CHROM. 5509

Thin-layer separation and detection of free estrogens

A variety of chromatographic techniques have been applied to analysis of estrogens. It has already been shown that estrogens which differ only by one double bond can be separated and quantitated by gas[1] and liquid chromatography[2]. The availability of uniform pre-coated thin-layer plates and versatile spectrodensitometers has also led to several publications of quantitative *in situ* thin-layer methods for some estrogen compounds[3–5]. The recently reported[4,5] thin-layer separation and quantitation of estrone, estradiol and estriol azobenzene-4-sulfonate and 1-dimethylaminonaphthyl-5-sulfonyl chloride derivatives is quite sensitive and precise; however, derivatization and extraction of estrogens prior to chromatography is rather time-consuming. Furthermore, since derivatization tends to obscure minor differences between the structures of closely related estrogens (*e.g.* estrone and equilin), separation of derivatized estrogens yields intrinsically lower selectivity. The above limitation is revealed by the observed relative R_F values of derivatized estrone and estradiol[4,5]; the degree of this separation is insufficient to quantitatively delineate a more complex mixture of estrogens containing estrone, equilin, equilenin and estradiol.

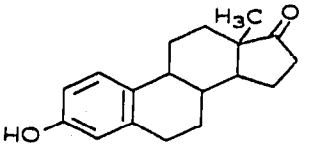
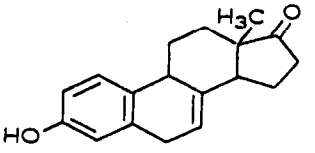
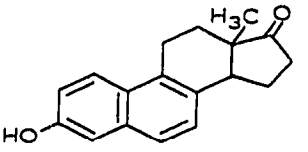
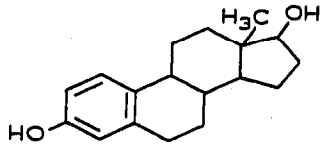
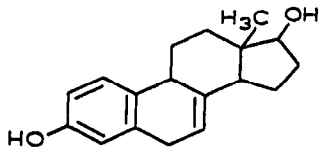
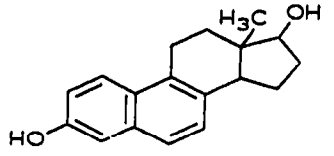
A mixture of the above estrogens in an underivatized state has been partially separated on a silica gel plate by a combination of two amine (containing triethylamine and/or collidine component) solvent systems which yielded different overlapping pairs of estrogens[6]. Thus, complete analysis of the mixture involved either two independent thin-layer chromatography (TLC) plates or one two-dimensional development. Since our quantitation is based on direct comparison of an unknown estrogen mixture with a known standard mixture, both applied to the same plate, it was desirable to develop a solvent system which would separate the entire mixture in one uni-dimensional sample channel.

This communication describes a continuous and dual elution TLC system each part of which separates a mixture of six naturally occurring estrogens[7,8]: estrone, equilin, equilenin, 17α-estradiol, 17α-dihydroequilin and 17α-dihydroequilenin (Table I). Several independent means of detection and quantitation were also investigated. A comparative study of the relative magnitude of the *in situ* densitometric response is presented. Since the chromatographic system is uni-dimensional, several unknown and reference samples may be chromatographed on one plate.

Experimental

Apparatus and materials. A dual-beam spectrophotometer (Model SD-3000), with a high-pressure mercury xenon arc source lamp, reflectance attachment, Model SDC-300 density computer and a 100 mV Honeywell recorder with a disc integrator was obtained from Schoeffel Instruments Corp. (Westwood, N.J., U.S.A.). Pre-coated silica gel (Q1) plates were supplied by Quantum Industries (Fairfield, N.J., U.S.A.). A rectangular ($8\frac{1}{2} \times 4\frac{1}{2} \times 8\frac{1}{2}$ in.) chromatographic tank which was obtained from Brinkmann Instruments Inc. (Westbury, N.Y., U.S.A.) was used for multiple elution chromatography. The continuous elution TLC apparatus (SAB-2852) was obtained from Shandon Scientific Company, Inc. (Sewickley, Pa, U.S.A.). All estrogen compounds were obtained from Ayerst Research Laboratories (Montreal, P.Q., Canada). All chemicals were reagent grade.

