Determination of Phenothiazine Compounds in Biologic Specimens by UV Spectrophotometry

JACK E. WALLACE and JOHN D. BIGGS

Abstract □ A UV spectrophotometric technique for specific determination of drugs that have the phenothiazine group as part of their molecular structure was developed. The method utilizes the oxidation capability of the cobalt (III) ion. The phenothiazines and their sulfoxide metabolites are oxidized to a product that is stable in a hexane–tertiary butanol mixture. No other group of alkaline-extractable compounds gives a UV-absorbing product by the conditions of the proposed method. The procedure is sensitive, and the spectra of the products are highly specific for the phenothiazine drugs. Analysis is linear over the concentration range of 0.5–50.0 mcg./ml. The distribution pattern of certain phenothiazine drugs in the rat is presented.

Keyphrases □ Phenothiazines, cobalt (III) oxidation products—UV spectrophotometric determination □ UV spectrophotometry—determination, phenothiazines in blood, urine, tissues □ TLC—identification, phenothiazines

Phenothiazine-type drugs have a high sensitivity for the absorption of UV radiations in the 250–260-nm. region of the spectrum. Numerous other alkaline-extractable drugs, as well as certain normal tissue components, also absorb radiations in this area with high efficiency. Therefore, direct UV spectrophotometric methods for the determination of phenothiazine drugs have little or no specificity (1–3). These drugs are often metabolized to their corresponding sulfoxide derivatives. Such metabolites have a high molar absorptivity for UV radiations but at wavelength maxima different from that of unchanged phenothiazine.

The sulfoxides were used by several investigators to establish, either by colorimetric or UV spectrophotometric methods, the presence of phenothiazine-type compounds in urine (1, 4, 5). Unless an elaborate separation of the phenothiazines from their metabolites is accomplished, the methods have limited application to quantitative analysis of the unchanged drugs. For specimens other than urine, identification of the sulfoxide as a mechanism to determine specifically the presence of phenothiazine compounds is unrewarding.

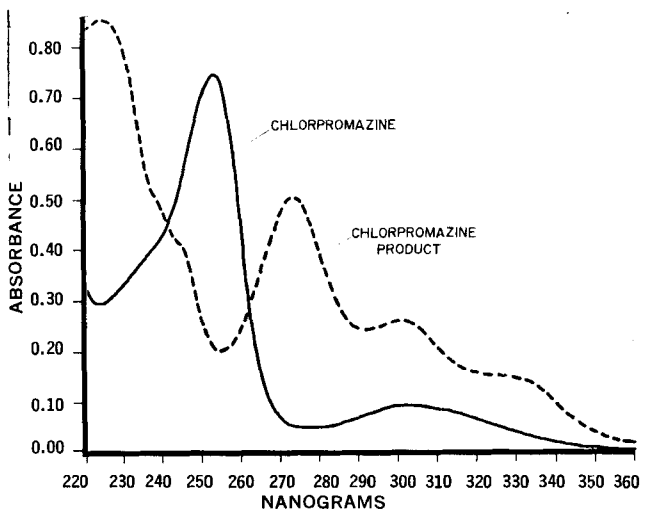

Figure 1—*UV absorption spectra of chlorpromazine in 0.5 N HCl (7.5 mcg./ml.) and of the chlorpromazine product in hexane containing 20% tertiary butanol (10.0 mcg./ml.).*

