Table II—Comparison between Dithiocarbamate Method and Annino's Method (7).

Preparation	Dithiocarbamate Method: Labeled Amount, mg./ml.	Dithiocarbamate Method: Amount Found, mg./ml.	Dithiocarbamate Method: Percent Recovery	Annino's[a] Method, Percent Recovery
1. Eye drops containing physostigmine salicylate, methylcellulose, sodium bisulfite, sodium chloride, chlorobutanol, and citric acid	5	5.046	102.0	100.0
2. Injection containing physostigmine salicylate, sodium bisulfite, and benzyl alcohol	4	3.41	85.5	86.9
3. Injection containing physostigmine salicylate, l-hyoscyamine hydrobromide, benzyl alcohol, and sodium chloride	0.6	0.591	98.66	98.5

[a] Calculated as physostigmine.

reference (4) stated that tertiary aliphatic amines, aromatic amines, and secondary aliphatic–aromatic amines give a negative test, several alkaloids and other substances with primary, tertiary, and quaternary nitrogen groups were found that give a positive test, including atropine, pilocarpine, benzethonium chloride, urea, and some amino acids. Substitution of sodium bicarbonate for ammonium hydroxide in the procedure increased the specificity, eliminating positive reactions with all these chemicals except pilocarpine. The use of the pH 8.6 buffer further increased the specificity, with only physostigmine giving the color.

Different amounts of physostigmine were determined using this method. Recovery data are listed in Table I.

Three commercial preparations containing physostigmine were analyzed using this method. The results are listed in Table II.

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Separation of Quingestanol Acetate from Sesame Oil Solution and Its Determination in Combinations with Ethinyl Estradiol or Quinestrol

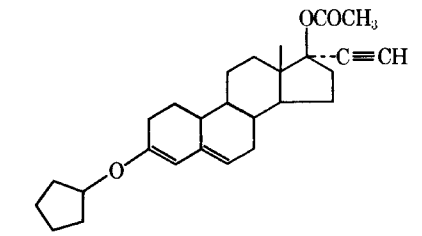
MELVIN H. PENNER, DIMITRI C. TSILIFONIS, and LESTER CHAFETZ

Abstract □ Quingestanol acetate, norethindrone acetate 3-cyclopentyl enol ether, was not separable from its sesame oil dosage form vehicle as the intact compound using chromatography procedures that depend on differences in polarity. The steroid can be retained quantitatively on a Florisil column treated with a relatively high concentration of ammoniacal silver nitrate and then quantitatively eluted with ethanolic ammonium chloride. The progestin can be determined selectively in the presence of its products of solvolytic or oxidative degradation by UV spectrophotometry after treatment with sodium borohydride; the chromophore of the decomposition products is eliminated by this process while that of the intact compound is unaffected. Quinestrol or ethinyl estradiol, which may be coformulated with the progestin, does not interfere with its determination. Methods for the quantitative determination of the declared steroids in quingestanol acetate formulations and for the estimation of degradation products of the progestin are presented.

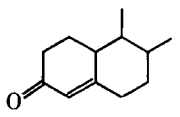
Keyphrases □ Quingestanol acetate capsules, combinations with ethinyl estradiol or quinestrol—separation, analysis □ Sesame oil formulations—separation of quingestanol acetate □ Column chromatography—separation, quingestanol acetate in capsules □ UV spectrophotometry—analysis, quingestanol acetate □ TLC—monitoring, quingestanol acetate separation

Quingestanol acetate (I), the cyclopentyl enol ether of norethindrone acetate (II), was shown to be a potent progestational and antiestrual agent (1, 2). As is typical of enol ethers, quingestanol acetate is sensitive to acid-catalyzed solvolysis. Both norethindrone acetate, the solvolysis product, and 6-hydroxynorethindrone acetate (III), formed by autoxidation, have been detected in aged samples of the drug and its dosage forms, but no other decomposition products have been observed. Optimum stability and activity of quingestanol acetate

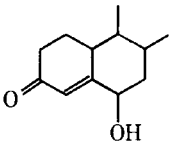
have been achieved by formulating it as sesame oil solutions, stabilized with piperidine (3) and enclosed in soft gelatin capsules.



