Identification and Determination of Synthetic Estrogens in Pharmaceuticals by High-Speed, Reversed-Phase Partition Chromatography

ROBERT W. ROOS

Abstract □ A high-speed, reversed-phase partition chromatographic method is described for the identification and quantitative determination of synthetic estrogens in pharmaceutical dosage forms. The method is applicable to diethylstilbestrol, dienestrol, hexestrol, benzestrol, chlorotrianisene, and the dipropionic acid esters of diethylstilbestrol and promethestrol. Prior to UV spectrophotometric detection at 254 nm, the synthetic estrogens are separated on either a chemically bonded hydrocarbon column or an ether partition column, utilizing 2-propanol–water mobile phase systems. Limits of detectability are at the nanogram level for diethylstilbestrol and dienestrol, the synthetic estrogens most commonly found in commercial preparations. Chromatographic studies were conducted after a number of the synthetic estrogens underwent photochemical change, and the synthetic estrogen 4,4′-stilbenediol esters underwent controlled ester hydrolysis. The chromatograms of the photochemical and hydrolysis degradation products were found to be useful for identification. For example, a scheme developed for the identification of diethylstilbestrol provides for the separation of three photochemical by-products: the *cis*-isomer of diethylstilbestrol, the phenanthrenedione (3,4,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene), and the phenanthrenediol (9,10-diethyl-3,6-phenanthrenediol). Application of the method to the analysis of synthetic estrogens in commercial preparations is described.

Keyphrases □ High-speed, reversed-phase partition chromatography—identification and determination of synthetic estrogens in pharmaceuticals □ Estrogens, synthetic—identification and determination in pharmaceuticals by high-speed, reversed-phase partition chromatography

Recent publications have shown the application of high-speed (pressure) liquid chromatography to the separation and/or quantitative determination of analgesics (1, 2), sulfonamides (3, 4), barbiturates (5, 6), steroids (7–9), benzodiazepines (10, 11), monosubstituted pyridine isomers (12), purine and strychnos alkaloids (13), and vitamins (14, 15). The technique appears most promising in providing for the separation of highly polar, nonvolatile, and thermally labile compounds. To extend the usefulness of this analytical method, this paper describes its potential application to the qualitative and quantitative determination of the synthetic estrogen family of drugs.

Various methods have been proposed for the quantitative determination of synthetic estrogens present in pharmaceutical dosage forms. Gottlieb (16), introducing a spectrophotometric method for the synthetic estrogenic 4,4′-stilbenediols—*viz.*, diethylstilbestrol, dienestrol, hexestrol, benzestrol, and promethestrol, based on the formation of their corresponding *o*-nitrosophenols, gave an excellent review of a number of conventional methods together with a discussion of their various shortcomings. The official compendia (17, 18) provide assays for the quantitative determination of five individual synthetic estrogens. These methods, although applicable to the products for which they are intended, are, in general, tedious or nonspecific and most often are not applicable when an unrelated drug, such as a sulfonamide, steroid, or barbiturate, is present in combination.

GLC has also been applied to the identification and quantitative determination of the synthetic estrogens. For example, Fricke *et al.* (19) described a method for the synthetic estrogen dienestrol when in combination with methyltestosterone; the method is based on the formation of a bis(trimethylsilyl) ether derivative. Other papers have also reported methods that use GLC, and most of these methods require the formation of derivatives.

The objectives of this paper are: *(a)* to show that the synthetic estrogens are amenable to separation, identification, and quantitative determination in pharmaceutical dosage forms, utilizing high-speed, reversed-phase partition chromatography; and *(b)* to indicate the potential use of photolysis and ester hydrolysis in synthetic estrogen identification studies.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a UV absorption photometer, set at 254 nm, was utilized throughout this study. The instrument provides for: *(a)* a maximum absorbance unit full-scale (aufs) setting of 0.5×10^{-2}, and *(b)* mobile phase degassing by vacuum. The UV (254 nm) irradiation unit applied in qualitative studies is that described in USP XVIII (17) for the quantitative determination of diethylstilbestrol.

Columns and Mobile Phases—Two chemically bonded, reversed-phase partition columns applied in achieving synthetic estrogen separations are commercially available from the instrument manufacturer and are 1-m × 2.1-mm i.d. × 0.6-cm (0.25-in.) o.d., precision bore stainless steel tubes, containing the following stationary phases: *(a)* Column 1, an octadecylsilane (hydrocarbon) chemically bonded to the surface of a controlled porous surface[2]; and *(b)* Column 2, an ether chemically bonded to the surface of a controlled porous surface[3]. Column 2 was the most utilized and was applied in quantitative analysis. The mobile phases used were simple combinations of 2-propanol[4] and water. Prior to use, the mobile phases were degassed by vacuum.

