the main product within the group of catecholoestrogens (Gelbke et al. 1977; Fishman 1976). This steroid is biosynthesized in the liver (Gelbke et al. 1977; Fishman 1976; Ball et al. 1976) and the brain (Fishman & Norton 1975; Fishman et al. 1976; Ball et al. 1978b; Ball & Knuppen 1978) and is excreted in high amounts in the urine of man (Ball et al. 1975; Ball et al. 1977; Chattoraj et al. 1978).

Our investigations concerning the interaction of catecholoestrogens and catecholamines on the cellular level (Gelbke et al. 1977) induced the hypothesis that the availability of catecholoestrogens in brain might be involved in the mechanism of regulation of hypothalamic releasing factor production and release.

Because of the possible physiological significance of catecholoestrogens for the regulation of the menstrual cycle methods for the determination of 2-OHOe in plasma (Yoshizawa & Fishman 1971; Emns et al. 1977; Ball et al. 1978a) and urine (Ball et al. 1975, 1977; Chattoraj et al. 1978) were elaborated quite recently.

As known from in vitro and in vivo studies the catecholoestrogens are rapidly metabolized by the catechol O-methyltransferase leading to the formation of the corresponding monomethyl ethers (Ball et al. 1976, for review cf. Gelbke et al. 1977). It seemed likely that the determination of catecholoestrogens alone did not allow a representative screening for catecholoestrogen production under various physiological and pathological conditions. As 2-OHOe, 2-Me, besides 2-OHOe, was found to be the main excretory product in the group of 2-substituted oestrogens in human urine, a method for the determination of 2-OHOe, 2-Me in plasma was developed.

MATERIALS AND METHODS

Steroids

2- and 4-hydroxyoestrogens were prepared according to Stubenrauch & Knuppen (1976) and the isomeric monomethyl ethers of 2-OHOe and 2-OHOe were synthesized by the method of Fishman (Fishman 1958; Fishman et al. 1960). All other steroids were purchased from E. Merck, Darmstadt, Germany. [6,7-3H2]2-OHOe, 2-Me was obtained by oxidation of [6,7-3H2]oestrone (specific radioactivity 44 Ci/mmmole) with Fremy's salt (Gelbke et al. 1973) and subsequent enzymatic methylation of the reaction product with a catechol O-methyltransferase preparation from rat liver using S-adenosyl methionine (Ball et al. 1972). The resulting [6,7-3H2]2-OHOe, 2-Me was separated from the isomeric by-product on formamide impregnated papers in the cyclohexane system; the purity was more than 96%.

Chemicals and solutions

All chemicals were purchased from E. Merck, Darmstadt, and were of analytical grade. Organic solvents were redistilled before use. Bovine serum albumin was obtained from Behringwerke (Marburg, Germany), complete Freunds adjuvant from Difco
Laboratories (Detroit, Mich., USA), pentobarbital from Deutsche Abbott (Ingelheim, Germany), Riafluor from New England Nuclear (Boston, USA), Instagel from Packard Instruments (Frankfurt, Germany) and lysozyme from Boehringer Mannheim (Germany). The charcoal suspension was prepared by continuously stirring 200 mg of dextran charcoal (Isotopendients West, Frankfurt, Germany) in 100 ml of buffer C (see below) at 0-4°C.

The standard ascorbic acid solution (buffer A) contained ascorbic acid (15 g) and glacial acetic acid (4 ml) in methanol (400 ml). The ascorbic acid buffer of pH 10.5 (buffer B) was prepared by dissolving ascorbic acid (30 g) and NaHCO₃ (45 g) in water (600 ml); shortly before use approximately 15 ml of 15 M NaOH were added. The ascorbic acid buffer of pH 7.4 (buffer C) contained ascorbic acid (4.4 g), EDTA (14.9 g) and lysozyme (2 g) in water (1 l); shortly before use approximately 75 ml of 1 M NaOH were added.

