Scope of Differential UV and Differential Fluorescence Assays for Phenothiazines: Comparison with Official Methods

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Abstract □ Operational difficulties have been encountered with many of the official phenothiazine assay procedures in this laboratory. Although some difficulties were corrected, the procedures still lack specificity. Accordingly, the general scope of differential UV and differential fluorescence methods was investigated. These specific methods are facile, reproducible, and applicable to both composite and single-tablet assays.

Keyphrases □ Phenothiazines—comparison of differential UV and differential fluorescence methods with official assays □ Differential UV and fluorescence spectrophotometry—analysis, phenothiazines, comparison with official methods

The 10-substituted phenothiazines constitute one of the largest drug classes in the official compendia (1, 2). Nineteen different phenothiazine bases of this type are represented by 74 entries in the USP and NF. A recent drug quality assurance program in this laboratory resulted in the semiautomated analysis of 291 phenothiazine samples, in a wide variety of formulations, from 14 manufacturers.

While checking the results from the automated analyzer by the official assay procedures, considerable difficulty was experienced. Many of the official procedures did not work well and had to be amended. Consequently, the general scope of a differential spectrophotometric assay for phenothiazines was investigated (3). Results from this method were compared with those from the automated method and from modifications of the official procedures.

BACKGROUND

The oxidative degradation of 10-substituted phenothiazines has been studied extensively (see Refs. 4 and 5 for recent reviews). In particular, electrochemical oxidation has been studied in great detail (6–8). It was demonstrated that a 10-substituent raises the oxidation potential and directs oxidation to the sulfur atom. Theoretical considerations supported by electron-spin resonance studies have rationalized this oxidation direction in terms of a steric effect on the phenothiazine electronic system (9, 10). Chemical oxidation in these systems also leads predominantly to sulfur oxidation (11). Among the chemical reagents used have been ozone (12), oxygen (13), sodium nitrite (14), dichromate (15), and hydrogen peroxide (16).

Despite the ease of oxidation of these compounds, specific assay procedures that are stability indicating are not abundant in the literature. Although it is stability indicating, Ryan's palladium complex method (17) has not been widely adopted. A quantitative TLC procedure was described for butaperazine (18), but it is too laborious for routine analysis. GLC (19–21) and high-pressure liquid chromatographic (22, 23) methods are not generally applicable to the entire class of 10-substituted phenothiazines in the presence of their degradation products.

With this background in mind, the following considerations of the official phenothiazine assays indicate the need for their revision. The compendial procedures are of three types: measurement at a single UV wavelength, difference measurements at two UV wavelengths, and measurement of the palladium chloride complex at visible wavelengths. The first method is nonspecific and, at the low wavelengths employed, is susceptible to excipient interference. The difference method apparently involves baseline correction for interfering excipients. Use of the selected correction wavelength (~275 nm) can lead to spurious results since sulfoxide impurities can interfere through their nearby absorption maximum (24). The third method, a variation of Ryan's palladium complexation method, is specific. However, this method has not been widely adopted by the compendia (only one-third of the phenothiazine drugs in this study are officially determined by this method). This lack of acceptance may be a result of poor complex solubility, low complex absorptivity, or unstable complexes. These considerations, as well as a recent report (25) that cited extensive degradation of phenothiazine liquid formulations, emphasize the need for a general, specific assay.

EXPERIMENTAL

Apparatus—A UV-visible double-beam spectrophotometer[1] equipped with 1-cm quartz cells was used for the manual assays. The automated system consisted of a liquid sampler, two proportioning pumps, and two manifolds[2]. This system was equipped with a recorder[3] and interfaced with a minicomputer[4]. Absorbances were measured automatically on a single-beam spectrophotometer[5]. Fluorescence intensities were measured automatically on a ratio fluorometer[6]. Both were determined in a 1-cm flowcell. Manual measurements of fluorescence intensities were taken on the same fluorometer utilizing 1-cm rectangular cells. Tablets were disintegrated in an ultrasonic generator[7] or, if possible, on a mechanical shaker[8].

Materials—Alcohol (95%), acetic acid, hydrochloric acid, *n*-heptane, and *n*-butanol were analytical reagent grade[9] and were used as received. Ammonium hydroxide, sodium nitrite, potassium chloride, sodium hydroxide, and ascorbic acid were ACS grade[10] and were used as received. Hydrogen peroxide (30%) was refrigerated and used as needed. Chromatographic siliceous earth[11] was acid-washed grade and was rewashed with 0.1 *N* HCl on the column.

Standards—All standards were pure drug substances obtained from manufacturers whose products were studied in the quality assurance program. Each drug substance was analyzed *versus* the appropriate USP or NF reference standard by the automated procedure. All substances gave assays in the range of 100 ± 0.4%.

Preparation of Reagents—To prepare 0.1 *N* HCl, concentrated hydrochloric acid (10 ml) was diluted to 1 liter with deionized water.

The peracetic acid oxidizing solution was prepared by pipetting 1.5 ml of 30% H_2O_2 into a 250-ml volumetric flask containing 200 ml of acetic acid. The flask was covered and heated on a steam bath for 1 hr. After

[1] Acta MIV, Beckman Instruments Inc., Fullerton, Calif.
[2] AutoAnalyzer, Technicon Instruments Co., Tarrytown, N.Y.
[3] Servoriter II, Texas Instruments Inc., Houston, Tex.
[4] Series 2116 minicomputer, Hewlett-Packard Inc., Palo Alto, Calif.
[5] PM2DL, Carl Zeiss Inc., New York, N.Y.
[6] Ratio-2 fluorometer, Farrand Optical Co., Valhalla, N.Y.
[7] Model 11, Heat Systems-Ultrasonic Inc., Plainview, N.Y.
[8] Wrist-action shaker, Burrell Co., Pittsburgh, Pa.
[9] Mallinckrodt Chemical Works, St. Louis, Mo.
[10] Fisher Scientific Co., Fair Lawn, N.J.
[11] Celite, Johns-Manville, Denver, Colo.