Solvents and Standards—During chromatographic elution behavior studies, methanol was used as the solvent for the synthetic estrogens. The synthetic estrogen 4,4′-stilbenediols were readily soluble in methanol; however, heating was required to achieve solution of the 4,4′-stilbenediol esters and chlorotrianisene. For quantitative studies, three solvent systems were utilized to both dissolve and extract the synthetic estrogens from pharmaceutical dosage forms; these were: *(a)* methanol, *(b)* methanol–ether (1:1), and *(c)* dimethylformamide. The synthetic estrogen standards were of laboratory working grade, with previous assay results

[1] Du Pont model 820, equipped with a model 410 UV detector, E. I. du Pont de Nemours and Co., Wilmington, Del.
[2] ODS-Permaphase, Du Pont.
[3] ETH-Permaphase, Du Pont.
[4] Fisher Spectranalyzed (A-419).

Table I—Summarized Sample Preparation

Sample Preparation	Sample Size[a], mg	Solvent	Vessel Size, ml	Vessel Type[b]	Amount of Solvent	Heating Required	Internal Standard	Aliquot, ml	Final Vessel Size, ml	Solvent
Diethylstilbestrol and dienestrol										
Tablets	2.5	Methanol	50	V.F.	*q.s.*	No	Phenothiazine[c,d]	—	—	—
Creams	1	Methanol + ether (1:1)	100	g.s.f.	50	Yes	Phenothiazine[c,d]	—	—	—
Injection[e]	10	Dimethylformamide + methanol (1:1)	100	V.F.	50 ml	No	Phenothiazine[c,d]	—	—	—
Suppositories	0.5	Methanol + ether (1:1)	50	g.s.f.	20 ml	Yes	Phenothiazine[c,d]	—	—	—
Chlorotrianisene soft capsules	100	Dimethylformamide	100	V.F.	*q.s.*	No	Phenothiazine[c,d]	10	50	Methanol
Promethestrol dipropionate tablets	10	Dimethylformamide + methanol (1:1)	50	V.F.	20 ml then *q.s.*	No	Chlorotrianisene[d,f]	—	—	—
Hexestrol tablets	25	Methanol	50	V.F.	*q.s.*	No	Benzestrol[g]	—	—	—
Benzestrol tablets	25	Methanol	50	V.F.	*q.s.*	No	Hexestrol[g]	—	—	—

[a] Representative sample accurately weighed to contain this much active ingradient. [b] V.F. = volumetric flask; g.s.f. = glass-stoppered flask. [c] Using 0.5 mg/ml in methanol, add an aliquot to contain an amount similar to amount of active ingredient in sample. [d] In preparation of standard solution, some heating may be necessary to aid dissolution. [e] Cottonseed oil formulation. [f] Add one-tenth the amount of active ingredient using 0.5 mg/ml in methanol. [g] Add internal standard directly to volumetric flask to provide a final concentration similar to that of the sample.

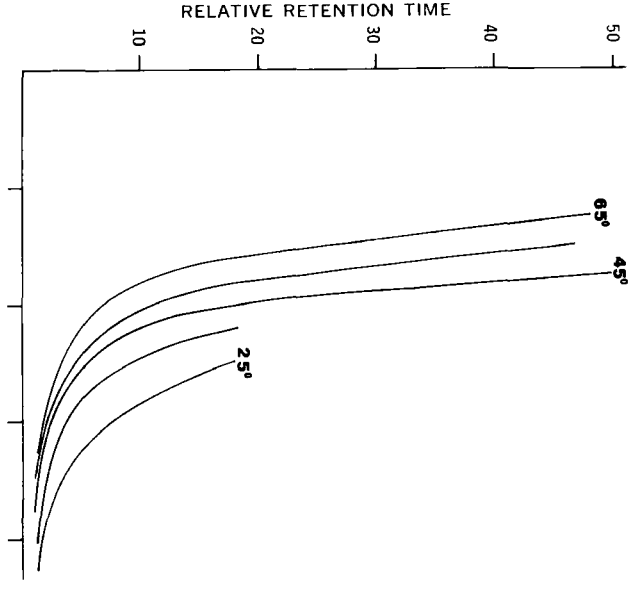

Figure 1—*Effects of mobile phase variation on the elution behavior, expressed in terms of relative retention times, of trans-diethylstilbestrol. Chromatography conditions were: Column 2; pressure at inlet, 1000 psig; flow rate, variable; column temperature, 25, 35, 45, 55, and 65°, respectively; and UV detector sensitivity, 254 nm, 0.16 aufs.*

meeting regulatory standards. Promethestrol was not available and was formed by heating promethestrol dipropionate in methanol–potassium hydroxide solution, a procedure taken from Gottlieb (16).

Qualitative Analysis—*Irradiation Studies*—Methanol solutions of *trans*-diethylstilbestrol (0.2 mg/ml), chlorotrianisene (0.2 mg/ml), diethylstilbestrol dipropionate (0.2 mg/ml), and promethestrol dipropionate (0.5 mg/ml) were irradiated with 254-nm radiation for 30–60 min. This procedure was repeated for *trans*-diethylstilbestrol using equal volumes of (*a*) methanol–1.8% aqueous dipotassium hydrogen phosphate, and (*b*) methanol–acetic acid. Further studies of *trans*-diethylstilbestrol after initial UV radiation involved the direct conversion of derived phenanthrenedione to phenanthrenediol in sodium bisulfite–hydrochloric acid solution.

