Table I—Retinoic Acid Assay Calibration

Number of Samples[a]	Retinoid[b] in Sample, ng	Retinoid Detected,[c] ng	Sample Concentration, ng/ml	Peak Height Ratio ± *SD* Retinoic Acid I	Retinoic Acid II
9	10	2.5	100	0.045 ± 0.015	0.055 ± 0.02
3	20	5.0	200	0.077 ± 0.002	—
9	40	10	400	0.143 ± 0.010	0.157 ± 0.02
3	60	15	600	0.217 ± 0.002	—
9	80	20	800	0.297 ± 0.016	0.308 ± 0.038
3	100	25	1000	0.375 ± 0.007	—
9	150	37.5	1500	0.601 ± 0.016	0.581 ± 0.037
3	300	75	3000	1.231 ± 0.037	—
9	450	112.5	4500	1.821 ± 0.048	1.594 ± 0.088
3	600	150	6000	2.431 ± 0.061	—
9	750	187.5	7500	3.021 ± 0.098	2.678 ± 0.103

[a] Samples of nine were determined over 3 weeks, three samples per week. [b] Contained in 0.1 ml of rat serum. [c] Detected quantity represents the calculated theoretical quantity of retinoid injected into the chromatograph.

front (Fig. 1B). Under these conditions, the retention times for I, retinol, and III were 3.0, 3.9, and 5.9 min, respectively. In addition to the spiked serum samples, a drug-free sample was run to determine if there were any absorbing substances in the region of I (Fig. 1A). Separation of II from the internal standard, retinol, and the solvent front was achieved under the same conditions as for I (Fig. 1C). The retention time for II was 2.6 min.

The results of calibration procedures for I and II in rat serum samples are shown in Table I. Linear regression analysis of chromatogram peak height ratios of sample to standard *versus* concentration showed correlation coefficients of 0.9999 for both I and II for a sample size of 10–750 ng (100–7500 ng/ml). At a sample size of 10 ng of retinoic acid, the standard deviations for data pooled from 3 different days were ±33% for I and ±36% for II and probably represent the lower working limits for the assay. At the 80-ng sample size, reproducibility was much better, with standard deviations of 7 and 13%, respectively, for the two acids. Single-day determinations usually showed lower standard deviations.

The peak height ratios of retinoids to the internal standard at each concentration for the serum and water samples were compared to determine the percent recovery of I and II from the serum samples. The recoveries of I and II were 102.9 ± 5.6% and 91.7 ± 5%, respectively.

Figure 2 shows the applicability of the assay to monitor serum levels in rats after single doses of the retinoids. Both serum level profiles showed log-linear elimination phases with correlation coefficients of ~0.99. The elimination half-lives of I and II were 0.58 and 0.92 hr, respectively. The elimination curve for I, possibly indicating saturation or storage, tends to confirm previous observations (7).

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Quantitative Analysis of Ethynodiol Diacetate and Ethinyl Estradiol/Mestranol in Oral Contraceptive Tablets by High-Performance Liquid Chromatography

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Received August 6, 1980, from the *Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, K1A 0L2, Canada.* Accepted for publication July 7, 1981.

Abstract □ A procedure is described for the assay of ethynodiol diacetate and ethinyl estradiol/mestranol by HPLC using two UV detectors at 210 and 280 nm. The system was acetonitrile 38% (v/v) in water as mobile phase on a 250 × 3.2-mm i.d. RP-2 column, with butylated hydroxytoluene as the internal standard. There was >99% recovery from synthetic preparations and the coefficient of variation was <2.0% for formulations.

