SYNTHESIS OF [$^2H_8$] ESTRADIOL, [$^2H_7$] ESTRONE, [$^2H_6$] 2-HYDROXYESTRONE AND [$^2H_6$] 4-HYDROXYESTRONE AS INTERNAL STANDARDS FOR SELECTED ION MONITORING

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ABSTRACT

The synthesis of [$^2H_8$] estradiol, [$^2H_7$] estrone, [$^2H_6$] 2-hydroxyestrone and [$^2H_6$] 4-hydroxyestrone from estrone (as a source) is described. The high isotopical purity renders the labelled compounds as suitable carriers and internal standards for quantitative gas chromatography - mass spectrometry. The content of protonium-form (i.e. natural) estrogens in the labelled derivatives ranged from 0.12 % to 2.58 %. The performance of these compounds in quantitative assays using selected ion monitoring has been established; and this allows the determination of estrogens from biological material in the lower picogram range.

INTRODUCTION

Combined gas chromatography-mass spectrometry (GC-MS) has been applied for quantitative evaluation of a variety of compounds in biological fluids and tissues, using the technique of selected ion monitoring (SIM) or mass fragmentography. Since 1970 methods have been described for steroid hormones [1] and prostaglandins [2] which use isotopically labelled substances as carriers and internal standards for the nonlabelled endogenous compounds. These "homogenous" standards offer certain advantages: although their chemical and chromato-

graphic properties are almost identical they can be easily separated from the non-labelled compounds in the mass spectrometer. As discussed in a recent review [3], the performance of the fragmentographic assay, particularly its sensitivity, depends highly on the stability and purity of the internal standard used.

RESULTS AND DISCUSSION

The main objective of the syntheses described in this paper was to obtain an estrogen standard with a high deuterium content and the lowest possible contamination by (natural) protonium-form. This was achieved by specific reduction of carbonyl functions and saturation of double bonds. The introduction of deuterium into 15-dehydroestrone (1) by NaB^2H_4, as the first step (Fig.1) was an adaption of a method described by Sondheimer et al [4]. The introduction of a Δ^6-double bond and its catalytic reduction later, followed well established procedures in estrogen chemistry [5]. Estradiol (7), as the final product of the deuterium labelling, contained two deuterium atoms more than expected from the synthetic pathway (Fig.1). It was assumed that, while preparing 6-dehydroestradiol (6), hydrogen atoms 2 and 4 had been exchanged for deuterium during the treatment with ^2HCl. These conditions have been described to yield 2,4-labelled estrogens with a good isotopic purity [6]. The fragmentation pattern of (7) supported this assumption (Fig.2). Further evidence arose from the fact that synthesis of labelled 2-hydroxyestrone (9) as

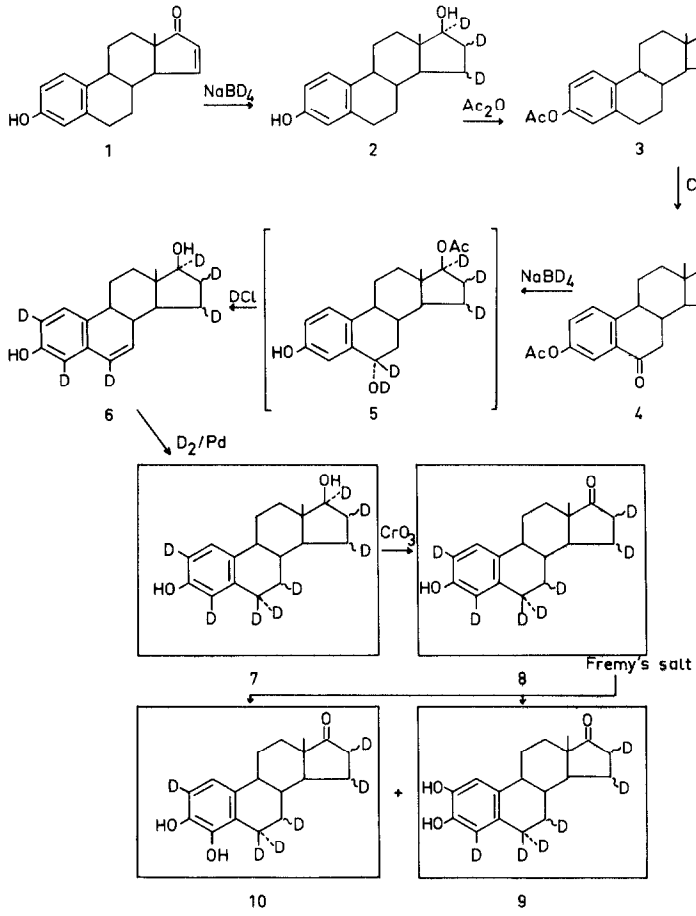

Fig. 1: Synthesis of Deuterium Labelled Phenolic Steroids.

well as 4-hydroxyestrone (10) from estrone (8), as indicated by the mass spectra (Fig.3), causes the loss of one deuterium atom - apparently from positions 2 or 4, respectively. The mass spectrum of 4-hydroxyestrone was omitted as it was virtually indistinguishable from that of 2-hydroxyestrone [7].