TABLE I

Trivial name	*Chemical name*	*Formula*
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one	
Equilin	3-Hydroxyestra-1,3,5(10),7-tetraen-17-one	
Equilenin	3-Hydroxyestra-1,3,5(10),6,8-pentaen-17-one	
Estradiol (α)	Estra-1,3,5(10)-triene-3,17(α)-diol	
Dihydroequilin (α)	Estra-1,3,5(10),7-tetraen-3,17(α)-diol	
Dihydroequilenin (α)	Estra-1,3,5(10),6,8-pentaen-3,17(α)-diol	

Methods

Sample application. The chromatographic plates were scored to yield 1 cm wide channels (Schoeffel Model SDA320 scorer was used for this purpose) and activated for 30 min in a 120° oven. A 1–0.1 mg/ml benzene–methanol (9:1) solution of a given estrogen was spotted under a stream of dry nitrogen on alternate channels of the plate.

Chromatography. Continuous elution was carried out at 4° in a refrigerator. The developing tray contained cyclohexane–chloroform–triethylamine–collidine (670:280:17:46) solvent while the bottom of the tank was covered by cyclohexane–chloroform–collidine (67:28:10) solvent. To reduce the humidity, a beaker containing silica gel desiccant was placed within the tank. The chromatographic plate was developed for 4–4½ h.

Multiple elution was carried out at room temperature in a saturated tank which also contained a silica gel desiccant in a 50-ml beaker. The plate was first developed in cyclohexane–chloroform–triethylamine–collidine (66:29:2:3) solvent. In

each development, the solvent front was allowed to reach the top of the plate. The plate was dried in air 10–15 min after the first development. The dual-development required approximately 4½ h.

Detection. The chromatographed plates were dried for 1 h at room temperature, then scanned at 280 mμ with the reflectance attachment. Subsequently, some plates were sprayed with 9 *N* sulfuric acid solution then heated for 7 min in a 125° oven and scanned within 24 h. To stabilize the colors, the sprayed plate was covered with another glass plate during storage. The remaining plates were sprayed with the *p*-nitrobenzenediazonium fluoroborate–acetic acid solution (500 mg of the diazonium compound in 100 ml of glacial acetic acid–water, 1:1) and scanned at any time, 1 h after spraying. The sulfuric acid-sprayed plates were scanned with the reflectance attachment set at 360 and 550 mμ and by transmittance at 550 mμ. It was not possible to scan by transmittance at 360 mμ. The *p*-nitrobenzenediazonium fluoroborate-sprayed plates were scanned by reflectance and transmittance at 435 mμ.

Results and discussion

Fig. 1 illustrates the chromatograms of the six estrogens separated by continuous development of the plate and scanned by reflectance at 280 mμ. The plate was then sprayed with sulfuric acid and scanned by reflectance at 360 and 550 mμ and transmittance at 550 mμ. Each scan corresponds to the same sample channel scanned