Hydrolysis Studies—Hydrolysis of the 4,4′-stilbenediol esters, *e.g.*, diethylstilbestrol dipropionate and promethestrol dipropionate, was carried out by adding an equal volume of 1.8% aqueous dipotassium hydrogen phosphate solution to the methanol solution of the ester.

Quantitative Analysis—Methods of analysis were developed for six synthetic estrogens included in this study and directly applied to their quantitative determination in commercial preparations (Table I). The methods are based on extraction and solubilization of the synthetic estrogens and the use of internal standards.

Chromatographic Conditions—The following were used: Column 2; mobile phase, 25% 2-propanol in water (v/v); column temperature, 60°; pressure at inlet, 1000 psig; and flow rate, 0.55 ml/min.

Sample Preparation—The various products and dosage forms were prepared as outlined in Table I.

Standard Solution—A standard solution, containing internal standard and sample synthetic estrogen in the same quantities chosen for the sample solution, was prepared concurrently.

Chromatography—Make preliminary injections of the standard solution to determine the UV detector setting (in terms of absorbance unit full-scale) and sample volume that provide an acceptable full-scale response. When the instrumental settings and sample volume (usually 4–10 μl) have been satisfactorily determined, inject a replicate number of sample volumes for quantitative analysis. Calculate the peak areas of both the internal stan-

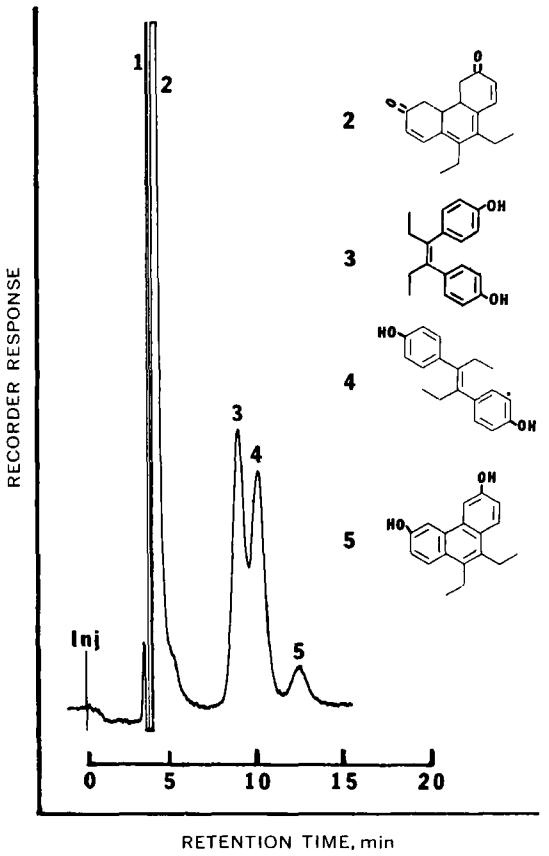

Figure 2—*Chromatogram of the photochemical by-products of* trans-*diethylstilbestrol. Key: peak 1, methanol solvent; peak 2, the phenanthrenedione (3,4,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene); peak 3,* cis-*diethylstilbestrol; peak 4,* trans-*diethylstilbestrol; and peak 5, the phenanthrenediol (9,10-diethyl-3,6-phenanthrenediol). Chromatography conditions were: Column 2; mobile phase, 20% 2-propanol in water; pressure at inlet and column temperature, 400 psig and 60°, respectively; and UV detector sensitivity, 254 nm, 0.04 aufs.*

dard and sample synthetic estrogen. In this work, the areas *(A)* were computed using the relationship $A = \frac{1}{2}bh$, where b = peak base, and h = peak height. Area was found proportional to concentration within ±50% of the quantity of synthetic estrogen chosen for analysis.

Calculations—The total quantity of sample synthetic estrogen taken for analysis (final volumetric or glass-stoppered flask) may be calculated using Eq. 1:

$$W_1 = \frac{A_1 A_3 W_2 W_4}{A_2 A_4 W_3} \qquad \text{(Eq. 1)}$$

where A represents peak area; W represents weight (milligrams); and subscripts 1, 2, 3, and 4 represent the sample synthetic estrogen, the standard synthetic estrogen, the internal standard in the standard solution, and the internal standard in the sample solution, respectively.

Calculation of the quantity of synthetic estrogen per dosage unit can be achieved by introduction of appropriate factors into Eq. 1.

RESULTS AND DISCUSSION

Column Characteristics and Elution Behavior—The reversed-phase partition chromatographic elution behavior on two chemically bonded columns, expressed in terms of relative retention times (methanol as 1.00) of 10 synthetic estrogens, is shown in Table II.

