significance for the comparison of the infusion values with the 100-mg dose. Post steady-state disappearance half-lives ranging from 0.67 to 1.03 hr were not different from the single-dose data, (the increase in the postinfusion plasma concentration seen in Fig. 5 was due to flushing of the catheter with saline at the time the infusion was stopped). No time-dependent change in clearance or half-life was exhibited over the 1-week period of the study.

In the rhesus monkey, progabide behaves as a medium extraction ratio drug with incomplete bioavailability and first-order disappearance kinetics. It exhibits nonlinearity in plasma binding *in vitro*. There was a tendency for systemic clearance to decrease within a two-fold dose range. Progabide exhibited no evidence of time dependency in clearance during chronic infusion.

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Determination of Ethinyl Estradiol in Solid Dosage Forms by High-Performance Liquid Chromatography

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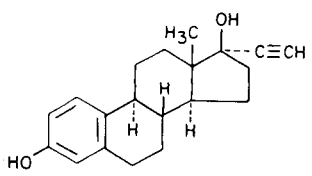
Abstract □ A rapid, reproducible high-performance liquid chromatographic system for the determination of ethinyl estradiol in solid dosage forms consisting of a reversed-phase column with a mobile phase of 0.05 *M* aqueous KH_2PO_4–methyl alcohol (2:3) and fluorescence detection has been developed. This stability-indicating method is applicable to tablets containing ethinyl estradiol alone or in combination with methyltestosterone and progesterones. The procedure has been used for the determination of ethinyl estradiol in single tablets, stability samples, and dissolution medium. Recovery of drug substance added to placebo was from 97.3 to 101.5% in stability and single-tablet assays, and 95.4 to 102.2% in dissolution assays. Reproducibility studies gave relative standard deviations of 0.4–2.2%.

Keyphrases □ Ethinyl estradiol—high-performance liquid chromatographic analysis, stability, content uniformity and dissolution assays □ High-performance liquid chromatography—analysis, ethinyl estradiol, fluorescence detection □ Estrogens—ethinyl estradiol, high-performance liquid chromatography, analysis of solid dosage forms

Ethinyl estradiol (I), a well known estrogen, is used in hormonal therapy, contraception, and certain cancer treatments. The steroid may be prescribed either alone, as in treatment of estrogen deficiency, or in combination with a progesterone in contraceptive formulations.

Current methods for the analysis of ethinyl estradiol include both wet chemical (1–4) and chromatographic methods. Among the latter are gas chromatography (GC) (5) and high-performance liquid chromatography (HPLC) with UV detection (6–10).



(I)

Determination of ethinyl estradiol by HPLC has been hampered by low sensitivity, as the administered dosages may be as low as 10 μg/tablet. To counter this lack of sensitivity, several investigators have proposed analyzing composite samples of up to 10 tablets (7, 8). While useful for stability assays, this approach is not suitable for either content uniformity or dissolution assays. Other investigators have increased detection sensitivity by using precolumn derivatization with dansyl chloride and fluorescence detection (11, 12). A much simpler approach is to use the native fluorescence of the phenolic ring of the steroid; this method has been used for normal phase HPLC of estrogens (13) and of ethinyl estradiol in cosmetics (14, 15).

A reversed-phase HPLC system with native fluorescence detection for the determination of ethinyl estradiol in solid dosage forms is described. The proposed method, which requires minimal sample preparation, is not only stability-indicating but also sensitive enough for use in content uniformity and dissolution assays.

EXPERIMENTAL

Materials—HPLC grade methyl alcohol[1], *o*-phenylphenol[2], and potassium phosphate monobasic crystals[2] (KH_2PO_4) were obtained from commercial sources. Ultrapure water was prepared by deionization, treatment for removal of organic compounds, and filtering[3].

Apparatus—The high-performance liquid chromatograph was equipped with a constant flow pump[4], an automatic injector[5], a fluores-

[1] Mallinckrodt, Inc., Paris, KY 40361.
[2] Matheson, Coleman and Bell, Cincinnati, OH 45212.
[3] Milli-Q Water Purification System, Millipore Corp., Bedford, MA 01730.
[4] Model M6000A Chromatography Pump, Waters Associates, Milford, MA 01757.
[5] WISP Model 710A Automatic Injector, Waters Associates, Milford, MA 01757.