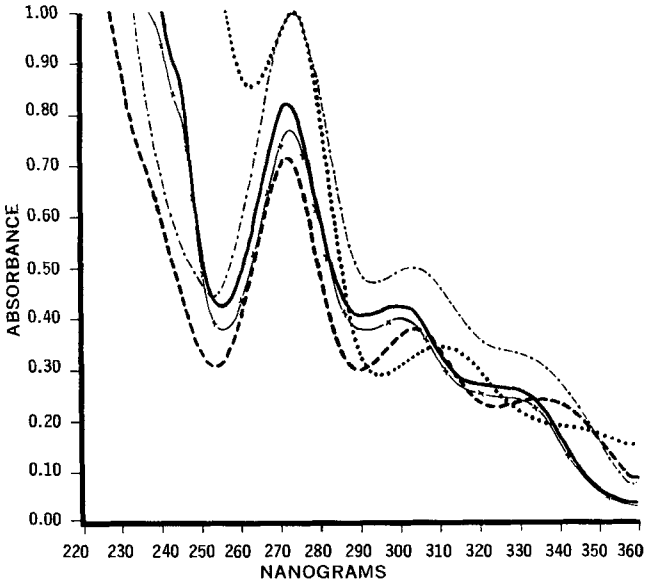

Figure 2—*UV absorption spectra of the prochlorperazine product (——), perphenazine product (X-X), trimeprazine product (-··-), propiomazine product (. . .), and the trifluoperazine product (- - -), all in hexane–tertiary butanol mixture, each corresponding to a phenothiazine concentration of 20 mcg./ml.*

The sulfoxide metabolites have excellent fluorescent characteristics, and several spectrophotofluorometric methods utilizing that property were described (6–9). The drug and the primary sulfoxide metabolite can be extracted simultaneously, the former oxidized by agents such as hydrogen peroxide, and the total phenothiazine content of the specimen measured spectrophotofluorometrically. Such procedures have proven to be useful and have improved the sensitivity for analysis of these compounds. However, only approximate identity for phenothiazine compounds can be established by that technique. In addition, previous reactions for the oxidation of phenothiazines to their corresponding sulfoxides are time and temperature dependent and nonstoichiometric. In a recent report, Kaul *et al.* (10) described a fluorometric method for determining chlorpromazine and its metabolites at the nanogram level in plasma and urine. This method, which utilizes a reaction of the phenothiazine with 5-dimethylaminonaphthylene-1-sulfonyl chloride, is apparently not applicable to the analysis of tissues or phenothiazines other than chlorpromazine.

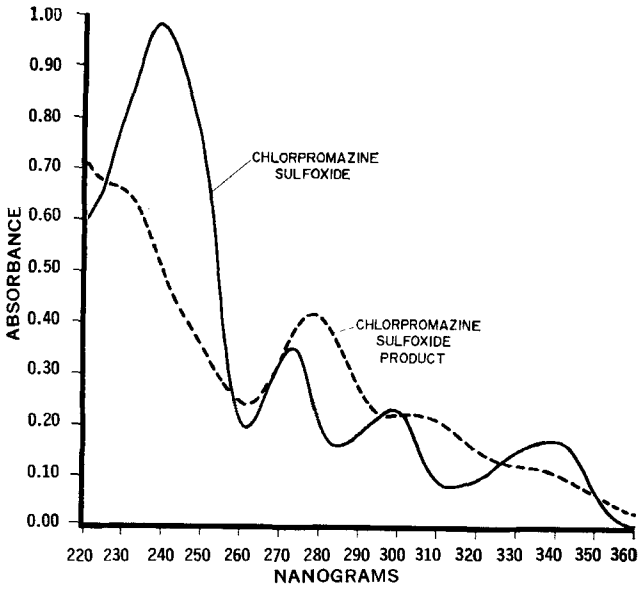

Figure 3—*UV absorption spectra of chlorpromazine sulfoxide in 0.5 N HCl and of the chlorpromazine sulfoxide product in hexane–tertiary butanol mixture, both corresponding to a chlorpromazine sulfoxide concentration of 10 mcg./ml.*

Methods for specific determination of amitriptyline[1] and imipramine[2], two drugs that have molecular structures closely related to that of the phenothiazine compounds, were published (11, 12). In extracts from biologic samples, the three pharmacologic agents are difficult to analyze by direct UV spectrophotometric scanning. In an intoxication involving more than one of the compounds, specific quantitative analysis in the past has been extremely complex. Prior to the series of investigations initiated at this laboratory, elaborate GLC techniques had to be employed for quantitative analysis of biologic extracts containing two or more of the compounds. Such an analysis is limited since many of the phenothiazines and, especially, sulfoxides are not easily made volatile (3, 13). In addition, a certain degree of specificity is lacking in GLC techniques. TLC analysis of urine samples can be misleading because of the variable chromogenic nature of the major metabolites of the phenothiazine that may appear on the chromatographic plate. In practice, phenothiazine substances are converted into sulfoxides during solvent-induced migration on the chromatographic plate (13). This method has been used primarily for identification of the drugs in dosage forms and has little applicability to the analysis of biologic specimens.

