The detector response with varying concentrations of terpin hydrate and biphenyl followed a linear relationship and the minimum measurable quantity was 5 nanograms.

The effect of column temperature on retention time was investigated and a linear relationship obtained (Fig. 2). The equation calculated by the method of least squares is $\log t_r = -0.014\ T_c + 4.7$, where t_r = retention time in seconds and T_c is the column temperature in °C. A plot of the log of the retention volume *versus* $1/T$, where T = absolute temperature, also yielded a linear relationship. The apparent ΔH_s was calculated from the equation $\log V_R = -\Delta H_s/2.3RT + C$ and found to be −12.4 Kcal./mole, where the slope of the resulting straight line was determined by the method of least squares.

A number of products containing terpin hydrate was assayed. The results and recovery data are presented in Table I. The precision is ±3%.

Under the conditions described, no interference in the analysis of the samples was encountered. Apparently dextromethorphan hydrobromide and codeine were not eluted, or were in too low a concentration to result in a measurable detector response. However, it should be pointed out that measurable interference due to glycerol will occur unless complete elution of this compound from the column is carried out prior to subsequent injections. The retention time of glycerol is approximately 12 min. Continued use of the column will result in the build-up of sugar which tends to skew the elution peaks and decrease the precision of the method. When this occurs, the column should be replaced.

SUMMARY

A gas–liquid chromatographic method for the determination of terpin hydrate in elixirs has been developed. The procedure involves diluting the sample with ethanol, adding an internal standard, biphenyl, and chromatography.

The precision of the developed method was ±3% and duplicate analyses can be completed within 1 hr.

Detection levels, interferences, and recovery data are presented.

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Keyphrases

Terpin hydrate elixirs
Codeine terpin hydrate elixir—analysis
Dextromethorphan terpin hydrate hydrobromide elixir—analysis
GLC analysis

Technical Articles

Unit Tablet Assay of 17α-Ethynylestradiol-3-methyl Ether (Mestranol) by a Direct Colorimetric and an Automated Fluorometric Method

By J. P. COMER, P. HARTSAW, and C. E. STEVENSON

Unit tablet assays of 17α-ethynylestradiol-3-methyl ether (mestranol) were obtained with optimum precision and accuracy by direct dissolution of sample tablets and standardized reference tablets in sulfuric acid–methanol (70:30) reagent. An automated method which utilized basic automatic analyzer (AutoAnalyzer) components and a Turner fluorometer was more rapid but less precise than the direct method.

THE ASSAY of low dosage combination steroid tablets has been a challenge to several analysts. Gänshirt and Polderman (1) separated ethinyl estradiol from tablet excipients and decomposition products by thin-layer chromatography (TLC), and measured the color formed with sulfuric acid–water (80:20) with a relative standard deviation (RSD) of ±3.2%. Comer (2) mentioned the utility of the sulfuric acid–methanol (70:30) reagent for the determination

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of 17α-ethynylestradiol-3-methyl ether (mestranol) in combination with 6-chloro-6-dehydro-17-α-acetoxy progesterone (chlormadinone acetate). The reagent had been used for several years for routine control assays of mestranol.

Schultz (3) compared a gas chromatography (GC) method and a TLC method for mestranol in the presence of chlormadinone acetate. Recoveries of 96.4 and 98.2% with a 1.5-hr. assay time by GC and 95.5% with a 3–4 hr. assay time for TLC were reported on tablets containing 80 mcg. of mestranol. The mestranol, separated by TLC, was measured at 278 mμ. Boughton *et al.* (4) formed the trimethylsilyl ethers of ethinyl estradiol and 17-β-hydroxy-6-α-methyl-17-(1-propynyl]-androst-4-en-3-one and separated them by GC. An accuracy of 98% with RSD of ±8.6% was reported for 100-mcg. ethinyl estradiol tablets by the GC method.

