SYNTHESIS OF [16,16,17α-$^2H_3$] ESTRADIOL AND [14α,15,15-$^2H_3$] ESTRONE OF HIGH ISOTOPIC PURITY[1]

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SUMMARY

The title compounds, each of high isotopic purity, were synthesized as internal standards for the mass spectral quantitation of estradiol and estrone. The [$^2H_3$] estradiol 3 was obtained from estrone (1) by alkaline H/^{2}H exchange of the C-16 protons and $LiAl^2H_4$ reduction of the C-17 keto function. The [$^2H_3$] estrone 16 was synthesized by a multistep sequence using deuterioboration of a C-14 double bond and a three step reductive deuteration of a C-15 keto function as the key steps for deuterium incorporation.

Key Words: [16,16,17α-$^2H_3$] Estradiol, [14α,15,15-$^2H_3$] Estrone; GC-MS quantitation

INTRODUCTION

Successful utilization of the combined gas chromatographic-mass spectrometric technique for quantitation of trace components in animal and human tissue relies upon the availability of suitable standards. It has been demonstrated that the accuracy and precision of this quantitation method is improved by the use of internal rather than external standards.[2] Of the various types of internal standards that have been employed[3], isotopically labeled analogs of the compound to be quantitated have several advantages which stem from the identical chemical and almost identical physical properties of the analyte and standard.[4] Our need to quantitate a few parts per trillion of estradiol (E2) and estrone (E1) in cattle tissue by the combined gas chromatographic-mass spectrometric technique therefore required the synthesis of isotopically labeled estrone and estradiol as internal standards. The availability of a variety of relatively low cost high isotopic purity deuterated reagents together with synthetic consideration led us to select deuterium as the isotopic label.

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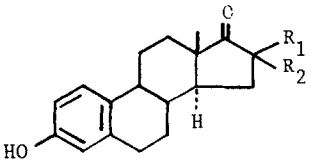
As a suitable internal standard for very high sensitivity analyses a deuterium labeled compound must meet certain requirements. In this quantitation method, the intensity of a characteristic ion of the analyte, e.g., the molecular ion (M), is compared to that of the corresponding ion M' of the internal standard. The accuracy of the method is improved when there is no contribution to M by the internal standard and none to M' by the analyte. For very high sensitivity analyses, the labeled compound is often added in large excess (100 fold) compared to the analyte in order to function both as carrier substance[5] and an internal standard. Suitable internal standards should therefore be of very high isotopic purity and should not contain any unlabeled (d_0) compound. In addition, since the estrogens have an M+2 ion of significant natural abundance, the internal standard should contain at least three deuterium atoms. In order to avoid any loss of deuterium from the internal standard during derivatization and GC-analysis these labels must be located in non-exchangeable positions.

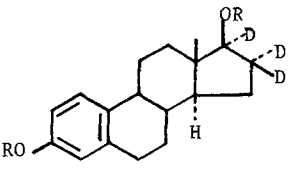
A variety of deuterium labeled estrogens have appeared in the literature. The syntheses of mono-deuterated estrogens with deuterium at C-2[6], 4[7], 14α[8], 15[8], 16[8], and 17[9] have been reported. Di-deuterio estrogens 2,4[10]; 6,7[11]; and 16,16[12], are also readily available. Tri-deuterio estrogens 4,16,16[13]; 6,6,9[13]; and 11,12,12[14], and a [9α,11,11,12,12-$^2H_5$] estrogen[14] have also been synthesized. However, none of the deuterium labeled compounds reported in the literature were of sufficiently high isotopic purity to function as internal standards for the quantitation of E1 and E2 in the very low picogram range. The requirements of a suitable internal standard outlined above in conjunction with various synthetic considerations led us to select [16,16,17α-$^2H_3$] estradiol (3) and [14α,15,15-$^2H_3$] estrone (16) as internal standards.

