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ACKNOWLEDGMENTS

The authors thank Ms. Rose Lancellotti and Ms. Theresia Mueller for assistance in the preparation of this manuscript.

Analysis of Dienestrol and Its Dosage Forms by High-Performance Liquid Chromatography

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Received September 22, 1977, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, Rutgers—the State University of New Jersey, Piscataway, NJ 08854.* Accepted for publication October 3, 1978.

Abstract □ A high-performance liquid chromatographic (HPLC) analysis is described for dienestrol as a drug substance and in cream, foam, and tablet dosage forms. After incorporation of the drug or dosage form into a solvent mixture containing an internal standard, biphenyl, an aliquot was chromatographed using a reversed-phase medium, followed by UV spectrophotometric detection at 254 nm. The response of the chromatographic system was linear over a concentration range corresponding to 50–200% of the labeled amount of dienestrol. Satisfactory accuracy and precision were confirmed by analyzing cream by the standard addition method. The advantages of the HPLC method are its simplicity, speed, and sensitivity, which permit direct analysis of single-dose quantities of dienestrol.

Keyphrases □ Dienestrol—analysis by high-performance liquid chromatography, in various dosage forms □ High-performance liquid chromatography—analysis, dienestrol in various dosage forms □ Estrogens, synthetic—high-performance liquid chromatographic analysis in various dosage forms

Dienestrol [4,4′-(1,2-diethylidene-1,2-ethanediyl)bisphenol, I] is a synthetic estrogenic compound used for the treatment of osteoporosis and estrogen deficiency. Because of its high level of potency, the dose used is very small. Therefore, analysis of pharmaceutical preparations requires not only separation of the compound from the dosage form matrix but also detection of microgram quantities of drug.

BACKGROUND

Separation of dienestrol from associated interfering substances has been accomplished by TLC (1), solvent extraction (2), open column chromatography (2, 3), and reversed-phase high-performance liquid chromatography (HPLC) at elevated temperatures (4). Quantitation has been obtained by UV spectrophotomety of dienestrol itself or of the indene formed by acid-induced isomerization (2). A recent publication (5) discussed the optimization of a normal-phase HPLC separation of dienestrol but not its applicability to pharmaceutical dosage forms.

The method of analysis official in NF XIV (6) for dienestrol cream requires a preliminary solvent extraction followed by lengthy column chromatography. The isolated phenolic fraction is then reacted with nitrous acid, and the nitrosophenols are determined polarographically (7). Although the method is sensitive, it is time consuming and subject to wide variation due to the number of manipulations required.

The purpose of this study was to use the inherent speed and sensitivity of HPLC to develop a simple, direct, accurate, and sensitive enough method for dienestrol to determine single-dose quantities. Such an analysis would be particularly applicable in quality assurance and stability testing situations.

The proposed method involves only dissolution of the sample, addition of the internal standard, and introduction of an aliquot of the resulting mixture onto a liquid chromatograph with a reversed-phase column and a UV detector operated at 254 nm. The accuracy and precision are comparable to those reported for other methods, but the time required is significantly less.

EXPERIMENTAL

Reagents and Chemicals—Dienestrol NF reference standard was used as received. Biphenyl[1] was recrystallized twice from ethanol, dried *in vacuo*, and stored in a vacuum desiccator, mp 69–71°.

Solvents—Acetonitrile[2] (99 mole % pure) was used as received.

Apparatus—The HPLC system consisted of a pump[3] and a UV monitor[4] operated at 254 nm. Samples were introduced using a loop injector[5] with a fixed volume of 20 μl. The loop injector was equipped with a sample prefilter (8). The output of the detector was displayed on a recorder[6] having a full-scale range of 10 mv. The output signal was integrated, and results were calculated using an electronic integrator[7]. The 25-cm × 4.6-mm i.d. stainless steel column contained reversed-phase packing material[8]. The mobile phase was pumped at a pressure of about 1600 psig, which resulted in a flow rate of 3.0 ml/min.

A mechanical shaker[9] was used in the extraction of samples.

Mobile Phase—The mobile phase, a mixture of acetonitrile and water (40:60), was degassed *in vacuo* prior to use.

Standard Solutions—*Dienestrol Standard Solution*—An accurately weighed 0.0500-g sample of dienestrol was transferred to a 100-ml volumetric flask and diluted to volume with acetonitrile.