The method proposed in this report is a specific and quantitative UV spectrophotometric technique for determining phenothiazine compounds in blood, urine, and tissues. The drugs are oxidized to sulfone or similar type derivatives whose spectra are different from that of the original compounds. No other drugs have been observed to interfere. Each phenothiazine and its sulfoxide metabolite give the desired product in reproducible amounts. Quantitative results are obtained if the type of phenothiazine is known. Rapid qualitative identification is best achieved by TLC (14).

[1] Elavil.
[2] Tofranil.

Table I—Standard Curve Data of Phenothiazine Reaction Products

Compound	$\lambda_{max.}$, nm.	Concentration, mcg./ml.	Absorbance[a]	Absorbance/Concentration
Chlorpromazine	273	5	0.24	0.048
		10	0.48	0.048
		15	0.72	0.048
		20	0.94	0.047
Perphenazine	272	5	0.20	0.040
		10	0.39	0.040
		15	0.61	0.041
		20	0.77	0.039
Prochlorperazine	272	5	0.21	0.042
		10	0.43	0.043
		15	0.63	0.042
		20	0.82	0.041
Propiomazine	273	5	0.26	0.052
		10	0.52	0.052
		15	0.76	0.051
		20	1.00	0.050
Pyrathiazine	273	5	0.15	0.030
		10	0.29	0.029
		15	0.44	0.029
		20	0.56	0.028
Thioridazine	276	5	0.15	0.030
		10	0.28	0.028
		15	0.43	0.029
		20	0.54	0.027
Trifluoperazine	272	5	0.19	0.038
		10	0.37	0.037
		15	0.60	0.040
		20	0.71	0.036
Trimeprazine	273	5	0.27	0.054
		10	0.51	0.051
		15	0.75	0.050
		20	0.98	0.049

[a] In hexane–tertiary butanol.

EXPERIMENTAL

Instrumentation—UV absorption data were obtained with a Beckman DK-2A ratio-recording spectrophotometer with linear presentation of the wavelength. A Beckman IR-9 spectrophotometer was used for IR absorption measurements.

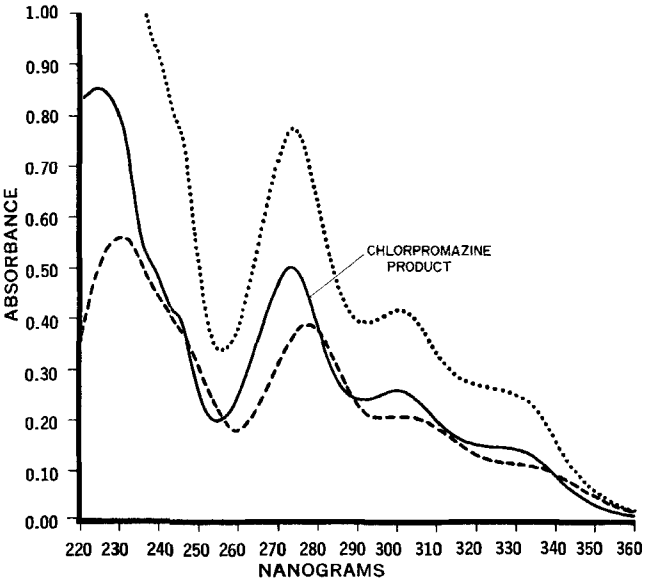

Figure 4—*UV absorption spectra of the chlorpromazine product, 10 mcg./ml., and of the chlorpromazine product from oxidation of drug isolated from rat liver (. . .) and rat urine (- - -), each in hexane–tertiary butanol mixture.*