Both columns were utilized previously for the separation of compounds of interest in pharmaceutical analysis. For example, Column 1 was found useful for the separation of several steroids (8), and Column 2 was useful for the separation of barbiturates (6). The value of chemically bonded stationary phases in comparison with stationary fluids that are retained by physical interaction was discussed by Kirkland (20). The most apparent advantages seem to be the elimination of precolumns and presaturated mobile phases. The mobile phases selected to achieve satisfactory migrations of the synthetic estrogens are simple combinations of 2-propanol and water.

Table II—Chromatographic Behavior of Synthetic Estrogens

Synthetic Estrogens	Relative Retention Times[a]		
	Column 1[b]		Column 2[c]
	15%[d]	30%[d]	20%[d]
Methallenestril	2.53	—[e]	1.14
Dienestrol	3.03	—[e]	5.10
Diethylstilbestrol[f]	3.62	—[e]	5.42
Hexestrol	3.78	—[e]	5.19
Benzestrol	9.20	—[e]	7.28
Promethestrol	14.3	—[e]	9.10
Dienestrol diacetate	20.2	1.70	5.32
Chlorotrianisene	—[g]	4.30	20.8
Diethylstilbestrol dipropionate	—[g]	5.22	13.8
Promethestrol dipropionate	—[g]	6.64	16.7
Methanol emergence, min	1.34	1.85	1.73
Pressure at inlet, psig	1000	1000	1000
Column temperature	50°	50°	70°

[a] Relative retention times are expressed relative to the solvent methanol as 1.00. [b] Chemically bonded hydrocarbon column (ODS-Permaphase, Du Pont). [c] Chemically bonded ether column (ETH-Permaphase, Du Pont). [d] Mobile phase: percentage of 2-propanol in water (v/v). [e] Less than 1.20. [f] Refers to *trans*-diethylstilbestrol. [g] Greater than 25.0.

The retention data (Table II) indicate that both partition columns provide for synthetic estrogen separations, with Column 1 permitting the greater degree of separability. However, peak tailing on Column 1 greatly limits its potential use, particularly in quantitative analysis. Comparison of the data between columns shows some elution order variations. For example, chlorotrianisene is shown to elute before the dipropionic acid esters of diethylstilbestrol and promethestrol on Column 1, whereas it elutes later on Column 2. Another important observation is the similarity in elution behavior of dienestrol and its diacetic acid ester, *e.g.*, dienestrol diacetate, on Column 2 in contrast to the wide difference in elution behavior on Column 1.

When utilizing Column 2, the column selected for most subsequent studies and applied in quantitative analysis, a further investigation was undertaken to determine the effect of 2-propanol content variation in the mobile phase on elution behavior. Figure 1 shows the relative retention times (relative to methanol as 1.00) of *trans*-diethylstilbestrol *versus* 2-propanol concentration in the mobile phase. The study was conducted at five different column temperatures, varying in 10° intervals, and relative retention times were calculated for successive 2.5% increments of increasing 2-propanol concentration. Although other synthetic estrogens are not shown, studies have indicated a similar response. The series of curves shown in Fig. 1 indicates that the analyst can rapidly estimate a mobile phase composition and a column temperature to provide a desired relative retention time.

Although the mobile phase may be varied, some considerations must be borne in mind relating to sensitivity. For example, increases in the 2-propanol concentration of the aqueous mobile phase result in a higher mobile phase viscosity and, therefore, in a loss of column efficiency. This loss in efficiency leads to a greater peak band width and a loss in peak height response. In addition, lowering of the 2-propanol concentration of the aqueous mobile phase was found to provide a greater peak height response for the synthetic estrogen hormones diethylstilbestrol and dienestrol. This increase in peak height response is directly related to the increase in molar absorptivity values at 254 nm for these synthetic estrogens; *i.e.*, the UV absorption curves are shifting to a longer wavelength, with a corresponding increase in absorbance at 254 nm.

Qualitative Analysis—*Photochemical Behavior*—A potentially

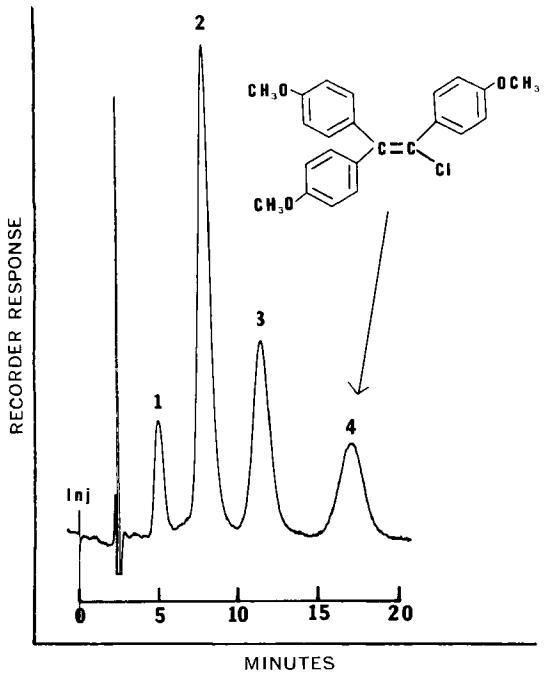

Figure 3—*Chromatogram of the photochemical by-products of chlorotrianisene. Peaks 1–3 are unidentified, and peak 4 is chlorotrianisene. Chromatography conditions were the same as in Fig. 2; however, pressure at inlet was 600 psig.*