SYNTHESIS

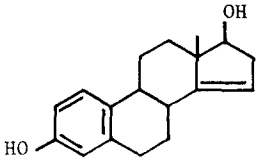
Estrone (1) was used as the starting material for the synthesis of the labeled compounds 3 and 16. Base catalyzed H/^{2}H exchange of 1 gave primarily the [16,16-$^2H_2$] estrone (2) together with small amounts of



1 $R_1 = R_2 = H$
2 $R_1 = R_2 = D$



3 R = H
4 R = $SiMe_3$



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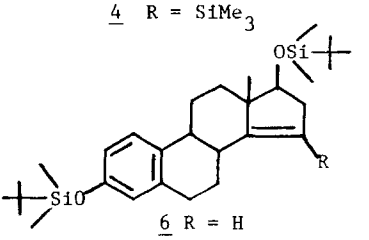
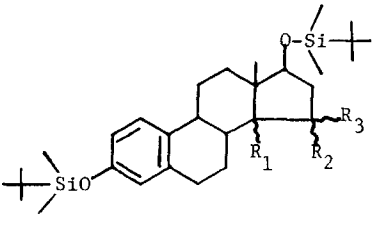
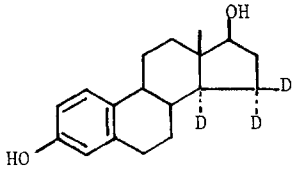


6 R = H
7 R = D



8 R_1 = α-D, R_2 = H, R_3 = α-OH
9 R_1 = β-D, R_2 = H, R_3 = β-OH
10 R_1 = α-D, R_2,R_3 = O
11 R_1 = α-D, R_2 = D, R_3 = α-OH
12 R_1 = α-D, R_2 = D, R_3 = β-OH
13 R_1 = α-D, R_2 = D, R_3 = OSO_2CH_3
14 $R_1 = R_2 = R_3$ = D



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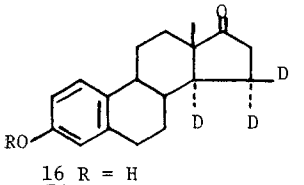

16 R = H
17 R = $SiMe_3$

components containing additional deuterium in ring-A. Reduction of the crude 2 with lithium aluminum deuteride[12] followed by acid catalyzed back exchange of any deuterium in ring-A gave the [16,16,17α-$^2H_3$] estradiol (3). The isotopic purity of [$^2H_3$] estradiol (3), determined by GC-MS of the bis-trimethylsilyl ether, 4, was found to be 3% d_4, 92% d_3, 3% d_2, 1% d_1, 0.1% d_0.

The key intermediate for introduction of deuterium into the D-ring of estrone is the tetraene 5 which was synthesized from estrone (1) by standard procedures[15]. Treatment of the protected tetraene-diol 6 with deuteriodiborane[16] followed by alkaline hydrogen peroxide oxidation of the intermediate borane gave a mixture of the alcohols 8 (86%) and 9 (14%) which were separated by chromatography. Alcohol 8 which has the natural configuration at C-14 was carefully oxidized with the Jones reagent to give the C-15 ketone 10 which, owing to the lability of the 14α-^2H towards exchange, was not purified. Reduction of 10 with lithium aluminum deuteride gave a mixture of the 15α and 15β-alcohols 11 and 12 which, although not required for subsequent reactions, could be separated by chromatography. Attempted conversion of this alcohol mixture into the corresponding tosylates was not successful, the 15β-alcohol being either unreactive or giving rise to elimination products under forcing conditions. Failure to form the tosylate of 12 is presumably a consequence of steric effects since the 15α-alcohol 11 is known to give the tosylate in good yield under similar conditions[8]. Treatment of the alcohols 11 and 12 with mesyl chloride and triethylamine[17], in which the reactive species is a sterically less demanding sulfene, gave primarily the elimination product presumably by base catalyzed elimination of the mesylate. However, substitution of the bulky base N,N-diisopropylethylamine for the triethylamine in the mesylation reaction gave a good yield of the mesylates 13. Reduction of the mesylates with lithium triethylborodeuteride[18] in tetrahydrofuran gave primarily the trideuterio-E2 derivative 14 (80%) together with the tetraene 7 (20%) which were separated by chromatography on silver nitrate impregnated silical gel. Removal of the protecting groups from 14 by treatment with aqueous acid gave

the estradiol 15 which was oxidized with the Jones reagent to afford the [14α,15,15-$^2H_3$] estrone (16).

