Isolation and Identification of 2-Hydroxyoestrone 3-Methyl Ether from the Urine of Pregnant Women

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Knuppen & Breuer (1966) have reported that 2-hydroxyoestrone, one of the main metabolites of oestradiol-17 β in man, is methylated *in vitro* to 2-methoxyoestrone as well as to 2-hydroxyoestrone 3-methyl ether by human liver. The monomethylated isomers of 2-hydroxyoestrone are formed in approximately equal amounts (Knuppen *et al.*, 1970a). More recently, these findings *in vitro* were confirmed by experiments *in vivo*; after oral administration of [4-¹⁴C]oestradiol-17 β to four male subjects, 2-methoxyoestrone and 2-hydroxyoestrone 3-methyl ether were detected as metabolites in urine (Knuppen *et al.*, 1970b). Since 2-methoxy oestrogens have quantitative importance in human pregnancy urine (Kraychy & Gallagher, 1958; Notchev & Stimmel, 1962), it was decided to make an intensive search in pregnancy urine for 2-hydroxyoestrone 3-methyl ether.

Specimens (24h collections) of late-pregnancy urine from three women were acidified to pH 4.8 with acetic acid, buffered to this pH by the addition of 0.1 vol. of 1M-sodium acetate buffer and incubated at 37°C with an extract from *Helix pomatia* [2ml, containing 100000 Fishman units (Talalay *et al.*, 1946) of β -glucuronidase and 50000 Whitehead units (Whitehead *et al.*, 1952) of sulphatase/ml] for a period of 48h; half of the total amount of enzyme used was added initially and half after 24h. The hydrolysed mixture was extracted twice with 0.67 vol. of ether; the extracts were combined and washed twice with 0.1 vol. of NaOH-NaHCO₃ buffer [0.11 vol. of 5M-NaOH and 0.89 vol. of 8% (w/v) NaOH₃; pH 10.5]. The ethereal phase was then washed once with 0.1 vol. of 8% (w/v) NaHCO₃ and twice with 0.25 vol. of water, dried over Na₂SO₄ and evaporated to dryness. The residue was dissolved in ethanol (5ml). After dilution with benzene (125ml) and light petroleum (b.p. 40–60°C; 125ml), the mixture was extracted twice with 0.5 vol. of water. The organic phase was dried over Na₂SO₄ and evaporated to dryness. The residue was chromatographed in benzene on a column of 3g of neutral alumina (M. Woelm, Eschwege, Germany), elution being carried out first with benzene (200ml); 2-methoxyoestrone and 2-hydroxyoestrone 3-methyl ether were then separated by careful elution of the column with

20ml portions of 0.2% ethanol in benzene (100ml) and 0.35% ethanol in benzene (100ml) under the conditions described by Knuppen & Breuer (1966). The fractions eluted by 0.2% ethanol in benzene were combined and evaporated, yielding fraction A-1; those eluted by 0.35% ethanol in benzene gave fraction B-1.

Portions of fractions A-1 and B-1 were subjected to chromatography on formamide-impregnated paper with cyclohexane. In fraction A-1 a spot was detected that corresponded in mobility to 2-methoxyoestrone (2.6cm/h), whereas in fraction B-1 a spot was found in the position of 2-hydroxyoestrone 3-methyl ether (mobility 2.1cm/h). The remainder of fraction B-1 was further purified by paper chromatography in the same system as described above. After elution with methanol, the 2-hydroxyoestrone 3-methyl ether-like material was distributed between ether and water phases. The ethereal phase was evaporated, yielding fraction B-2.

Fraction B-2 was then further purified by t.l.c. on Kieselgel HF₂₅₄ (E. Merck A.-G., Darmstadt, Germany) with the solvent system ethyl acetate-cyclohexane (1:1, v/v). The area containing the 2-hydroxyoestrone 3-methyl ether-like material was eluted with redistilled ethyl acetate. After evaporation, the residue was treated with *NN*-bis(trimethylsilyl)trifluoroacetamide for 30min at room temperature. The compound was analysed by using a gas-liquid chromatograph-mass spectrometer (LKB 9000) under the conditions described by Stárka *et al.* (1969). The mass spectra and retention times [10.5min; column conditions: 3% OV-7 on Chromosorb WHP (100–120 mesh), in glass tubing (0.2cm $\times$ 180cm); flash heater temp. 245°C; column temp. 230°C; helium gas flow rate 35ml/min] of the derivatives of 2-hydroxyoestrone 3-methyl ether and compound B-2 isolated from the pregnancy urine were identical. Prominent peaks at *m/e* 372 (*M*), 357 (*M*–15) and 342 (*M*–30) were seen in the spectra of the trimethylsilyl ether derivatives.

The results reported here show the presence of 2-hydroxyoestrone 3-methyl ether in extracts of enzymically hydrolysed late-pregnancy urine. The concentration of 2-hydroxyoestrone 3-methyl ether in pregnancy urine was found to be approx. one-twentieth of that of the isomeric 2-methoxyoestrone; this finding confirms the divergence between the results *in vitro*, where the two isomeric monomethyl

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ethers of 2-hydroxyoestrone were formed in equal amounts, and those obtained *in vivo* (Knuppen *et al.*, 1970a,b).

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