I



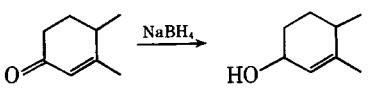
II



III

Because of the very lipophilic character imparted to the progestin molecule by the cyclopentyl enol ether function, all previous attempts to separate quingestanol acetate from sesame oil were unsuccessful. The drug migrated with the oil in some systems and decomposed in others. Its more polar decomposition products, however, could be separated from the oil by any of several paper chromatographic or TLC methods (4–9). A paper chromatographic method, similar to that described by Talmage *et al.* (4) for progesterone 3-cyclopentyl enol ether, was used in these laboratories for several years. In this procedure, two aliquots of diluted sesame oil solution are subjected to paper chromatography, one directly and the other after acid hydrolysis. The difference in the values obtained by elution and colorimetric measurement of the zones corresponding to norethindrone acetate in the two chromatograms is taken as the measure of intact quingestanol acetate, and the decomposition products are measured by elution and colorimetry of the appropriate zones in the chromatogram from the dilution obtained without preliminary hydrolysis. Although this method affords reliable results in the hands of an experienced worker, it is time consuming and requires meticulous technique.

Görög (10) described a differential UV spectrophotometric procedure for Δ^4-3-ketosteroids, applicable in the presence of other types of steroids and some oil vehicles. The absorbance of an aliquot of an alcoholic dilution of steroid added to decomposed sodium borohydride is determined *versus* another where the UV chromophoric system has been destroyed by reduction with sodium borohydride (Scheme I).



Scheme I

Quingestanol acetate has a UV chromophore system similar to those of its decomposition products (Fig. 1). The enol ether function, however, is stable in the alkaline medium afforded by the reducing agent. Unlike its decomposition products, its UV absorption is unaffected by treatment with borohydride. Thus, the intact drug can be determined selectively in the presence of its degradation products. Unfortunately, sesame oil also exhibits a differential spectrum in the borohydride procedure, so the method is inapplicable to quingestanol acetate dosage forms. A simple procedure was developed in which the oil solution was hydrolyzed with alcoholic hydrochloric acid; the norethindrone acetate, formed by hydrolysis of the enol ether, was separated from the oil by partition between aqueous methanol and isooctane, and it was determined by Görög's method. This assay surmounted many of the problems encountered with the paper chromatographic procedure, but it shared the failing that it provided only an indirect measure of intact drug.

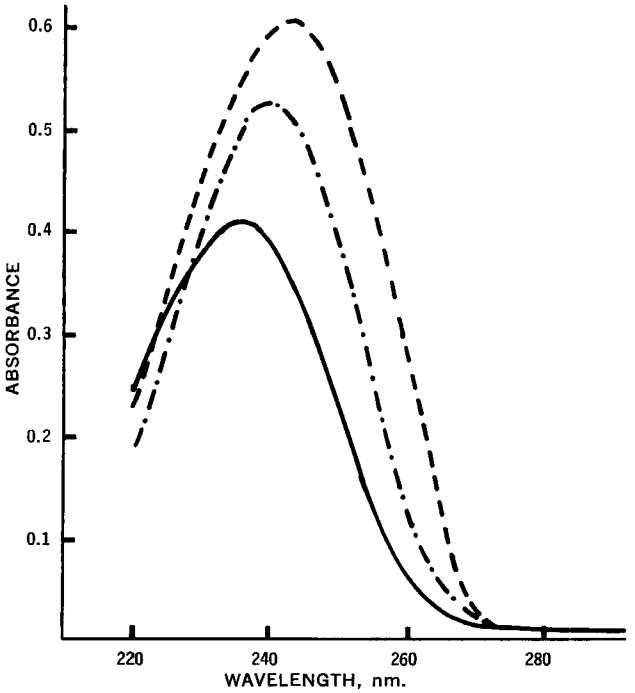


Figure 1—*Spectra of quingestanol acetate (- - -), norethindrone acetate (·-·), and 6β-hydroxynorethindrone acetate (——), all at concentrations of about 10 mcg./ml. in alcohol.*