The isotopic purity of 16 was determined by GC-MS analysis of the trimethylsilyl ether 17. The mass spectral data indicated the isotopic purity to be 3.2% d_2, 96% d_3, and 0.7% d_4. The data also indicated the presence of approximately 1% of a component with a molecular weight identical to that of unlabeled estrone. However, in view of the isotopic purity of the deuterated reagents used in this synthesis, together with statistical considerations it is unlikely that this component corresponds to unlabeled estrone. It is suggested that the sample of estrone 16 contains 1% of dideuterio mono-unsaturated estrone. All attempts to remove this contaminant by chromatography and recrystallization have been unsuccessful. Since the composition of a sample of 16 remains constant during analysis, the interference from the 1% of contaminant can easily be corrected for without any effect on the precision and accuracy of the quantitation method.

EXPERIMENTAL

The 60-MHz nuclear magnetic resonance (NMR) spectra were recorded on a Varian Associates A-60 spectrometer using $CDCl_3$ as solvent and Me_4Si as internal standard. Resonances associated with dimethyl-tert-butylsilyl groups are not reported.

Low resolution electron impact mass spectra were recorded on a Varian MAT 311A spectrometer interfaced with an SS-100 data system. GC-MS was performed on a 3'3% OV-17 column at 235° mounted in a Varian 2740 gas chromatograph which was interfaced with the mass spectrometer via an all glass jet separator.

Melting points were determined on a Thomas-Hoover "Uni-Melt" capillary melting point apparatus and are uncorrected.

Chromatography was performed on Silica Gel GF-254, Type 60 using the Chromatotron supplied by Harrison Research[19].

[16,16,17α-$^2H_3$] Estradiol (3): Sodium (100 mg, 4.3 mg-atoms) was added, under a nitrogen atmosphere, to a solution of estrone (1) (100 mg, 0.37 mmol) in a mixture of MeOD (10 ml) and D_2O (1 ml). The reaction

mixture was heated under reflux for 6 hr and then concentrated to half its volume. More MeOD (10 ml) was added and the solution heated under reflux for a further 6 hr. The reaction mixture was allowed to cool to room temperature and then brought to pH 7 by dropwise addition of 1 M-hydrochloric acid. The reaction mixture was extracted with chloroform (3 x 20 ml) and the combined extracts washed sequentially with 5% sodium bicarbonate, water, and saturated sodium chloride. After drying over anhydrous sodium sulfate, removal of the solvent gave [16,16-$^2H_2$] estrone (2) (93 mg) as a white solid, mp 253-256°C; mass spectrum m/z 272 (M^+), 1% d_0, 3% d_1, 88% d_2, 7% d_3, 1% d_4. The estrone 2 (91 mg,0.33 mmol) in anhydrous tetrahydrofuran (5 ml) was treated at 18°C with $LiAl^2H_4$ (30 mg, 0.71 mmol). After 12 hr the excess of reducing agent was destroyed by dropwise addition of saturated sodium sulfate solution. The resultant precipitate was removed by filtration and washed well with methanol. Removal of the solvent gave the crude estradiol 3 which was dissolved in methanol (4 ml):water (1 ml) and heated under reflux for 6 hr with concentrated hydrochloric acid (2 drops). The reaction mixture was diluted with water and the precipitated estradiol removed by filtration. Purification of the crude products by chromatography using benzene:acetone (4:1) as solvent gave 3 (60 mg) as needles from aqueous methanol: mp 174-176°C [Lit.[20] mp 174°C[21]].

Treatment of 3 (1 mg) with N,O-bis-(trimethylsilyl)-trifluoroacetamide/1% trimethylchlorosilane (BSTFA/1% TMCS) (50 µl) gave [16,16,17α-$^2H_3$] estradiol 3,17-bis-trimethylsilyl ether 4 which was not isolated. GC-MS showed a single GC peak: m/z 419 (M^+); 3% d_4, 92% d_3, 3% d_2, 1% d_1, 0.1% d_0.