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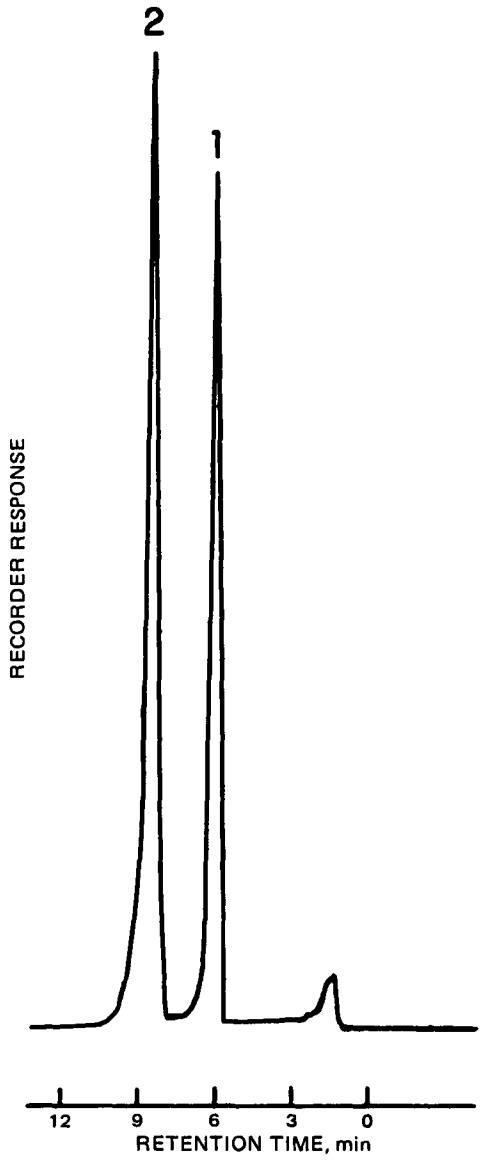


Figure 1—*High-performance liquid chromatogram of ethinyl estradiol and internal standard. Key: 1, o-phenylphenol; 2, ethinyl estradiol.*

cence detector (excitation at 280 nm, emission at 330 nm) containing a xenon lamp source[6], and a chart recorder[7]. The stainless steel column (25 cm × 4.6-mm i.d.) was packed with 10-μm, irregularly shaped, totally porous silica particles bonded with a C-8 hydrocarbon phase[8]. A 2.0-μm filter[9] was placed in-line before the column. A data acquisition system[10] was used for peak processing.

Chromatographic Conditions—The mobile phase was 0.05 *M* aqueous KH_2PO_4, filtered through a 0.45-μm filter[11]–methyl alcohol (2:3) and degassed for 20 min by using an ultrasonic water bath. The flow rate through the HPLC system was 2.0 ml/min. Helium was bubbled through the mobile phase constantly while the system was running.

Internal Standard Solution—A 0.1-mg/ml solution of *o*-phenylphenol was prepared in mobile phase. This solution was diluted to 1.0 μg/ml with mobile phase.

Standard Solution—Approximately 25 mg of ethinyl estradiol standard was accurately weighed, transferred to a 100-ml volumetric flask, and dissolved and diluted to 100 ml with methyl alcohol. This solution was diluted exactly 5:50 in methyl alcohol. A 2.00-ml aliquot of this solution and 5.00 ml of a 1.0-μg/ml internal standard solution were added to a 50-ml volumetric flask. Approximately 23 ml of methyl alcohol was added to the flask and diluted to volume with 0.05 *M* aqueous KH_2PO_4 to a final concentration of 1 μg/ml of ethinyl estradiol and 0.1 μg/ml of internal standard. A 50-μl aliquot was injected into the liquid chromatograph.

Determination of Ethinyl Estradiol in Solid Dosage Forms—Tablets containing 0.02 mg of ethinyl estradiol were assayed by adding single tablets to individual 50-ml centrifuge tubes containing 4.00 ml of aqueous 0.05 *M* KH_2PO_4. The sample was rotated for 15 min. A 2.00-ml aliquot of internal standard and 4 ml of methyl alcohol were added to the sample, which was again rotated for 15 min and centrifuged. The super-

[6] Model 650-10LC Fluorescence Detector, Perkin-Elmer Corp., Norwalk, CT 06856.

