tested contained, in addition to lipases, other hydrolytic enzymes of pancreatic, plant, or microbial origin mixed with lactose and smaller quantities of lubricants. The data were analyzed statistically, and excellent agreement between the two methods was observed (Table III). The reproducibility of the method was evaluated by performing repeated analysis of the same enzyme solution. The precision of the method was determined from the variability obtained upon repeated analysis of lipase standard or samples. A standard deviation of 0.76, with a coefficient of variation of 3.05%, was obtained.

CONCLUSION

The advantages of a fluorometric procedure are many. In contrast to earlier methods, the fluorometric assay is more sensitive and requires smaller amounts of substrate and enzyme. The lipolytic measurements can be made at such a great dilution that interfering enzymes and other excipients are frequently diluted out. 4-Methylumbelliferone laurate was found to be a most suitable substrate for the fluorometric assay, because it was unaffected by a number of pharmaceutical agents and enzymes tested.

The method shows a high degree of precision and accuracy. The procedure is simple and requires little preparation. The substrate is stable under the experimental conditions and can be stored frozen for long periods. The method may be valuable in determining content uniformity of pharmaceutical preparations containing lipase.

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▲ To whom inquiries should be directed.

Sensitive Colorimetric Determination of Isoniazid

HENRY S. I. TAN

Abstract □ A sensitive colorimetric method was developed for the assay of isoniazid. The method is based on measuring the absorbance at 467 nm. of the orange-colored product produced by the interaction of isoniazid with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in the presence of sodium tetraborate in absolute methanol. The Beer–Lambert law was obeyed over the concentration range of 0.3–5 mcg./ml, and the color was stable for at least 90 min. The coefficient of variation as determined on nine replicate samples containing 2 mcg. isoniazid/ml. was 0.87%. Applied to the assay of isoniazid tablets and syrups, the method gave results comparable to those obtained by the USP XVIII method. It also afforded improvements in sensitivity, ease, and speed over the official method. It was established that the major metabolites of isoniazid (acetylisoniazid and isonicotinic acid) produced no color with the reagent under the proposed experimental conditions.

Keyphrases □ Isoniazid—colorimetric analysis, compared to compendial method □ Colorimetry—analysis, isoniazid, compared to compendial method

Isoniazid (isonicotinyl hydrazine, I) is a remarkably effective drug which is now considered a primary drug for the chemotherapy of tuberculosis. It is not surprising that the determination of this drug has been, and is still, the subject of much investigation (1, 2). The need for further investigation is evidenced by the fact that many of the assay procedures reported appear not to be very satisfactory. For example, the current official assay method for isoniazid employs an iodometric titration in the presence of sodium bicarbonate (3). This method, originally developed by Canbäck (4), involves a slow reaction; 90 min. is needed for the completion of the reaction before a back-titration of the excess iodine can be performed. In addition, at several stages during the assay period, loss of iodine by volatilization can occur. The USP XVIII method for the assay of isoniazid tablets is time consuming and laborious; *at least* 2.5 hr. is required for the extraction of the drug from the tablet mass, evaporation of the extract, and completion of the reaction (5).

Several methods for the analysis of isoniazid in the microgram range have been developed using 1-chloro- or 1-fluoro-2,4-dinitrobenzene as the reagent (6, 7). Reisch *et al.* (8) recently reported the use of a similar reagent for visualization of isoniazid spots on thin-layer chromatoplates. This reagent, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (II)[1], gave a colored product

[1] NBD Chloride, Aldrich Chemical Co., Milwaukee, Wis.