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Table I—Analysis Parameters for the Modified Official Assay

Drug	Extraction Aliquot Concentration	Assay Concentration, μg/ml
Prochlorperazine maleate or edisylate	3 mg of salt/10 ml	12
Chlorpromazine hydrochloride	2 mg of salt/15 ml	20
Promazine hydrochloride	4 mg of salt/10 ml	40
Trimeprazine tartrate	1.5 mg of salt/10 ml	6

standing overnight at room temperature, the solution was diluted to volume with acetic acid. This solution was discarded after 1 week.

A solution of 0.025% ascorbic acid in 0.1 *N* HCl was prepared by dissolving 250 mg of ascorbic acid in 1 liter of 0.1 *N* HCl.

Three hundred milliliters of 95% alcohol was diluted to 1 liter with pH 2.2 buffer (26) to prepare a solution of alcohol and pH 2.2 buffer.

Equal volumes of *n*-butanol and *n*-heptane were mixed to prepare heptane–butanol (1:1).

The 0.5% $NaNO_2$ oxidizing solution was prepared by dissolving 1 g of sodium nitrite in 200 ml of deionized water.

A solution of 0.1 *N* NaOH was prepared by diluting 5.3 ml of 50% NaOH (w/v) to 1 liter with deionized water.

Modified Official Assay Procedure—Except for the following changes, the official procedures were used as given.

Class I Compounds—Prochlorperazine maleate or edisylate, chlorpromazine hydrochloride, promazine hydrochloride, and trimeprazine tartrate were assayed. To prepare single tablets for assay, the tablet was disrupted ultrasonically in 0.1 *N* HCl. To prepare sample composites, the average tablet weight was determined, and the tablets were ground in a mortar and pestle to pass through a 60-mesh screen. The appropriate weight of tablet powder then was shaken with 0.1 *N* HCl in a volumetric flask (Table I), diluted to volume with 0.1 *N* HCl, and filtered through paper. The first 10 ml of the filtrate was discarded.

An aliquot of the filtrate of the concentration specified in Table I was transferred to a separator. Water (125 ml) and saturated sodium chloride (5 ml) were added, and the solution was made basic with ammonium hydroxide (3 ml). The pH was checked to confirm that the solution was strongly alkaline. The solution then was extracted with four portions of heptane (40, 25, 25, and 25 ml). The combined heptane extracts were extracted with 0.1 *N* HCl (4 × 25 ml), and the combined hydrochloric acid extracts were diluted to the final concentration specified in Table I. The standards were prepared in 0.1 *N* HCl to the same final concentration as that expected for the assay.

Immediately after preparation of the assay and standard solutions, the absorbance was determined as specified in the individual official monograph. With the exception of prochlorperazine maleate and edisylate, all assay and standard operations were carried out in low-actinic glassware. In addition, prochlorperazine maleate and edisylate standards and trimeprazine tartrate standards were carried through the extraction procedure to remove the counterions, which are UV absorbing.

Class II Compounds—Fluphenazine hydrochloride, thiethylperazine maleate, and trifluoperazine hydrochloride were assayed. Sample preparation was the same as that described for the Class I compounds. For thiethylperazine maleate, 20 mg of drug was transferred to a separator. Ten milliliters of 0.1 *N* HCl, 15 ml of water, and 5 ml of saturated sodium chloride were added, after which the procedure in NF XIV was followed. For trifluoperazine hydrochloride tablets, the official NF XIV procedure was used.

For trifluoperazine hydrochloride liquid formulations, an aliquot containing 2 mg of drug dissolved in 10 ml of 0.1 *N* HCl was transferred to a separator. Fifteen milliliters of water, 5 ml of saturated sodium chloride, and 3 ml of stronger ammonia were added. Then the solution was extracted with four portions of heptane (40, 25, 25, and 15 ml). The combined heptane extracts were extracted with 0.1 *N* HCl (4 × 25 ml), and the combined acid extracts were diluted to a final concentration of 10 mg/ml with 0.1 *N* HCl. The absorbance was measured at the wavelength specified in the monograph *versus* a blank of 0.1 *N* HCl. A standard of the same final concentration as expected for the sample was prepared, and its absorbance was read concomitantly. All operations were performed in low-actinic glassware.

For fluphenazine hydrochloride, the official USP procedure was used but the extraction solvent was changed from the specified *n*-hexane to *n*-hexane–*n*-butanol (98:2).