Internal Standard Solution—An accurately weighed 0.0500-g sample of biphenyl was transferred to a 100-ml volumetric flask and diluted to volume with acetonitrile.

Diluted Internal Standard Solution—Exactly 5.0 ml of internal standard solution was transferred to a 250-ml volumetric flask and diluted to volume with acetonitrile.

Dienestrol Drug Substance—*Standard Preparation*—Exactly 2.0 ml of dienestrol standard solution and 2.0 ml of internal standard solution were transferred to a 100-ml volumetric flask and diluted to volume with acetonitrile. A 20-μl sample was introduced into the liquid chromatograph. The areas under the two peaks were measured, and the ratio, R_s, was calculated by dividing the area of the dienestrol peak by the area of the internal standard peak.

Assay Preparation—About 50 mg of dienestrol was weighed and transferred to a 100-ml volumetric flask and diluted to volume with ac-

[1] Eastman Kodak Co., Rochester, N.Y.
[2] Fisher Scientific Co., Fair Lawn, N.J.
[3] Constametric IIG, Laboratory Data Control, Riviera Beach, Fla.
[4] Model 1203, Laboratory Data Control.
[5] Model HPSV-20, Spectra-Physics.
[6] Omniscribe model 521I-151, Houston Instruments, Austin, Tex.
[7] Model 3380A, Hewlett-Packard, Avondale, Pa.
[8] Partisil PXS-1025 ODS, Whatman, Clifton, N.J.
[9] Eberbach Corp., Ann Arbor, Mich.

0022-3549/79/0400-0508$01.00/0

etonitrile. Exactly 2.0 ml of this solution and 2.0 ml of internal standard solution were transferred to a 100-ml volumetric flask and diluted to volume with acetonitrile.

Assay—A 20-μl sample was introduced into the liquid chromatograph. The areas under the two peaks were measured, and the ratio, R_u, was calculated by dividing the area of the dienestrol peak by the area of the internal standard peak. The quantity, in micrograms of dienestrol present in the sample, was calculated by the formula:

$$\text{dienestrol } (\mu g) = W_s(R_u/R_s) \qquad \text{(Eq. 1)}$$

where R_u and R_s are as defined previously and W_s is the weight, in micrograms, of dienestrol present in 10.0 ml of the standard preparation.

Dienestrol Cream—*Standard Preparation*—The procedure was as given under *Dienestrol Drug Substance.*

Assay Preparation—An accurately weighed quantity of the cream, equivalent to not less than 100 μg of dienestrol, was transferred to a 25-ml glass-stoppered flask, and 10.0 ml of diluted internal standard solution was added and agitated on a mechanical shaker for 15 min. The resulting suspension was poured into a 15-ml conical centrifuge tube and centrifuged at high speed for 5 min.

Assay—The procedure was as given under *Dienestrol Drug Substance.*

Dienestrol Foam—*Standard Preparation*—The procedure was as given under *Dienestrol Drug Substance.*

Assay Preparation—The dienestrol container was shaken vigorously, the actuator was depressed, and a portion of the contents was discharged into a 125-ml glass-stoppered flask. The flask was immersed in a 60° water bath, and the contents were swirled until the entrapped propellant was dispelled and there was no further reduction in volume. Then the procedure was as given under *Dienestrol Cream Assay Preparation.*

Assay—The procedure was as given under *Dienestrol Drug Substance.*

Dienestrol Tablets—*Diluted Biphenyl Solution*—Exactly 5.0 ml of internal standard solution was transferred to a 250-ml volumetric flask and diluted to volume with mobile phase.

Standard Preparation—Exactly 2.0 ml of dienestrol standard solution and 2.0 ml of internal standard solution were transferred to a 100-ml volumetric flask and diluted to volume with mobile phase. The preparation continued as directed under *Dienestrol Drug Substance Standard Preparation,* beginning with: "A 20-μl sample was introduced . . . ."

Assay Preparation—A single dienestrol tablet was weighed and transferred to a glass-stoppered flask. An exactly measured quantity of diluted biphenyl solution was added such that the final concentration of dienestrol was nominally 10 μg/ml. The solution was agitated on a mechanical shaker for 15 min or until the tablet had completely disintegrated. The resulting suspension was poured into a 15-ml conical centrifuge tube and centrifuged at high speed for 5 min.

