ISOLATION OF 16α-HYDROXY-2-METHOXYESTRONE FROM RAT BILE

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ABSTRACT

A metabolite of 2,3-dihydroxyestra-1,3,5(10)-trien-17-one-6,7-^{3}H isolated from rat bile, was partially characterized by mass spectrometry as a methyl ether of 2,3,16-trihydroxyestra-1,3,5(10)-trien-17-one. The α configuration of the 16-hydroxy function was established by chromatographic comparison of the sodium borohydride reduced metabolite with synthetic 2-methoxyestra-1,3,5(10)-triene-3,16α,17β-triol and 2-methoxyestra-1,3,5(10)-triene-3,16β,17β-triol. The methyl group was located on the C-2 position by comparison with authentic 2- and 3- monomethyl ethers of 2,3-dihydroxyestra-1,3,5(10)-trien-17-one following pyrolytic removal of the 16α-hydroxyl group.

3,16α-dihydroxy-2-methoxyestra-1,3,5(10)-trien-17-one was found to constitute 2% and 15% of the biliary radioactivity following administration of estrone-6,7-^{3}H and 2,3-dihydroxyestra-1,3,5(10)-trien-17-one-6,7-^{3}H respectively.

INTRODUCTION

In various *in vitro* (1,2) and *in vivo* (3) studies of estrogen metabolism in the rat, highly polar metabolites have been detected which thus far remain unidentified. Although 2-oxygenated products of radio-

labelled estradiol predominate in bile, the "polar" fraction constitutes a significant portion of the excreted dose (3). The observed characteristics of this fraction are suggestive of a polyhydroxylated metabolite or metabolites.

The present report describes the isolation and identification of a 2,16α-dihydroxylated metabolite from rat bile which may bear some relevance in studies of the in vivo "polar" metabolites.

EXPERIMENTAL

General

Infrared spectra were recorded with a Perkin-Elmer model 237B double-beam spectrophotometer. NMR spectra were determined with a Varian A-60A spectrometer for solutions in deuteriochloroform with tetramethylsilane as internal standard. Mass spectra were determined with a Hitachi-Perkin-Elmer RMS4 spectrometer using the direct insertion probe. Gas-liquid chromatography (GLC) was carried out with a model 810 F and M chromatograph equipped with a stream splitter. The column was 5% OV-210 (8ft x 4 mm i.d.); carrier gas helium; flow rate 60 ml/min; column temperature 240°. Thin layer chromatography (TLC) was performed on 0.4 mm silica gel coated plates (Kieselgel N, Macherey, Nagel and Co., Germany). Phosphomolybdic acid in methanol was used for colour development. Radioactivity was assayed in a Packard liquid scintillation spectrometer using Bray's reagent (4).

Synthesis of steroidal compounds

The 2- and 3-monomethyl ethers of 2,3,16α-trihydroxyestra-1,3,5(10)-trien-17-one were synthesized according to the method of Leeds et al. for the preparation of ring D glycols (5). The 2- or 3-methyl ether of 2,3-dihydroxyestra-1,3,5(10)-trien-17-one was treated with isopropenyl acetate in the presence of catalytic amounts of toluene-p-sulfonic acid to produce

the methyl ether of estra-1,3,5(10),16-tetraene-2,3-17-triol diacetate. The enol acetate was converted to the 16α,17α-epoxy derivative with dilute chloroperbenzoic acid in benzene. Acid hydrolysis of the epoxide yielded the 2- or 3-monomethyl ether of 2,3,16α-trihydroxyestra-1,3,5(10)-trien-17-one. The product was isolated by TLC using the solvent system chloroform:ethanol, 9:1 and crystallized from benzene-methanol. The following characteristics were found for the 2-methyl ether: m.p. 210-211°; $\lambda(KBr)_{max}$ 3440, 2920, 2850 and 1740 cm^{-1}; NMR δ 6.77; $[\alpha]_D^{22}$ + 174.8° (dioxane). Anal Calcd. for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 72.13; H, 7.70. For the 3-methyl ether the following were found; m.p. 208.5-210°; NMR δ 6.86, 6.58; $[\alpha]_D^{22}$ + 185.2° (dioxane). Anal. Calcd. for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 71.94; H, 7.65.

For the synthesis of 2-methoxy-16-epiestriol (2-methoxyestra-1,3,5(10)-triene-3,16β,17β-triol), 3,16α-dihydroxy-2-methoxyestra-1,3,5(10)-trien-17-one was initially rearranged to 16-keto-2-methoxyestradiol-17β (3,17β-dihydroxy-2-methoxyestra-1,3,5(10)-trien-16-one) by treatment with 2.5 N NaOH under nitrogen at room temperature. Subsequent reduction with sodium borohydride provided 2-methoxy-16-epiestriol which was isolated by TLC (chloroform-ethanol; 90:10) and crystallized from chloroform-hexane; m.p. 233.5-235.5°; $[\alpha]_D^{22}$ + 101.7°. Anal. Calcd. for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.48; H, 8.33.

