Journal of Chromatography, 212 (1981) 239–244
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,882

Note

High-performance liquid chromatography of naturally occurring estrogens

JIANN-TSYH LIN* and ERICH HEFTMANN
Plant Physiology and Chemistry Research Unit, Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, CA 94710 (U.S.A.)
(Received April 13th, 1981)

High-performance liquid chromatography (HPLC) of steroids has recently been reviewed[1]. Systematic studies of the behavior of natural estrogens in HPLC which have appeared since then include two on equine estrogens[2,3], and one on catechol estrogens[4]. In connection with our studies on steroid metabolism in plants[5,6], we have also developed a chromatographic method for the separation of free naturally occurring estrogens. By combining both adsorption and reversed-phase partition HPLC, the identification of most of the metabolites of interest to us has become possible[6]. The present study deals with 24 estrogens.

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. The adsorption column was a 250 × 4.6 mm I.D. stainless-steel chromatography tube (Altex, Berkeley, CA, U.S.A.), packed with Zorbax BP-SIL (7–8 μm; DuPont, Wilmington, DE, U.S.A.). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (7–8 μm, DuPont). The columns were packed in our laboratory. The packing method, detector, recorder, solvents[7], pump and sample injection valve[8] were as previously described. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Our results are summarized in Table I. Being phenolic steroids, the estrogens were detected at 280 nm near their λ_{max}. The 24 estrogens in Table I were arranged in order of increasing polarity in adsorption chromatography and were divided into four groups: monools, diols, triols and tetraols. The number and locations of hydroxyl groups in estrogens play the most important role in their separation by HPLC. Addition of a keto group or double bond to an estrogen molecule does not increase

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

0021-9673/81/0000–0000/$02.50 © 1981 Elsevier Scientific Publishing Company

TABLE I

RETENTION TIMES OF ESTROGENS

Conditions: 1: see Fig. 1; 2: see Fig. 3; 3: see Fig. 4; 4: see Fig. 2.

Compounds	*Retention time (min)*			
	1	*2*	*3*	*4*
Monools				
17-Deoxoestrone		3.25	>75	
Estrone		9.5	32	
6-Dehydroestrone		9.5	28.5	
Equilin		9.5	28.5	
Equilenin		10.5	25.5	
Diols				
Estradiol-17α		16.25	25.5	
Estradiol-17β	4.75	17.75	23	
2-Hydroxyestrone		19.5*	16	
6-Dehydroestradiol		20.25	18.5	
17α-Dihydroequilin		22.5	18.5	
3,16α-Estradiol		22.5	21	
17α-Dihydroequlenin		24.25	16	
16-Ketoestradiol		27.5	6.75	22.5
16α-Hydroxyestrone		30.75	7.25	25.5
6-Ketoestradiol	6.75	32	7.25	25.5
Triols				
16-Epiestriol	8.25		9.25	
17-Epiestriol	8.25		13.5*	
2-Hydroxyestradiol	8.25*		13.5	
Estriol	11		4.25	13
6α-Hydroxyestradiol	11		4.25	10
16,17-Epiestriol	12.75		4.25	13
6-Ketoestriol	16.75 (15)**		2.5	6.25
Tetraols				
2-Hydroxyestriol	19.25*		3.5	7.75
6α-Hydroxyestriol	28		2.5	4

* Broad peaks.

** Sample also gave a minor peak at 15 min.

the polarity in adsorption chromatography as much as in reversed-phase partition chromatography. On the other hand, epimeric estrogens are generally better resolved in adsorption than in reversed-phase systems. The fact that the elution sequence of estrogens in reversed-phase partition chromatography is not exactly the reverse of that in adsorption chromatography makes the two HPLC systems complement each other even more.