Assay—The procedure was as given under *Dienestrol Drug Substance.*

System Suitability Test—Five injections of the standard preparation were chromatographed as directed under *Dienestrol Cream.* In a suitable system, dienestrol exhibits one major peak with a retention time of about 0.71 relative to the internal standard and the resolution factor (9) between dienestrol and the internal standard is not less than 2.5. The relative standard deviation of the ratio of the peak areas of dienestrol to the peak areas of the internal standard should be not more than 2%.

RESULTS AND DISCUSSION

Mobile Phase—The suggested mobile phase, acetonitrile–water (40:60), was used for most of the work reported here. However, when new columns or those from manufacturers[10] other than the one cited under *Experimental* were used, adjustments were necessary in the relative proportions of the mobile phase components to achieve acceptable separations. Mobile phases containing as much as 55% acetonitrile were used with some columns with satisfactory results.

Higher proportions of acetonitrile reduced the retention times and, consequently, the time for each analysis, but an undesirable reduction in the resolution of dienestrol and the internal standard may result if the amount of acetonitrile is excessive. The suggested mobile phase should be used when required for sample dissolution because of the limited solubility of dienestrol in aqueous media.

Chromatographic Response—The typical response of dienestrol and the internal standard to the chromatographic system is shown in Fig. 1. This chromatogram was obtained from the analysis of dienestrol cream containing 80.1 μg/g of dienestrol.

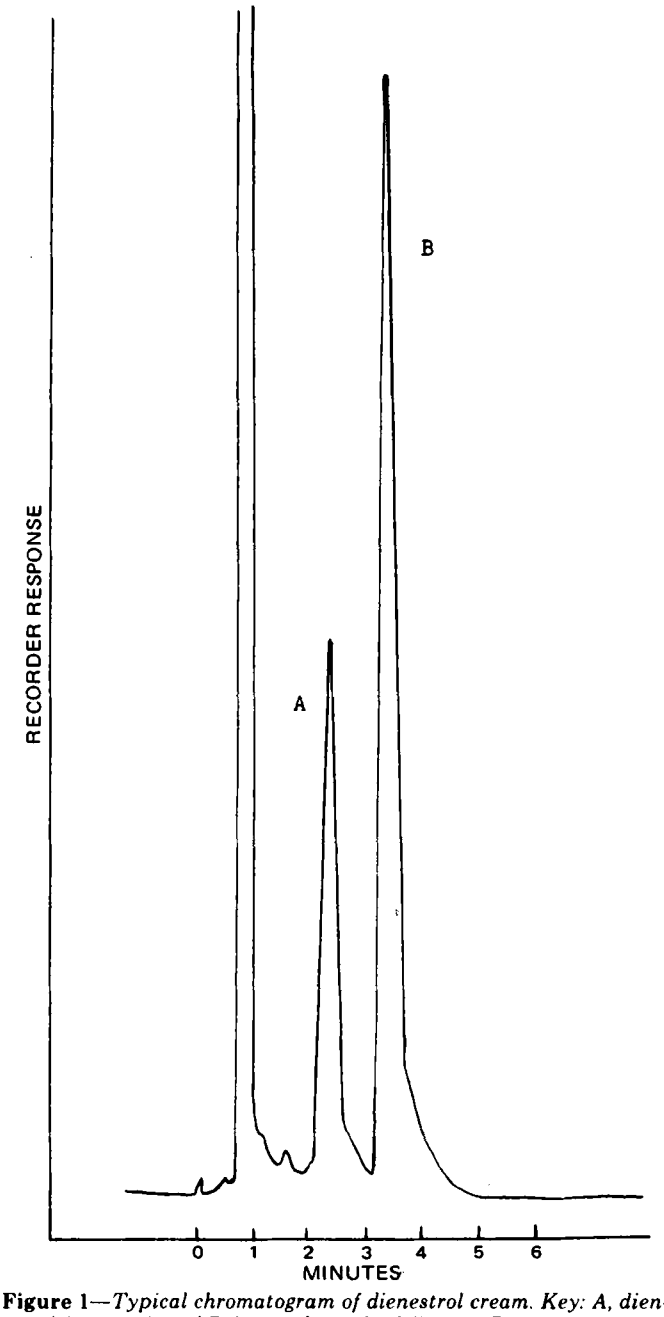

Figure 1—*Typical chromatogram of dienestrol cream. Key: A, dienestrol (0.160 μg); and B, internal standard (0.2 μg). Detector range was 0.032 aufs.*