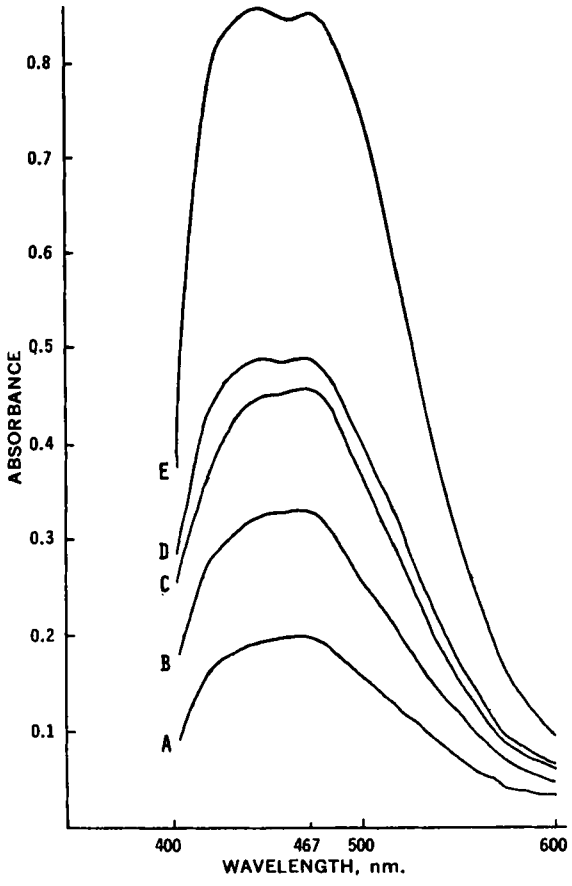


Figure 1—*Absorption spectra of reaction product at various isoniazid concentrations (micrograms per milliliter). Key: A, 2.0; B, 3.3; C, 4.5; D, 5.0; and E, 9.0.*

with isoniazid. The color of the product seemed to be pH dependent.

This work served as the basis for the present study, in which II in alkaline medium was used for the quantitative colorimetric assay of isoniazid.

EXPERIMENTAL[2]

Materials and Reagents—The following were used:

Isoniazid—Highest purity grade isoniazid[3] recrystallized from 80% methanol and dried under reduced pressure for 24 hr., m.p. 172–172.5°.

Methanol—Acetone-free absolute methanol containing 0.008% water[4].

Isonicotinic Acid—Isonicotinic acid[5], m.p. 310–315°.

1-Isonicotinyl-2-acetylhydrazine—Prepared by a slightly modified procedure of Fox and Gibas (9). To a stirred solution of 4 g. isoniazid in 25 ml. warm glacial acetic acid was added dropwise 3 ml. of acetic anhydride. The mixture was heated in a boiling water bath for 30 min., cooled, and evaporated under reduced pressure. Upon treatment of the residue with 35 ml. benzene, a white precipitate (4.5 g.) was formed. Recrystallization from isopropyl alcohol–ethyl acetate afforded fine needles, m.p. 161–162.5° [lit. (9) m.p. 162–163°].

[2] A Beckman Acta V UV-visible double-beam spectrophotometer (Beckman Instruments, Fullerton, Calif.), with 1-cm. Bausch and Lomb cells and a slit width of 0.36 mm., was used for the absorption measurements. A Blue M Magniwhirl model MW 1110A-1 constant-temperature water bath (Blue M Electric Co., Blue Island, Ill.) and a Mettler type H18 analytical balance (Mettler Instrument Corp., Princeton, N. J.), were also utilized in the assay procedure.

[3] Fisher Scientific Co., Pittsburgh, Pa.

[4] Matheson, Coleman and Bell, Norwood, Ohio.

[5] Aldrich Chemical Co., Milwaukee, Wis.