In contradistinction to chromatographic methods dependent on polarity differences for separation, the chromatographic methods for 17-ethynyl steroids reported by Ercoli *et al.* (11) depend on a specific structural feature. They reported that chromatographic adsorbents impregnated with metallic silver or silver nitrate bound 17α-ethynyl steroids so that other lipoidal materials could be washed away. They described silvered silica gel G for TLC or Florisil for column chromatography, prepared by reducing ammoniacal silver nitrate with glucose, and silica gel G plates impregnated with silver nitrate solution. The silvered materials gave variable results in a number of trials in this laboratory. Kulkarni and Goldzieher (12), however, reported that the silvered Florisil column provided quantitative recoveries of ethinyl estradiol after elution with ethanolic ammonium chloride solution. We reasoned that the efficiency of the preferential adsorption of ethynyl steroids on silvered chromatographic adsorbents depends on the number of sites of silver ion present, and

that variable results in adsorbing these compounds could be ascribed to too efficient reduction of silver ion to metallic silver. Based on this rationale, we found that quingestanol acetate could be retained quantitatively on a Florisil column impregnated with ammoniacal silver ion, desorbed by elution with ammonium chloride in alcohol, and determined by an extension of the Görög method. This report describes methods for the determination of quingestanol acetate and its combinations with ethinyl estradiol or quinestrol in soft gelatin capsule dosage forms.

EXPERIMENTAL

Reagents and Supplies—The absolute methanol and ethanol, acetone, sodium borohydride, sulfuric acid, silver nitrate, ammonium chloride, ammonium hydroxide, and sodium sulfate used were reagent grade chemicals. Phillips "pure" grade isooctane was found satisfactory for use in colorimetry of the estrogens. The steroids used were quingestanol acetate and quinestrol reference standards[1], ethinyl estradiol reference standard USP, norethindrone acetate NF[2], and 6β-hydroxynorethindrone acetate[2]. Dosage forms declaring quingestanol acetate alone, quingestanol acetate with ethinyl estradiol, and progestin with quinestrol were available as clinical test supplies and stability samples.

The separators used were fitted with Teflon stoppers and stopcocks. Disposable glass columns[3], 0.65 cm. (0.25 in.) i.d. and 30.5 cm. (12 in.) long, were used for chromatography. Confirmatory TLC experiments were run using chromatoplates[4] coated with 0.25-mm. layers of alumina F. The plates were activated before use by heating at 105° for 30 min. UV spectrophotometry was performed using 1-cm. silica cells in a Beckman DU fitted with the Gilford model 222 modification or a Cary model 14 recording instrument.

Assay of Quingestanol Acetate Capsules—*Column Adsorbent Preparation*—Transfer about 10 g. of Florisil, 100–200 mesh, to a 250-ml. conical flask containing 100 ml. of distilled water. Add 50 ml. of a 2 in 100 solution of silver nitrate in equal volumes of water and concentrated ammonium hydroxide; stopper the mixture, and shake it mechanically for 30 min. Filter it through a büchner funnel, transfer the solid to a crystallizing dish, and dry it overnight at 105°. Protect the adsorbent from light by storage in a low actinic vessel.

Ethanolic Ammonium Chloride—Dissolve 100 mg. of ammonium chloride in 5 ml. of water. Dilute the solution to 500 ml. with absolute ethanol.

Benzene–Acetone Solvent—Mix equal volumes.

Standard Preparation—Dissolve an accurately weighed quantity of quingestanol acetate reference standard in benzene–acetone. Dilute the solution quantitatively and stepwise with this solvent mixture to obtain a concentration of about 0.6 mg./ml.