Table II—Drugs Found Not to Interfere[a] with the Cobalt (III) Oxidation Method for Determining Phenothiazine Compounds[b]

Amitriptyline	Imipramine	Phenethylamine
Amphetamine	Isoniazid	Phenelzine
Atropine	Lidocaine	Phenindamine
Azacyclonol	Meclizine	Phenmetrazine
Benzphetamine	Mepenzolate	Phenylpropanolamine
Brucine	Meperidine	Phenyltoloxamine
Captodiamine	Meprobamate	Phenyramidol
Carphenazine	Mescaline	Picrotoxin
Carbinoxamine	Methadone	Pilocarpine
Chlophedianol	Methapyrilene	Pipenzolate
Chlorcyclizine	Methoxamine	Procaine
Chlordiazepoxide	Methoxyphenamine	Procyclidine
Chlorpheniramine	4-Methyl-2,5-di-methoxy-amphetamine	Propoxyphene
Chlorphentermine		Pyrrobutamine
Clemizole		Quinidine
Cyclizine	Methylphenidate	Reserpine
Dextromethorphan	Morphine	Strychnine
Diethylpropion	Nicotine	Sulfadiazine
Diphenhydramine	Orphenadrine	Thenyldiamine
Diphenylpyraline	Papaverine	Thonzylamine
Doxylamine	Pargyline	Trichlormethiazide
Ephedrine	Phenacemide	Trimethadione
Epinephrine	Phenacetin	Tripelennamine
Ethoheptazine	Phenaglycodol	Triprolidine
Hydroxyzine	Phendimetrazine	Yohimbine

[a] Optical density less than 0.01 for 10 mcg./ml. of each drug in solvent mixture of 20% tertiary butanol in hexane. [b] Absorbance measured at 273–275 nm.

Method—Five- to ten-milliliter volumes of blood, serum, or urine are placed in a separator and made strongly alkaline (pH 10.0) by the addition of 6 *N* NaOH. For tissue analysis, 5–10-g. amounts are homogenized and 10 *N* NaOH is added to achieve a normality of 2.0 (8). The tissue mixture is hydrolyzed in a boiling water bath for 5 min., removed, and allowed to cool in an ice bath. At this point, all specimens are extracted by vigorous shaking for 3 min. with 50–200 ml. of a solvent system of *n*-heptane (ACS grade) containing 4% tertiary butanol[3]. The aqueous phase is removed and discarded, after which the organic solvent is filtered through a fast-flowing filter paper and washed with a 0.5 *N* NaOH solution. Complete recovery of the heptane–alcohol mixture is not necessary, but the recovered volume is recorded and the solvent loss is included in the final calculations.

Ten milliliters of 2.5 *N* hydrochloric acid solution is added to the recovered solvent phase, and the mixture is shaken vigorously in a separator for 3 min. After phase separation, 9 ml. of the aqueous acid layer is transferred to a 250-ml. round-bottom flask with a ground-glass neck for attachment to a water-cooled reflux condenser. To the flask is also added a magnetic Teflon-coated stirring bar, 10 ml. of an 80:20 mixture of spectroquality *n*-hexane[4] and tertiary butanol (specifications as listed previously), and 250 mg. of cobalt (III) oxide, Co_2O_3[5]. The mixture is refluxed by utilization of a heating mantle[6] with 40 v. applied. Constant stirring of the flask contents is maintained by means of a magnetic mixer.

After 30 min. of refluxing, the reaction flask is cooled to room temperature and the solvent layer is removed. The latter is scanned in a recording UV spectrophotometer between the wavelengths of 360 and 220 nm. Two or more maxima are identified if the drug product concentration in the final solution is equivalent to at least 2.5 mcg./ml. of phenothiazine. To enhance sensitivity further, the hexane–tertiary butanol mixture can be concentrated to 0.5–1.0 ml., utilizing a rotary vacuum evaporator, and the absorption determined in a microcell.