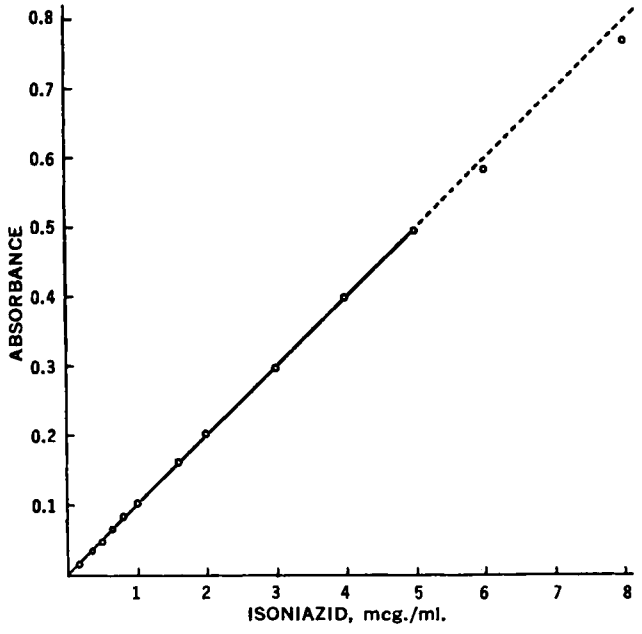


Figure 2—*Relationship between absorbance and concentration of isoniazid.*

Color Reagent—A 0.10% (w/v) solution of II (m.p. 97–99°)[6] in methanol. This reagent is stable for 2 weeks if kept under refrigeration.

Borax Solution—A 2.5% solution of sodium tetraborate decahydrate (ACS grade)[3] in methanol.

Preparation of Standard Curve—A stock solution of isoniazid was prepared by dissolving 50.0 mg. of isoniazid in 100 ml. of methanol. Further dilutions were made to obtain standard solutions, each containing 1.6, 2.0, 3.2, 4.0, 5.0, 6.4, 8.0, 10.0, 12.0, 16.0, 20.0, 30.0f 40.0, 50.0, 60.0, 80.0, and 100.0 mcg. isoniazid/ml. One milliliter or each solution was utilized for color development as described unde, *Assay Procedure for Isoniazid.* The stock and standard solutions must be freshly prepared.

Assay Procedure for Isoniazid—Pipet 1.0 ml. of a methanolic solution containing 3–50 mcg. of isoniazid into a 10-ml. glass-stoppered volumetric flask. To this solution, add successively 1 ml. of color reagent and 2 ml. of borax solution. Dilute the mixture to the mark with methanol. Stopper the flask tightly and place in a constant-temperature water bath at 60 ± 1° for 1 hr. Allow the mixture to cool to room temperature by immersing the flask in a tap-water bath (3–5 min.). Adjust the volume with methanol, if necessary, and measure the absorbance at 467 nm. against a blank prepared as already described but omitting the isoniazid. Obtain the amount of isoniazid present from a calibration curve.

Assay Procedure for Isoniazid Tablets—Weigh and finely powder 20 tablets. Weigh accurately a portion of powder, equivalent to about 100 mg. of isoniazid, and transfer to a 100-ml. volumetric flask. Add methanol to the mark and shake well for about 5 min. Filter the mixture quickly through a dry filter into a dry flask, rejecting the first 10 ml. of filtrate and keeping the funnel covered during the filtration. Pipet 1.0 ml. of the filtrate into a 50-ml. volumetric flask and make up to volume with methanol. Pipet 1.0 ml. of the resulting solution into a 10-ml. glass-stoppered volumetric flask and proceed as described in the *Assay Procedure for Isoniazid*, beginning with: "To this solution, add . . .."

Assay Procedure for Isoniazid Syrups—Transfer an accurately measured volume of syrup, equivalent to about 100 mg. of isoniazid, to a 100-ml. volumetric flask. Add methanol to the mark and shake well for about 1 min. Pipet 1.0 ml. of the resulting solution into a 50-ml. volumetric flask and make up to volume with methanol. Pipet 1.0 ml. of the latter solution into a 10-ml. glass-stoppered volumetric flask and proceed as described in the *Assay Procedure for Isoniazid*, beginning with: "To this solution, add . . .."

Effect of Reagents on Color Formation—*Color Reagent*—A series of 10-ml. glass-stoppered volumetric flasks, each containing 1.0 ml. of a methanolic solution of isoniazid (20 mcg./ml.), was prepared. Varying volumes of 0.05% II in methanol were added to each flask.