Assay Preparation—Determine the average weight of the contents of not fewer than 20 capsules. Weigh an amount equivalent to about 3 mg. of quingestanol acetate into a 5-ml. volumetric flask, and dilute the sample to volume with benzene–acetone.

Procedure—Place a pledget of glass wool at the bottom constriction of each of two chromatography columns. Percolate slurries of 1.0 g. of adsorbent in 15 ml. of benzene–acetone into each of the columns, allowing the solvent to descend into the top of the adsorbent beds. Wrap the columns with aluminum foil to shield them from light.

Concomitantly transfer 2.0 ml. of the assay preparation and 2.0 ml. of the standard preparation to the columns. Allow them to percolate into the upper portion of the adsorbent; successively wash the columns with single 50-ml. portions of benzene–acetone and 10-ml. portions of absolute ethanol. Discard the washings. Elute the columns with ethanolic ammonium chloride, receiving the eluates in 50-ml. volumetric flasks, until the receiving vessels are nearly to volume. Adjust the eluates to the mark with eluant.

[1] Warner-Lambert.
[2] Warner-Vister Steroid Research Institute, Casatenovo (Como), Italy.
[3] Bio-Rad Laboratories.
[4] Analtech, Inc.

Table I—Spectral Data for Quingestanol Acetate and Its Decomposition Products in Ethanol

Steroid	$\lambda_{max.}$, nm.	a	ϵ
Quingestanol acetate	243	48.8	20,000
Norethindrone acetate	238	50.1	17,000
6β-Hydroxynorethindrone acetate	236	41.2	14,660

Pipet 10-ml. portions of the eluates from the assay preparation and the standard preparation and 10 ml. of ethanolic ammonium chloride into each of three 25-ml. volumetric flasks charged with 10 mg. of sodium borohydride. Allow the mixtures to stand for 30 min.; then dilute the solutions to the mark with absolute alcohol. Determine the absorbance of the solutions from the assay preparation and the standard preparation in 1-cm. cells at the wavelength of maximum absorbance (about 243 nm.), using the diluted ethanolic ammonium chloride solution as the blank. Calculate the quantity of $C_{27}H_{36}O_3$, in milligrams per capsule, from the formula $5C(A_U/A_S)(B/SW)$, where C is the concentration, in milligrams per milliliter, of quingestanol acetate reference standard in the standard preparation; A_U and A_S are the absorbances of the solutions from the assay preparation and the standard preparation, respectively; B is the average weight, in milligrams, of the capsules; and SW is the weight, in milligrams, of the sample taken for assay.

Assay of Quingestanol Acetate–Quinestrol Capsules—*Methanol–Sulfuric Acid*—Cautiously add sulfuric acid, in small increments and with mixing, to 30 ml. of chilled anhydrous methanol in a 100-ml. volumetric flask. Adjust to room temperature, dilute with sulfuric acid to volume, and mix.

Standard Preparation—Dissolve accurately weighed amounts of quingestanol acetate and quinestrol reference standards in benzene–acetone solvent, and dilute the solution quantitatively and stepwise to obtain concentrations of about 0.5 mg./ml. of quingestanol acetate and 0.4 mg./ml. of quinestrol.

Assay Preparation—Determine the average weight of the contents of not fewer than 20 capsules. Weigh an amount of capsule contents equivalent to about 5 mg. of quingestanol acetate into a 10-ml. volumetric flask, and dilute the sample to volume with benzene–acetone solvent.

Procedure for Quingestanol Acetate Determination—Follow the *Procedure* given under *Assay of Quingestanol Acetate Capsules*, through: "Discard the washings." Elute the columns with ethanolic ammonium chloride, receiving the eluates in 100-ml. volumetric flasks, until the receiving vessels are nearly to volume. Adjust the eluates to the mark with eluant.

