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CHROMATOGRAPHIC PROPERTIES OF THE EPIMERIC ESTRIOLS

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SUMMARY

Thin-layer and gas chromatographic methods to separate the epimeric estriols are described. Gas chromatography of the acetates provided separation of all four. Thin-layer chromatography on Silica Gel G separated the *cis* from the *trans* estriols. The *trans* epimers separated only on silica gel modified with ammonium bisulfate. The acetonides separated well on both thin-layer and gas chromatography.

INTRODUCTION

Interest in the epimeric estriols was stimulated by the isolation of 16-epiestriol from monkey urine[1]. This substance was shown to be a major metabolite of both estrone and estradiol-17β in the rhesus monkey. Proof of the structure required an investigation of the chemistry and chromatographic behavior of the four epimeric estriols. The results of these experiments and the use of a new thin-layer medium are described in this paper. Methods involving both gas (GC) and thin-layer chromatography (TLC) will separate the epimers of estriol and the other common estrogens including the 2-hydroxy derivatives.

MATERIALS AND METHODS

All solvents for TLC were of analytical grade and redistilled before use. The thin-layer plates were all of Silica Gel G. These included Silica Gel G unmodified, Silica Gel G impregnated with phosphomolybdic acid[2] and Silica Gel G to which alcoholic ammonium bisulfate had been added[3].

The estrogens as listed below were obtained as follows: Estrone, estradiol-17β and estriol (16α,17β) and 16-epiestriol (16β,17β) were purchased from Sigma Chemical Company, St. Louis, Mo. 3,16α,17α-Trihydroxy-1,3,5,(10)-estratriene (17-epiestriol) (16α,17α) and 3,16β,17α-trihydroxy-1,3,5,(10)-estratriene (16,17-diepiestriol) (16β,17α) were generous gifts from Dr. MAX N. HUFFMAN, Creighton University, Omaha, Nebr. All of these materials on both TLC and GC showed little or no impurity and

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were used without further purification. Solutions of these steroids were made in acetone solution to give 1 μg/μl. Spotting from these solutions for TLC was performed with 10-μl Unimetric microsyringes. The chromatograms were developed in the designated solutions in the ascending manner until the solvent had reached 1 cm from the top edge of the 20 × 20 cm plates. The solvent systems used for TLC are: (A) 15% ethanol in benzene; (B) 20% ethanol in benzene; (C) 10% ethanol in benzene; (D) 2% ethanol in benzene; (E) 3% ethanol in benzene.

GC was performed with a Glowall Model 210 chromatograph which contained a detector with a 22.5 μCi radium foil. A 6 ft. × 4 mm glass coil column, containing Supelcoport (Supelco Inc., Bellefonte, Pa.) coated with 5% of OV-210 and 2½% of OV-1 together, was used for the free estrogens. For acetylated estrogens the same column packed with Supelcoport with 5% OV-1 was required. Operating conditions were: oven and detector temperature 245°, with an argon gas flow of 60 ml/min at 30 p.s.i. for both columns.

The retention times in GC were calculated as mm from the injection peak to the peak of the curve representing the eluted compound. All relative retention times were calculated from estrone or estriol as 100.

The acetates were made by dissolving the steroid (100 μg) in 6 drops of pyridine and then adding 3 drops of acetic anhydride. After remaining for 18 h at room temperature the reaction mixture was evaporated by a stream of nitrogen while warming the containers in a water bath. The acetonides were made by a modification of the method of HUFFMAN AND LOTT[4]. To the steroid (100 μg) in 1 ml of acetone was added 0.5 ml of acetone saturated with dry HCl. (The acetone was saturated with HCl while cooled in an ice bath.) After 15 min at room temperature, the HCl was neutralized with 5% sodium bicarbonate. The acetonide was extracted with ether after removal of acetone *in vacuo*. These conditions gave essentially complete reaction of the steroid with the ketone; no peak other than the acetonide was seen after GC.

RESULTS

Gas chromatography

The free *cis*-estriols are not amenable to GC because of decomposition at the high temperatures required. The separation of these free estriols was not possible by GC. The *cis* epimers presented flattened and broad peaks in the recorder tracings while the *trans* epimers gave symmetrical peaks but did not separate. The presence of the 2-hydroxy increased the retention time of the estrogens. In contrast, the acetates of all four epimeric estriols showed sharp symmetrical peaks and separated well as indicated in Table I.

The acetonides of the epimeric *cis*-estriols had retention times shorter than that of the free estriols. The *trans* epimers did not react with the acetone. The relative retention time for estriol-16α,17α was 57, while that for the β,β-epimer was 68 compared to free E_3. Table II gives the relative retention time of the acetonides of the two epimers with two different packings in the GC columns in comparison with the unreacted steroids.

TABLE I

GC RELATIVE RETENTION TIMES FOR ESTROGENS

Calculated as mm from injection peak to the peak of the curve.