Keyphrases □ Oral contraceptives—quantitative analysis of ethynodiol, estradiol, and mestranol by high-performance liquid chromatography □ Ethynodiol—quantitative analysis by high-performance liquid chromatography, oral contraceptive tablets □ Ethinyl estradiol—quantitative analysis by high-performance liquid chromatography, oral contraceptive tablets □ Mestranol—quantitative analysis by high-performance liquid chromatography, oral contraceptive tablets □ High-performance liquid chromatography—quantitative analysis of ethynodiol, estradiol, and mestranol in oral contraceptive tablets

Ethynodiol diacetate is a synthetic steroid showing progestogenic activity. It is used in oral contraceptives in admixture with either ethinyl estradiol or mestranol. Compendial procedures (1–3) are limited to raw material or to single ingredient formulations, except the USP XX (1) which describes the analysis of the ethynodiol diace-

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Table I—Capacity Factor (K^1) for the Steroids and Other Substances

Substance	K^1
Ethinyl estradiol	3.85
Mestranol	8.08
Ethynodiol diacetate	22.65
Butylated hydroxytoluene	16.54
Unknown No. 1	8.33
Unknown No. 2	11.62
Ethynodiol diacetate acidic degradation product	27.82
Ethynodiol diacetate basic degradation products	1. 3.57 2. 8.62

tate–ethinyl estradiol mixtures.

Ethynodiol diacetate has been quantitatively assayed by argentimetric titration of its ethinyl group (2), by UV determination after its acidic conversion to a conjugated diene (1), by colorimetric reaction with antimony trichloride (3), and by GLC analysis (4).

The estrogenic components have been analyzed using such methods as argentimetry (2), colorimetry (1, 3, 5), fluorometry (2), UV spectrophotometry (6), GLC (6), TLC with fluorescent detection (7), and high-performance liquid chromatography (HPLC) (8–10).

This paper describes a simple, rapid HPLC procedure to assay quantitatively mixtures of ethynodiol diacetate with either ethinyl estradiol or mestranol using simultaneous measurements of UV absorption at 210 and 280 nm.

EXPERIMENTAL

Apparatus—A modular HPLC system consisting of a pump[1] operated at 1.75 ml/min, two variable-wavelength UV detectors (set at 210 and 280 nm, respectively)[2,3], and a 7000-psi loop injector[4] (equipped with a 20-μl loop) were used. The column (250 × 3.2-mm i.d.) was ethylsilane chemically bonded to totally porous, irregularly shaped microparticulate silica[5].

Peak retention times and areas were obtained with two reporting integrators[6].

Reagents—Ethynodiol diacetate, ethinyl estradiol, and mestranol were USP reference standards. Butylated hydroxytoluene[7] was recrystallized from methanol, acetonitrile was HPLC grade[8], and water was double-distilled in glass.

Mobile Phase—Acetonitrile 38% (v/v) in water (filtered through a membrane[9] and degassed while filtering under a vacuum not exceeding 500 mm Hg) was used.

Internal Standard Solution—A solution of butylated hydroxytoluene in aqueous acetonitrile (80% v/v) was prepared at a concentration of 50 μg/ml.

Standard Preparations—*Ethynodiol Diacetate–Ethinyl Estradiol Standard*—A solution of ethynodiol diacetate with ethinyl estradiol in internal standard solution was prepared at the same concentration as in the sample preparation (based on label claim).

Ethynodiol Diacetate–Mestranol Standard—A solution of ethynodiol diacetate with mestranol in internal standard solution was prepared at the same concentration as in the sample preparation (based on label claim).

Sample Preparation—Not less than 20 tablets were weighed and finely powdered (60 mesh). An amount of powder equivalent to one tablet was accurately weighed into a 15-ml centrifuge tube with a polytef-lined cap. Two milliliters of internal standard solution were added. The tube was capped and vigorously shaken for 30 min. The tube was then centrifuged to obtain a clear solution.

