THIN LAYER CHROMATOGRAPHY OF THE FOUR ESTRIOL EPIMERS AND THEIR METHYL ETHERS.

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Diczfalusy and Halla[1] have reported that 16-epiestriol, when methylated with $(CH_3)_2SO_4$ in borate buffer according to the procedure of Brown[2], gave two peaks in countercurrent distribution, and they identified these peaks as being due to the mono- and dimethyl ethers of 16-epiestriol. In the course of studies on the occurrence of 16-epiestriol in avian urine, we have observed that the product of methylation of this compound by Brown's method gave three separate spots when subjected to thin layer chromatography in a number of different solvent systems. When estriol, 16,17-epiestriol and 17-epiestriol were treated in a similar manner, it became clear that the two methylated cis-glycols, i.e. 16-epiestriol and 17-epiestriol, gave three spots on chromatograms, while the two methylated trans-glycols gave single spots.

This communication describes the thin layer chromatography of the four estriol epimers and their methyl ethers, and the isolation and partial characterization of the three methylated compounds of 16-epi-estriol by formation of derivatives and their subsequent chromatography on silica gel G.

EXPERIMENTAL AND RESULTS

Materials and methods

All solvents were redistilled before use. Absolute ethanol (Con-

solidated Alcohols Ltd., Toronto) was refluxed over sodium hydroxide pellets and zinc dust and twice distilled. Benzene (thiophene free) and absolute methanol, both "spectroanalyzed" grade, were obtained from Fisher Scientific Co. Ethyl acetate A.R., chloroform A.R., and cyclohexane "Laboratory Reagent" grade were obtained from British Drug Houses Ltd. (BDH). Dimethyl sulphate (BDH) was redistilled under reduced pressure. Silica gel G (Merck, Darmstadt) and the Desaga outfit for thin-layer chromatography were purchased from Brinkmann Instruments, Inc., Great Neck, New York.

Thin layer chromatography. Glass plates (20 x20 cm) were coated with a layer (0.25 mm thick) of silica gel G, a slurry of which was prepared by vigorously shaking 30 g of the adsorbent with 60 ml of distilled water for 1 minute in a glass stoppered 250 ml Erlenmeyer flask. The plates were then left on the bench for 30 minutes, activated at 100°C for 1 hour and stored in a desiccator over dry silica gel.

Samples (1-15 μg) in absolute ethanol were applied in successive small portions by means of micropipettes (to prevent undue spreading of the spots) approximately 2.5 cm from the bottom edge and 2-4 cm from the side edges of the plates. This procedure was found necessary in order to obtain reproducible R_f values. Three sides of the chromatography chambers were lined with filter papers which dipped into the solvent. The plates were developed at room temperature. When the solvent front ascended to the line drawn across the plates, they were then removed from the chamber, dried and sprayed with a 2% solution of sulphuric acid in aqueous ethanol, followed by heating in an oven at 100°C for 20 minutes (Lisboa and Diczfalusy[3]).

Methylation. The crystalline estrogens were dissolved in ethanol and

TABLE I.

Average R_f values of the four estriol epimers and their methyl ethers on thin layers of silica gel G. The figures in brackets denote standard deviations of a single estimate.

Compound	S_1	S_2	S_3	S_4
estriol	0.09 (0.02)	0.06 (0.01)	0.05 (0.00)	0.17 (0.01)
16,17-epiestriol	0.08 (0.01)	0.06 (0.01)	0.07 (0.01)	0.20 (0.02)
16-epiestriol	0.12 (0.01)	0.13 (0.01)	0.16 (0.02)	0.32 (0.02)
17-epiestriol	0.15 (0.02)	0.16 (0.01)	0.19 (0.02)	0.36 (0.02)
estriol-3-methyl ether	0.17 (0.02)	0.12 (0.01)	0.07 (0.00)	0.20 (0.01)
16,17-epiestriol-3-methyl ether	0.16 (0.02)	0.11 (0.01)	0.10 (0.01)	0.24 (0.02)
16-epiestriol-3-methylether	0.21 (0.05)	0.23 (0.02)	0.20 (0.02)	0.35 (0.05)
16-epiestriol dimethyl ether (M2)	0.60 (0.03)	0.71 (0.03)	0.48 (0.03)	0.61 (0.03)
16-epiestriol dimethyl ether (M3)	0.66 (0.03)	0.76 (0.03)	0.54 (0.03)	0.65 (0.03)
17-epiestriol-3-methyl ether	0.28 (0.03)	0.31 (0.02)	0.24 (0.01)	0.41 (0.03)
17-epiestriol dimethyl ether 1	0.64 (0.02)	0.79*(0.02)	0.51 (0.03)	0.63 (0.02)
17-epiestriol dimethyl ether 2	0.69 (0.02)	0.79*(0.02)	0.56 (0.02)	0.68 (0.03)