Heusser (5) separated mestranol and chlormadinone acetate on Silica Gel G using ether–cyclohexane (8:2) as the solvent. After extraction of the silica gel the mestranol was determined colorimetrically with 1.5% titanium chloride in sulfuric acid solution. Using composite samples of 20 tablets containing 100 mcg. of mestranol, Heusser obtained an accuracy of 97.9% and a RSD of ±4.2% from 20 determinations. Tsilifonis and Chafetz (6) reported on a similar method for ethinyl estradiol. Cali and Khoury (7) then presented their studies on an automated method for ethinyl estradiol and mestranol. This paper presents a direct method and an automated method which offered advantages in economy, precision, and accuracy over previous methods for unit tablet assays.

EXPERIMENTAL

Direct Method—Many problems normally involved in the quantitative measurement of microgram amounts of tablet ingredients were simplified by the utilization of the properties of a simple tablet formulation of the two steroids with sodium bicarbonate. During the assay development, it was found that when unit tablets were ground prior to sampling, adsorption of mestranol to the grinding vessel became a significant error. Dissolution of the whole tablet in an aqueous solution and subsequent addition of the color reagent resulted in considerable retardation of color formation. The dissolution of the whole tablet directly in the color reagent was achieved. However, quantitation by the addition of mestranol standards dissolved in methanol to the color reagent proved unsuccessful due to a dilution of the color reagent and the change in the sulfuric acid–methanol concentrations. Color development of dry residue mestranol standards gave erratic results. Quantitation was obtained when a tablet of known concentration was dissolved under the same conditions as sample tablets.

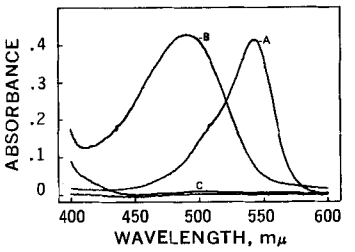

Fig. 1—Spectra in sulfuric acid–methanol (70:30) reagent. Key: A, *mestranol tablet at 5° for 2 hr.;* B, *chlormadinone acetate tablet at 25° for 2 hr.;* C, *chlormadinone acetate tablet at 5° for 2 hr.*

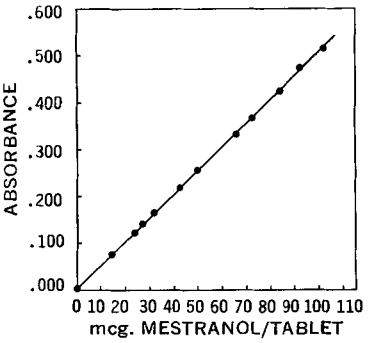

Fig. 2—Absorbance versus *concentration.*

The method thus evolved in which a weighed single tablet is placed into a 25-ml. conical flask containing 20 ml. of the sulfuric acid–methanol (70:30) color reagent which is situated in an ice bath equipped with a magnetic stirring assembly. The solution is stirred with a magnetic stirring bar, and the mestranol color developed simultaneously as the tablet dissolves. The absorbance of the clear solution containing the completely reacted tablet is then measured at 545 mμ *versus* a reagent blank in 5-mm. cells. The spectrum is shown in curve A of Fig. 1. When the color reaction is carried out in the presence of chlormadinone acetate, the mestranol color development process must be carried out at a reduced temperature or a positive interference occurs due to a reaction of chlormadinone acetate with the color reagent. Spectra B and C show the interference of chlormadinone acetate at 25° and 5°.

The linearity of the color reaction was determined by placing weighed portions of reference tablets into the color reagent. The tablets had previously been standardized in mcg. steroid/Gm. by a manual multiple chloroform extraction technique performed by several analysts on several days. The data in Fig. 2 illustrate the conformance to Beer's law. The RSD of the dozen replicates listed in Table I was 1.05% including tablet heterogeneity.