Estrogen	*3½% OV-210*[a] *+ 1½% OV-1*	*5% OV-1*[a]
Estrone (E_1)	100 (12 min)	
2-OH-Estrone[b]	205	
Estradiol-17β (E_2)	80	
2-OH-estradiol-17β	158	
Estriol-16α,17β (E_3)	146	
2-OH-estriol-16α,17β	290	
Estriol-16β,17α	148	
E_1-acetate		100
2-OH-E_1-acetate		194
E_2-acetate		138
2-OH-E_2-acetate		272
E_3-acetate-16α,17β		234
2-OH-E_3-acetate-16α,17β		462
E_3-acetate-16β,17β		277
E_3-acetate-16α,17α		286
E_3-acetate-16β,17α		270

[a] For description of column packings see text.
[b] 2-OH = 2-hydroxy.

TABLE II

GC RELATIVE RETENTION TIMES OF ESTRIOL ACETONIDES

Free estriol = 100.

Estrogen acetonide	*3.5% OV-210 + 1.5% OV-1*	*5% OV-1*
E_3-16α,17β (N.A.)[a]	100	100
E_3-16β,17β	90	68
E_3-16α,17α	74	57
E_3-16β,17α (N.A.)	104	96

[a] N.A. = no acetonide was formed.

TABLE III

TLC R_F VALUES OF ESTROGENS

Estrogen	*System A, plain plate*	*System B, H_2SO_4 plate*[a]	*System A, PMA plate*[b]
E_1	0.72	0.56	0.61
2-OH-E_1	0.54	0.44	0.43
E_2	0.60	0.46	0.55
2-OH-E_2	0.44	0.40	0.37
E_3-16α,17β	0.35	0.09	0.21
2-OH-E_3-16α,17β	0.26	0.26	0.12
E_3-16α,17α	0.47	0.44	0.19
E_3-16β,17β	0.47	0.18	0.19
E_3-16β,17α	0.35	0.09	0.23

[a] Silica gel with ammonium bisulfate.
[b] Silica gel with phosphomolybdic acid.

Thin-layer chromatography

TLC methods have provided a wide variety of possibilities for separation of estrogens. None of the conventional systems[5,6] so far studied will separate the four epimeric estriols individually. The *cis* can be separated from the *trans* epimers in conventional systems. The separations with varying solvent and plate conditions are shown in Table III. Of the three different types of media available only the modified silica gel was capable of separating the epimeric estriols. The two *trans* epimers could be separated on plain silica gel from the two *cis* epimers. On the PMA modified silica gel the *cis* isomers traveled slower than the *trans* epimers. The two *cis* epimers showed a very wide separation in the silica gel to which NH_4HSO_4 had been added. The two *trans* epimers were not separated although they were separated from the *cis* epimers. The epimeric estriols also have mobilities close to that of 2-hydroxy-estrone and 2-hydroxy-estradiol-17β, in unmodified silica gel. As shown in Table III by modification of the silica gel separation of these compounds is more easily achieved.

TABLE IV

TLC R_F VALUES OF ESTRIOL ACETONIDES

Estrogen acetonide	*System C, plain plate*	*System A, H_2SO_4 plate*	*System C, PMA plate*
E_3-16α,17β (NA)[a]	0.06	0.10	0.05
E_3-16α,17α	0.41	0.41	0.38
E_3-16β,17β	0.41	0.24	0.38
E_3-16β,17α (NA)	0.06	0.10	0.05

[a] NA = no acetonide was formed.

The separation of the acetonides of the epimeric estriols followed the same pattern as the free epimers. Separation of the two *cis*-acetonides was achieved in the silica gel modified with NH_4HSO_4 as shown in Table IV. Table V indicates that estrogen acetates can be separated by TLC on silica gel. There is some overlapping of mobilities. Of particular interest is that after acetylation the two *cis* epimers no longer separated on the modified silica gel.

TABLE V

TLC R_F VALUES OF ESTROGEN ACETATES

Estrogen acetate	*System D, plain plate*	*System E, H_2SO_4 plate*	*System E, PMA plate*
E_1-Ac[a]	0.53	0.46	0.37
2-OH-E_1-diAc	0.27		
E_2-diAc	0.41	0.52	0.49
2-OH-E_2-triAc	0.38		
E_3-16α,17β-triAc	0.38	0.52	0.35
2-OH-E_3-tetraAc	0.28		
E_3-16β,17β-triAc	0.47	0.41	0.28
E_3-16α,17α-triAc	0.47	0.41	0.28
E_3-16β,17α-triAc	0.54	0.52	0.35

[a] Ac = acetate.

DISCUSSION

The literature lists many conditions and solvent systems for TLC separation of steroids. LISBOA[6] has compiled a list of estrogens and solvent systems which will separate them. Few, if any of the conventional systems will separate all of the estrogens in a single operation.

The use of modified adsorbents such as the silica gel containing bisulfate or phosphomolybdic acid may provide a means for more specific separations in situations such as existing in the problem of separation of the epimeric estriols. GC along with TLC provides a useful tool for separation and quantitation of many steroids. The separation of the estriol acetates was readily achieved.

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