[1] Constametric II, Laboratory Data Control, Riviera Beach, FL 33404.
[2] Spectromonitor I, Laboratory Data Control, Riviera Beach, FL 33404.
[3] Schoeffel model SF 770, Westwood, NJ 07675.
[4] Rheodyne septumless valve injector model 7120, Berkeley, CA 94710.
[5] RP-2 Express series, Altex Inc., Berkeley, CA 94710.
[6] HP 3385A automation systems, Hewlett-Packard, Avondale, PA 19311.
[7] Koch-Light Laboratory, Colnbrook, England S13 0B7.
[8] Fisher Scientific, Fair Lawn, NJ 07410.
[9] FH 0.2 μm, Millipore Inc., Bedford, MA 01730.

Table II—Standard Curves for Ethinyl Estradiol, Mestranol, and Ethynodiol Diacetate

Steroid	Weight Range Injected, mg	Wavelength, nm	Curve, $y = mx + b$	Correlation Coefficient
Ethinyl estradiol	0.2–1.2	210	0.827×-0.003	0.9999
		280	0.835×-0.022	0.9998
Mestranol	0.2–1.2	210	$0.895 \times +0.002$	0.9996
		280	0.784×-0.013	0.9998
Ethynodiol diacetate	2.0–12	210	0.303×-0.007	0.9999

Recovery Study—Sufficient inert materials (4.67 g of lactose, 4.67 g of cornstarch, 0.5 g of povidone, 0.1 g of calcium stearate) to make a total weight of 10 g (after drying) were added to a solution in 10 ml of alcohol of about 2.5 mg of ethinyl estradiol, 5.0 mg of mestranol, and 50.0 mg of ethynodiol diacetate, each accurately weighed. This mixture was shaken to homogenize, then dried at room temperature using reduced pressure.

A 200-mg portion of this synthetic preparation was treated as in sample preparation.

Procedure—Aliquots (20 μl) of standard preparation or sample preparation (synthetic preparation) were successively injected into the chromatograph. The peak area ratios of ethynodiol diacetate and the estrogen to the internal standard were calculated on the 210-nm detector and the peak area ratio of the estrogen to internal standard was calculated on the 280-nm detector for both standard and sample preparations. The quantities of active ingredients per tablet were calculated using the following formula:

$$C_u = 2 \times C_s \times \frac{R_u}{R_s} \times \frac{W_t}{W_u} \qquad \text{(Eq. 1)}$$

where:

C_u = active ingredient per tablet, milligrams
C_s = concentration of active ingredient in standard preparation, milligrams per milliliter
R_u = area ratio of active ingredient to internal standard in sample preparation
R_s = area ratio of active ingredient to internal standard in standard preparation
W_u = weight of sample taken, milligrams
W_t = average weight per tablet, milligrams

RESULTS AND DISCUSSION

All chromatograms were as expected with respect to the shape of the peaks, and complete baseline resolution was achieved between the solvent front, ethinyl estradiol, mestranol, internal standard, and ethynodiol diacetate (Table I). An unidentified impurity definitely not either of the monoacetates (as determined by basic degradation) or either of the diene degradation products (as determined by acidic degradation), was detected while analyzing the ethynodiol diacetate–ethinyl estradiol formulations. It had a retention close to that of mestranol. Several solvent systems on different reversed-phase columns failed to improve separation. Spectrometric study showed its absorption maximum was at ~235 nm with no absorption at 280 nm. This impurity was found in all commercial formulations studied (two manufacturers), and it appeared to be an ethynodiol diacetate degradation product.

To overcome the problem of the same impurity (No. 1, Table I) appearing in ethynodiol diacetate–mestranol formulations, detection using 280-nm wavelength seemed more appropriate for the estrogenic component. However, since ethynodiol diacetate has no measurable absorbance at 280 nm, it was necessary to use 210 nm for its determination. A second impurity (K^1 = 11.62) did not conflict with other components in the mixture.

Linearity of response *versus* concentration was studied for the three steroids at both wavelengths (Table II). All standard curves were found to be linear in the concentration ranges studied and passed close to the origin. Their correlation coefficients were nearly ideal (>0.9996).