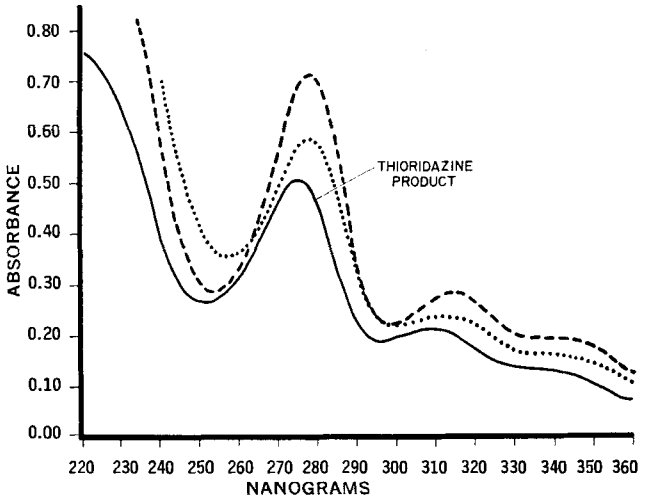

Figure 5—*UV absorption spectra of thioridazine product, 18 mcg./ml., and of the thioridazine product from oxidation of drug isolated from human liver (. . .) and human lung (- - -), each in hexane–tertiary butanol mixture; liver level, 21 mcg./g.; lung level, 23 mcg./g.*

[3] Baker Analyzed Reagent, No. 9056 or equivalent.

Table III—Recovery of Chlorpromazine after *In Vitro* Addition to Serum, Urine, and Homogenized Liver

Amount Added, mcg./ml. (g.)	Recovery, Mean ± Standard Deviation (mcg./ml.)[a] Serum	Urine	Homogenized Liver
50	—[b]	49.0 ± 0.9	48.9 ± 0.8
25	24.7 ± 0.7	24.5 ± 0.6	24.7 ± 0.5
10	9.4 ± 0.5	9.3 ± 0.4	9.7 ± 0.4
5	4.8 ± 0.3	4.5 ± 0.2	—[b]
2.5[c]	2.4 ± 0.2	2.4 ± 0.3	2.4 ± 0.2
1.0[c]	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
0.5[c]	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.05
Average recovery	94.1%	93.6%	95.8%

[a] Six determinations for each mean value. [b] Not determined. [c] Tertiary butanol–hexane mixture concentrated.

Table IV—Distibution of Phenothiazine Compounds[a] in the Rat

Tissue[b,c]	Chlorpromazine	Prochlorperazine	Propiomazine	Thioridazine	Trifluoperazine
	mcg./ml. or g.				
Blood	1.23	0.86	0.20	0.84	0.77
Brain	8.85	4.58	1.64	6.50	8.27
Fat	4.24	3.47	5.00	4.40	4.10
Kidney	37.83	17.21	10.65	20.17	16.56
Liver	58.48	19.08	14.71	26.43	27.60
Lung	85.45	43.93	29.20	52.64	50.81
Muscle	4.54	2.79	2.51	1.48	3.05

[a] Each phenothiazine was administered in oral doses at 25 mg./kg. body weight. [b] Animals sacrificed after 4 hr. [c] For each drug, the results are based on analysis of specimens from the same rat.

RESULTS

Phenothiazine compounds, after oxidation with cobalt (III) in dilute hydrochloric acid, give reproducible amounts of a product with unique UV absorption spectra. Figure 1 illustrates the spectrum of the product from chlorpromazine. Spectra of the oxidation products obtained from several phenothiazine compounds (Fig. 2) have only minor differences. Standard curve data for eight frequently utilized phenothiazines are shown in Table I. The sulfoxides are also oxidized to similar products, as demonstrated by the oxidation of the sulfoxide of chlorpromazine (Fig. 3). Phenothiazines oxidized after extraction from the tissues and urine of animals that have received the drug yield spectra nearly identical to those observed for the product from the pure drug (Figs. 4 and 5).