useful approach in the rapid qualitative analysis (in addition to their normal retention time) of a number of synthetic estrogens, *e.g.*, diethylstilbestrol, chlorotrianisene, and the dipropionic acid esters of diethylstilbestrol and promethestrol, is their chromatographic behavior after undergoing photochemical change.

Figure 2 presents a chromatogram of a methanolic solution of *trans*-diethylstilbestrol which was irradiated by UV light. Five peaks are noted. The solvent methanol (peak 1) eluted very rapidly, followed closely by a tall peak (peak 2) which was found to be the phenanthrenedione (3,4,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene). The doublet corresponds to *cis*- (peak 3) and *trans*- (peak 4) diethylstilbestrol, and the final small peak (peak 5) is a phenanthrenediol (9,10-diethyl-3,6-phenanthrenediol).

The nature of the photochemical changes brought about by the irradiation of diethylstilbestrol in solution has been carefully studied (21, 22). In fact, formation of both the phenanthrenedione (22) and the phenanthrenediol (23) has been the basis of quantitative methods of analysis for this particular synthetic estrogen. Diethylstilbestrol, usually present as the *trans*-isomer, is induced to equilibrate with the higher energy *cis*-isomer, which then rearranges to yield phenanthrenedione and, finally, phenanthrenediol. The presence of each of these compounds in this study was corroborated as follows. The phenanthrenedione and phenanthrenediol were identified by elution behavior studies of reference standards prepared by previously reported methods (21, 23) and, in addition, by collecting each compound after chromatographic separation and performing a UV spectral comparison with the reference standards. The *cis*-diethylstilbestrol was identified by an elution behavior comparison with that of a reference standard, prepared by allowing a pure reference standard of *trans*-diethylstilbestrol in chloroform to equilibrate between the two isomers (24).

Variation in solution composition results in a different chromatographic pattern. In the system composed of equal volumes of methanol and aqueous 1.8% dipotassium hydrogen phosphate, a weakly basic medium, the chromatogram shows three peaks: a single peak for the phenanthrenedione and a doublet once again corresponding to *cis*- and *trans*-diethylstilbestrol. No peak for the phenanthrenediol is present, as expected (21). When diethylstilbestrol in acetic acid–methanol solution is irradiated, the chromatogram shows the presence of the peaks corresponding to the *cis*- and *trans*-isomers of diethylstilbestrol and a greatly increased peak height response for the phenanthrenediol. The conversion of *trans*-diethylstilbestrol to the phenanthrenediol in acetic acid was reported previously (25). Thus, in summary, it is noticed that in basic methanol solution, irradiation favors formation of the phenanthrenedione, and in an acidic medium, phenanthrenediol forms upon irradiation.

If the identification of *trans*-diethylstilbestrol at submicrograms levels is to be carried out by UV conversion to phenanthrenedione, difficulties are encountered. Since phenanthrenedione elutes relatively close to the solvent front, it will not be observed in most cases owing to interferences. However, the dione mobility may be altered and the resultant peak separated from the solvent front by using a 5–10% 2-propanol mobile phase system together with a temperature adjusted to provide for a desired retention time. In addition, a favorable change—*viz.*, stronger photometric absorption, would result if the detector was used at 280 nm since a phenanthrenedione maximum occurs near this wavelength.

A practical approach for the identification of *trans*-diethylstilbestrol at submicrogram levels is based on the preparation of the phenanthrenediol directly from the phenanthrenedione. After irradiation of a methanol or basic buffered methanol solution of *trans*-diethylstilbestrol to yield phenanthrenedione, the solution is acidified with hydrochloric acid and a quantity of sodium bisulfite (approximately 100 mg) is added. The resulting solution is heated on a steam bath until the yellow color disappears and there is no odor of sulfur dioxide (usually 10 min). Under these conditions, phenanthrenediol is formed in almost quantitative yield.