For adsorption chromatography of the more polar estrogens, *n*-hexane–ethanol (9:1) was used as the eluent (Fig. 1). This system resolved three of the four epimeric estriols: 16-epiestriol, estriol and 16,17-epiestriol. However, it did not separate 16-epiestriol from 17-epiestriol. This pair was resolved by reversed-phase partition chromatography with acetonitrile–water (35:65) as eluent (Table I), but the

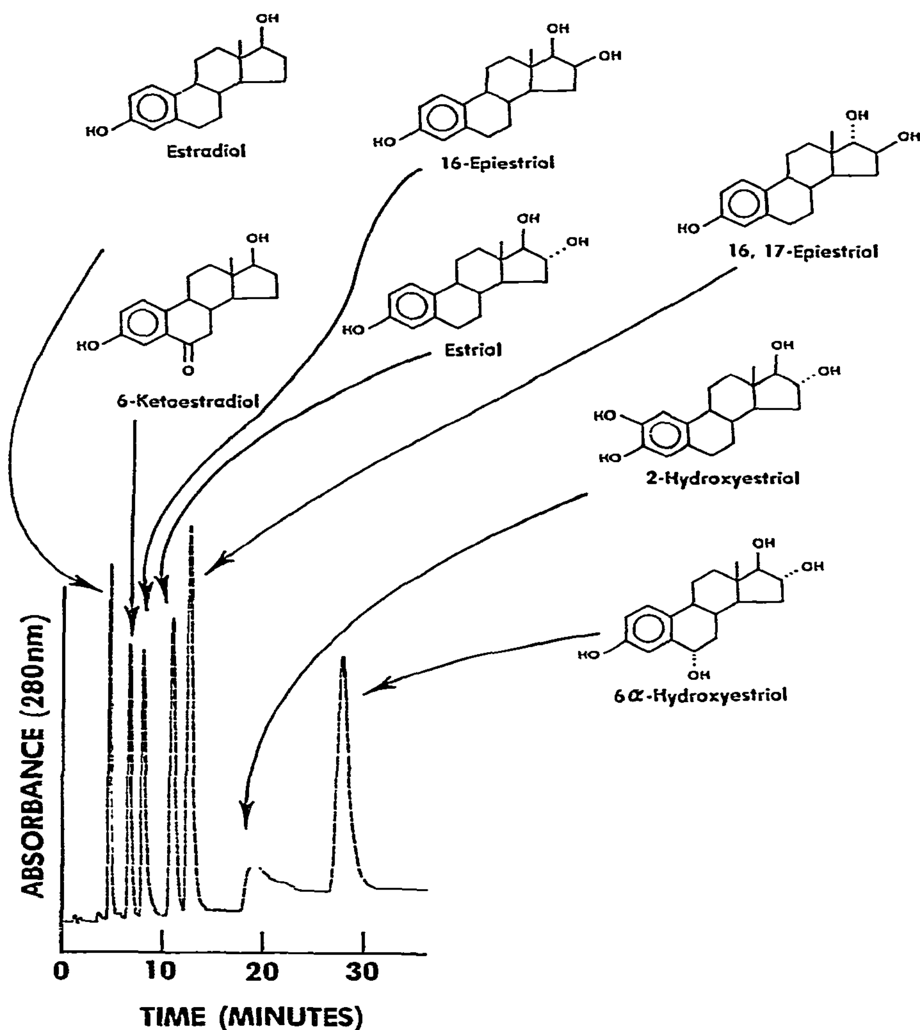

Fig. 1. Adsorption chromatogram of the more polar estrogens. Between 1.5 μg (estradiol) and 10 μg (6α-hydroxyestriol) of estrogens, dissolved in about 50 μl of the eluent, were chromatographed on a column of Zorbax BP-SIL, 250 × 4.6 mm I.D. Eluent, *n*-hexane–ethanol (9:1); flow-rate, 2 ml/min; pressure, 200 p.s.i. Detector at 280 nm; range, 0.05; time constant, 1.0. Recorder speed, 12 cm/h; span, 10 mV.

other epimers could not be separated this way. On the other hand, the position isomers, estriol and 6α-hydroxyestradiol, which could not be separated by adsorption chromatography, were resolved by reversed-phase partition chromatography (Fig. 2). 16-Epiestriol, 17-epiestriol and 2-hydroxyestriol are less polar than the other triols in Table I. Apparently, vicinal hydroxyl groups can form hydrogen bonds when they have the same orientation, and this makes the molecule less polar.