Class III Compounds—Triflupromazine hydrochloride, perphenazine,

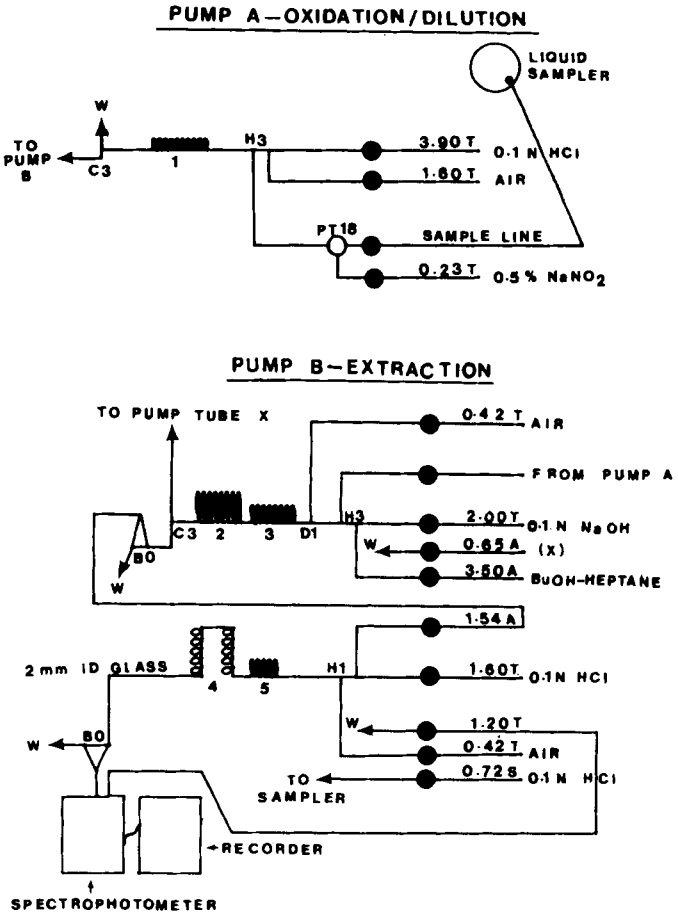

Figure 1—*Diagram of automated analytical system for phenothiazines. Pump tube sizes are listed in milliliters per minute. Key for pump tube materials: T, Tygon; S, silicone; and A, Acidflex. All connectors, mixing coils, and separators are identified with the Technicon designation, but similar units by other suppliers may be used. Key for mixing coils: 1, Technicon 105-0085; 2, eight turns of 2.4-mm (0.095 in.) i.d. Teflon tubing wrapped around a 38.1-mm (1.5-in.) diameter core; 3, Technicon 116-101-2 (beaded coil); 4, Technicon 157-0248; and 5, Technicon 116-0127-05 (1 mm i.d.). All points marked W go to the waste container. All connecting tubing should be as short as possible. The spectrophotometer is equipped with a 1-cm flowcell.*

and carphenazine maleate were assayed. The official procedures were used.

Automated Method—To prepare tablet samples, one tablet was placed in a vial and a sufficient amount of 0.025% ascorbic acid in 0.1 *N* HCl was added to yield the final drug concentration specified in Table II. The capped vial was shaken mechanically until tablet disintegration commenced. Then the vial was transferred to an ultrasonic bath, and the bath was operated until disintegration was complete. The vial was shaken mechanically for 30 min. After the solids settled, the supernate was decanted into a sample cup.

To prepare fluphenazine hydrochloride tablet samples, one tablet was placed in a volumetric flask chosen such that its volume yielded a final concentration of 50 μg/ml. Deionized water was added to equal 5% of the total flask volume. The flask was shaken mechanically until the tablet began to disintegrate. Then the flask was transferred to an ultrasonic bath, and the bath was operated until tablet disintegration was complete. The flask was diluted to volume with 95% alcohol and mixed. After the solids settled, the supernate was decanted into a sample cup.

To prepare the liquid formulation samples, a suitable aliquot was transferred in a "to contain" pipet to a volumetric flask. The pipet and volumetric flask were chosen such that the final assay concentration corresponded to that specified in Table II. The pipet contents were washed into the volumetric flask with 0.025% ascorbic acid in 0.1 *N* HCl, and the solution was diluted to volume with the same solvent. The standard solutions were prepared in this solvent at the same final concentration as that expected for the sample assay.

Table II—Differential UV and Semiautomated Analysis Parameters

Drug	Automated[a] Wavelength, nm	Automated[a] Final Concentration, μg/ml	Differential UV[b] Wavelength, nm	Differential UV[b] Final Concentration, μg/ml
I Carphenazine maleate	251	500	372	208
II Chlorpromazine hydrochloride	241, 298	200	344	40
III Promazine hydrochloride	241, 298	200	342	40
IV Thiethylperazine maleate	276	200	350	83
V Thioridazine hydrochloride	276, 342	200	348	40
VI Triflupromazine hydrochloride	273	400	347	40
VII Trimeprazine tartrate	271	100	342	42
VIII Fluphenazine hydrochloride	256	50	349	4.2
IX Methdilazine hydrochloride	251	160	341	58
X Prochlorperazine maleate	257	100	342	42
XI Trifluoperazine hydrochloride	257	40	349	4.0
XII Perphenazine	255	80	343	33

[a] Drugs I–VII were oxidized and double extracted. Drugs IX–XII were extracted and determined directly. For VIII in tablet strengths of ≥2.5 mg, the extracted drug was determined at a concentration of 50 μg/ml. Tablets containing ≤1 mg were oxidized, double extracted, and determined fluorometrically at a concentration of 10 μg/ml. [b] Drugs VIII and XI were analyzed by differential fluorescence; all others were analyzed by the differential UV method.

The assay (Fig. 1) was carried out by placing the sample and standard solutions in 3-ml polystyrene cups on a liquid sampler set at 24 samples/hr with a sample-to-wash ratio of 4:1. The cups were filled in the following order: three cups of standard, five cups of sample, one cup of standard, five cups of sample, *etc.* Two cups of standard were placed at the end of each run.

For drugs requiring oxidation and dilution, the sample was withdrawn and segmented with air, and 0.1 *N* HCl and 0.5% $NaNO_2$ (if necessary) were added. The solution then was mixed, resampled, and made basic with 0.1 *N* NaOH; heptane–butanol was added. After segmentation with air, the solution was mixed in a beaded coil, and the phases were allowed to coalesce in a phasing coil. Then the phases were separated. The organic phase was segmented with air and mixed with 0.1 *N* HCl. The phases were separated again. The drug was extracted into 0.1 *N* HCl, and the absorbance of the aqueous phase was read at the specified wavelength in a spectrophotometer equipped with a 1-cm flowcell.