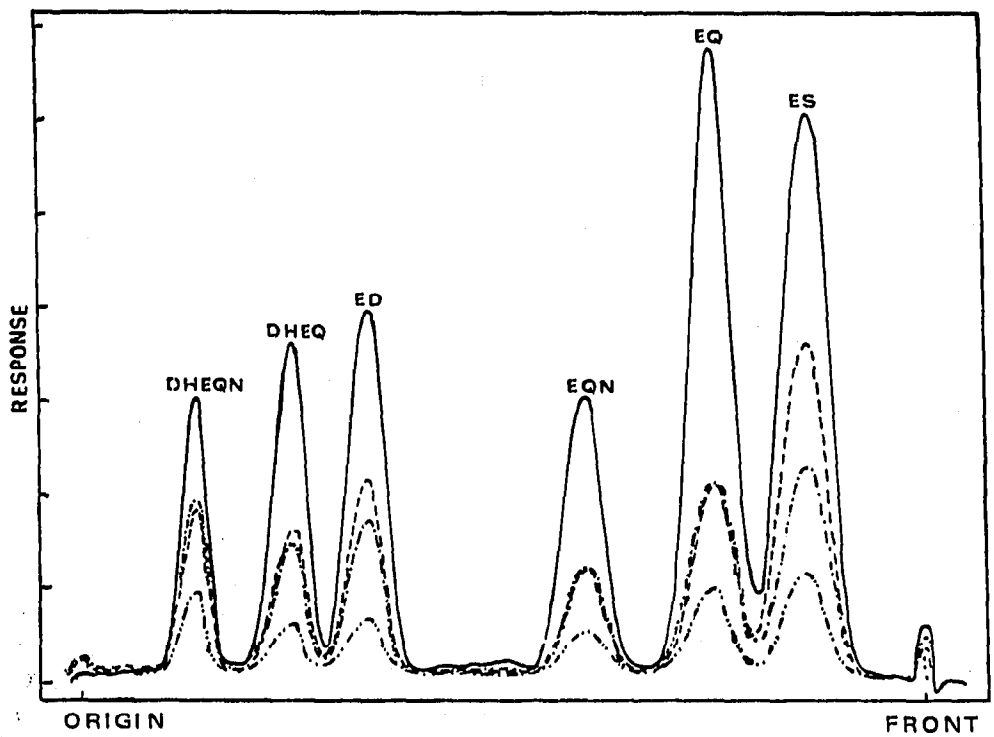


Fig. 1. Separation of estrogens by continuous development. Sample: 10.0 μg of estrone (ES), 8.0 μg of equilin (EQ), 2.0 μg of equilenin (EQN), 3.5 μg of 17α-estradiol (ED) [illegible] μg of 17α-dihydroequilin (DHEQ) and 2.0 μg of 17α-dihydroequilenin (DHEQN). Detection density range 0.4; (–·–·–) reflectance at 280 mμ, (–·–·–) reflectance at 360 mμ after sulfuric acid spray, (– – – –) reflectance at 550 mμ after sulfuric acid spray, (————) transmittance at 550 mμ after sulfuric acid spray.

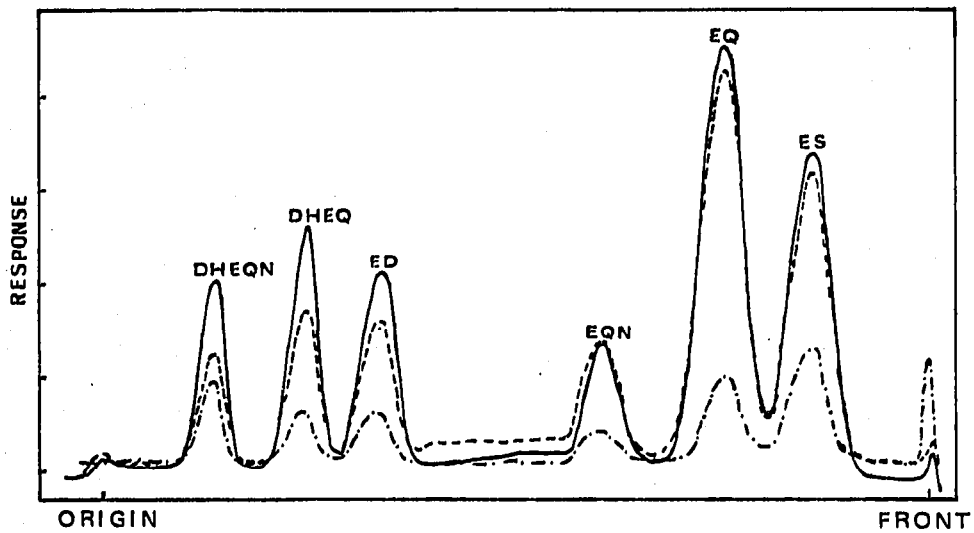


Fig. 2. Separation of estrogens by continuous development. Sample: as specified under Fig. 1. Detection: density range 0.4; (– · – · – · –) reflectance at 280 mμ, (– – – –) transmittance at 435 mμ after *p*-nitrobenzenediazonium fluoroborate spray, (————) reflectance at 435 mμ after *p*-nitrobenzenediazonium fluoroborate spray.