For adsorption chromatography of the less polar estrogens *n*-hexane–ethanol (97:3) was used. This eluent separated monools and diols very well (Fig. 3). However, this system was incapable of separating estrone from 6-dehydroestrone and 17α-

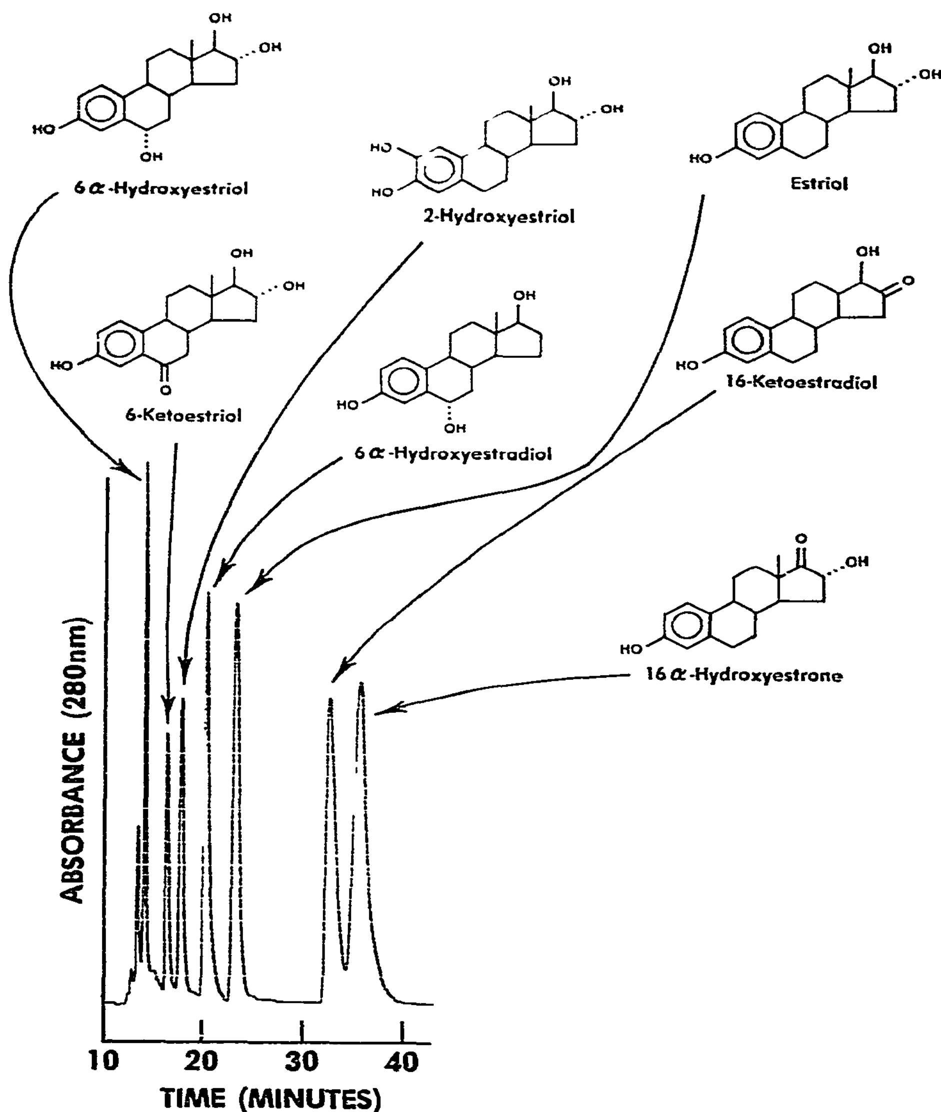

Fig. 2. Reversed-phase partition chromatogram of the more polar estrogens. Between 1 μg (6α-hydroxyestriol) and 5 μg (16α-hydroxyestrone) of estrogens, dissolved in about 50 μl of the eluent, were chromatographed on a column of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, acetonitrile–water (25:75); pressure, 700 p.s.i. Other conditions as in Fig. 1.

dihydroequilin from 3,16α-estradiol. These two pairs of estrogens could be separated by reversed-phase partition chromatography with acetonitrile–water (35:65) as the eluent (Table I). On the other hand, this reversed-phase partition system did not separate the following three pairs of estrogens: equilenin from estradiol-17α, 6-dehydroestradiol from 17α-dihydroequilin and 16α-hydroxyestrone from 6-ketoestradiol, whereas these three pairs could be resolved by the adsorption system (Fig. 3).