Table I—Assay of Solutions of Known Concentrations of Isoniazid at Different Levels

Amount Weighed, mg.	Analyzed at Concentration Level, mcg./ml.	Amount Found, mg.		Percent Recovery		
		Proposed Method[a]	USP XVIII Method	Proposed Method		USP XVIII Method
				Individual Samples	Average	
50.0	0.32	49.5		99.1	100.4	
	0.64	50.8		101.6		
50.0	0.64	50.8		101.6	101.4	
	1.6	50.6		101.2		
100.1	0.5	99.0		98.9	100.4	
	1.0	102.0		101.9		
	4.0	100.6		100.5		
100.0	0.5	100.0		100.0	100.0	
	4.0	100.9		100.9		
	5.0	99.2		99.2		
199.9	1.0	196.0		98.0	98.8	
	2.0	198.0		99.0		
	4.0	198.5		99.3		
200.0	1.0	202.0		101.0	100.9	
	2.0	203.0		101.5		
	4.0	200.0		100.0		
98.9			98.2			99.3
100.2			99.5			99.3
Overall percent recovery					100.3	
Standard deviation					0.88	

[a] Each value is the average of three replicate assays.

After 2 ml. of borax solution was added to each flask and the mixture was diluted to the mark with methanol, the flasks were warmed at 60 ± 1° for 1 hr. The flasks were cooled to room temperature, the volume of the solution was adjusted, if necessary, and the absorbance was measured at 467 nm. against a blank[6].

Borax Solution—A series of 10-ml. glass-stoppered volumetric flasks, each containing 1.0 ml. of a methanolic solution of isoniazid (20 mcg./ml.) and 1 ml. of color reagent, was prepared. Varying volumes of 2.5% borax solution were added to each flask, and the experiment was completed as described for the color reagent. The absorbance of each solution was measured at 467 nm. against a blank[6].

Effect of Isonicotinic Acid and Acetylisoniazid on Assay Results of Isoniazid—Two solutions of isonicotinic acid in methanol containing 20 and 40 mcg./ml., respectively, were prepared. Two solutions of acetylisoniazid in methanol containing 20 and 40 mcg. acetylisoniazid/ml., respectively, were also prepared. One milliliter of each solution was assayed as described under *Assay Procedure for Isoniazid.*

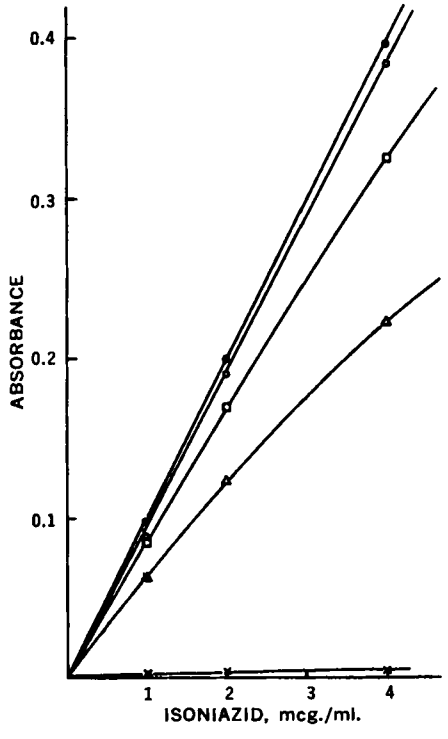


Figure 3—*Effect of heating on color development. Key: ×, 30 min. at room temperature; △, 30 min. at 50°; □, 60 min. at 50°; ○, 30 min. at 60°; and ●, 60 min. at 60°.*

[6] Each blank contained the same amount of II and sodium tetraborate as in the solution studied but contained no isoniazid.