Estra-1,3,5(10),14-tetraene-3,17β-diol Bis(dimethyl-tert-butylsilyl) Ether (6): To a solution of estra-1,3,5(10),14-tetraene-3,17β-diol (5)[15] (2 g, 7.4 mmol) in dimethylformamide (16 ml) were added imidazole (7.9 g, 0.12 mol) and dimethyl-tert-butylsilyl chloride (6.3 g, 42 mmol). After 2 hr the reaction mixture was diluted with ether (150 ml), washed with water, dried over anhydrous sodium sulfate and concentrated to give an oil which solidified

on standing. Recrystallization from methanol gave 6 (3.5 g) as colorless plates: mp 105-107°C [Lit.[15] mp 107-109°C].

Hydration of 6: A solution of boron trifluoride etherate (17 g, 0.12 mol) in anhydrous ether (50 ml) was added dropwise, with stirring under nitrogen, to an ice cold mixture of 6 (2.5 g, 5 mmol) and $LiAl^2H_4$ (5 g, 0.12 mol) in anhydrous ether (80 ml). The reaction mixture was allowed to warm to room temperature and stirred for a further 1 hr. The excess of reducing reagent was destroyed by dropwise addition of saturated sodium sulfate solution. The precipitate was removed by filtration and washed well with ether. The ethereal solution was washed sequentially with 5% sodium bicarbonate, water, and brine, and dried over anhydrous sodium sulfate. Removal of the solvent gave a yellow oil which was dissolved in tetrahydrofuran (80 ml) and treated with hydrogen peroxide (29 ml of 30%) and 2.5 M sodium hydroxide (43 ml) at 0° for 12 hr. The reaction mixture was diluted with water and extracted with ether (3 x 100 ml). The combined organic extracts were washed with 5% sodium sulfite, water, and brine, dried over anhydrous sodium sulfate and concentrated to give a colorless oil. The crude product was chromatographed on silica gel (100 g) using hexane-ethyl acetate (10:1) as eluant. The more polar [14α-^2H] 15α-hydroxyestradiol 3,17-bis(dimethyl-tert-butysilyl) ether (8) (1.5 g) was obtained as colorless plates from methanol:mp 148- 149°C; NMR δ 0.76 (3H,s,18-H_3), 3.90 (1H, t, J=8Hz, 17α-H), 4.15 (1H, q, J=9, 3.5 Hz, 15β-H), 6.5-6.75 (2H, 2- and 4-H), 7.12 (1H, d, J=8Hz, 1-H); mass spectrum m/z 517 (M^+). [Lit.[8] mp 147-149°C]. The [14β-^2H] 15β-hydroxyestradiol 3,17-bis(dimethyl-tert-butylsilyl) ether (9) (240 mg) was obtained as needles from methanol mp 168-170°C; NMR δ 1.08 (3H, s, 18-H_3), 3.65 (1H, d, J=4 Hz, 17α-H), 4.26 (1H, d, J=8 Hz, 15α-H), 6.50-6.75 (2H, 2- and 4-H), 7.12 (1H, d, J=8Hz, 1-H); mass spectrum m/z 517 (M^+). [Lit.[8] mp 171-175°C].

[14α,15-$^2H_2$] 15-Hydroxyestradiol 3,17-Bis(dimethyl-tert- butylsilyl) Ethers (11) and (12): Jones reagent (chromic acid in aqueous acetone) was added dropwise to an ice cold solution of 8 (1.5 g) in acetone (50 ml) until a permanent yellow coloration was obtained. The reaction mixture was diluted