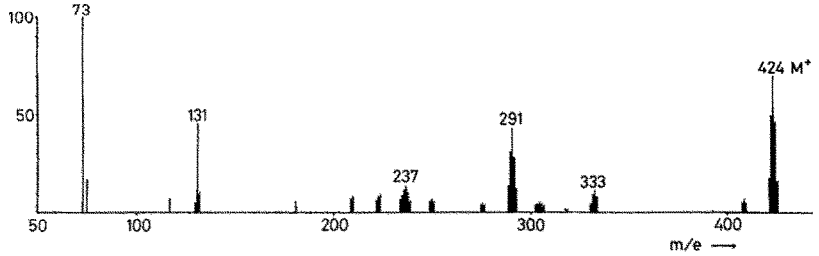

Fig. 2: Mass Spectrum of Deuterium Labelled Estradiol (7) (TMS-Derivative). For conditions see text.

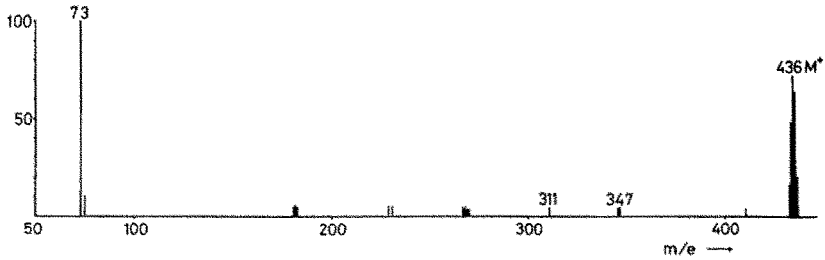

Fig. 3: Mass Spectrum of Deuterium Labelled 2-Hydroxyestrone (9) (TMS-Derivative). For conditions see text.

The content of non-labelled steroids in the deuterium-labelled products, which eventually determined the detection limit of the mass spectrometric assays, was calculated from the labelled compounds' mass spectra i.e. 0.148 % for (7), 0.120 % for (8), 2.56 % for (9) and 2.58 % for (10). The results indicate that only minute amounts of the protonium-

form are left in the labelled compounds which in turn render the latter suitable as standards for mass fragmentography [8].

EXPERIMENTAL

Estrone was kindly donated by Schering AG, Berlin. Deuterium labelled reagents (isotopic purity > 99 %) were purchased from E. Merck, Darmstadt. Deuterium gas (isotopic purity 99.7 %) was supplied by Messer-Griesheim, Düsseldorf. UV spectra were recorded on a Beckman Acta III spectrophotometer.

Mass spectra were taken on a LKB 2091 gas chromatograph-mass spectrometer (LKB instruments, Bromma, Sweden) using a glass column (0.2 x 150 cm) filled with 3 % OV-3 on 100/120 chromosorb WHP (W. Günther Analysentechnik, Düsseldorf) under the following conditions: Injector temp. 240°C, column temp. 220°C, separator temp. 245°C, ion source temp. 250°C, carrier gas flow 25 ml He/min, ionization energy 70 eV with 50 µA emission current, scan speed from m/e 50 to 450 in 6 s, filters 200 Hz, and slits adjusted to a resolution of approximately 1000.

15-Dehydroestrone (1). 15-Dehydroestrone was prepared from 16α-bromoestrone according to Cantrall et al. [9]. Prior to use its purity was checked by paper chromatography using the system formamide/chlorobenzene (R_F = 0.58 as compared to R_F = 0.67 for estrone).

[15,16,17α-$^2H_3$]Estradiol (2). 350 mg of (1) was dissolved in CH_3O^2H (50 ml) and treated with NaB^2H_4 (1 g) at 20°C for 12 h. The reaction mixture was acidified with acetic acid and diluted with water (50 ml). After evaporation of the methanol the mixture was extracted with ethyl acetate (3 x 50 ml). The extract was washed with water and the solvent was evaporated. Recrystallization of the residue from methanol/water yielded 320 mg of white crystals. MS : mol. weight 275.