To determine the linearity of the chromatographic response, a calibration curve was run in which the internal standard concentration was maintained constant while the dienestrol concentration was varied. The resulting response was linear (r = 0.999) over a range corresponding to quantities of dienesterol that would be obtained in the analysis of dosage forms containing 50–200% of the label claim. The intercept was essentially zero. The limit of sensitivity, defined as that quantity of dienestrol which, when injected on the column, gave a symmetrical peak with a height at least twice that of the noise level, was 1 ng.

No degradation of column performance was noted even after hundreds of injections. As a precautionary measure, the column was rinsed thoroughly with acetonitrile at the end of each day and kept filled with acetonitrile.

Analysis of Dosage Forms—Table I gives the results obtained from the analysis of some commercially available dosage forms containing dienestrol. In all cases except that of the cream, the amount recovered was essentially that claimed to be present by the manufacturer. The precision was also satisfactory, as indicated by the standard deviations

[10] μBondapak C18, Waters Associates, Milford, MA 01757; Datasorb ODS, 10 mm × 25 cm, Laboratory Data Control.

Table I—Determination of Dienestrol by HPLC

Drug Entity	Label Claim	Mean	*SD*	n^a
Powder	—	99.91[b]	1.02	4
Cream	100 μg/g	78.46	1.52	9
Foam	100 μg/g	104.83	0.50	6
Tablet	100 μg/tablet	100.07	4.48	10

[a] Number of samples analyzed. [b] Percent of label claim.

Table II—Single-Tablet Analysis of 0.1-mg Dienestrol Tablets

	Tablet Weight, g	Dienestrol Found, % of claim
	0.1614	101.71
	0.1499	103.83
	0.1395	97.08
	0.1722	100.88
	0.1487	97.23
	0.1550	99.83
	0.1626	106.47
	0.1546	90.53
	0.1626	99.35
	0.1606	103.80
Mean	0.1567	100.07
RSD, %	5.87	4.48

Table III—Analysis of Dienestrol Cream by HPLC with Standard Addition

Dienestrol Added[a], μg/g	Dienestrol Found	Standard Recovered[a]
0	78.46	—
25	24.59	98.4
50	50.44	100.9

[a] Percent of amount added.

of 1.52% or less except with tablets. Since all analyses of tablets were done on single tablets (Table II), the variation in tablet weight, which was 5.87 (% *RSD*) for the samples taken, was superimposed on the usual analytical variation due to weighing, pipetting, and diluting. If the results were corrected to a constant tablet weight or if a composite sample were analyzed, the variability would probably be less than that reported.

The low recoveries obtained from cream samples were assumed initially to be the result of incomplete extraction of dienestrol from the cream base. Various techniques, including heat, longer extraction times, and different mixtures of solvents, were employed to improve recoveries, but none increased the quantity of dienestrol recovered. Therefore, it was decided to verify the accuracy of the results by the method of standard addition (10). Three sets of samples of dienestrol cream were weighed and extracted in the usual manner with acetonitrile containing 10 μg/ml of internal standard and 0, 25, and 50 μg of dienestrol/10 ml.

The results obtained on the cream samples containing no added dienestrol confirmed the earlier analyses (Table III). Recovery of the added dienestrol was 98.4% for the 25-μg samples and 100.9% for the 50-μg samples. Therefore, it was concluded that the analytical results accurately reflected the true content of dienestrol in the sample used.

Because of the proven photochemical instability of synthetic estrogens of similar chemical structure (4), samples should be analyzed promptly after dissolution. A sample of dienestrol standard solution that had been left exposed to ambient light for 4 days showed no chromatographic peak at the dienestrol retention time. Instead, three smaller peaks with lower retention times were observed. They did not interfere with the dienestrol or internal standard peaks and, therefore, did not detract from the specificity of the method.

Other UV-absorbing ingredients in several formulations were benzoic acid, methylparaben, propylparaben, and nonoxynol. Qualitative analysis of these compounds showed no interference with dienestrol or the internal standard.

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ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Thomas Medwick for helpful discussions and to Theresa A. Folinusz for technical assistance.