For routine control purposes, nine sample tablets and three reference tablets are run simultaneously in a special stirrer—ice bath apparatus illustrated in Fig. 3. The stirrers are a series of 12 small clock

TABLE I—PRECISION DATA ON UNIT TABLET ASSAYS

Colorimetric Method Abs./Gm. Tablet	Automated Method Peak Ht./Gm. Tablet
3.45	3.93
3.46	3.85
3.49	3.84
3.49	3.88
3.46	4.00
3.52	3.96
3.52	3.86
3.49	3.95
3.42	3.88
3.49	3.85
3.40	3.90
3.46	3.91
Av. 3.47	Av. 3.90
RSD 1.05%	RSD 1.30%

Fig. 3—Magnetic stirrer–ice bath apparatus.

motors with attached magnets. The motors are mounted on the bottom of an inverted aluminum cake pan. Nonmagnetic metal grids were placed in an upright pan to separate the flasks (8). The individual tablet weights and absorbance values are submitted to a computer and various parameters printed out. The data in Table II are a condensation of computer information from several of these nine tablet replicates on various lots of tablets fron export areas. These are expressed in terms of the RSD of mcg. of mestranol/Gm. tablet to remove variance of tablet weight. The comparison statistic is the assay value of a conventional extraction assay on a composite of 10 tablets. The direct method described yielded about six samples/hour/analyst counting reagent preparation and clean-up time and produced the optimum accuracy and precision over many other methods evaluated for unit tablet assay for control purposes.

Automated Method—An automated procedure was desired that would minimize variation and permit a greater number of unit tablet assays. Cali and Khoury (7) outlined an automated fluorometric assay using 90% sulfuric acid. Jakovljevic (9) had previously utilized the author's color reagent (70:30) for the fluorometric assay of mestranol in urine.

The excitation spectrum at an emission of 556 mμ and the emission spectrum at an excitation of 526 mμ for the automated sulfuric acid reagent for mestranol are shown in Fig. 4. This method utilizes basic automatic analyzer[1] components and a Turner model 111 fluorometer with a flow cell door conversion kit.[2]

The description below follows the flow diagram in Fig. 5. A weighed tablet is dropped into the blender of the Solid prep unit,[2] and 120 ml. diluent [H_2O–denatured alcohol (100 parts alcohol + 5 parts methanol) 85:15] are added. Dissolution of the tablet in the blender occurs, and an air segmented sample is withdrawn. The air is then debubbled at the A_2 glass fitting. An unsegmented portion of the sample stream is pumped to a 0.038 in. (0.965 mm.) i.d. Teflon mixing coil where it is segmented into a stream of ethylene dichloride. This coil was described by Kuzel (10). It consists of 36 loops of Teflon tubing wound around an empty chart paper roller. After leaving the first Teflon extraction coil, the aqueous portion is desegmented from the ethylene dichloride stream by pumping all of the aqueous phase along with a portion of the ethylene dichloride to waste from a C_2 glass fitting. The remainder of the ethylene dichloride is forced by difference into a 36 loop 0.053 in. (1.35 mm.) i.d. Teflon mixing coil where it is segmented into a stream of H_2SO_4. Upon leaving the second Teflon extraction coil, the ethylene dichloride is desegmented from the H_2SO_4 stream in a modified fluorometric flow cell and the fluorescence of the H_2SO_4 is measured in the Turner instrument. The shape of this flow cell is indicated in the flow diagram. The system operates at a rate of 20 determinations/hour.

Interferences—An interesting quality control problem was solved by a study of interference on the color reaction. A lot of tablets from a foreign production facility assayed low by the direct colorimetric method but assayed correctly by the manual extraction procedure. The actual raw material excipients used in the lot, or water up to 5%, in the tablets did not retard the color reaction. Sulfite, thiosulfate, phosphate, chloride, bicarbonate, and sodium ions alone in small amounts caused no problems. Exhaustive analyses of the tablets for many components showed no significant difference over normal tablets except for nitrate content. Normal tablet values were less than 1 p.p.m. nitrate, and the troublesome lot 6 p.p.m. Experiments were made to evaluate the effect of nitrate. Aliquots of $NaNO_3$ were evaporated along with solutions containing 80 mcg. of mestranol or ethinyl estradiol. Bicarbonate and color reagents were added and the color developed for 1 hr. The effect of nitrate on fluorescence on 1 mcg. of mestranol was studied in a similar manner excluding the bicarbonate. Fluorescence measurements were made on a model 110 Turner fluorophotometer using No. 58 Kodak Wratten and 1-60 Corning primary filter and a No. 22 Kodak Wratten secondary filter. Since the retardation of the reactions by nitrate was confirmed, the search for its source in the tablet was continued.