2-Methoxyestriol (2-methoxyestra-1,3,5(10)-triene-3,16α,17β-triol) was synthesized by a method adapted from Nambara et al. (6). Estriol 3-methyl ether was acetylated and subjected to Friedel-Crafts acylation to provide the 2-acetyl derivative. Removal of the 3-methyl group with hydrogen bromide in acetic acid gave 2-acetylestriol 16,17-diacetate. Treatment with benzyl chloride and potassium carbonate yielded the 3-benzyl ether which was subjected to Baeyer-Villager oxidation with 3-chloroperbenzoic acid to afford the 2-acetoxy compound. Following removal of the acetates by alkaline hydrolysis, the product was treated with methyl sulfate to yield the 2-methoxy estriol 3-benzyl ether. The 2-methoxyestriol obtained upon hydrogenation over palladium on charcoal was isolated by TLC (chloroform-ethanol; 90:10); m.p. 214-215° (lit m.p. 215-218°) (7).

2-Hydroxyestrone (2,3-dihydroxyestra-1,3,5(10)-trien-17-one) and 2-hydroxyestrone-6,7-^{3}H were prepared from estrone and estrone -6,7-^{3}H (sp.a. 40 C/mM; New England Nuclear, Boston, Mass.) by a previously described method (8).

Isolation of Metabolite

One-fifth of a μC of 2-hydroxyestrone-6,7-^{3}H (sp.a. 0.16 μC/μmole) was administered subcutaneously to each of nine female Wistar rats weighing approximately 220 gm. Bile was collected over a period of 7 h as previously described (3) and the samples were pooled, made 50% with respect to ethanol and kept at 5°C for 30 min prior to centrifugation. The dried supernatant was taken up in water and passed through an Amberlite XAD-2 resin (Rohm and Hass, Philadelphia, Pa.) column (9). The methanol eluate was further chromatographed on a column of DEAE-Sephadex (A-25, Pharmacia (Canada) Ltd., Montreal, Que.) utilizing a linear gradient of 0-0.8 M NaCl (10). The total radioactive eluate was pooled and reprocessed through the Amberlite XAD-2 column. The conjugate fraction was incubated with 500 U of Ketodase (Warner-Chilcott Labs., New York, N.Y.) per ml in 0.1 M sodium acetate buffer (pH 5.0) at 37° for 18 h and the hydrolyzed steroid extracted with 3 x 15 ml of chloroform. The extract was dried over sodium sulfate and subjected to TLC in the system chloroform-ethanol, 95:5. The appropriate radioactive spot was eluted and the product acetylated prior to final purification by GLC.

RESULTS AND DISCUSSION

Mass spectral analysis of the acetylated metabolite collected following GLC demonstrated its molecular weight to be 400. Fragment ions at m/e 244 and 137 indicated the presence of an additional hydroxy function on ring D (11) and a methyl group on ring A respectively. Moreover, its relative instability, as evidenced by altered TLC mobility following mild alkali treatment in air, suggested

that the compound might be a ring D α-ketol. Subsequent mass spectral analysis of synthetic 3,16α-dihydroxy-2-methoxyestra-1,3,5(10)-trien-17-one diacetate (Fig. 1) in fact demonstrated the essential similarity of its spectrum to that of the isolated metabolite.

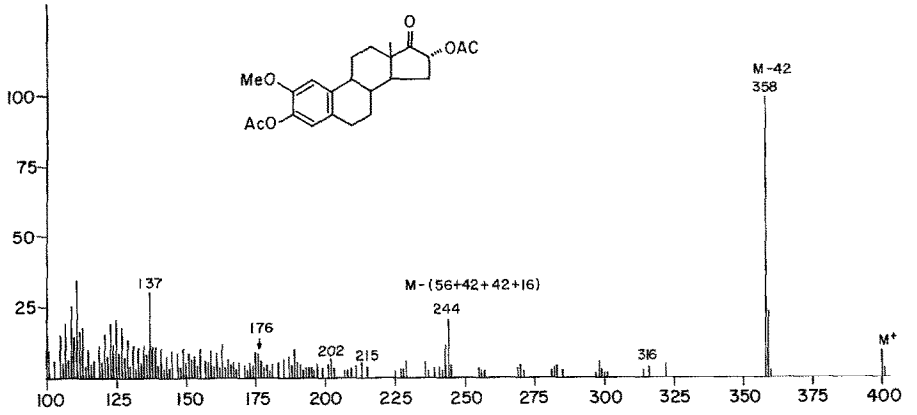

Fig. 1. Mass spectrum of 16α-hydroxy-2-methoxyestrone diacetate.

The base peak occurs in the spectrum at 358 as a result of loss of a ketene fragment from the aromatic acetate. Fragment ions at m/e 244, 215, 202 and 176, characteristic of estrogens, have been described by Budzikiewitcz et al (11). The ion at m/e 244, which was critical in locating the position of the extra hydroxyl group in the metabolite, results from a loss of the D ring. Fragment ions equivalent to m/e 137 in the present compound were found in all estrogens analyzed by us and

were indicative of a ring A fragment.