For drugs requiring only extraction, the oxidation–dilution manifold was not used. The sample was introduced directly into the extraction manifold at the resample pump tube and made basic with 0.1 *N* NaOH, and heptane–butanol was added. After segmentation with air, the solution was mixed in a beaded coil, and the phases were allowed to coalesce in a phasing coil. The phases were separated. The organic phase was segmented with air and mixed with 0.1 *N* HCl. The phases were separated again. The drug was extracted into 0.1 *N* HCl, and the absorbance of the aqueous phase was read at the specified wavelength in a spectrophotometer equipped with a 1-cm flowcell.

Differential UV Method—The chromatographic columns for single-tablet and composite assays were prepared as follows. For 10-mg and higher strength tablets, a 17 × 250-mm column[12] plugged with glass wool was used. Acid-washed siliceous earth (3 g) was added and tamped down. The column was topped with a glass wool plug. Then 60 ml of 0.1 *N* HCl was passed through the column, and the wash was discarded. The column then was ready for use. For tablets of <10-mg strength, this procedure was modified to use a 13 × 200-mm column[12] packed with 1 g of acid-washed siliceous earth.

Single-tablet assays for promazine hydrochloride, chlorpromazine hydrochloride, triflupromazine hydrochloride, thioridazine hydrochloride, and thiethylperazine maleate were conducted as follows. One tablet was disintegrated in 60 ml of 0.1 *N* HCl in an ultrasonic bath. The tablet suspension was shaken for 1 hr on a mechanical shaker. Composites were prepared by determining the average tablet weight and then grinding the tablets to pass through a 60-mesh screen. A sufficient amount of tablet powder was weighed accurately to contain 10–12.5 mg of phenothiazine salt, to which 60 ml of 0.1 *N* HCl was added. The tablet suspension was shaken mechanically for 1 hr. A standard solution was prepared in 0.1 *N* HCl such that 60 ml contained an accurately known amount (10–12.5 mg) of standard.

Single-tablet, composite, and standard solutions were prepared in the following manner. Sixty milliliters of solution containing 10–12.5 mg of phenothiazine salt was passed through a chromatographic column into a 100-ml volumetric flask. Residual drug was washed through the column with three portions (20, 15, and 15 ml) of 0.1 *N* HCl. The combined eluate was diluted to 100.0 ml with 0.1 *N* HCl. Peracetic acid (5.0 ml) was added to 20.0 ml of the eluate[13], and the solution was mixed and diluted to 50.0 ml with 0.1 *N* HCl.

[12] No. K-420530 or equivalent, Kontes, Evanston, Ill.

Table III—Phenothiazine Sulfoxide Absorptivities[a]

Sulfoxide	Wavelength, nm	Δ Absorptivity[b]	Absorptivity[c]
Prochlorperazine	342	7.95 (3)[d]	9.70
Perphenazine	343	12.0 (5)	14.8
Fluphenazine[e]	349	9.16 (4)	11.0
Chlorpromazine	344	13.8 (8)	18.1
Thioridazine	348	10.9 (8)	13.1
Triflupromazine	347	11.7 (3)	14.0
Carphenazine	372	2.05 (6)	5.44
Thiethylperazine	350	6.87 (2)	8.47
Methdilazine	341	16.2 (1)	18.2
Promazine	342	16.0 (1)	18.8
Trimeprazine	342	14.1 (1)	15.5
Trifluoperazine[e]	349		11.5

[a] Based on grams of phenothiazine salt per liter (1-cm cell). [b] Measured *versus* a blank containing an equal concentration of unoxidized drug. [c] Measured *versus* a solvent blank. [d] Number of determinations. [e] Assayed fluorometrically.

The absorbance of the oxidized composite assay solution was read rapidly *versus* that of a sample blank prepared from 20.0 ml of the assay eluate and 5.0 ml of acetic acid diluted to 50.0 ml with 0.1 *N* HCl. The absorbance of an oxidized standard solution, prepared from 20.0 ml of the standard eluate and 5.0 ml of peracetic acid diluted to 50.0 ml with 0.1 *N* HCl, was measured concomitantly *versus* that of a standard blank prepared from 20.0 ml of the standard eluate and 5.0 ml of acetic acid diluted to 50.0 ml with 0.1 *N* HCl. The absorbance of oxidized single-tablet assay solutions was measured rapidly *versus* that of a solvent blank prepared from 5.0 ml of acetic acid diluted to 50.0 ml with 0.1 *N* HCl. The absorbance of an oxidized standard solution was measured concomitantly *versus* that of a solvent blank. The wavelengths of the assay determination and final assay concentrations are given in Table II. Phenothiazine sulfoxide absorptivities are listed in Table III.

Single-tablet solutions of perphenazine were prepared by disintegrating one tablet in 25 ml of 0.1 *N* HCl in an ultrasonic bath. Composite solutions were prepared by grinding 20 tablets to pass through a 60-mesh screen and adding one tablet weight of composite powder to 25 ml of 0.1 *N* HCl. The single-tablet or composite suspension was shaken mechanically for 1 hr. The suspension was diluted, if necessary, to 80 μg/ml with 0.1 *N* HCl. A perphenazine standard solution of 80 μg/ml in 0.1 *N* HCl was prepared. Then 25 ml of 0.1 *N* HCl containing 2 mg of drug or standard was passed through a chromatographic column into a 50-ml volumetric flask.