**Chromatographic methods**

Paper chromatography (2043b Mgl paper, Schleicher & Schüll, Dassel, Germany) was carried out on formamide/ascorbic acid impregnated papers (Gelbke & Knuppen 1972) with cyclohexane (PC I) and on ascorbic acid impregnated papers in the system methanol/water:benzene/n-hexane (70:30:10:90 by vol.) (PC II). Gas liquid chromatography was performed with a Pye gas chromatograph, Series 104 equipped with a flame ionization detector. Before gas liquid chromatographic analyses the steroids were converted to their trimethylsilyl ethers using N-methyl-N-trimethylsilyl trifluoroacetamid (Machery & Nagel, Düren, Germany). Mass spectra were recorded with a gas chromatograph-mass spectrometer LKB 2091 (LKB-producer, Stockholm) as described previously (Hoppen & Siekmann 1974).

**Preparation of 2-OHOr; 2-Me-17-(O-carboxymethyl)oxime**

A solution of 1.66 mmole 2-OHOr; 2-Me and 2.97 mmole carboxymethylhydroxylamine-HCl dissolved in 40 ml of ethanol containing 1.7 ml of ascorbic acid buffer (pH 10.5; buffer B) was refluxed for 4 h under nitrogen. After cooling the ethanol was removed in vacuo and 2 ml of water were added to the oily residue. After the addition of a few drops of acetic acid the mixture was extracted three times with ethyl acetate, the combined organic phases were washed with water until free of acid and then evaporated in vacuo. The residue was recrystallized from ethyl acetate-petroleum ether (40-60°C) yielding fine needles (m. p. 105-108°C). The ultraviolet spectrum in pH 8.5 Tris buffer exhibited absorption at 287 nm (ε 4683).

**Preparation of the steroid-protein conjugate**

The 2-OHOr; 2-Me-17-oxime was coupled with bovine serum albumin according to the technique described by Rao (1974) with the modification that dialysation of the steroid-protein conjugate was performed against 2 x 10 l of a 0.001 % ascorbic acid solution (pH 7.0).

**Determination of the 2-OHOr; 2-Me content of the steroid-protein conjugate**

By ultraviolet spectral analysis 30 steroid residues per mole of albumin were determined. In order to obtain definite proof whether the steroid-protein conjugate contained intact 2-OHOr; 2-Me the steroid was liberated and analyzed. Therefore, 2.85 mg of the steroid-protein conjugate were refluxed for 5 h in 10 ml of glacial acetic acid containing 200 mg of KI and 0.5 ml of a 0.1 M Na₂S₂O₃-solution; 1 ng of [6,7-³H₂]-
2-OHE$_2$ Me was added to correct for procedural losses. After cooling a further portion of 1 ml of the 0.1 M Na$_2$S$_2$O$_7$-solution was added and the acetic acid was removed in vacuo. The residue was dissolved in 2 ml of 1 M HCl containing 3 mg of ascorbic acid and the mixture was then extracted twice with 5 ml of ethyl acetate. The organic phase was washed three times with a 1% aqueous ascorbic acid solution and water; after drying with Na$_2$SO$_4$ the ethyl acetate was evaporated to dryness in vacuo. The residue was silylated as described previously (Happen & Siekmann 1974). By gas chromatography and gas chromatography-mass spectrometry 2-OHE$_2$ Me was definitely identified; the quantitative data obtained showed that at least 19 moles of intact 2-OHE$_2$ Me had been coupled to 1 mole of albumin.

**Immunisation and blood collection**

Two mg of the lyophilized steroid-protein conjugate were dissolved shortly before injection in 0.5 ml of Tris buffer (0.025 M, pH 7.4, containing 1% ascorbic acid) and emulsified in 1 ml of Freund's complete adjuvant. Immunization was carried out by injecting 0.35 ml of this emulsion into each of the four foot pads of randomly bred male rabbits (2-2.5 kg). Boostering was accomplished in the same manner 3 and 5 weeks later. Blood was obtained from the carotid vein prior to and 2, 4, 6, 8, 10 and 12 weeks following the first injection of antigen. Antibody titers as determined by crude 2-OHE$_2$ Me binding assays were maximal after 8-12 weeks. At that stage the rabbits were anaesthetized with pentobarbital and bled after fixing a canula in the carotid artery. Serum samples (70-110 ml from each animal) were stored at -20°C or lyophilized.