[7] Linear Model 485 Recorder, Linear Instruments Corp., Irvine, CA 92664.

[8] LiChrosorb RP8 Column, E. M. Laboratories, Inc., E. Merck, Elmsford, NY 10523.

[9] Model 7302 Column Inlet Filter, 2 μm, Rheodyne Incorporated, Cotati, CA 94928.

[10] PDP 11/34 minicomputer utilizing peak-112 Software, Digital Equipment Corp., Maynard, MA 01754.

[11] Millipore Filter, Type HA, Millipore Corp., Bedford, MA 01730.

Table I—Recovery of Ethinyl Estradiol Added to Tablet Placebos for Content Uniformity and Stability Assays

Formulation	Placebo, mg	Ethinyl Estradiol Added, mg	Ethinyl Estradiol Found, mg	Recovery, %
0.02 mg/tablet, placebo	181.0	0.0215	0.0216	100.5
	240.5		0.0216	100.5
	301.7		0.0213	99.1
	360.7		0.0213	99.1
	424.5		0.0213	99.1
0.02 mg/tablet, placebo[a]	230.5	0.0195	0.0195	100.0
	290.7		0.0198	101.5
	347.7		0.0194	99.5
	418.9		0.0194	99.5
0.05 mg/tablet, placebo	180.7	0.0538	0.0537	99.8
	241.5		0.0539	100.2
	300.2		0.0541	100.6
	361.0		0.0535	99.4
	421.1		0.0530	98.5
0.05 mg/tablet, placebo[a]	181.2	0.0488	0.0475	97.3
	242.0		0.0476	97.5
	307.3		0.0484	99.2
	360.7		0.0485	99.4
	422.3		0.0481	98.6
0.5 mg/tablet, placebo	121.1	0.538	0.545	101.3
	160.3		0.544	101.1
	201.2		0.545	101.3
	239.8		0.544	101.3
	280.3		0.540	100.4
0.5 mg/tablet, placebo[a]	120.6	0.516	0.520	100.8
	160.9		0.519	100.6
	200.8		0.512	99.2
	240.9		0.517	100.2
	280.8		0.513	99.4

[a] Heated 75°, 2 weeks.

Table II—Reproducibility of Ethinyl Estradiol Determinations for Content Uniformity and Stability Assays

Tablet Strength	Day	Assay, % Label			
0.02 mg/tablet		Lot a	Lot b	Lot c	Lot d[a]
	1	107.4	108.2	108.0	110.3
	2	108.6	110.2	109.9	107.7
	3	111.4	108.1	107.8	106.5
	4	108.1	—	—	106.1
$\bar{X}$		108.9	108.8	108.6	107.6
RSD, %		1.6	1.1	1.1	1.7
0.05 mg/tablet		Lot e	Lot f	Lot g	Lot h[a]
	1	109.0	105.4	105.3	108.1
	2	110.2	106.3	105.3	103.0
	3	108.2	104.5	106.5	105.6
	4	—	—	—	107.5
$\bar{X}$		109.1	105.4	105.7	106.0
RSD, %		0.9	0.8	0.7	2.2
0.5 mg/tablet		Lot i	Lot j	Lot k	Lot l[a]
	1	105.3	104.6	103.4	102.7
	2	105.7	105.8	101.5	103.3
	3	106.1	102.8	102.1	103.1
	4	—	—	—	99.4
$\bar{X}$		105.7	104.4	102.3	102.2
RSD, %		0.4	1.4	1.0	2.1

[a] Heated 75°, 2 weeks.