IR spectra of chlorpromazine, chlorpromazine sulfoxide, and the chlorpromazine product show several distinct differences (Figs. 6–8). Most significant are the IR absorption changes in the 1000–1200-

[4] Fisher Scientific Co., No. H-334 or equivalent.
[5] Alfa Inorganics, Ventron Division, Beverly, Mass., No. 23129.
[6] Glas-Col Apparatus Co., Terre Haute, Ind.

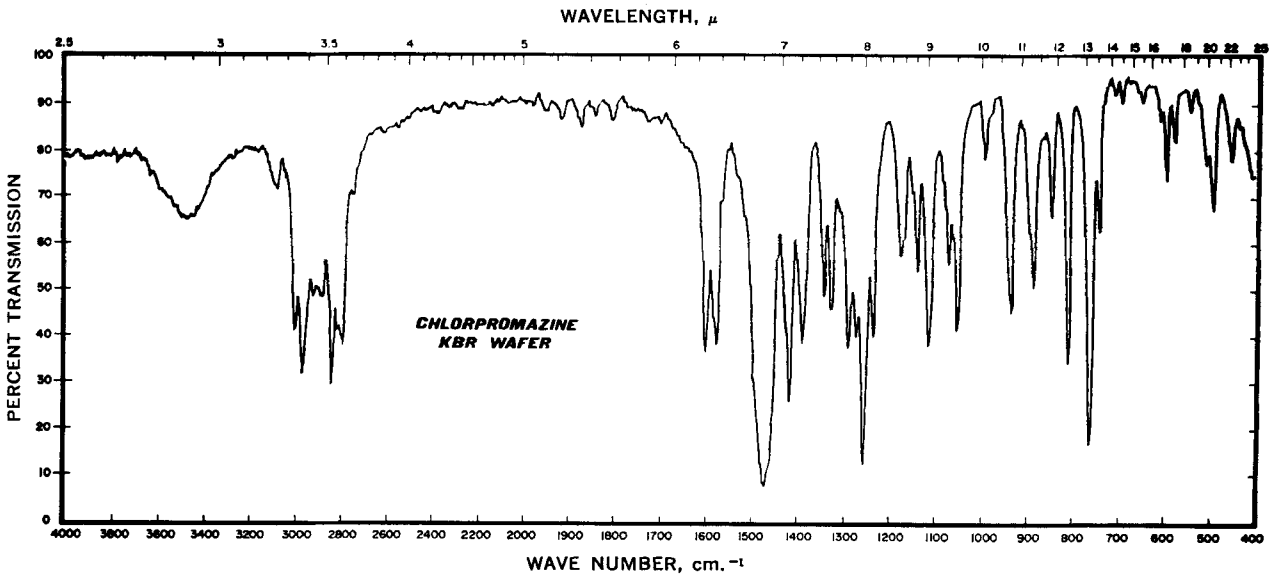

Figure 6—*IR spectrum of chlorpromazine, 2 mg. in 400 mg. potassium bromide.*

cm.$^{-1}$ region. The spectra for the sulfoxide show the characteristic strong absorption for that functional group at 1040–1050 cm.$^{-1}$ (15); yet in the cobalt oxidation product, a strong band at 1130–1150 cm.$^{-1}$ (15) characteristic of sulfone absorption is observed.

Many nitrogen-containing organic bases were investigated for possible interference with qualitative or quantitative determination of phenothiazines by the proposed method. After cobalt oxidation, the compounds listed in Table II provided no significant absorption (less than 0.02 optical density) within the wavelength range of 240–360 nm.

The recovery of chlorpromazine after *in vitro* addition to whole blood, urine, and homogenized liver is summarized in Table III. Freshly made standard aqueous solutions of chlorpromazine hydrochloride were added to the biologic material to provide concentrations of 5, 10, 25, and 50 mcg./ml. (g.). The mixtures were allowed to incubate at room temperature for 30 min. prior to analysis. Ten milliliters of blood, 20 ml. of urine, or 10 g. of homogenized liver with added drug was carried through the procedure as described previously. Recoveries greater than 92% were obtained.