Generally, reversed-phase partition chromatography was superior to adsorp-

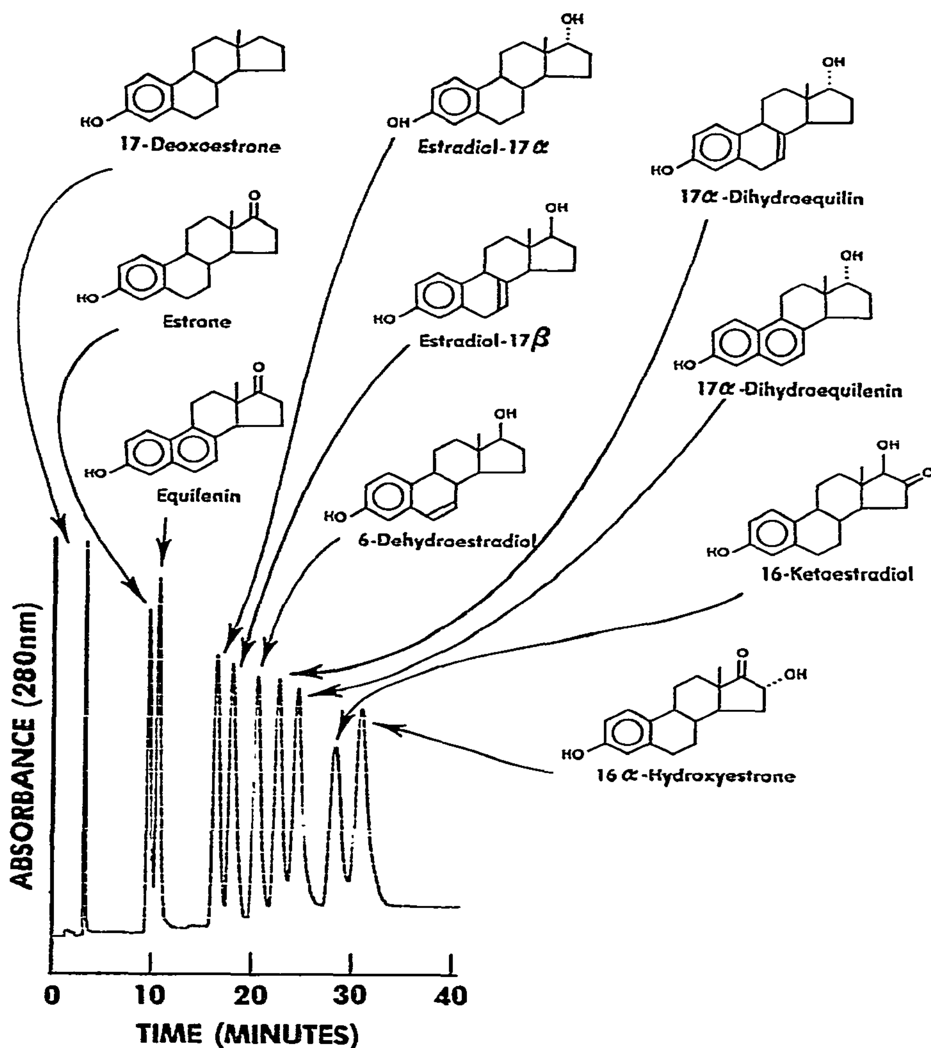

Fig. 3. Adsorption chromatogram of the less polar estrogens. Conditions as in Fig. 1, except between 1 μg (17-deoxoestrone) and 10 μg (16α-hydroxyestrone) of estrogens and an eluent of *n*-hexane–ethanol (97:3) were used.

tion chromatography in the separation of estrogens differing from each other by a double bond, *e.g.*, estrone and equilin (or 6-dehydroestrone) (Fig. 4). As for the relative polarity of the hydroxyl groups at C-16 and C-17, previously published generalizations[9] seem to hold. Estradiol-17β was found to be more polar than estradiol-17α and 3,16α-estradiol was more polar than estradiol-17β. Since 16,17-epiestriol is more polar than estriol, we predict that 3,16β-estradiol will be found to be more polar than 3,16α-estradiol. Thus, the polarity of hydroxyl groups at C-16 and C-17 increases in the order: $17\alpha < 17\beta < 16\alpha < 16\beta$. 6-Ketoestradiol is more polar than 16-ketoestradiol in adsorption chromatography, but less polar in reversed-phase partition chromatography.