S_1: Benzene 90 parts - absolute methanol 10 parts.
S_2: Chloroform 95 parts - absolute ethanol 5 parts.
S_3: Ethyl acetate 50 parts - cyclohexane 50 parts.
S_4: Ethyl acetate 50 parts - cyclohexane 45 parts-absolute ethanol 5 parts.

* did not separate in this system.

methylated by the procedure described by Brown[2]. Estriol yielded a product which gave R_f values identical with those given by reference estriol-3-methyl ether (Sigma Chemicals Co.) in three solvent systems. None of the methylation products of the estriol epimers gave a positive reaction with the Folin and Ciocalteu reagent, which fact indicated the absence of a free phenolic group. All of the methylation products of the estriol epimers gave positive Kober reactions. The R_f values obtained for the phenolic estriol epimers and their methyl ethers in four systems are given in Table I.

Isolation and characterization of the three methylated compounds of 16-epiestriol. The methylated 16-epiestriol in absolute ethanol was chromatographed on silica gel G. Usually 10 spots were applied on a

plate which was then developed in chloroform - ethanol (97.5 : 2.5). In this solvent system the three methylated compounds of 16-epiestriol, designated M1, M2 and M3, gave R_f values of 0.13, 0.57 and 0.62 respectively. Upon completion, the two terminal strips were sprayed with the 2% H_2SO_4 reagent while the rest of the plates was protected by a plastic plate. Three strips of the blank area corresponding to the stained spots on each side of the chromatoplates were scraped off, transferred into a sintered glass funnel and eluted with methanol. Care was taken to leave a sufficiently wide area (about 1 cm) between the second and third marked strips containing M2 and M3. The eluates of 30 chromatoplates were pooled, the solvent was evaporated under N_2 and the white material thus obtained recrystallized three times from aqueous methanol. The crystals were dried over $CaCl_2$ for 48 hours and weighed. The yields were 1.5 mg of M1, 1.0 mg of M2 and 0.8 mg of M3.

Melting points. These were determined with the Fisher-Johns melting point apparatus. All values are uncorrected for emergent stem. M1 melted at 141-142°; this value is identical with that reported by Huffman and Darby[4] for the monomethyl ether of 16-epiestriol. The melting point of M2 was 134.5-136° and that of M3 was 121-124°.

Acetonides of 16-epiestriol and M1. The procedure was similar to that used by Marrian and Bauld[5]. Small samples (0.1-0.2 mg) of 16-epiestriol, M1, M2 and M3 were dissolved in 0.05-0.1 ml of anhydrous acetone (prepared from BDH., A.R. grade) containing 1% (w/v) of HCl. The solutions were shaken intermittently at room temperature for 30 minutes, after which 1 ml of iced water was added. Only 16-epiestriol and M1 yielded white fluffy precipitates. This was filtered off in a small sintered glass funnel, washed with several 0.5 ml portions of water, dried in vacuo over $CaCl_2$ for 24 hours and recrystallized from aqueous ethanol.

16-Epiestriol acetonide melted slowly between 90° and 97°, resolidified over the range 125-132° and melted again at 183-184.5° (cf.Marrian and Bauld[5]). The remainder of each crystalline acetonide sample was dissolved in ethanol and chromatographed in four systems. (Table II.) M2 and M3 were recovered and chromatographed in the same systems but no change in the R_f values was observed.