![Binding curve of 2-methoxyoestrone. For each point (ten determinations) the average value and the standard deviation are given.](Fig. 1.)
Table 1.
Cross-reactivity of steroids and catecholamines with the antiserum.

<table>
<thead>
<tr>
<th>Substance tested</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₈-steroids</td>
<td></td>
</tr>
<tr>
<td>2-OHOC₁ 2-Me</td>
<td>100</td>
</tr>
<tr>
<td>2-methoxyoestradiol</td>
<td>44</td>
</tr>
<tr>
<td>2-hydroxyoestrone</td>
<td>0.3</td>
</tr>
<tr>
<td>oestrone</td>
<td>0.28</td>
</tr>
<tr>
<td>oestradiol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>2-hydroxyoestrone 3-methyl ether</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>2-hydroxyoestradiol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>4-hydroxyoestrone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>4-hydroxyoestradiol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>oestriol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>2-hydroxyoestriol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>4-hydroxyoestriol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>ethynyl-oestradiol 3-methyl ether</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>oestrone sulphate</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>C₁₉-steroids</td>
<td></td>
</tr>
<tr>
<td>testosterone, androstenedione, dehydroepiandrosterone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>C₂₁-steroids</td>
<td></td>
</tr>
<tr>
<td>progesterone, cortisol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>epinephrine, metanephrine</td>
</tr>
</tbody>
</table>

**Characterization of the antiserum**

The antiserum was diluted with the ascorbic acid buffer pH 7.4 (buffer C). At a final dilution of 1:27 500 approximately 55% of 55 pg of [6,7-³H₂]2-OHOC₁ 2-Me (8 nCi) were bound. The affinity constant, calculated according to Feldmann & Rodbard (1971), was found to be 8.2 x 10⁹ l/mole. The standard curve obtained with the antiserum diluted 1:27 500 is given in Fig. 1. Cross-reactivity was determined by comparing the concentrations of unlabelled 2-OHOC₁ 2-Me and test-haptens necessary for the displacement of 50% of the antibody-bound [6,7-³H₂]2-OHOC₁ 2-Me: test-haptens were added up to amounts of 100 ng (Table 1).

**Assay procedure**

To avoid decomposition of 2-OHOC₁ 2-Me in EDTA plasma samples 0.1 ml of an aqueous ascorbic acid solution (3%) was added to 1 ml of plasma directly after centrifugation. Under these conditions the content of 2-OHOC₁ 2-Me did not change within at least 6 months when stored at -20°C. Even repeated freezing and thawing (up to 5 times) had no influence on the stability.

Duplicate aliquots of these plasmas (25–600 μl) were extracted 2 times with 1.5 ml of ether/n-hexane (1:1). The recovery of undecomposed endogenous 2-OHOC₁ 2-Me, as monitored by the recovery of [6,7-³H₂]2-OHOC₁ 2-Me pre-incubated for 30 min at 37°C with plasma was about 95%.
After addition of 0.6 ml water, 40 mg KI, 30 µl Na$_2$S$_2$O$_3$ (0.1 M) and 200 µl concentrated HCl, the aqueous phases remaining after ether/n-hexane extraction were heated for 60 min at 100°C. After cooling, another portion of Na$_2$S$_2$O$_3$ (150 µl) was added and the hydrolysates extracted 2 times with 2.5 ml benzene/ethyl acetate (1:2) each. The extracts, washed once with 3 ml of ascorbic acid buffer (buffer B) and 3 ml acetic acid (10%) and stabilized by the addition of 30 µl of buffer A, were then evaporated under nitrogen and redissolved in 1 ml of ethanol. The recovery of endogenous 2-OHOe$_1$ 2-Me, estimated by the recovery of [6,7-$^3$H$_2$]2-OHOe$_1$ 2-Me added after hydrolysis, was about 87%.