[15,16,17α-$^2H_3$]Estradiol-3,17β-diacetate (3). 320 mg of (2) was treated with a mixture of pyridine/acetic acid anhydride (3 : 2; 20 ml) at 20°C overnight. The reaction mixture was diluted with water (100 ml) and extracted with ethyl acetate (3 x 50 ml). The extract was washed with 0.1 M HCl and water and the solvent was evaporated. After recrystallization of the residue from methanol/water, 300 mg of white crystals were obtained. MS : mol. weight 359.

[15,16,17α-$^2H_3$]6-Ketoestradiol (4). 300 mg of (3) was dissolved in 1.2 ml of acetic acid and treated with a solution of CrO_3 (250 mg) in water (0.22 ml) and acetic acid (1.6 ml) at 20°C for 24 h. Ethanol (1 ml) was added and the mixture was diluted with water (5 ml) and extracted with ether (3 x 10 ml). The etheral layer was washed with sat. $NaHCO_3$-solution (3 x 2 ml) and a mixture of 1 M Na_2CO_3 sat. $NaHCO_3$-solution (3 : 1, 6 x 2 ml), then with water until neutral. The ether was evaporated yielding 130 mg of dry residue. The crude product was dissolved in 10 ml of ethanol and 1 ml of acetic acid and, after addition of 1 g of Girard-T reagent, refluxed for 1 h. After cooling, the reaction mixture was poured into ice-water (50 ml) containing 1 g Na_2CO_3. The mixture was then extracted with ether (2 x 50 ml) yielding 20 mg of non-ketonic products. The aqueous layer was acidified with 1 M HCl and again extracted with ether (2 x 50 ml) yielding 80 mg of crude ketone. The product was treated with 5 % KOH in methanol at 20°C for 12 h. After dilution with water (50 ml) and neutralization with acetic acid the mixture was extracted with ether (2 x 25 ml). Evaporation of the solvent left 70 mg of a brown semicrystalline product. Further purification was achieved by column chromatography on 15 g of neutral Al_2O_3 (Woelm, Eschwege, activity II-III) using benzene/ethanol (98 : 2, by vol.) as solvent. Yield: 60 mg.

[2,4,6,15,16,17α-$^2H_6$]6-Dehydroestradiol (6). 60 mg of (4) was dissolved in CH_3O^2H (5 ml) and treated with NaB^2H_4 (150 mg) at 20°C for 12 h. Without isolating the crude 6α-hydroxyestradiol (5) dehydration was achieved by adding ^2HCl (1 ml of a 37 % sol. in ^2H_2O) and refluxing for 1 h. After cooling, the mixture was diluted with H_2O (20 ml) and extracted with ethyl acetate (2 x 20 ml). The combined organic layers were washed with H_2O until neutral. The solvent was evaporated yielding 45 mg of crude (6).

[2,4,6,6,7,15,16,17α-$^2H_8$]Estradiol (7). 40 mg of (6) was dissolved in $^2H_2$ saturated ethyl acetate (2 ml) and added to a suspension of palladium/charcoal (5 mg) in ethyl acetate (2 ml) which had been saturated with $^2H_2$ prior to use. Deuteration was accomplished by treating with $^2H_2$ under atmospheric pressure for 2 h. The catalyst was removed by filtration and the crude product crystallized from ethyl acetate/petroleum ether (2 : 1, by vol.). Yield: 35 mg; MS : mol. weight 280; mol. weight of the TMS-derivative 424 (Fig.2).

[2,4,6,6,7,15,16-$^2H_7$]Estrone (8). 30 mg of (7) was oxidized with Jones reagent according to [10] and the resulting 17-keto compound was recrystallized from methanol/water (4 : 1, by vol.) MS : mol. weight 277, mol. weight of the TMS-derivative 349. Yield: 24 mg.

[4,6,6,7,15,16-$^2H_6$]2-Hydroxyestrone (9) and [2,6,6,7,15,16-$^2H_6$]4-Hydroxyestrone (10). 20 mg of (8) was oxidized with Fremy's salt according to Gelbke et al. [11]. The reaction products were separated by preparative paper chromatography using the system formamide/chlorobenzene-ethyl acetate (3 : 1, by vol.) under the protection of ascorbic acid. Zones containing labelled 2-hydroxyestrone (9), 4-hydroxyestrone (10) and non-converted estrone (8) were eluted separately. (9) was further purified on a column (1 cm I.D.) filled with silica gel (Kieselgel 60, 1.5 g) and ascorbic acid impregnated silica gel (5.5 g); (9) was eluted between 25 and 50 ml of the solvent system n-hexane/chloroform/acetic acid (4 : 4 : 1, by vol.) and was recrystallized from ether containing a few drops of acetic acid. MS : mol. weight of the TMS-derivative of (9) 436 and of (10) 436.

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