Nitrate was not observed in any of the tablet raw materials. The source of nitrate was found to be the glassine paper used in manufacturing at the foreign facility. Typical assays for nitrate for foreign glassine paper were 18–21 p.p.m. and for domestic glassine paper 0.1 p.p.m. The colorimetric nitrate assays (11) were confirmed by a

[1] AutoAnalyzer, Technicon Corp., Chauncey, N. Y.
[2] Technicon Corp., Chauncey, N. Y.

TABLE II—DATA ON ROUTINE CONTROL SAMPLES

Lot	Tablets, Mestranol: Direct Method RSD[c]	Tablets, Mestranol: Av. mcg./Gm.	Tablets, Mestranol: Manual Method,[b] mcg./Gm.	Tablets Mestranol Plus Chlormadinone OAc: Direct Method[a] RSD[c]	Tablets Mestranol Plus Chlormadinone OAc: Av. mcg./Gm.	Tablets Mestranol Plus Chlormadinone OAc: Manual Method,[b] mcg./Gm.
1	.628%	750	758	.301	781	778
2	.527	773	776	.631	772	768
3	.780	798	802	.366	772	765
4	.795	786	798	.400	763	780
5	.219	805	801	.212	768	768

[a] Data from 9 tablet replicates using 3 standardized tablets as reference. [b] Data from a single assay on a 10-tablet composit sample. [c] Relative standard deviation.

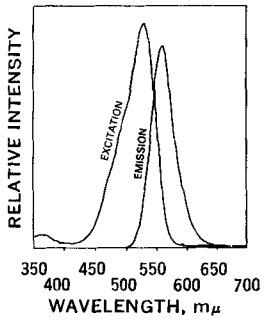

Fig. 4—Excitation and emission spectra.

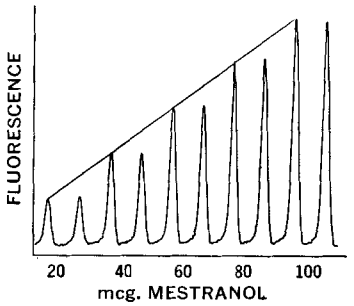

Fig. 6—Peak height versus *concentration for automated assay.*

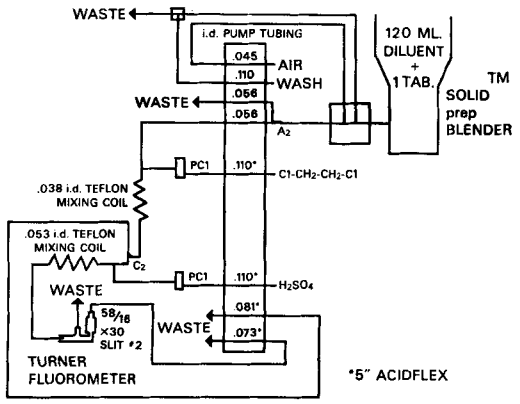

Fig. 5—Flow chart for automated assay.

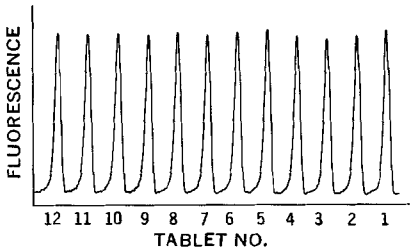

Fig. 7—Precision of automated assay.

polarographic procedure (12). When the source of nitrate was removed, the assay results returned to normal values. Hydrogen peroxide and nitrites also inhibited color formation but these were not found in tablets. Related steroids such as estradiol methyl ether, estrone methyl ether, and estrone produced insignificant positive interference when added in amounts up to 500 mcg. per tablet.