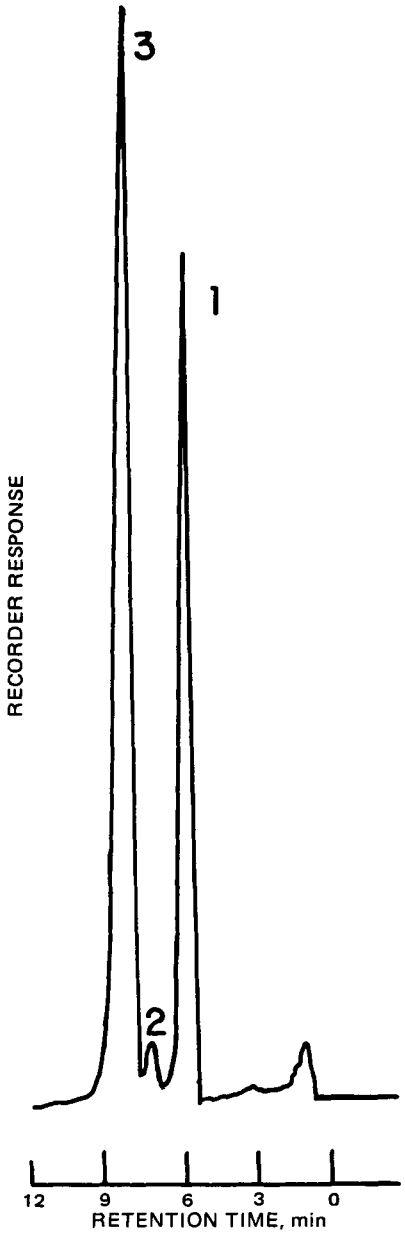


Figure 2—*High-performance liquid chromatogram of ethinyl estradiol tablet extract. Key: 1, o-phenylphenol; 2, butylparaben; 3, ethinyl estradiol.*

nate was transferred to a 25-ml volumetric flask. The samples were extracted two more times with ~5 ml of mobile phase and a 10-min rotation. The supernates were combined and a 50-μl aliquot injected into the liquid chromatograph. The ethinyl estradiol content was calculated as follows:

mg of ethinyl estradiol/tablet =

$$(R_{sam}/R_{std}) \times (W_{std}) \times (V_{sam}/V_o) \qquad \text{(Eq. 1)}$$

where:

R_{sam} = ratio of ethinyl estradiol to *o*-phenylphenol peak heights in the sample chromatogram;
R_{std} = peak height ratio in the standard chromatogram;
W_{std} = weight of ethinyl estradiol in the reference standard solution;
V_{sam} = total volume of sample solution;
V_o = total volume of *o*-phenylphenol in the sample solution.

This sample preparation allows for the determination of ethinyl estradiol in higher strength dosage forms. Tablets containing up to 0.5 mg of ethinyl estradiol may be assayed by adjusting extraction volumes and internal standard concentration to give final assay concentrations of 1 μg/ml of ethinyl estradiol and 0.1 μg/ml of internal standard.

Table III—Recovery of Ethinyl Estradiol Added to Formulation Placebos for Dissolution Assay

Formulation	Ethinyl estradiol Added, mg	Ethinyl estradiol Found, mg	Recovery, %
0.02 mg/tablet, 300-mg placebo	0.0102	0.0101	99.0
	0.0153	0.0154	100.7
	0.0205	0.0198	96.6
	0.0256	0.0258	100.8
0.05 mg/tablet, 300-mg placebo	0.0259	0.0248	95.7
	0.0388	0.0387	99.7
	0.0518	0.0508	98.1
	0.0647	0.0617	95.4
0.5 mg/tablet, 200-mg placebo	0.256	0.260	101.6
	0.384	0.380	99.0
	0.511	0.522	102.2
	0.639	0.610	95.5

Accelerated Degradation Studies—Accelerated degradation studies were conducted by heating the ethinyl estradiol drug substance at 178° for 16 hr as described previously (16), and heating the ethinyl estradiol tablets for 2 weeks at 75°.

Dissolution Assay Studies—A solution of 2 μg/ml of *o*-phenylphenol was prepared in methyl alcohol as the internal standard solution.

Approximately 25 mg of ethinyl estradiol standard was accurately weighed, transferred to a 50-ml volumetric flask, and dissolved and diluted to volume with methyl alcohol. This standard solution was diluted exactly 2:50 in methyl alcohol. A 1.00-ml aliquot of this solution and 1.00 ml of the 2 μg/ml internal standard were added to 900 ml of ultrapure water[3].

Determination of Ethinyl Estradiol in Dissolution Medium—Single tablets containing 0.02 mg of ethinyl estradiol were placed in 900 ml of ultrapure water[3] along with 1.00 ml of 2 μg/ml of the internal standard solution. Dissolution of the tablet was conducted at 37° for 1 hr. The samples and standards were then treated identically. The standard solutions and dissolution medium were filtered through a 1.2-μm filter[12], the filtrates equilibrated to room temperature, and the entire contents of each filtrate concentrated onto an activated C-18 cartridge[13] by using a vacuum to draw the solution through the cartridges. The cartridge was eluted with 20-ml 0.05 *M* aqueous KH_2PO_4–methyl alcohol (2:3) and a volume of 50 μl injected into the liquid chromatograph. The ethinyl estradiol content was calculated from Eq. 1.