Pipet 10-ml. portions of the eluates from the assay preparation and the standard preparation and 10-ml. of ethanolic ammonium chloride into each of three 25-ml. volumetric flasks charged with 10 mg. of sodium borohydride. Allow the solutions to stand for 30 min.; then determine the absorbance of the solutions from the assay preparation and the standard preparation in 1-cm. cells at the wavelength of maximum absorbance (about 243 nm.), using the ethanolic ammonium chloride solution as the blank. Calculate the quantity of $C_{27}H_{36}O_3$, in milligrams per capsule, from the formula $10C(A_U/A_S)(B/SW)$, where the symbols have the definitions given previously.

Procedure for Quinestrol Determination—Pipet 3.0-ml. portions of the eluates from the assay preparation and the standard preparation into 125-ml. separators. Add 25 ml. of water and 4 drops of concentrated hydrochloric acid to each, and extract the mixtures with 30

Table II—Recovery of Quingestanol Acetate Added to Sesame Oil

Milligrams Added	Milligrams Found	Percent Recovered
3.30	3.22	97.6
	3.26	98.6
	3.37	102.0
3.09	3.09	100.0
	3.08	99.6
3.05	3.00	98.3
	3.08	100.9
	3.02	99.1
		Mean = 99.7
		RSD = ±1.4%

ml. of isooctane. Filter the upper layers through beds of anhydrous sodium sulfate supported on a filter funnel, wash the sodium sulfate layer with an additional 5 ml. of isooctane, and collect the filtrates in scrupulously dry 60-ml. separators. Add 5.0 ml. of methanol–sulfuric acid, and shake vigorously for 2 min. Allow the layers to separate, and withdraw about 4.5 ml. of the colored lower phase. Transfer 4.0-ml. portions to dry glass-stoppered centrifuge tubes, add exactly 0.5 ml. of anhydrous methanol to each, mix well, and clarify by centrifugation. Concomitantly determine the absorbances of the solutions from the assay preparation and from the standard preparation in 1-cm. cells in a suitable spectrophotometer at the wavelength of maximum absorbance (about 540 nm.). Calculate the quantity of $C_{25}H_{32}O_2$, in milligrams per capsule, by the formula $10C(A_U/A_S)(B/SW)$, where C is the concentration, in milligrams per milliliter, of quinestrol in the standard preparation, and the other symbols have the definitions given previously.

Assay of Quingestanol Acetate–Ethinyl Estradiol Capsules—*Standard Preparation*—Dissolve accurately weighed amounts of quingestanol acetate reference standard and ethinyl estradiol reference standard USP in benzene–acetone solvent. Dilute the solution quantitatively and stepwise to obtain concentrations of about 0.8 mg./ml. of quingestanol acetate and about 0.08 mg./ml. of ethinyl estradiol.

Assay Preparation—Determine the average weight of the contents of not fewer than 20 capsules, and weigh an amount equivalent to about 4 mg. of quingestanol acetate into a 5-ml. volumetric flask. Dilute the contents to volume with benzene–acetone solvent.

Procedure for Quingestanol Acetate Determination—Follow the *Procedure* given under *Assay of Quingestanol Acetate Capsules*, but transfer 3.0-ml. volumes of the assay preparation and the standard preparation to the columns. Continue through: "Adjust the eluates to the mark with eluant."

Pipet 5-ml. portions of the eluates from the assay preparation and the standard preparation and 5-ml. of ethanolic ammonium chloride into each of three 25-ml. volumetric flasks. Follow the *Procedure* given under *Assay of Quingestanol Acetate Capsules*, beginning with: "Allow the mixtures to stand for 30 min...."

Procedure for Ethinyl Estradiol Determination—Pipet 4.0-ml. portions of the eluates from the assay preparation and the standard preparations into 125-ml. separators. Add 25 ml. of water and 2 ml. of 12 N sulfuric acid to each, and extract with 25 ml. of carbon tetrachloride. Filter the lower layers through beds of anhydrous sodium sulfate supported on a filter funnel, wash the funnels with 25-ml. portions of isooctane, and combine the filtrates in clean, dry, 125-ml. separators. Follow the *Procedure for Quinestrol Determination*, beginning with: "Add 5.0 ml. of methanol–sulfuric acid...." Calculate the quantity of $C_{20}H_{24}O_2$, in milligrams per capsule, by the formula $5C(A_U/A_S)(B/SW)$, where C is the concentration, in milligrams per milliliter, of ethinyl estradiol in the standard preparation; A_U and A_S are the absorbances of the solutions from the assay preparation and the standard preparation, respectively; and B and SW have the definitions given previously.