Table III shows the accuracy of the procedure for a synthetic preparation. Recovery was >98% for each steroid at its determination wavelength, and reproducibility was excellent (*RSD* <1.3%).

Quantitative analysis of three commercial dosage forms are listed in Table IV. All results are within compendial limits (90–110%). Coefficients of variation are <2.0%. A difference of 2.4% is reported for mestranol

Table III—Recovery of Steroids from a Synthetic Preparation

Steroid	Added, μg	Recovery at 210 nm[a] μg	%	CV	Recovery at 280 nm[a] μg	%	CV
Ethinyl estradiol	51.86	50.98	98.3	1.3	51.96	100.2	1.1
Mestranol	100.23	98.97	99.0	1.0	99.78	99.8	1.1
Ethynodiol diacetate	1015.5	1008.6	99.3	0.7	—	—	—

[a] Average of five determinations.

Table IV—Assay of Commercial Tablets[a]

Steroid	Wavelength Setting, nm	Product A % Label Claimed	CV	Product B % Label Claimed	CV	Product C % Label Claimed	CV
Ethinyl estradiol	210	99.9	1.2	101.3	1.3	—	—
	280	101.3	1.9	102.5	1.8	—	—
Mestranol	210	—	—	—	—	103.2	1.3
	280	—	—	—	—	100.8	0.8
Ethynodiol diacetate	210	96.5	0.9	95.2	0.9	92.4	0.5

[a] Average of 10 determinations.

analyses between the two wavelengths used, whereas analysis of the synthetic formulation revealed a variation of 0.8%, which is relatively minor. Variations reported for ethinyl estradiol (~1.5%) are the same for tablets and the synthetic formulation.

CONCLUSIONS

This HPLC procedure, using dual wavelength detection, is fast and accurate. It has been specially designed for single dosage form analysis as required in the USP–NF content uniformity test, and would advantageously replace the long, open-column chromatography procedure.

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Effect of the pH–Zero Point of Charge Relationship on the Interaction of Ionic Compounds and Polyols with Aluminum Hydroxide Gel

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Abstract □ The adsorption of magnesium nitrate, docusate sodium, and mannitol by chloride-containing aluminum hydroxide gel or aluminum hydroxycarbonate gel can be directly related to the surface charge characteristics of the aluminum hydroxide gel as determined by the pH–zero point of charge (ZPC) relationship. Magnesium cation is completely adsorbed under pH conditions where the gel has a negative surface charge, *i.e.*, when the pH is above the ZPC. Docusate sodium is more strongly adsorbed when the pH–ZPC relationship causes the surface charge of aluminum hydroxycarbonate gel to be positive indicating adsorption of the docusate anion. However, adsorption also occurred when the pH was above the ZPC suggesting that adsorption of the hydrophobic portion of docusate anion by van der Waals forces also contributes to the overall adsorption mechanism. Mannitol is adsorbed under all pH conditions. However, greater adsorption occurs when the pH is above the ZPC. Maximum hydrogen bonding is believed to occur when mannitol acts as the proton donor and the negative aluminum hydroxycarbonate gel surface serves as the proton acceptor.

Keyphrases □ Aluminum hydroxide—gel, adsorption of ionic compounds and polyols, effect of pH–zero point of charge □ Aluminum hydroxycarbonate—gel, adsorption of ionic compounds and polyols, effect of pH–zero point of charge □ Adsorption—ionic compounds and polyols to aluminum hydroxide and aluminum hydroxycarbonate, effect of pH–zero point of charge

The zero point of charge (ZPC) is an important property of colloidal systems possessing a pH-dependent surface charge. The ZPC is the pH at which the net surface charge is zero; at this pH the densities of the positive and negative charges are equal. The apparent surface charge can be controlled by adjusting the pH to be either below or above the ZPC to produce a positive or negative surface charge, respectively (1). A recent study demonstrated the impor-

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