A second compound whose photochemical behavior was studied is chlorotrianisene. Figure 3 is a chromatogram of this drug after irradiation. The first peak to emerge is methanol; peaks 1, 2, and 3 represent unidentified decomposition products, and peak 4 is the parent compound which could be totally converted into other products with continued irradiation. Although no effort was made to identify the irradiation products, studies indicated that the compound contributing to peak 3 is a secondary photochemical by-product of chlorotrianisene and is formed directly from the compound contributing to peak 2. Therefore, peak 3 will be the dominant peak when chlorotrianisene is allowed to undergo con-

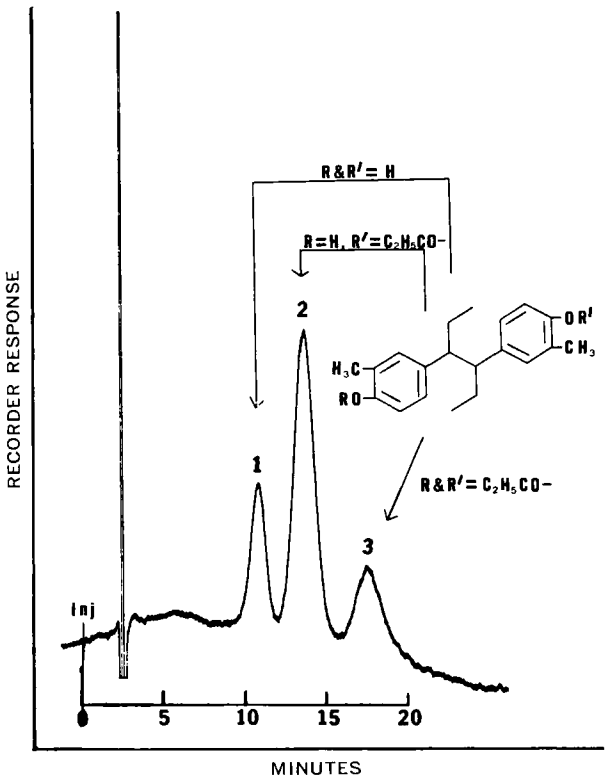

Figure 4—*Chromatogram of the ester hydrolysis decomposition products of promethestrol dipropionate. Peak at 2.5 min is the solvent methanol, and peaks 1–3 are identified as noted. Chromatography conditions were the same as in Fig. 3.*

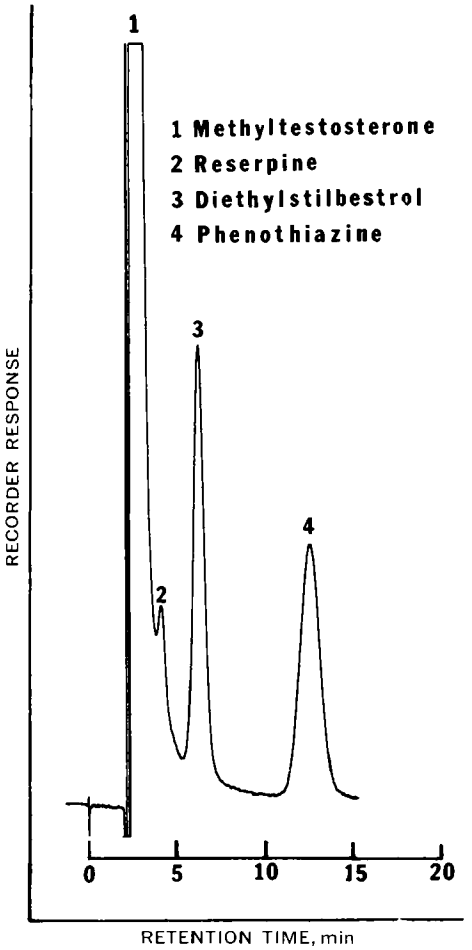

Figure 5—*Chromatogram of a methanol extract of a commercial preparation, including the internal standard phenothiazine. Chromatography conditions were: Column 2; mobile phase, 25% 2-propanol in water; pressure at inlet and column temperature, 600 psig and 50°, respectively; and UV detector sensitivity, 254 nm, 0.08 aufs.*

tinued irradiation in methanol. At any rate, the chromatographic pattern is characteristic and useful for identification.

Chromatographic studies of the dipropionic acid esters of diethylstilbestrol and promethestrol after irradiation in methanol also indicated the potential use of this approach in their identification. In each case, the synthetic estrogen underwent photochemical change and provided two additional peaks, *i.e.*, one peak immediately before and one immediately after the parent compound. Each peak provided a greater peak height response than the parent compound, therefore indicating an indirect route to higher sensitivity. Dienestrol, hexestrol, and benzestrol did not appear suitable for this type of study due to their lack of useful chromatographic change following UV exposure.

Ester Hydrolysis—In addition to the photochemical behavior studies of promethestrol dipropionate and diethylstilbestrol dipropionate, chromatographic studies of these synthetic estrogens were conducted while undergoing controlled ester degradation. Each synthetic estrogen was dissolved in methanol, and an equal volume of 1.8% aqueous dipotassium hydrogen phosphate was added to provide for partial degradation prior to chromatographic study. Figure 4 shows a chromatogram produced after promethestrol dipropionate underwent decomposition for 1 hr. In addition to some quantity of promethestrol dipropionate (peak 3), two additional compounds corresponding to promethestrol (peak 1) and the intermediate promethestrol monopropionate (peak 2) are present. Since the normal route of degradation of phenolic esters is ester hydrolysis, the detection of both of these compounds could be useful in stability studies of standard materials or dosage forms. Diethylstilbestrol dipropionate was found to undergo