RESULTS AND DISCUSSION

The orange-colored product obtained from the interaction between isoniazid and II in alkaline medium shows an absorption peak at 467 nm. in its absorption spectrum (Fig. 1). At concentrations above 3 mcg. isoniazid/ml., another peak appears around 445 nm. This absorption maximum shifts gradually toward lower wavelengths as the concentration of isoniazid is increased. As might be expected, absorbances measured at this second peak are not proportional to the concentrations of isoniazid. At 467 nm., however, the procedure as described shows adherence to the Beer–Lambert law up to 5 mcg. isoniazid/ml. A typical standard curve is shown in Fig. 2. Under the experimental conditions described in the assay procedure, the molar absorptivity, ϵ, is 13,715 l. mole^{-1} cm.$^{-1}$. Since isoniazid has a molecular weight of 137.15, an absorbance value of 0.100 corresponds to a concentration of 1 mcg./ml.

The interaction between isoniazid and II in the presence of sodium tetraborate in methanol was favored by heating. The time and tem-

Table II—Reproducibility of Color Development of Replicate Isoniazid Samples Containing 2 mcg./ml.

Solution	Absorbance at 467 nm.
1	0.200
2	0.197
3	0.202
4	0.200
5	0.201
6	0.202
7	0.202
8	0.200
9	0.198
Average	0.200
Standard deviation	0.0017
Coefficient of variation	0.87%

Table III—Assay of Isoniazid Tablets and Syrups[a]

Dosage Form	Manufacturer	Claim, mg./Tablet or mg./ml.	Found mg./Tablet or mg./ml.		Percent of Claim	
			Proposed Method	USP XVIII Method	Proposed Method	USP XVIII Method
Tablet	A	100	97.9	99.4	97.9	99.4
Tablet	B	100	98.3	98.9	98.3	98.9
Tablet	C	300	287.4	289.7	95.8	96.6
Syrup	D	10	9.81	9.97	98.1	99.7
Syrup[b]	—	10	9.82	9.79	98.2	97.9

[a] Tablets: average of duplicate assays. Syrups: average of triplicate assays. [b] Isoniazid, 1.00 g.; water (40°), 3 ml.; and sufficient syrup USP to make 100 ml.

perature of heating affected the color formation. At room temperature, hardly any color was formed (Fig. 3). Since heating at 60° required about 60 min. to obtain satisfactory absorbance values, attempts were made to speed up the reaction time by increasing the reaction temperature. Heating at temperatures above the boiling point of methanol presented problems since screw-capped tubes must be used to prevent significant evaporation of the solvent. Furthermore, heating at these high temperatures caused a shift in the absorption maximum to 462.5 nm. At this wavelength, adherence to the Beer–Lambert law could not be observed. Therefore, 60° was chosen as the heating temperature.

The time of heating was determined by following the color development. Absorbance values increased through 70 min. and then decreased. It was found that a heating period of 1 hr. at 60 ± 1° was quite satisfactory. The color produced appeared to be stable for at least 90 min. after the heating. Under these conditions the reference blank showed a pale-yellow coloration.

Gosh and Whitehouse (10) reported the determination of certain amino acids with II in aqueous solutions. When using water or 95% ethanol as solvents for the assay of isoniazid with II, it was found that the color formation was greatly accelerated. However, variations in color intensity occurred with the use of these solvents. The presence of water in the system caused nonreproducibility of the results. In these cases, a deep-yellow color was obtained with the reference blank.

The effect of the concentrations of reagent and sodium tetraborate on color formation was also studied. Higher absorbance values were obtained with the increasing amount of II used. At the same time the reference blank also exhibited a more intense yellow color. This caused a decrease in the sensitivity of the proposed procedure and made the determination of isoniazid at the lower limits of the concentration range obeying the Beer–Lambert law less reliable. The presence of more than 5 mg. sodium tetraborate/ml. in the assay solution caused a decrease in the absorbance value. In view of these results, 1 ml. 0.10% II and 2 ml. 2.5% sodium tetraborate were selected as optimum concentrations for the assay.