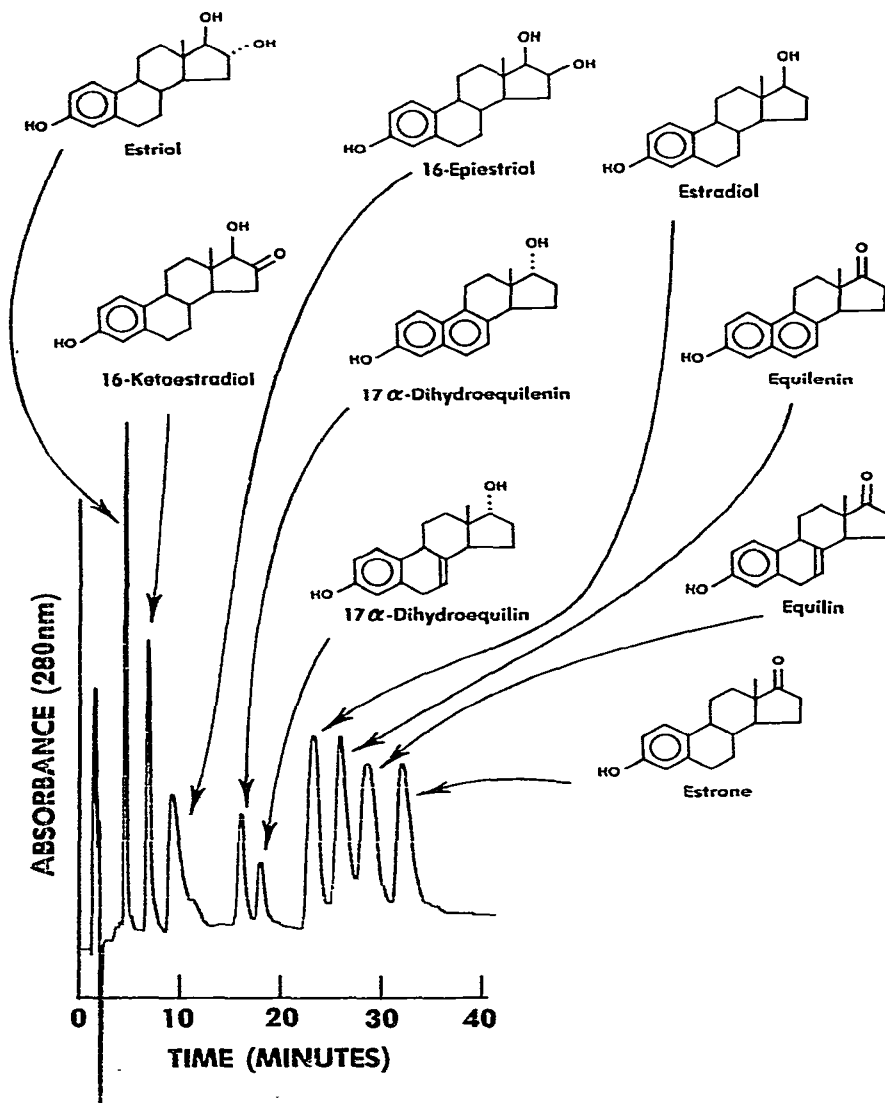

Fig. 4. Reversed-phase partition chromatogram of the less polar estrogens. Between 1 μg (estriol) and 3 μg (estrone) of estrogens, dissolved in about 50 μl of methanol, were chromatographed on a column of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, acetonitrile–water (35:65); pressure, 700 p.s.i. Other conditions as in Fig. 1.

REFERENCES

1 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
2 G. Capitano and R. Tscherne, *J. Pharm. Sci.*, 68 (1979) 311.
3 R. W. Roos, *J. Ass. Offic. Anal. Chem.*, 63 (1980) 80.
4 K. Shimada, T. Tanaka and T. Nambara, *J. Chromatogr.*, 178 (1979) 350.
5 J.-T. Lin, W. M. Proebsting and E. Heftmann, *Phytochemistry*, 18 (1979) 1667.
6 J.-T. Lin and E. Heftmann, *Phytochemistry*, in press.
7 J.-T. Lin, E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 190 (1980) 169.
8 J.-T. Lin, W. D. Nes and E. Heftmann, *J. Chromatogr.*, 207 (1981) 457.
9 E. Heftmann, *J. Liquid Chromatogr.*, 2 (1979) 1137.