RESULTS AND DISCUSSION

The direct colorimetric method for mestranol using a reference standard tablet has proved to be a very precise and accurate method for routine control (Table II) with a relative standard deviation often less than 1% including tablet heterogeneity but excluding tablet weight variance. The interference of chlormadinone acetate is insignificant if the temperature is held below 5°. The use of the special stirrer shown in Fig. 3 allowed a multiple assay at a rate of about six samples/hour/analyst.

The fluorescence of mestranol in sulfuric acid which is more sensitive than the color reaction was used as the means of measurement for automation. The interferences caused by nitrate by direct dissolution of the tablet led to the solvent extraction scheme as outlined in the flow diagram of the automated method. The relative fluorescence intensity was found to have a linear relationship to mestranol concentration as illustrated by the straight line connecting the peak heights in Fig. 6. The mestranol was added in solution in varying amounts to the sample cups. A run of 12 reference standard tablets, shown in Fig. 7, produced a RSD of 1.3% including tablet heterogeneity. These values are listed in Table I.

The retardation of the color and fluorescence reaction if nitrate is present is significant in con-

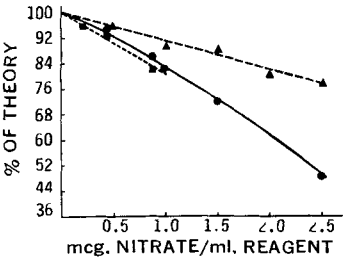

Fig. 8—Effect of nitrate on reactions. Key: ▲, mestranol fluorometric assay; ●, mestranol colorimetric assay; ■, ethinyl estradiol colorimetric.

centrations above 0.1 mcg./ml. of reagent as shown in Fig. 8.

SUMMARY

The utility of H_2SO_4 to produce color or fluorescence of α-ethinyl estradiol and derivatives has been established by several authors (1, 2, 5–7). When used in conjunction with a standardized reference tablet, the method yields optimum precision and accuracy for mestranol. The fluorescence measurement is more sensitive and more suitable for automation but less precise than the color measurement. Nitrate, nitrite, and hydrogen peroxide retard the reaction with sulfuric acid.

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Keyphrases

17α-Ethynylestradiol-3-methyl ether (mestranol) tablets
Colorimetric analysis
Sulfuric acid–methanol color reagent
Automated fluorometric analysis
Fluorescence produced by sulfuric acid
Nitrate effect on analysis accuracy

Notes

Preparation of Some Phenyl Pyridyl Ethers with Antifungal and Antibacterial Properties

By RICHARD O. MUHLHAUSER* and EUGENE C. JORGENSEN†

2-Methyl-4-chlorophenyl-4′-pyridyl ether (II) and 2-chlorophenyl-4′-pyridyl ether (III) were prepared by condensation of *N*-pyridyl-4-pyridinium chloride hydrochloride (I) with appropriate phenols. These compounds were found to be effective as antifungal agents but were less effective as antibacterial agents. Compound II had the greatest antifungal activity and the least toxicity.

As PART of a program dealing with the synthesis of thyroxine analogs, a series of phenyl pyridyl ethers (II–VI) has been prepared. Two of these compounds, 2-methyl-4-chlorophenyl-4′-pyridyl ether (II) and 2-chlorophenyl-4′-pyridyl ether (III), were found to have antifungal and antibacterial activity.

The synthetic sequence utilized is shown in Scheme I.

N-Pyridyl-4-pyridinium chloride hydrochloride (I) was prepared as described by Jerchel (1). Compounds II–V were then prepared by condensing (I) with the appropriate phenols as described by Jerchel (2). Compound VI was prepared from V by acid hydrolysis using a mixture of concentrated hydrochloric and glacial acetic acids. Pertinent data are listed in Table I.

Nuclear magnetic resonance and infrared spectral studies supported the structures assigned to com-

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