To the test tubes containing the dried residues of the total extracts (determination of the unconjugated 2-OHOe$_1$ 2-Me) or of 1/10 of the hydrolysate extracts (determination of the conjugated 2-OHOe$_1$ 2-Me) 8 nCi [6,7-$^3$H$_2$]2-OHOe$_1$ 2-Me (55 pg), dissolved in 0.1 ml of ascorbic acid buffer pH 7.4 (buffer C), 0.1 ml of the antiserum (dilution 1:5500), and 0.3 ml buffer C were added (final dilution of the antiserum 1:27 500). After incubation for 4 h at 0°C, 0.5 ml of the dextran/charcoal suspension was added and the mixture incubated for further 10 min at 0°C. After centrifugation (10 min; room temperature) 0.3 ml of the supernatant was assayed for radioactivity.

Miscellaneous
Melting points were determined with a microscope hot-stage and are uncorrected. Ultraviolet spectra were recorded with a Shimadzu double beam spectrophotometer UV-200. A BF 5003 liquid scintillation spectrometer was used for the measurement of radioactivity. The scintillation cocktails were Riafluor for aqueous samples and Instagel for organic solutions.

RESULTS

Reliability criteria
In addition to the cross-reactivity studies the specificity of the assay was checked by the introduction of a paper-chromatographic step (PC II. 2-OHOe$_1$ 2-Me $R_F = 0.56$, 2-OHOe$_2$ 2-Me, $R_F = 0.23$; cf. methods). The content of unconjugated 2-OHOe$_1$ 2-Me of three different plasma samples measured directly was 104, 135 and 200 pg and after additional paper chromatography 95, 138 and 190 pg.

The solvent blank (< 2 pg), the buffer blank (< 25 pg) and the coefficient of variation of the standard curve allowed a lower limit of detection of 25 pg.

The accuracy of the assay was checked by the addition of definite amounts (12.5, 25, 50, 100 and 200 pg) of 2-OHOe$_1$ 2-Me to human plasma (0.5 ml). The pg-amounts of 2-OHOe$_1$ 2-Me (unconjugated), as determined 4-fold by radioimmunoassay, were found to be 62.5 (no addition), 76 (12.5 pg added), 88 (25 pg added), 120 (50 pg added), 158 (100 pg added) and 250 (200 pg added), respectively (linear regression: $y = 0.93x + 66$; $r = 0.998$). The determination of 2-OHOe$_1$ 2-Me (unconjugated and conjugated) in different plasma volumes (during cycle: 0.2–0.6 ml; during pregnancy: 0.025–0.200 ml) showed a linear relationship between the assayed amount of 2-OHOe$_1$ 2-Me and the plasma volume (coefficient of correlation $> 0.993$).
Table 2.
Concentrations of 2-methoxyoestrone (pg/ml) in human plasma.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age</th>
<th>Number</th>
<th>2-OHOe1 2-Me</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unconjugated</td>
</tr>
<tr>
<td>Children</td>
<td>5–9</td>
<td>6</td>
<td>61 ± 16</td>
</tr>
<tr>
<td>Men</td>
<td>21–40</td>
<td>6</td>
<td>74 ± 13</td>
</tr>
<tr>
<td>Men</td>
<td>51–70</td>
<td>6</td>
<td>109 ± 44</td>
</tr>
<tr>
<td>Women</td>
<td>21–40</td>
<td>14</td>
<td>131 ± 40</td>
</tr>
<tr>
<td>Women</td>
<td>51–70</td>
<td>7</td>
<td>102 ± 26</td>
</tr>
<tr>
<td>Women, follicular</td>
<td>21–40</td>
<td>6</td>
<td>96 ± 19</td>
</tr>
<tr>
<td>Women, peri-ovulat.</td>
<td>21–40</td>
<td>6</td>
<td>181 ± 27</td>
</tr>
<tr>
<td>Women, luteal</td>
<td>21–40</td>
<td>6</td>
<td>152 ± 22</td>
</tr>
<tr>
<td>Women, pregnant</td>
<td>21–40</td>
<td>20</td>
<td>3980 ± 1030</td>
</tr>
</tbody>
</table>