Residual drug was washed from the column with three portions (10, 10, and 5 ml) of 0.1 *N* HCl. The combined eluate was diluted to 50.0 ml with 0.1 *N* HCl, and 20.0 ml of the eluate was oxidized with 4.0 ml of peracetic acid. The absorbance of the composite assay solution was measured rapidly at 343 nm *versus* a sample blank prepared from 20.0 ml of the same column eluate and 4.0 ml of acetic acid. The absorbance of an oxidized standard solution was measured concomitantly *versus* that

[13] For thiethylperazine maleate assays, 25.0 ml of the column eluate and 5.0 ml of peracetic acid were used.

Table IV—Percent Recovery of Phenothiazine Salts from Synthetic Formulation Mixtures by the Differential UV–Differential Fluorescence Method

Drug	Percent Recovered[a]	*RSD*, %	Simulated Dosage Strength, mg
Thiethylperazine maleate	99.5	0.93	10
Prochlorperazine maleate	100.2	0.81	5
Chlorpromazine hydrochloride	100.8	0.73	25
Thioridazine hydrochloride	99.0	0.43	10
Perphenazine	100.3	0.42	16
Carphenazine maleate	99.5	0.41	12.5
Promazine hydrochloride	99.8	0.41	25
Trifluprom azine hydrochloride	100.1	0.38	10
Trimeprazine tartrate	100.3	0.21	2.5

[a] Average of eight determinations.

Table V—Precision Study on Composite of 100-mg Chlorpromazine Hydrochloride Tablets

Percent of Label Claim		
Automated[a]	Modified Official	Differential
101.6		
99.7		98.5
98.2	99.2	98.5
99.2	99.8	99.0
100.0	100.3	99.1
99.5	96.1	99.9
99.3	100.1	99.1
99.1	98.7	98.0
99.4	100.3	98.0
Mean (*RSD*, %)		
99.6 (0.92)	99.2 (1.42)	98.8 (0.65)

[a] One sample run on each of 9 days.

of a standard blank prepared from 20.0 ml of the standard eluate and 4.0 ml of acetic acid. The absorbance of the oxidized single-tablet solution and the absorbance of an oxidized standard were measured *versus* a solvent blank prepared from 20.0 ml of 0.1 *N* HCl and 4.0 ml of acetic acid.

Single-tablet solutions of trimeprazine tartrate were prepared by disintegrating one tablet in 25 ml of 0.1 *N* HCl in an ultrasonic bath. Composite solutions were prepared by grinding 20 tablets to pass through a 60-mesh screen and adding one tablet weight of composite powder to 25 ml of 0.1 *N* HCl. The single-tablet or composite suspension was shaken mechanically for 1 hr. A trimeprazine tartrate standard solution of 100 μg/ml in 0.1 *N* HCl was prepared. Then 25 ml of the standard or assay solution containing 2.5 mg of drug was passed through a chromatographic column into a 50-ml volumetric flask. The assay proceeded as directed in the perphenazine assay beginning with the washing of residual drug. The absorbance was read at 342 nm.

Single-tablet solutions of methdilazine hydrochloride were prepared by disintegrating one tablet in 25 ml of 0.1 *N* HCl in an ultrasonic bath. Composite solutions were prepared by grinding 20 tablets to pass through a 60-mesh screen and adding one tablet weight of composite powder to 25 ml of 0.1 *N* HCl. The single-tablet or composite suspension was shaken mechanically for 1 hr. A methdilazine hydrochloride standard solution of 144 μg/ml was prepared in 0.1 *N* HCl. Then 25 ml of the standard or assay solution containing 3.6 mg of methdilazine hydrochloride was passed through a chromatographic column into a 50-ml volumetric flask. The assay proceeded as directed in the perphenazine assay beginning with the washing of residual drug. The absorbance was read at 341 nm.

Single-tablet solutions of prochlorperazine maleate were prepared by disintegrating one tablet in 50 ml of 0.1 *N* HCl in an ultrasonic bath. Composite solutions were prepared by grinding 20 tablets to pass through a 60-mesh screen and adding one tablet weight of composite powder to 50 ml of 0.1 *N* HCl. The single-tablet or composite suspension was shaken mechanically for 1 hr. The suspension was diluted, if necessary, to 100 μg/ml with 0.1 *N* HCl. A prochlorperazine maleate standard solution (100 μg/ml) was prepared in 0.1 *N* HCl. Then 50 ml of the standard or assay solution containing 5 mg of prochlorperazine maleate was passed through a chromatographic column into a 100-ml volumetric flask. The column was washed with three portions (15, 15, and 10 ml) of 0.1 *N* HCl. The combined eluate was diluted to 100.0 ml with 0.1 *N* HCl, and the assay proceeded as directed in the perphenazine assay beginning with the oxidation of the eluate. The absorbance was read at 342 nm.

Single-tablet solutions of carphenazine maleate were prepared by disintegrating one tablet in 25 ml of 0.1 *N* HCl in an ultrasonic bath. Composite solutions were prepared by grinding 20 tablets to pass a 60-mesh screen and adding one tablet weight of the composite powder to 25 ml of 0.1 *N* HCl. The single tablet or composite suspension was shaken mechanically for 1 hr. A carphenazine maleate standard solution (500 μg/ml) was prepared in 0.1 *N* HCl. Then 25 ml of the standard or the assay solution containing 12.5 mg of carphenazine maleate was passed through a chromatographic column into a 50-ml volumetric flask. Residual drug was washed from the column with three portions (15, 15, and 10 ml) of 0.1 *N* HCl. The combined eluate was diluted to 50.0 ml with 0.1 *N* HCl, and the assay proceeded as directed in the perphenazine assay beginning with the oxidation of the eluate. The absorbance was read at 372 nm.