The distribution of the drugs among the various tissues of the rat, 4 hr. after the animals received orally 25 mg./kg. of body weight, is shown in Table IV. The levels of the drugs in the tissues, in decreasing order, were: lung, liver, kidney, brain, fat, muscle, and blood. The procedure of this report gives, after oral ingestion, distribution ratios in agreement with those previously published (16) for intramuscular injection of the drug. With the exception of the brain, the findings are in agreement also with levels observed by Salzman and Brodie (1) in dog tissues after intravenous injection of chlorpromazine.

DISCUSSION

Cobalt (III) oxidation permits rapid identification of phenothiazine drugs, even in a biologic extract containing a mixture of several pharmacologic compounds. It affords a useful UV spectrophotometric technique for measuring phenothiazines as characteristic oxidation products. In certain instances, some indication of the type of phenothiazine can be elucidated. With each drug the product can be distinguished from the unoxidized phenothiazine. The efficiency of the products for absorbing UV radiations is slightly less than that of the original phenothiazines, yet the specificity of the spectrophotometric analysis is greatly enhanced. Concentration of the hexane–tertiary butanol mixture permits the method to approach a sensitivity limit of 0.25 mcg./ml. (g.) if 10 ml. of urine, bile, or blood or 10 g. of tissue are utilized (Table III). Extracts from urine or partially decomposed tissues containing considerable amounts of compounds absorbing in the 250–270-nm. region can be analyzed with no difficulty. Systems that utilize hydrogen peroxide oxidation for fluorometric analysis are more sensitive, but considerable interference due to Raman scattering (7) is encountered when low con-

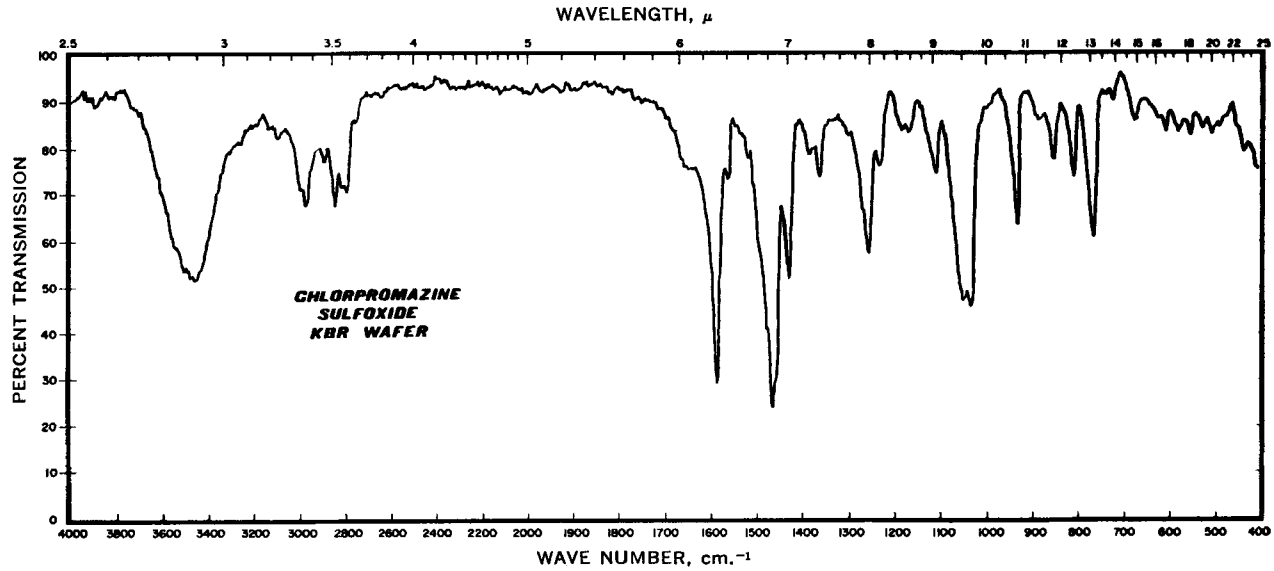

Figure 7—*IR spectrum of chlorpromazine sulfoxide, 1 mg. in 400 mg. potassium bromide.*