The stability of the color reagent solution was evaluated by recording its UV spectrum (λ_{max} 335 nm.) over an extended period. Molar absorptivities remained unchanged for 14 days, indicating that the reagent preparation, when kept refrigerated, could be kept for 2 weeks without significant deterioration.

As little as 0.16 mcg. isoniazid/ml. can be detected by the proposed procedure. The method, however, is reliable only for concentrations above 0.2 mcg./ml. The percent recoveries of known samples analyzed at 0.16- and 0.20-mcg. isoniazid/ml. levels were only 87.5 and 95.0%, respectively, with relative standard deviations from the mean of 38.5 and 3.5%, respectively. Very good recovery data were obtained for the concentration levels of 0.3–5 mcg./ml. The recovery experiments were performed by dissolving an accurately weighed amount of about 50 mg. isoniazid in 100 ml. methanol. Appropriate dilutions were then prepared to afford different concentrations (Table I) which were assayed by the proposed method. The recovery experiments were also conducted on 100 and 200 mg. isoniazid. Data presented in Table I indicate the accuracy of the method; the results were reproducible and quantitative. It was found that slight changes in absorbance reading can result in fairly substantial changes in percent recovery. The overall percent recovery for the six samples was 100.3%, with a standard deviation of ± 0.88%. In comparison, the average percent recovery for two samples analyzed by the USP XVIII method was 99.3%.

The precision of the analytical method was determined by running replication studies on nine 1.00-ml. aliquots of a standard isoniazid solution containing 2.0 mcg./ml. These solutions were assayed by the proposed procedure. The coefficient of variation for the nine replicate samples was 0.87% (Table II).

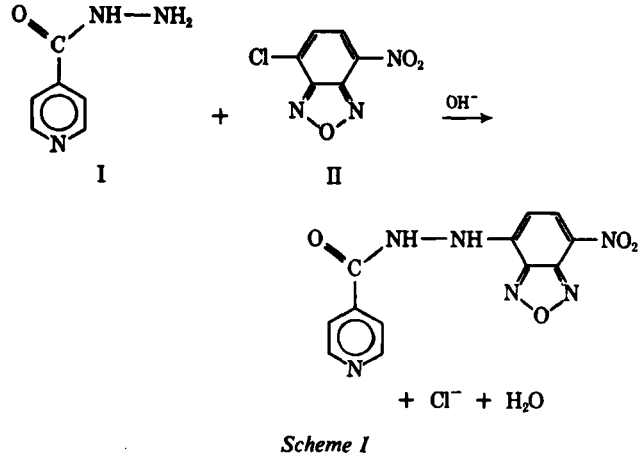
Results obtained by applying the assay procedure to commercially available isoniazid tablets are presented in Table III. Comparison of the experimental data with those obtained by the official method shows a relatively good correlation. The proposed extraction of the drug by shaking the tablet mass with methanol at room temperature for 5 min. appeared to be complete. In contrast, the official method requires heating on a steam bath for 30 min. Generally speaking, the present method offers ease and speed over the official method.

Table III also shows that the proposed colorimetric procedure is applicable for the assay of isoniazid syrups. The results of the proposed method are in agreement with those obtained by the USP XVIII procedure. Since the amount of water in the final assay solution is very low due to the two-step dilution procedure, it does not interfere with the reaction. Commercial Syrup D (Table III) also contained a flavoring agent and a yellow dyestuff. It appeared that these agents do not affect the absorbance measurements.

Several workers (6, 11) have suggested that, analogous to the König reaction, 1-chloro- or 1-fluoro-2,4-dinitrobenzene reacts with the pyridine nitrogen of isoniazid to form a polymethine dyestuff. The structurally related II may be expected to react with isoniazid in a similar fashion. Present studies revealed that neither acetylisoniazid nor isonicotinic acid produced any color with II under the experimental conditions described (Table IV). This would indicate that a free amino function in the molecule is necessary for the reaction with II. These findings are in agreement with those of Poole and Meyer (7), who stated that the reactivity of 1-fluoro-2,4-dinitrobenzene with the pyridine nitrogen of isoniazid is not probable.