Liquid formulations of chlorpromazine hydrochloride and thioridazine hydrochloride were assayed as follows. An aliquot of liquid containing 100 mg of drug was transferred in a "to contain" pipet to a suitable volumetric flask, and the pipet contents were rinsed into the flask with 0.1 *N* HCl. The solution was diluted quantitatively and stepwise to a final concentration of 50 μg/ml. Standard solutions of thioridazine hydrochloride and chlorpromazine hydrochloride were prepared at a concentration of 50 μg/ml. Then 20.0-ml aliquots of the thioridazine hydrochloride assay solution were pipetted into each of two flasks; 5.0 ml of peracetic acid was pipetted into one flask, and 5.0 ml of acetic acid was pipetted into the other flask. The absorbance of the oxidized thioridazine hydrochloride solution was read at 348 nm *versus* that of the sample blank. The absorbance of the oxidized thioridazine hydrochloride standard solution was measured concomitantly *versus* that of a standard blank. The absorbance of oxidized chlorpromazine hydrochloride assay and standard solutions was measured at 344 nm *versus* that of their appropriate blanks.

To assay liquid formulations of prochlorperazine maleate, an aliquot of liquid containing 10 mg of drug was transferred in a "to contain" pipet to a suitable volumetric flask. The pipet contents were rinsed into the flask with 0.1 *N* HCl, and the solution was diluted quantitatively and stepwise with 0.1 *N* HCl to a final concentration of 50 μg/ml. A standard solution of prochlorperazine maleate was prepared having a final concentration of 50 μg/ml. Then 20.0-ml aliquots of sample and standard solutions were oxidized with 5.0-ml aliquots of peracetic acid. The absorbance of oxidized standard and oxidized sample solutions was measured concomitantly *versus* that of their appropriate blanks at 342 nm.

Differential Fluorometric Method—The fluorescence of oxidized phenothiazines was reported previously (27), but an assay was not developed at that time. Excitation occurs at 365 nm[14]; emission is at 405 nm[15]. A linear relationship between the meter response and the concentration change was demonstrated in the concentration range of interest.

After insertion of the proper excitation and emission filters, the apertures, range, and sensitivity were set to yield a meter deflection of ~90% with an oxidized drug solution. With these instrumental settings, the fluorescence of the freshly oxidized standard, sample, and blank solutions was measured.

To assay 0.25-mg fluphenazine hydrochloride tablets, one tablet was disintegrated in 25 ml of pH 2.2 buffer in an ultrasonic bath. Then 15.0 ml of 95% alcohol[16] was added and shaken mechanically for 1 hr. The solution was diluted to 50.0 ml with pH 2.2 buffer and filtered through paper[17]; the first 10 ml was discarded. Then 25.0-ml aliquots of the filtrate were pipetted into each of two flasks. One aliquot was oxidized with 5.0 ml of peracetic acid, and 5.0 ml of acetic acid was added to the other aliquot. The fluorescence of the sample and blank solutions was determined in 1-cm rectangular cells. The fluorescence of the oxidized standard and standard blank solutions was measured concomitantly.

For tablets containing 1, 2.5, 5, or 10 mg of fluphenazine hydrochloride or trifluoperazine hydrochloride, one tablet was disintegrated in 60 ml of pH 2.2 buffer in an ultrasonic bath. Then 30.0 ml of 95% alcohol was added, and the solution was shaken mechanically for 1 hr. The solution was diluted to 100.0 ml with pH 2.2 buffer and filtered through paper[17]; the first 10 ml was discarded. An aliquot containing 0.5 mg of drug was pipetted into a 100-ml volumetric flask and diluted to volume with alcohol

[14] Filter 5860, Corning Glass Works, Corning, N.Y.

[15] Ditric Optics Inc., Marlboro, Mass. A narrow band filter reduces sample blanks about 75%.

[16] Alcohol hinders the disintegration and must be added after disintegration is complete. The final concentration of alcohol is important since it enhances the fluorescence intensity.

[17] No. 42, Whatman Inc., Clifton, N.J.

Table VI—Comparative Analysis of Commercial Solid Formulations

Drug and Dosage Strength	Assay Type	Percent of Label Claim		
		Differential	Automated	Modified Official
Perphenazine, 2 mg	Tablet	105.5 (10)[a]	—	106.5 (3)
	Composite	106.6 (5)	100.7 (2)	103.7 (4)
Promazine hydrochloride, 10 mg	Tablet	99.2 (20)	102.5 (10)	101.3 (1)
	Composite	100.6 (6)	—	—
Thioridazine hydrochloride				
10 mg	Tablet	98.8 (20)	—	95.8 (3)
	Composite	100.9 (2)	96.5 (10)	96.0 (2)
200 mg	Composite	96.1 (4)	—	100.2 (2)
Triflupromazine hydrochloride				
10 mg	Tablet	105.9 (20)	105.8 (10)	109.2 (2)
	Composite	106.6 (2)	—	—
50 mg	Composite	99.5 (9)	99.7 (2)	100.6 (4)
Prochlorperazine maleate				
5 mg	Tablet	102.1 (10)	96.8 (10)	97.8 (2)
	Composite	101.9 (2)	—	—
75 mg	Composite[b]	98.2 (2)	101.5 (2)	101.3 (1)
Trimeprazine tartrate, 2.5 mg	Tablet	100.8 (10)	99.6 (10)	104.5 (2)
	Composite	101.5 (5)	101.1 (2)	101.3 (4)
Carphenazine maleate				
12.5 mg	Tablet	104.7 (10)	102.0 (10)	109.2 (7)
50 mg	Composite	99.9 (4)	100.4 (2)	100.1 (6)
Methdilazine hydrochloride				
3.6 mg	Tablet	94.9 (10)	—	—
8.0 mg	Composite	96.5 (5)	97.3 (5)	99.9 (5)
Thiethylperazine maleate, 10 mg	Tablet	100.7 (10)	97.9 (10)	101.9 (10)
	Composite	97.8 (5)	98.0 (2)	98.1 (4)
Chlorpromazine hydrochloride, 100 mg	Tablet	102.2 (10)	—	—
	Composite	98.8 (8)	99.6 (9)	—
Trifluoperazine hydrochloride, 1 mg	Tablet	98.2 (7)	98.1 (10)	102.0 (4)
	Composite	102.1 (5)	100.3 (2)	97.9 (4)
Fluphenazine hydrochloride				
5 mg	Tablet	97.7 (10)	95.3 (10)	—
	Composite	100.1 (3)	96.1 (2)	96.1 (3)
1 mg	Tablet	96.8 (10)	96.8 (20)	—
0.25 mg	Tablet	96.7 (20)	92.6 (20	—