Table III—Analysis of Synthetic Estrogens in Commercial Preparations

Sample	Declared	Found, % Liquid Chromatography[a]	Found, % Alternate Method
Dienestrol tablets	0.1 mg/tab	97.9	96.8[b]
Dienestrol tablets	10.0 mg/tab	98.8	98.1[c]
Dienestrol cream	0.1 mg/tab	107.0	109.0[d]
Diethylstilbestrol tablets	0.25 mg/tab	97.2	96.0[e]
Diethylstilbestrol injection	5.0 mg/ml	101.2	99.0[f]
Diethylstilbestrol lotion	0.70 mg/g	100.0	98.6[e]
Diethylstilbestrol suppositories	0.1 mg/sup	104.0	102.0[g]
Chlorotrianisene capsules	72.0 mg/cap	99.7	99.2[h]
Hexestrol tablets	3.0 mg/tab	98.1	98.7[c]
Benzestrol tablets	5.0 mg/tab	96.2	95.0[i]
Promethestrol dipropionate tablets	1.0 mg/tab	101.0	103.0[j]

[a] Chemically bonded ether column (Column 2, ETH-Permaphase); mobile phase, 25% 2-propanol in water (v/v); column temperature, 60°; pressure at inlet, 1000 psig; internal standards: (*a*) phenothiazine for dienestrol, diethylstilbestrol, and chlorotrianisene, (*b*) benzestrol for hexestrol, (*c*) hexestrol for benzestrol, and (*d*) chlorotrianisene for promethestrol dipropionate. [b] NF XIII, p. 229. [c] Extraction and UV. [d] NF XIII, p. 228. [e] USP XVIII, p. 189 (modified). [f] USP XVIII, p. 188. [g] USP XVIII, p. 188 (modified). [h] NF XIII, p. 163. [i] NF XIII, p. 76. [j] Extraction, hydrolysis, and UV.

degradation in the same solvent medium, with quantitative conversion to diethylstilbestrol in 1.3 hr. As could be expected, the intermediate diethylstilbestrol monopropionate separated. The solvent system used for the hydrolytic conversion of the diester to diethylstilbestrol is suitable for the irradiation studies previously described if further identity is desired.

Quantitative Analysis—Six different synthetic estrogens were quantitatively determined in pharmaceutical dosage forms in this study. The commercial preparations were selected to be representative of the various dosage forms currently marketed.

The results for the quantitative analysis of the various dosage forms containing synthetic estrogens are presented in Table III. In all cases except three, the liquid chromatographic results are somewhat higher than the alternative method. Considering the relatively small quantities of drug in most dosage forms, the maximum difference of 1.9% is reasonable. Where the drug dosage is large, as in chlorotrianisene capsules, the results are closer (0.5%). Area integrations were carried out manually, but an electronic integrator could minimize integration error and variation.

The most difficult experimental tasks are the extraction and solubilization of the synthetic estrogens prior to chromatographic injection. The solvent required varies and depends on both the dosage form and the individual synthetic estrogen under study. For example, dienestrol in solid dosage forms, *i.e.*, a tablet powder, could readily be extracted and placed in solution with methanol. When present in a cream, however, dienestrol required an equal mixture of ether–methanol. The ether was necessary to solubilize the cream base, thereby preventing any undissolved dienestrol from remaining in the base.

Various internal standards were used for the quantitative determination of the synthetic estrogens. For example, phenothiazine was selected for diethylstilbestrol, dienestrol, and chlorotrianisene; hexestrol for benzestrol; benzestrol for hexestrol, and chlorotrianisene for promethestrol dipropionate. Although phenothiazine is not the ideal internal standard owing to its photochemical instability, its delayed elution position relative to diethylstilbestrol and dienestrol is desirable.

Although both chromatographic columns were found useful in providing separations of the synthetic estrogens, Column 2 was selected for quantitative studies. The primary reasons for its selection were: *(a)* all of the synthetic estrogens were eluted in a reasonable time for quantitative analysis, and *(b)* the peaks in

each case were symmetrical, whereas the peaks showed some tailing on Column 1. The column selected is thermally stable at elevated temperatures and is often used at temperatures up to 75°.

Limits of detectability for the two most common synthetic estrogens, diethylstilbestrol and dienestrol, reached the nanogram level. In fact, when 1 ng of each compound was injected onto the chromatographic column, using a 20% 2-propanol–water mobile phase and a column temperature of 70°, a peak height response twice that of the noise level was observed. However, there are indications that Column 1 might prove more useful in submicrogram identification studies, since the synthetic estrogens can be eluted using a mobile phase containing a higher percentage of water, a solvent composition trend resulting in increasing photometric absorption.

Figure 5 shows an actual separation of the synthetic estrogen hormone diethylstilbestrol from two other drugs, methyltestosterone and reserpine, which are sometimes found in combination. In this particular case the methyltestosterone was present in approximately a 20-fold excess. The chromatogram shows the relative position of phenothiazine internal standard.