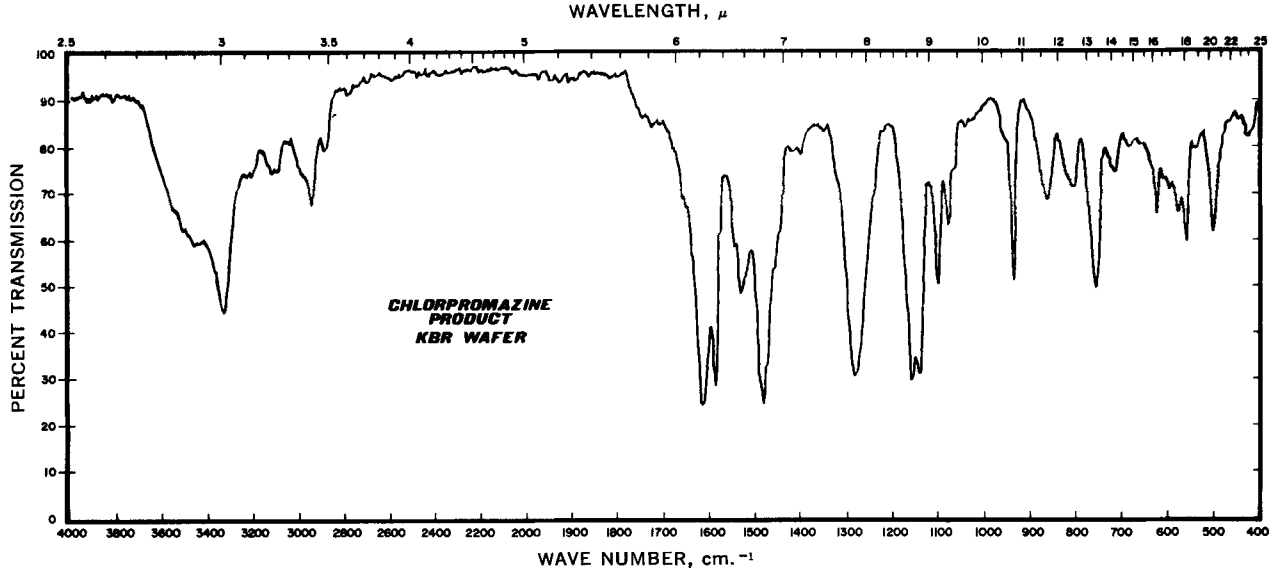

Figure 8—*IR spectrum of chlorpromazine oxidation product, 1 mg. in 400 mg. potassium bromide.*

centrations of the drugs are determined. This type of interference is detrimental in the determination of certain 2-position-substituted phenothiazines which have emission maxima near 380 nm. and excitation maxima in the range of 350 nm. (17). The close proximity of the two maxima can be separated by lowering the excitation wavelength but this significantly decreases sensitivity. The fluorescent characteristics of the products from cobalt (III) oxidation of the phenothiazines will be presented in a subsequent report.

In contrast to phenothiazine compounds and their sulfoxide derivatives, it has been impossible to obtain GLC data on the cobalt (III) oxidation products, using OV-1 or OV-17 liquid phases at temperatures up to 340°. This is apparently the result of the low volatility and high polarity of the sulfone compounds.

Since concentrations of the drug are much higher in urine than in blood, the former is routinely used for toxicologic analysis. With the proposed technique, the analyst is not hindered by variable amounts of sulfoxide derivative in the urine. A high order of specificity is provided by the inability of other alkaline-extractable drugs to form UV-absorbing derivatives after oxidation with the cobalt system (Table II).

IR data suggest the oxidized products to be sulfone derivatives. Their exact chemical structures are not known, but this information is not essential for the effectiveness of the procedure. The structural elucidation of the products and the cobalt (III) reaction mechanism will be the subjects of a subsequent report.

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