[a] Number of determinations. [b] Controlled-release capsule.

in pH 2.2 buffer. Then 25.0-ml aliquots were pipetted into each of two flasks. One aliquot was oxidized with 5.0 ml of peracetic acid, and 5.0 ml of acetic acid was added to the other aliquot. A standard assay and standard solution blank were prepared in the same manner. The fluorescence of each solution was determined concomitantly.

To run composite assays, the average tablet weight was determined. Then 20 tablets were ground to pass through a 60-mesh screen. One tablet weight of powder was used in a manner similar to the single-tablet procedure.

To assay liquid formulations of trifluoperazine hydrochloride, an aliquot of liquid containing 50 mg of drug was transferred in a "to contain" pipet to a suitable volumetric flask. The pipet contents were rinsed into the flask with alcohol in pH 2.2 buffer. The solution was diluted quantitatively and stepwise with this solvent to a final concentration of 5.0 μg/ml. A standard trifluoperazine hydrochloride solution was prepared with this solvent at a concentration of 5.0 μg/ml. Then 25.0-ml aliquots of the sample and standard solutions were oxidized with 5.0-ml portions of peracetic acid. The fluorescence of the sample and standard solutions was read rapidly in 1-cm rectangular cells. Sample and standard blanks were prepared from 25.0 ml of solution and 5.0 ml of acetic acid. The fluorescence of each blank was determined, and the sample and standard fluorescences were corrected.

RESULTS AND DISCUSSION

Validation Tests—A tablet placebo was prepared from the known formulation ingredients. To this placebo was added an aliquot containing an amount of drug corresponding to the label declaration. This synthetic mixture then was subjected to the appropriate differential UV or differential fluorescence composite assay. Recovery results are listed in Table IV.

Modified Official Method—The most serious problem encountered with the official assay methods involved the ether extraction steps (Class I compounds only; thioridazine hydrochloride and trifluoperazine hydrochloride ether extractions did not exhibit this problem). The ether extraction procedures require an aeration step to remove residual ether from the final dilute acid extract. Aeration generated colored solutions, and these solutions exhibited badly distorted UV curves (28). The substitution of nitrogen gas for air did not alleviate the problem. It is well known that hydroperoxides rapidly oxidize phenothiazines to their sulfoxides; thus, ether peroxide impurities were suspected (4). To avoid having to purify ether continuously, heptane was used as the solvent. This choice of solvent solved the problem, but mixtures of heptane and dilute acid are prone to emulsion.

The Class II phenothiazines were extracted incompletely by the official procedures. The simple expedient of using saturated sodium chloride solution solved this problem. In this way, the extraction partition coefficient for thiethylperazine maleate was raised 100-fold. In addition, the fluphenazine assays required the addition of 2% *n*-butanol to the *n*-hexane extraction solvent.

The official procedures worked quite well for the Class III compounds, but carphenazine–palladium complexes are very unstable.

The amendments to the official procedures, although improving their utility, do not make these methods specific.

Semiautomated Method—This procedure was developed initially without an oxidation step, but some dosage levels were too low to permit UV measurement. For most of these drugs, oxidation with 0.5% $NaNO_2$ raised absorbances to convenient working levels, but single tablets containing 0.25 or 1.0 mg of fluphenazine hydrochloride could not be assayed by oxidation followed by UV analysis. These tablet strengths eventually were assayed by oxidation followed by fluorometric determination.

With these modifications, the automated system was applicable to every type of formulation encountered. In most cases, coefficients of variation were as good as or better than those of the modified official method. Table V contains a typical comparative precision study. Since the automated procedure, like the official and modified official methods, employs a UV solvent blank, this method also is nonspecific if impurities are present.

Differential UV and Differential Fluorescence Methods—During the differential UV oxidation studies, it was noted that sulfoxide absorbances declined slowly with time; but it has been demonstrated that for each drug in this study, the ratio of standard and sample absorbances is constant for at least 3 hr after oxidation. In addition, absorbances and fluorescence intensities usually rise and reach a maximum value that is

Table VII—Comparative Analysis of Commercial Liquid Formulations

Drug and Dosage Strength	Percent of Label Claim		
	Differential	Automated	Modified Official
Chlorpromazine hydrochloride concentrate, 100 mg/ml	99.0 (8)[a]	99.6 (2)	101.6 (1)
Trifluoperazine hydrochloride concentrate, 10 mg/ml	97.8 (5)	101.2 (2)	98.8 (2)
Prochlorperazine maleate concentrate, 10 mg/ml	96.1 (2)	97.5 (2)	100.2 (1)
Thioridazine hydrochloride concentrate, 100 mg/ml	97.9 (2)	99.3 (2)	99.7 (1)

[a] Number of determinations.

sufficiently stable for measurement in ~3 min after oxidation. This rise apparently is due to the expulsion of air bubbles formed as a result of mixing acetic acid and dilute hydrochloric acid.