Acetylation. Samples (0.2 mg) of 16-epiestriol and of the three methylated compounds were each dissolved in 0.2 ml of anhydrous pyridine (BDH., A.R. grade). To each was added 0.1 ml of acetic anhydride (BDH., A.R. grade), and the solutions were left to stand at laboratory temperature overnight. 10 ml of iced water containing 0.5 ml of 6N H_2SO_4 was then added to each and the solutions were extracted with three fresh portions of ethyl acetate. This was followed by washing with 4% NaOH (until the organic phase was basic) and three times with 10 ml of water to remove

TABLE II.

Average R_f values of 16-epiestriol derivatives on silica gel G. Figures in brackets denote standard deviations of a single estimate.

Derivative	S_1	S_2	S_3	S_4
Triacetate	0.67 (0.02)	0.78 (0.02)	0.60 (0.04)	0.66 (0.03)
Acetylated monomethyl ether(M1)	0.71 (0.03)	0.79 (0.02)	0.60 (0.04)	0.66 (0.02)
Acetylated dimethyl ether(M2)	0.74 (0.03)	0.79 (0.02)	0.62 (0.02)	0.71 (0.03)
Acetylated dimethyl ether(M3)	0.75 (0.02)	0.80 (0.03)	0.65 (0.02)	0.74 (0.02)
Acetonide of monomethyl ether	0.78 (0.03)	0.82 (0.02)	0.71 (0.02)	0.75 (0.02)
Acetonide	0.47 (0.03)	0.53 (0.05)	0.63 (0.02)	0.69 (0.02)

See Table I. for solvents.

alkali. The solvent was evaporated under reduced pressure and the product recrystallized from aqueous ethanol. The acetylated samples were then dried, taken up in ethanol and chromatographed on silica gel G. The R_f values of the acetylated 16-epiestriol were compared with those of 16-epiestriol-16-^{14}C-triacetate previously crystallized to constant specific activity and were found to be identical in three systems tested. The mobilities of all three methylated compounds were increased by acetylation (Table II.), thus showing that each possessed at least one free hydroxyl group.

DISCUSSION

The results provide strong evidence that while methylation of estriol and 16, 17-epiestriol by the procedure of Brown yields only the corresponding 3-methoxy compounds, the methylation of 16-epiestriol or of 17-epiestriol yields three methyl ethers, all of which can be separated by thin layer chromatography on silica gel G in a suitable solvent system. The methylated compound of 16-epiestriol that exhibited the lowest R_f values (M1) was characterized as the 3-methyl ether by (i) melting point, (ii) negative phenol reaction, (iii) acetonide formation and (iv) chromatographic behaviour. The fact that neither M2 nor M3 (the two fast-moving methylated compounds) formed acetonides and that both acetylated as well as giving negative phenol reactions, strengthens the assumption that both these derivatives of 16-epiestriol were dimethyl ethers. The available evidence was insufficient to distinguish which of the two dimethyl ethers of 16-epiestriol was the 3,16-dimethyl ether and which was the 3,17-dimethyl ether.

Although the lack of sufficient material precluded a more extended study of the three methylated compounds of 17-epiestriol, the

close chromatographic analogy with the methyl ethers of 16-epiestriol suggested that the spot of lowest R_f was due to 17-epiestriol-3-methyl ether and that the other two spots were due to the dimethyl ethers. It may be of interest to note that the two dimethyl ethers of 17-epiestriol did not separate in the system chloroform-ethanol (95 : 5).

Applications of Brown's method for estrogens to material in which the proportion of either of the two cis-glycols may be significant relative to the proportion of estriol must obviously take account of the different behaviour of these compounds on methylation.

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FOOTNOTE

The systematic names of the estrogens discussed in this work are as follows: Estra-1,3,5(10)-trien,3,16α,17β-triol (Estriol); Estra-1,3,5(10)-trien,3,16β,17β-triol (16-epiestriol); Estra-1,3,5(10)-trien,3,16α,17α-triol (17-epiestriol); Estra-1,3,5(10)-trien,3,16β,17α-triol (16,17-epiestriol).