The metabolite was further found to have mobilities identical to synthetic 16α-hydroxy-2-methoxyestrone (3,16α-dihydroxy-2-methoxyestra-1,3,5(10)-trien-17-one) in several TLC systems, paper chromatography and GLC. The radioactive compound eluted from TLC co-crystallized with the synthetic compound to constant specific activity.

In an experiment in which tracer doses of estrone-6,7-H^3 were administered, the metabolite was isolated from bile following precipitation of lipids with ethanol, hydrolysis with Ketodase and multiple development in TLC system chloroform-ether; 95:5. Carrier 16α-hydroxy-2-methoxyestrone was added to the material eluted from TLC and the mixture divided into several portions. One portion was treated with alkali in air. A second portion was subjected to $NaBH_4$ reduction and a third portion was treated with alkali under nitrogen prior to $NaBH_4$ reduction (Fig. 2). Alkali treatment in air resulted in an altered mobility of coloured spots in both TLC (chloroform-ethanol; 90:10) and paper chromatography (Whatman #1; Formamide: benzene) and radioactive mobility was found to be similarly altered. Mass spectral analysis demonstrated the product to have a molecular weight of 314 indicating that the compound had undergone oxidation to a diketone.

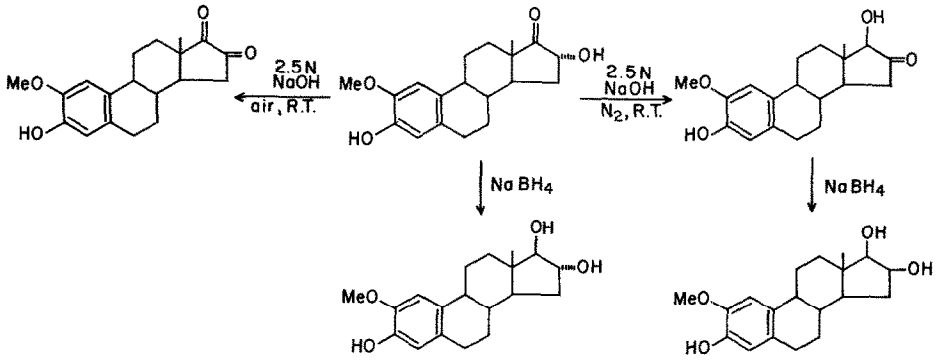

Fig. 2. Products of alkali treatment and reduction of metabolite.

TLC (chloroform-ethanol; 90:10) of the reduced metabolite with appropriate standards demonstrated that the major part of the radioactivity was associated with 2-methoxyestriol. Treatment with alkali under nitrogen prior to reduction resulted in the radioactivity being associated mainly with 2-methoxy-16-epiestriol. In the latter instance, rearrangement is presumed to have occurred to 16-keto-2-methoxyestradiol-17β which upon reduction formed the 16β-hydroxy compound. Thus, although the results do not preclude the occurrence of the 16β-hydroxy epimer, the predominant α configuration in the isolated metabolite was established.

Since the 2- and 3-monomethyl ethers of 2,3,16α-trihydroxyestra-1,3,5(10)-trien-17-one are not separated in any of the systems utilized, these two compounds were added to the metabolite and the mixture subjected to dehydration to the monomethyl ethers of 2-hydroxyestrone by pyrolysis with potassium hydrogen sulfate (12). Reduction of the double bond introduced at C-15 was effected by catalytic hydrogenation over palladium/charcoal. TLC separation (multiple runs in chloroform) of the products demonstrated the major part of the radioactivity to be associated with the 2-methyl ether.

16α-hydroxy-2-methoxyestrone was found to represent approximately 2% and 15% of the excreted radioactivity following administration of estrone-6,7-^{3}H and 2-hydroxyestrone-6-7-^{3}H respectively. There was no significant difference in the amounts excreted over a dose range of estrone from tracer quantities to 10 μg per animal.

2-Hydroxylation has been shown to be quantitatively of primary importance in the rat *in vivo* (3,13, 14). Several investigators have also reported a variable degree of 16-hydroxylation (16α-hydroxyestrone (3,16α-dihydroxyestra-1,3,5(10)-trien-17-one) and estriols) *in vitro* (1, 15), although Jellinck and Garland were unable to detect any estriol in their studies with liver microsomes (2). In the present study, there appeared to be some 16α-hydroxyestrone and estriol

although the quantities were less than 0.5% of the excreted radioactivity.

A 2,16-dihydroxylated metabolite, 2-methoxy-estriol, initially detected in small quantities in human urine (7), has been isolated following incubation of estriol with rat liver slices (16). This compound was, however, not detected in the present study.

That 16 hydroxylation can occur in the rat *in vivo* is of interest with regard to investigations of potential polyhydroxylated biliary metabolites. Since the "polar" metabolites, some of which are presumably polyhydroxylated, are elevated under certain instances of altered hepatic function (3), characterization studies of this nature are of continuing interest.

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