Estimation of Norethindrone Acetate and 6β-Hydroxynorethindrone Acetate—*Standard Preparation*—Dissolve equal and accurately weighed quantities of authentic specimens of norethindrone acetate and 6β-hydroxynorethindrone acetate in a mixture of equal volumes of acetone and methanol. Dilute the solution quantitatively and stepwise to obtain a series of solutions containing 100-, 80-, 60-, 40-, and 20-mcg./ml. concentrations of each of the decomposition products of quingestanol acetate.

Assay Preparation—Transfer an aliquot of the eluant obtained in the assay, equivalent to about 1 mg. of quingestanol acetate, to a 50-ml. glass-stoppered, round-bottom flask; evaporate it to dryness under reduced pressure. Carefully dissolve the residue in 0.5 ml. of a mixture of equal volumes of acetone and methanol.

Procedure—At equidistant points along a line 2.5 cm. from the lower edge of a 20 × 20-cm. TLC plate, coated with a 0.25-mm. layer of aluminum oxide F_{254} and previously activated by heating at 105° for 30 min., spot 50 μl. each of the standard preparations (corresponding to 1, 2, 3, 4, and 5 mcg. of each standard) and 50 μl. of the assay preparation (corresponding to 100 mcg. of quingestanol acetate). Place the plate in a chamber lined with filter paper on three sides and previously equilibrated with a 5 in 200 solution of anhydrous methanol in benzene. Allow the solvent front to ascend about 15 cm. above the line of application. Remove the plate from the chamber, mark the front, and dry it at 105° for 5 min. View the chromatogram under shortwave UV light in a suitable apparatus; compare the intensity of the norethindrone acetate and 6β-hydroxynorethindrone acetate zones, if present in the lanes for the assay preparation spot, with the zones for the standard preparations. Norethindrone acetate and 6β-hydroxynorethindrone acetate appear as UV-absorbing zones at R_f values of about 0.55 and 0.25, respectively.

Table III—Recovery of Quingestanol Acetate and Quinestrol Added to Sesame Oil

Quingestanol Acetate			Quinestrol		
Milligrams Added	Milligrams Found	Percent Recovered	Milligrams Added	Milligrams Found	Percent Recovered
5.26	5.30	100.9	4.12	4.19	101.7
5.12	5.13	100.1	4.10	4.17	101.9
	5.13	100.1		4.21	102.5
5.11	5.13	100.3	4.12	4.18	101.6
	5.13	100.3		4.22	102.3
	5.09	99.7		4.12	100.0
	5.09	99.7		4.14	100.4
	Mean =	100.2		Mean =	101.5
	RSD =	±0.4%		*RSD* =	±0.9%

RESULTS AND DISCUSSION

Selectivity of the Method—The elution of the column was monitored by TLC. No steroid could be detected in the washings of the column until it was eluted with ethanolic ammonium chloride. Quingestanol acetate was eluted as a single compound, having identical R_f with quingestanol acetate reference standard. Subjecting a portion of the column effluent to hydrolysis before TLC led to formation of norethindrone acetate, a further confirmation that quingestanol acetate was eluted as the intact enol ether.