SUMMARY

The method proposed provides for the direct isolation of the synthetic estrogens without derivatization and appears applicable when these compounds are present in various dosage forms. Studies have indicated that compounds often present in combinations will not interfere with the method. In addition, the chromatographic patterns that result when synthetic estrogens undergo photochemical or hydrolytic change may be used for identification as an adjunct to the retention time.

REFERENCES

(1) R. L. Stevenson and C. A. Burtis, *J. Chromatogr.*, **61**, 253(1971).

(2) R. A. Henry and J. A. Schmit, *Chromatographia*, **3**, 116(1970).

(3) T. C. Kram, *J. Pharm. Sci.*, **61**, 254(1972).

(4) R. B. Poet and H. H. Pu, *ibid.*, **62**, 809(1973).

(5) M. W. Anders and J. P. Latorre, *Anal. Chem.*, **42**, 1430(1970).

(6) R. W. Roos, *J. Pharm. Sci.*, **61**, 1979(1972).

(7) S. Siggia and R. A. Dishman, *Anal. Chem.*, **42**, 1223(1970).

(8) R. A. Henry, J. A. Schmit, and J. F. Dieckman, *J. Chromatogr. Sci.*, **9**, 513(1971).

(9) J. A. Mollica and R. F. Strusz, *J. Pharm. Sci.*, **61**, 444(1972).

(10) C. G. Scott and P. Bonner, *J. Chromatogr. Sci.*, **8**, 446(1970).

(11) D. J. Weber, *J. Pharm. Sci.*, **61**, 1797(1972).

(12) C. P. Talley, *Anal. Chem.*, **43**, 1512(1971).

(13) C. Y. Wu and S. Siggia, *ibid.*, **44**, 1499(1972).

(14) G. J. Krol, C. A. Mannan, F. Q. Gemmill, Jr., G. E. Hicks, and B. T. Kho, *J. Chromatogr.*, **74**, 43(1972).

(15) R. C. Williams, J. A. Schmit, and R. A. Henry, *J. Chromatogr. Sci.*, **10**, 494(1972).

(16) S. Gottlieb, *J. Amer. Pharm. Ass., Sci. Ed.*, **36**, 379(1947).

(17) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970.

(18) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970.

(19) F. L. Fricke, S. M. Walters, and W. T. Lampkin, *J. Pharm. Sci.*, **57**, 868(1968).

(20) J. J. Kirkland, *J. Chromatogr. Sci.*, **9**, 206(1971).

(21) T. D. Doyle, N. Filipescu, W. R. Benson, and D. Banes, *J. Amer. Chem. Soc.*, **92**, 6371(1970).

(22) D. Banes, *J. Ass. Offic. Agr. Chem.*, **44**, 323(1961).

(23) E. J. Umberger, D. Banes, F. M. Kunze, and S. H. Colson, *ibid.*, **46**, 471(1963).

(24) B. S. Rutherford, *J. Ass. Offic. Anal. Chem.*, **53**, 1242(1970).

(25) P. Hugelshofer, J. Kalvoda, and K. Schaffner, *Helv. Chim. Acta*, **43**, 1322(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 3, 1973, from the *Food and Drug Administration, Department of Health, Education, and Welfare, Brooklyn, NY 11232*

Accepted for publication November 20, 1973.

Presented in part at the 86th annual meeting of the Association of Official Analytical Chemists (Abstract 177), October 11, 1972.

The author thanks Dr. Thomas Medwick, Science Advisor, Food and Drug Administration, New York District, and Professor of Pharmaceutical Chemistry, Rutgers—The State University, New Brunswick, N.J., for his assistance in the preparation of this paper.

Specific Analysis for Homatropine Methylbromide in Syrups

F. F. CANTWELL ˣ, M. DOMJAN, and C. F. HISKEY

Abstract □ A sensitive, specific, and stability-indicating method is described for the analysis of homatropine methylbromide in complex pharmaceutical syrups. The procedure involves the isolation of methylhomatropinium ion on a cation-exchange resin column followed by on-column hydrolysis to mandelate ion by treatment with sodium hydroxide. The alkaline eluate is washed with chloroform and acidified. Ceric reagent is added to oxidize mandelic acid to benzaldehyde, which is then extracted into isooctane and measured by UV spectrophotometry. The procedure is routinely applied to complex syrups and is suitable for analyzing aged syrup samples.

Keyphrases □ Homatropine methylbromide—analysis in complex pharmaceutical syrups, ion-exchange separation and UV spectrophotometry □ Column chromatography, ion exchange—analysis of homatropine methylbromide in complex pharmaceutical syrups □ UV spectrophotometry—analysis, homatropine methylbromide in complex pharmaceutical syrups

The requirements of an analytical method for homatropine methylbromide (I) in pharmaceutical syrups include both sensitivity and selectivity. The drug is presently marketed in doses as low as 0.03 mg/ml and in formulations containing several other active ingredients, preservatives, buffers, flavors,