Differential UV assays results were unaffected by 18 commonly used excipients. However, the differential fluorescence method was affected by one excipient, dibasic calcium phosphate. This problem was corrected by changing the fluorescence assay solvent from 0.1 *N* HCl to pH 2.2 buffer containing ~30% alcohol. Comparative assay results for the three methods are summarized in Tables VI and VII.

CONCLUSIONS

Previously published work on the phenothiazine differential oxidation method suggested its general applicability (3). The inclusion of a siliceous earth filtration step and the introduction of a fluorometric procedure for lower formulation strengths make the method facile and accurate. The method was shown to be reproducible to concentrations as low as 1 μg/ml. The procedure was applied to tablets, sustained-release preparations, concentrates, and syrups. Recoveries from known mixtures ranged from 99 to 101%. These results and the specificity of the differential oxidation procedure suggest its superiority to the official assay procedures.

REFERENCES

(1) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975.

(2) "The National Formulary," 14th ed., Mack Publishing Co., Easton, Pa., 1975.

(3) A. G. Davidson, *J. Pharm. Pharmacol.*, **28**, 795 (1976).

(4) S. P. Massie, *Chem. Rev.*, **54**, 797 (1954).

(5) C. Bodea and I. Silberg, in "Advances in Heterocyclic Chemistry," vol. 9, A. R. Katritzky, Ed., Academic, New York, N.Y., 1968, p. 321.

(6) J. P. Billon, *Ann. Chim. (Paris)*, **7**, 183 (1962).

(7) T. N. Tozer and L. D. Tuck, *J. Pharm. Sci.*, **54**, 1169 (1965).

(8) P. Kabasakalian and J. McGlotten, *Anal. Chem.*, **31**, 431 (1959).

(9) J. P. Malrieu and B. Pullman, *Theoret. Chim. Acta*, **2**, 293 (1964).

(10) A. Pullman, *J. Chim. Phys.*, **61**, 1666 (1964).

(11) H. Gilman and R. D. Nelson, *J. Am. Chem. Soc.*, **75**, 5422 (1953).

(12) M. Nakanishi and N. Kuriyama, Japanese pat. 15,617 (1961); through *Chem. Abstr.*, **56**, 12908i (1962).

(13) G. P. Brown, J. W. Cole, and T. I. Crowell, *J. Org. Chem.*, **20**, 1772 (1955).

(14) D. Simov Antonov, *Izv. Khim. Inst., Bulgar. Nauk.*, **5**, 51 (1957); through *Chem. Abstr.*, **55**, 1655d (1961).

(15) C. Bodea and M. Raileany, *Ann. Chem.*, **614**, 171 (1958).

(16) L. K. Turner, *J. Forensic Sci.*, **4**, 39 (1963).

(17) J. A. Ryan, *J. Am. Pharm. Assoc., Sci. Ed.*, **48**, 240 (1959).

(18) A. J. Kapadia, M. A. Barber, and A. E. Martin, *J. Pharm. Sci.*, **59**, 1476 (1970).

(19) S. Cooper, J. Albert, R. Dugal, and M. Bertrand, *J. Chromatogr.*, **150**, 263 (1978).

(20) E. C. Dinovo, L. A. Gottschalk, B. R. Nandi, and P. G. Geddes, *J. Pharm. Sci.*, **65**, 667 (1976).

(21) S. H. Curry, *Anal. Chem.*, **40**, 1251 (1968).

(22) R. G. Muusze and J. F. K. Huber, *J. Chromatogr. Sci.*, **12**, 779 (1974).

(23) D. H. Rodgers, *ibid.*, **12**, 743 (1974).

(24) A. DeLeenheer, *J. Assoc. Off. Anal. Chem.*, **56**, 105 (1973).

(25) A. G. Davidson, *J. Pharm. Pharmacol.*, **30**, 410 (1978).

(26) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., p. 761.

(27) J. B. Ragland and V. J. Kinross-Wright, *Anal. Chem.*, **36**, 1356 (1964).

(28) R. D. Kirchhoefer and J. W. Myrick, *J. Assoc. Off. Anal. Chem.*, **61**, 1519 (1978).

Pyridones as Potential Antitumor Agents II: 4-Pyridones and Bioisosteres of 3-Acetoxy-2-pyridone

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Abstract □ Pyridone structural requirements for activity against murine P-388 leukemia have been extended to isosteric analogs of 3-hydroxy-4-pyridone, a compound previously found to have activity. An amino group can be substituted for the 3-hydroxyl function with retention of activity. A sulfur, but not an amino function, can replace the lactam oxygen in the 2-position. Relocation of the lactam oxygen from the 2- to the 4-position in the pyridine ring also produces active pyridones, including 2-methyl-3-acetoxy-4-pyridone. This compound, which has a T/C value of 179%, is the most active material discovered thus far in the pyridone studies.

Keyphrases □ Pyridones—4-pyridones and bioisosteres of 3-acetoxy-2-pyridone, synthesis and evaluation for antitumor activity □ Antitumor agents, potential—4-pyridones and bioisosteres of 3-acetoxy-2-pyridone, synthesis and evaluation for activity □ Structure–activity relationships—4-pyridones and bioisosteres of 3-acetoxy-2-pyridone, synthesis and evaluation for antitumor activity

It was reported recently that 3-hydroxy- (I) and 3-acetoxy-2-pyridone (II) have reproducible activity against murine P-388 lymphocytic leukemia (1). Eight additional derivatives of 3- and 5-hydroxy-2-pyridone also were active, and a tentative structure–activity relationship was established between antitumor activity and the position of the hydroxyl group relative to the 2-pyridone lactam function. The present investigation extends the study of

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