This sample preparation also allows for the determination of higher levels of ethinyl estradiol in dissolution medium. Tablets containing up

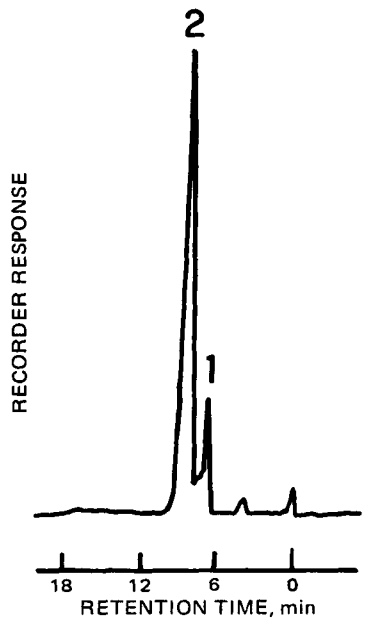


Figure 3—*High-performance liquid chromatogram of ethinyl estradiol drug substance subjected to accelerated degradation. Key: 1, unidentified decomposition product; 2, ethinyl estradiol.*

[12] Millipore Filter, Type RA, Millipore Corp., Bedford, MA 01730.
[13] Sep-Pak C-18 Cartridges, Waters Associates, Milford, MA 01757.

Table IV—Reproducibility of Ethinyl Estradiol Determinations in Dissolution Medium

Aliquot	Assay, mg recovered		
	Tablet, Strength A	Tablet, Strength B	Tablet, Strength C
1	0.0228	0.0492	0.363
2	0.0225	0.0502	0.360
3	0.0230	0.0508	0.357
4	0.0228	0.0491	0.355
5	0.0232	0.0495	0.372
6	0.0229	—	0.367
$\bar{X}$	0.0228	0.0498	0.362
RSD, %	1.0	1.6	1.8

to 0.5 mg of ethinyl estradiol were assayed by adjusting the internal standard concentration and dilutions to give approximate concentrations of 1 μg/ml of ethinyl estradiol and 0.1 μg/ml of the internal standard.

RESULTS AND DISCUSSION

Content Uniformity and Stability Assays—To develop a rapid, reproducible HPLC method for the determination of ethinyl estradiol, sample preparation was kept as simple as possible. In this method the tablet matrix is disintegrated by mixing with aqueous buffer, after which methyl alcohol is added to solubilize the drug substance; the final extracting solution has the same solvent composition as the mobile phase. Under these conditions, two extractions are sufficient to remove more than 99% of both active and internal standard from the tablet matrix; however, a third extraction was added to ensure quantitative recoveries.

To simplify the assay methods, ethinyl estradiol was detected by its native fluorescence rather than by precolumn fluorescence labeling. Both ethinyl estradiol and the internal standard, *o*-phenylphenol, have UV absorption maxima around 280 nm as well as from 200 to 220 nm. Excitation at either of these maxima produces broad ethinyl estradiol emission at 310 nm and *o*-phenylphenol emission at 340 and 420 nm. Adequate sensitivity and selectivity was maintained for these compounds by monitoring emission at 330 nm with a 20-nm spectral band width. While excitation can be done at either 220 or 280 nm, excitation at 220 nm leads to interferences of two types. First, more compounds absorb at 220 than at 280 nm, leading to a loss in detection specificity. Second, impurities in the mobile phase fluoresce to give a significant background. Large amounts of UV absorbing material, such as the methyltestosterone encountered in some formulations, absorb enough radiation to decrease the background, giving negative peaks which are potential chromatographic interferences. Both of the above detection interferences are of significantly lower magnitude when using excitation at 280 rather than 220 nm.

Due to the low levels of ethinyl estradiol and high detection sensitivity, care must be taken to avoid interferences due to fluorescent impurities. Glassware must be thoroughly cleaned and dried, avoiding any surfactants which could leave residues of fluorescing compounds on the glass. In the course of this work, it was also noted that spurious peaks arose from the use of latex dropper bulbs; particulate matter from the bulbs caused severe chromatographic interferences.