Fig. 2.
Plasma concentrations of 2-methoxyoestrone in a normal menstrual cycle
(●, unconjugated; ○, conjugated).
A 30-fold determination of plasma samples containing different amounts of 2-OH\(\text{OEO}_1\) 2-Me (89, 135, 560 and 800 pg/ml of unconjugated 2-OH\(\text{OEO}_1\) 2-Me and 1480 and 5260 pg/ml of conjugated 2-OH\(\text{OEO}_1\) 2-Me) resulted in coefficients of variation of \(<10\%\) (unconjugated: 9.6, 6.9, 4.5, 4.4; conjugated: 7.5, 9.2). For the interassay precision as determined from pool plasma (unconjugated 89 pg/ml; conjugated: 1900 pg/ml) coefficients of variation of \(<20\%\) (unconjugated: 19.6; conjugated 9.2) were calculated.

**Concentrations of 2-OH\(\text{OEO}_1\) 2-Me**

The concentrations of 2-OH\(\text{OEO}_1\) 2-Me in plasma are given in Table 2 and Fig. 2.

**DISCUSSION**

After the preparation of a steroid-protein conjugate containing intact 2-methoxyoestrone, an antiserum was obtained which allowed the determination of 2-methoxyoestrone in human plasma with high specificity and sensitivity. Cross-reactivity to a considerable extent was only found with 2-methoxyoestradiol. Nevertheless, no significant differences between the radioimmunologically determined amounts of 2-methoxyoestrone either without or with an inserted paperchromatographic step – known to separate 2-methoxyoestradiol and 2-methoxyoestrone – were found. Consequently it is believed that the concentration of unconjugated 2-methoxyoestradiol is definitely lower than that of 2-methoxyoestrone. Therefore, the determination of 2-methoxyoestrone by the method described above and the determination of 2-hydroxyoestrone by the method reported previously (Ball et al. 1978a) allow quantitative evaluations of total catecholoestrogen concentrations in human plasma.

The concentrations of unconjugated 2-methoxyoestrone in human plasma were slightly higher than those reported for 2-hydroxyoestrone (Ball et al. 1978a). It is noteworthy that the concentrations increase between the groups of children, young and elderly men whereas there is only a slight decrease between the groups of cycling and post-menopausal women.

During the menstrual cycle the pattern of unconjugated 2-methoxyoestrone followed that of oestrone and oestradiol. Of special interest is the fact that for 2-methoxyoestrone extremely high values were found during the second trimester of pregnancy, values which reached even the range of oestriol concentrations (Ahmed & Kellie 1973). In contrast to the high concentrations of unconjugated 2-methoxyoestrone relative low values have been reported for unconjugated 2-hydroxyoestrone during the same period of pregnancy (Ball et al. 1978a). Regarding the postulated hypertensive effects of catecholoestrogens (cf. Gelbke et al. 1977) the extensive methylation of the active catecholoestrogens formed may prevent the occurrence of hypertension during normal pregnancy.
In contrast to unconjugated 2-methoxyoestrone conjugated 2-methoxyoestrone was in the same order of magnitude in all the groups investigated. Even during pregnancy a 5 times increase only was observed. This resulted in quite different ratios of conjugated to unconjugated methyl ether (men 17.8; cycling women 7.9; pregnant women 1.5). Comparing these ratios of 2-methoxyoestrone for cycling and pregnant women to those of 2-hydroxyoestrone (ratio of conjugated to unconjugated 2-hydroxyoestrone: approximately 20, to be published) the lower conjugation of 2-methoxyoestrone is obvious. This confirmed previous \textit{in vitro} studies (Ball et al. 1974) which had shown that 2-hydroxyoestrone – compared to its monomethyl ether – is the better substrate for conjugating enzyme systems.

**ACKNOWLEDGMENT**

This investigation was supported by the Deutsche Forschungsgemeinschaft.

**REFERENCES**


Received on December 11th, 1978.