with a large excess of ether and filtered through Florosil. The ethereal solution was washed with 10% sodium bicarbonate, water, and brine, dried over anhydrous sodium sulfate and concentrated to give [14α-^{2}H] 15-keto-estradiol-3,17-bis(dimethyl-tert-butylsilyl) ether (10) as a colorless solid. The crude ketone 10 in anhydrous ether (50 ml) was treated at 0° with $LiAl^2H_4$ (300 mg). After 1 hr, work up as described for 3 gave a mixture of the C-15 alcohols 11 and 12 (1.4 g) as a crystalline solid. A small sample (50 mg) of this mixture was chromatographed using hexane-ethyl acetate (9:1) as eluant. The less polar 15β-alcohol 12 (35 mg) was obtained as needles from aqueous methanol mp 167-169°C; NMR 1.03 (3H, s, 18-H_3), 3.6 (1H, t, J=8Hz, 17α-H), 6.5-6.75 (2H, 2 and 4-H), 7.12 (1H, d, J=8Hz, 1-H); mass spectrum m/z 518 (M^+). The 15α-alcohol 11 (9 mg) was obtained as needles from aqueous methanol mp 147-149°C; NMR as for 8 but no signal at 4.15 due to the 15β-H. Mass spectrum m/z 518 (M^+).

[14α,15,15-$^2H_3$] Estradiol Bis(dimethyl-tert-butylsilyl) Ether (14): N,N-diisopropylethylamine (400 µl, 3.3 mmol) and methanesulfonyl chloride (180 µl, 2.2 mmol) were simultaneously added dropwise to an ice cold solution of a mixture of the C-15 alcohols 11 and 12 (850 mg, 1.6 mmol) in anhydrous dichloromethane (10 ml). After 0.5 hr the reaction mixture was diluted with dichloromethane, and washed with ice cold water, 10% hydrochloric acid, saturated sodium bicarbonate, and brine. After drying over anhydrous sodium sulfate removal of the solvent gave the mesylates 13 as a colorless oil. The mesylates 13 were dissolved in anhydrous tetrahydrofuran (25 ml) and treated dropwise at 0°C with $LiEt_3B^2H$ (8 ml of a 1 M solution). After 2 hr, the reaction mixture was allowed to warm to room temperature and was then stirred for a further 12 hr. The excess of reducing agent was destroyed by dropwise addition of water. The organoborane was oxidized with 3 N sodium hydroxide (1.5 ml) and hydrogen peroxide (30%, 1.5 ml). The reaction mixture was extracted with ether (3 x 100 ml) and the combined extracts washed with water and brine, dried over anhydrous sodium sulfate and concentrated to give a white solid which was chromatographed on silica gel impregnated with 5% of silver nitrate using the Chromatotron. The silyl ether 14 (340 mg) was

obtained as needles from methanol; mp 128-130°C; mass spectrum m/z 503 (M^+). The minor product of the reaction [15-^{2}H] estra-1,3,5(10),14-tetraene-3,17-diol bis(dimethyl-tert-butylsilyl) ether (7) (85 mg) was obtained as needles from methanol mp 103-106°C; NMR as for 6 but no 15-H; mass spectrum m/z 499 (M^+).

[14α,15,15-$^2H_3$] Estradiol (15): The silyl ether 14 (300 mg) in acetone (25 ml) was treated with 5N-HCl (4 ml) for 5 hr. The reaction mixture was neutralized by addition of 5% sodium bicarbonate solution, concentrated to half its volume and extracted with ethyl acetate. The combined extracts were washed with water and brine, dried over anhydrous sodium sulfate and concentrated to give crude 15. Chromatography using hexane-ethyl acetate (1:1) gave 15 (148 mg) as needles from ethyl acetate; mp 172-175°C; mass spectrum m/z 275 (M^+). [Lit.[20] mp 174°C[21]].

[14α,15,15-$^2H_3$] Estrone (16): Estradiol (15) (148 mg) was oxidized with the Jones reagent as described for 8. The crude trideuterio-estrone obtained was purified by chromatography using benzene-ether (3:1) as eluant to afford 16 (101 mg) as cubes from ethyl acetate, mp 252-254°C; mass spectrum m/z 273 (M^+) [Lit.[22] mp 254-255°C[21]].

Treatment of 16 (0.5 mg) with BSTFA (50 µl) gave [14α,15,15-$^2H_3$] estrone 3-trimethylsilyl ether (17) which was not isolated. GC-MS showed a single GC peak; m/z 345 (M^+); 3.2% d_2, 96.1% d_3, 0.7% d_4.

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