In the described method, ethinyl estradiol elutes with a retention time of 9 min and the internal standard elutes at 6 min (Fig. 1). Butylparaben, a tablet-coating excipient, chromatographs at 8 min and does not interfere with the assay. A sample chromatogram is shown in Fig. 2.

Ethinyl estradiol, when subjected to the extreme temperature conditions of 178° for 16 hr used in a previous study (16), degraded ~25%. The degradation products are separated from both ethinyl estradiol and the internal standard as shown in Fig. 3. A discussion of the degradation of ethinyl estradiol has been previously reported (16).

The linearity of the chromatographic system was demonstrated within the range of 0.61–1.42 μg/ml of ethinyl estradiol standard. Response *versus* concentration data gave a correlation coefficient of 0.9995 and an estimated relative standard deviation of linearity of 1.4%. Linearity of the sample preparation was tested over the range of 60–140% of the assay sample size for tablets containing 0.02 mg of ethinyl estradiol and yielded a correlation coefficient of 0.9999 and an estimated relative standard deviation of linearity of 0.64%. Linearity of the sample preparation was also demonstrated for high strength dosage forms of ethinyl estradiol tablets. The correlation coefficients obtained from these experiments were no less than 0.9994. No statistically significant bias was observed in any of the linearity experiments when extrapolated to zero sample size.

The accuracy of the content uniformity–stability method was demonstrated by the addition of ethinyl estradiol to varying amounts of placebo. As seen in Table I, recovery of ethinyl estradiol from the placebo is excellent, ranging from 97.3 to 101.5%.

The precision of the method was tested by performing replicate assays on several sample batches over a period of several days (Table II). The relative standard deviations of the assays were <2% for all normal batches, but increased slightly for heated samples.

Table V—Retention Times for Compounds Commonly Found in Combination with Ethinyl Estradiol

Compound	Relative Retention Time
Ethinyl Estradiol	1.00
Norethindrone	1.00
Norgestrel	1.49
Methyltestosterone	1.57
Norethindrone acetate	2.15
Ethynodiol diacetate	[a]

[a] No peak observed.

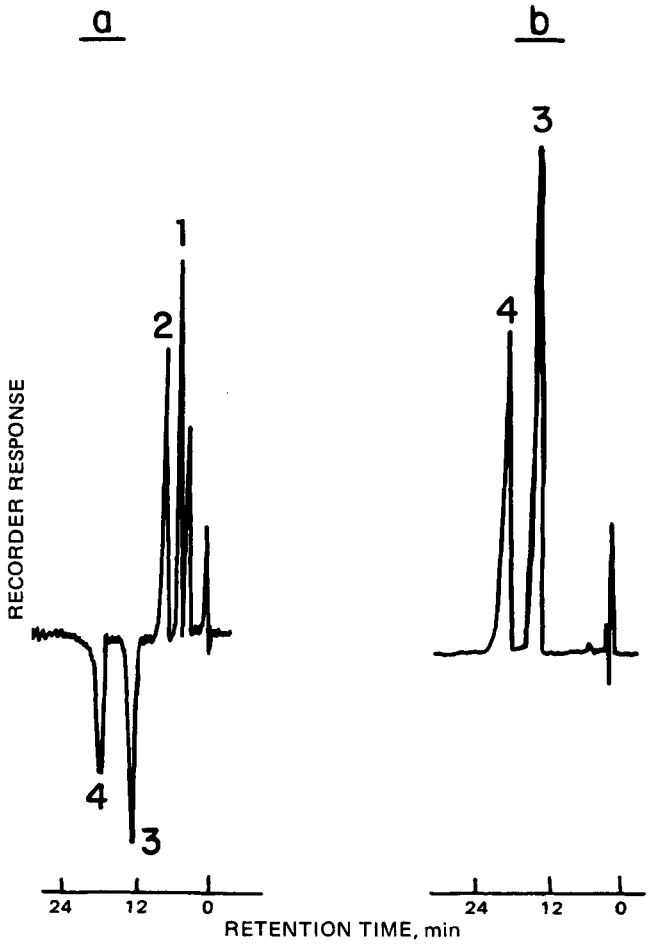
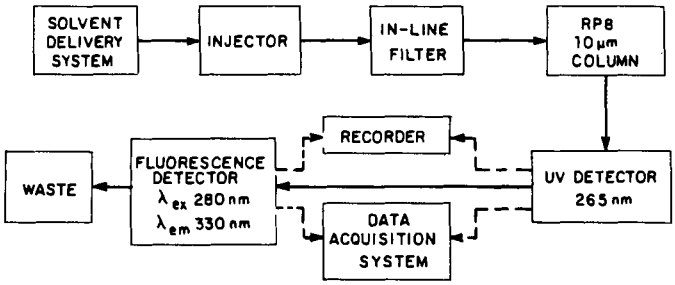