UV spectra of quingestanol acetate and its two degradation products are shown in Fig. 1. The spectral data obtained for the three compounds are presented in Table I. It is evident from these data that decomposition of quingestanol acetate could be detected by direct UV only if it had proceeded to a significant extent, and the amount of decomposition would be underestimated. Use of sodium borohydride reduction, however, eliminates interference by the degradation products without affecting the spectra of the enol ether. Neither of the estrogens, ethinyl estradiol or quinestrol, interferes at the wavelength of maximum absorbance for quingestanol acetate. Since the UV spectra of estrogens are not affected by treatment with borohydride, one could eliminate interferences by such compounds, if they were observed, by differential measurement. That is, pipet two aliquots of column effluent into separate volumetric flasks. Then:

1. Add sodium borohydride to the first, destroy excess reducing agent, and dilute to volume.
2. Hydrolyze quingestanol acetate to norethindrone acetate with alcoholic hydrochloric acid, neutralize, add sodium borohydride, destroy excess borohydride, and dilute to volume.
3. Determine the absorbance of the solution prepared in Step 1 *versus* the solution prepared in Step 2.

The selectivity of the methods used for the estrogens was reported previously by Chafetz *et al.* (13). The TLC procedure provided for estimation of the quingestanol acetate decomposition products was employed to confirm decomposition and to determine whether degradation, when observed, was due to hydrolysis or oxidation.

Table IV—Recovery of Quingestanol Acetate and Ethinyl Estradiol Added to Oil

Quingestanol Acetate			Ethinyl Estradiol		
Milligrams Added	Milligrams Found	Percent Recovered	Milligrams Added	Milligrams Found	Percent Recovered
3.51	3.45	98.1	0.361	0.361	100.0
	3.51	100.0		0.367	101.8
4.08	4.07	99.7	0.400	0.393	98.4
	4.04	98.9		0.402	100.4
	4.02	98.6		0.395	98.7
	Mean =	99.1		Mean =	99.9
	RSD =	±0.8%		*RSD* =	±1.4%

Table V—Assay Values on Some Quingestanol Acetate Formulations

Steroid Declared	mg./Capsule	Storage	Found, mg./Capsule
Quingestanol acetate	0.750	Initial	0.772, 0.769, 0.775, 0.766
Quingestanol acetate	0.30	19 months, RT	0.305, 0.308, 0.305, 0.305
Quingestanol acetate	2.5	Initial	2.64, 2.56, 2.45
With quinestrol	2.0		2.09, 2.08, 2.08
Quingestanol acetate	0.50	28 months, RT	0.474, 0.470
With ethinyl estradiol	0.05		0.054, 0.055

Precision and Recovery—Analytical samples for each formulation were prepared by dissolving accurately weighed amounts of the steroids in benzene–acetone, pipeting aliquots to volumetric flasks containing 1 ml. of sesame oil, and diluting the solutions to volume with the benzene–acetone solvent mixture. The procedures described were used to obtain the data in Tables II, III, and IV. Overall, the recovery of quingestanol acetate was 99.7%. Recoveries of the estrogens were 101.5% for quinestrol and 99.9% for ethinyl estradiol.

Application to Laboratory Formulations—Typical data obtained with formulations available as clinical supplies or as stability testing samples are presented in Table V.

SUMMARY

The problem of separating intact quingestanol acetate from its sesame oil vehicle had proved intractable owing to the very lipophilic character imparted to the steroid molecule by its 3-cyclopentyl enol ether function. A reinvestigation of the chromatographic methods described by Ercoli *et al.* (11), which provided selective adsorption of 17α-ethynyl steroids on silvered or silver nitrate impregnated supports, led to the finding that reliable separations of 17α-ethynyl steroids from other lipids could be obtained using a relatively high concentration of ammoniacal silver nitrate adsorbed on Florisil. After desorption with ethanolic ammonium chloride, quingestanol acetate is determined selectively by UV spectrophotometry, using a modification of the Görög technique (10). The UV chromophore of the degradation products is destroyed by reduction with sodium borohydride without affecting the spectra of the enol ether conjugated system in the intact compound. Coformulated quinestrol or ethinyl estradiol is not affected by this treatment; however, they do not interfere. Procedures for the determination of quingestanol acetate, estimation of its products of hydrolytic and oxidative decomposition, and selective determination of coformulated estrogens in dosage forms are presented.

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