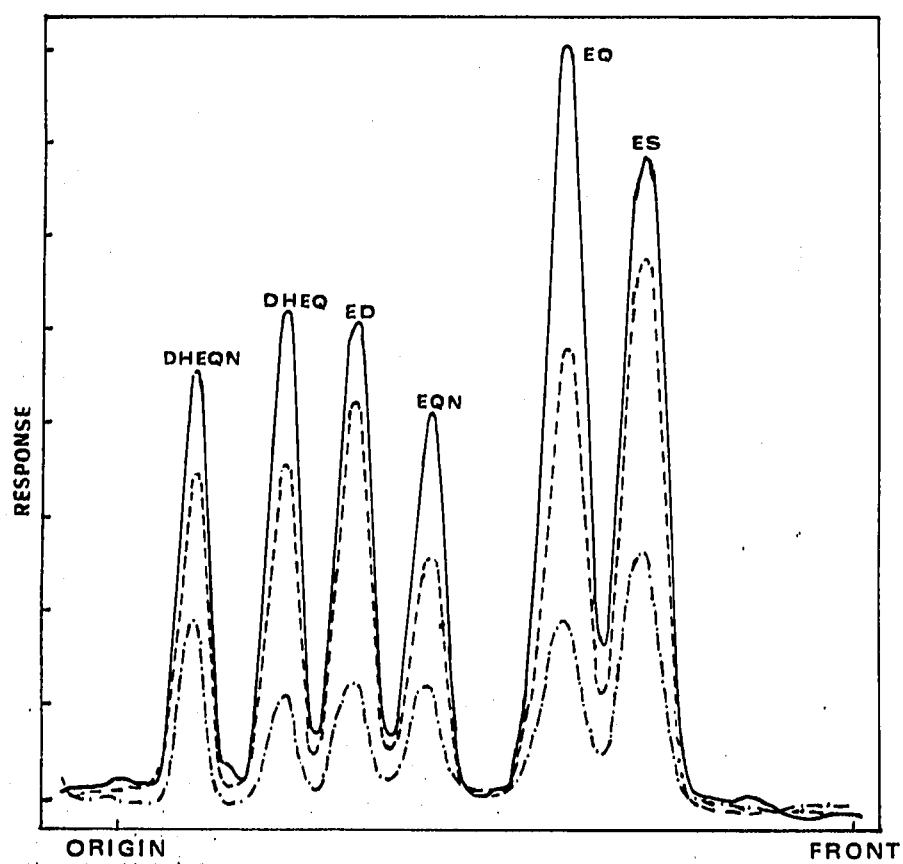


Fig. 3. Separation of estrogens by multiple development. Sample: as specified under Fig. 1. Detection: density range 0.4; (– · – · – · –) reflectance at 280 mμ, (– – – –) reflectance at 550 mμ after sulfuric acid spray, (————) transmittance at 550 mμ after sulfuric acid spray.

before or after sulfuric acid treatment. Fig. 2 illustrates another sample channel also chromatographed by continuous development and scanned by reflectance at 280 mμ, but subsequently sprayed with *p*-nitrobenzenediazonium fluoroborate and scanned by reflectance and transmittance at 435 mμ. Fig. 3 illustrates the chromatograms of the six estrogens separated by multiple development of the plate and scanned as described above under Fig. 1. Since equal volumes of the standard solution of mixed estrogens were spotted on each channel and scanned at the optical density range (0.4) of the spectrodensitometer, the sensitivity range of each scan can be expanded by a factor of 4.

The results observed lead to the following conclusions: (1) Sulfuric acid treatment followed by transmittance scan at 550 mμ yielded the most sensitive response. By expanding the scale one could quantitate less than 1 μg of estrone and less than 0.1 μg of equilenin. In contrast, the reflectance scan at 550 mμ is approximately half as sensitive. The advantage of transmittance over reflectance scanning has already been shown by application of the KUBELKA–MUNK theory[9,10]. It has also been illustrated by empirical application of spectrodensitometry to transmittance and reflectance scanning[11,12]. Our results with the sulfuric acid-treated plates conform to the previously observed results.

(2) However, comparison of the transmittance with the reflectance mode of detection of the *p*-nitrobenzenediazonium fluoroborate-sprayed plates does not reflect the same relationship as that observed with the sulfuric acid-treated plates. One possible explanation of this observation is based on the fact that the fluoroborate spray does not penetrate the silica gel layer as well as the sulfuric acid spray and thus only the upper surface layers of the plate are derivatized. Since the upper surface layers of the plate may contribute relatively more to the observed reflectance signal than to the observed transmittance signal, incomplete derivatization of the lower layers would tend to diminish the transmittance signal more than the reflectance signal. In this connection, it is of interest to note that chromatography and densitometry of derivatized steroids[12] did not lead to the discrepancy which we observed with the fluoroborate spray; in this case transmittance yielded greater response than reflectance. This discrepancy will be investigated further.

(3) Comparison of Fig. 1 with Fig. 3 indicates that the continuous development yields significantly higher separation efficiency with no increase of development time. Furthermore, continuous development requires less analyst attention and plate manipulation and thus is less prone to reproducibility complications.

The selection of continuous and multiple mode of plate development was made on the basis of preliminary results observed with single elution development. Preliminary results indicated that considerable improvement in the degree of separation was obtained when the single elution was replaced by either continuous or multiple elution development.

As specified under *Methods*, all separations presented in this communication were obtained with Quantum (Q1) plates; other commercial TLC plates did not yield equivalent results. Neither was it possible to obtain an equivalent separation on TLC plates which were prepared in this laboratory with E. Merck Silica Gel G.

The quantitative aspect of the above procedures is being examined by statistical analysis. A detailed presentation of the quantitative data will be published at a later date.

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