Figure 4—*High-performance liquid chromatogram of tablets containing both ethinyl estradiol and methyltestosterone, (a) fluorescence; (b) ultraviolet. Key: 1, o-phenylphenol; 2, ethinyl estradiol; 3, methyltestosterone; 4, progesterone (internal standard).*

Table VI—Reproducibility of Ethinyl Estradiol Determinations in the Presence of Methyltestosterone

Sample	Assay, % Label		
	Lot A[a]	Lot B[a]	Lot C[b]
1	109.9	108.0	103.7
2	112.1	105.3	104.6
3	109.9	105.0	—
$\bar{X}$	110.6	106.1	104.2
RSD, %	1.3	1.7	—

[a] 0.04 mg of ethinyl estradiol, 10 mg of methyltestosterone/tablet. [b] 0.02 mg of ethinyl estradiol, 5 mg of methyltestosterone/tablet.



Scheme A—High-performance liquid chromatographic system for simultaneous determination of ethinyl estradiol and methyltestosterone.

Dissolution Assays—The medium chosen for the dissolution assays was ultrapure distilled water, from which ions and organic compounds had been removed[3]. Ultrapure water must be used to prevent concentration of any fluorescent impurities onto the cartridge[13].

Precision of ethinyl estradiol recovery from dissolution assay medium was demonstrated by preparing eight standard solutions as described previously and assaying these according to the HPLC method described. These eight standards produced a relative standard deviation of 2.1%.

Since relatively large volumes of liquid were passed through the cartridges[13], some breakthrough of ethinyl estradiol and internal standard was expected. Standard solutions were concentrated onto cartridges[13] series so that any breakthrough from one cartridge would be concentrated onto a second cartridge. The breakthrough observed was 5.7% for ethinyl estradiol and 6.6% for the internal standard. These losses were considered acceptable since both standard and sample solutions are concentrated onto the cartridges and lose equal amounts of material. The breakthrough percentage is constant over a wide range of sample size as demonstrated by recovery studies of ethinyl estradiol and internal standard from dissolution medium. Over the range of 10–500 ng/ml of ethinyl estradiol, peak height ratios were obtained with a relative standard deviation of 1.0%.

The accuracy of the method was demonstrated by the addition of varying amounts of ethinyl estradiol to a constant amount of placebo. The recovery of ethinyl estradiol for three different tablet strengths is seen in Table III. The recovery of ethinyl estradiol ranged from 95.4 to 102.2% with no statistically significant bias when extrapolated to zero sample size.

To avoid the differences of tablet-to-tablet variations, precision of the recovery of ethinyl estradiol from dissolution media was tested on single tablet equivalents of tablet grinds. The largest relative standard deviation observed is 1.8% (Table IV).

Separation of Ethinyl Estradiol from Other Drug Substances—This HPLC method is also applicable to the analysis of ethinyl estradiol in the presence of other drug substances. Various drug substances commonly found in combination with ethinyl estradiol were chromatographed by using the proposed HPLC system with UV detection at 220 nm. As the relative retention times in Table V indicate, norgestrel, methyltestosterone, and norethindrone acetate are all well separated from ethinyl estradiol; norethindrone coeluted with ethinyl estradiol and ethynodiol diacetate did not appear to elute from the column.

Assays for ethinyl estradiol were performed on tablets containing both ethinyl estradiol (0.04 mg/tablet) and methyltestosterone (10 mg/tablet). The chromatographic system for this analysis is given in Scheme A. The relative standard deviation of the determination of ethinyl estradiol in the presence of methyltestosterone was not greater than 1.9%, as shown in Table VI for replicate sample preparations. Figure 4 indicates that methyltestosterone causes no chromatographic interference in the assay for ethinyl estradiol.

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