Table IV—Effect of Acetylisoniazid and Isonicotinic Acid on Assay Results of Isoniazid

Compound	Concentration Assayed, mcg./ml.	Absorbance at 467 nm.
Acetylisoniazid	2.0	0.000
Acetylisoniazid	4.0	0.000
Isonicotinic acid	2.0	0.001
Isonicotinic acid	4.0	0.000



Scheme I

Therefore, it is concluded that the reaction between isoniazid and II under the experimental conditions of the assay method likely proceeds as depicted in Scheme I.

The present method can be modified to make it applicable to the determination of *free* isoniazid in biological fluids since none of the most important metabolites of isoniazid that have been identified (acetylisoniazid, isonicotinic acid, isonicotinylglycine, pyruvic acid isonicotinylhydrazine, and α-oxoglutaric acid isonicotinylhydrazine) (12) possesses a free amino function in the molecule. Further studies are in progress, and the results will be reported at a later date.

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Potentiometric Titration of Antithyroid Drugs with Mercuric Acetate Solution

SERGIO PINZAUTI▲, VITTORIO DAL PIAZ, and ENZO LA PORTA

Abstract □ A simple and accurate potentiometric titration procedure for the analysis of five antithyroid drugs is described. The drugs dissolved in aqueous solution at particular pH values are titrated with 0.01 *M* mercuric acetate. The end-point is determined potentiometrically by the use of three different indicating electrodes (mercury drop, amalgamated gold, and silver). Quantitative recoveries are reported.

Keyphrases □ Thyroid inhibitors—analysis, potentiometric titration with mercuric acetate □ Methimazole—analysis, potentiometric titration with mercuric acetate □ Thiobarbituric acid—analysis, potentiometric titration with mercuric acetate □ Thiouracil—analysis, potentiometric titration with mercuric acetate □ Methylthiouracil—analysis, potentiometric titration with mercuric acetate □ Propylthiouracil—analysis, potentiometric titration with mercuric acetate □ Potentiometric titrimetry—analysis of five thyroid inhibitors using mercuric acetate □ Mercuric acetate—used in potentiometric titration of five thyroid inhibitors

Methimazole (1-methylimidazole-2-thiol), 2-thiobarbituric acid, 2-thiouracil, 6-methyl-2-thiouracil, and 6-propyl-2-thiouracil are commonly employed in treatment of hyperthyroidism. Many types of titrimetric procedures for their determination have been proposed (1–15). The mercurimetric method of Abbot (4) was the basis in the BP monographs (16) for the determination of 6-methyl-2-thiouracil and 6-propyl-2-thiouracil. Accordingly, thiouracil (0.35 g.) is dissolved in an excess of sodium hydroxide and the resulting disodium salt is titrated in a buffered acetate system with mercuric acetate (0.05 *M*); the end-point is determined by the use of diphenylcarbazone as the indicator. Although satisfactory results are attainable by this method, the indicator change is not sharp and the procedure is not suitable for other antithyroid thiols, *e.g.*, methimazole.

In the present study, a simple, rapid, and accurate direct potentiometric titration is reported for the determination of methimazole USP, thiouracil, methylthiouracil BP, propylthiouracil USP and BP, and 2-thiobarbituric acid. The use of three different indicating electrodes is described.

EXPERIMENTAL

Apparatus—Titrations were performed potentiometrically with a pH meter[1] equipped with a mercury drop electrode (17) and mercurous sulfate electrode[2]. An amalgamated gold electrode (a gold flag dipped into metallic mercury for 20 sec.) or a silver electrode (a silver coil) was also employed as indicating electrodes. The mercury drop and amalgamated gold electrodes can be used for many titrations if rinsed with water between titrations.

Reagents and Solutions—Pure drug samples (listed in Table I) were obtained from commercial sources. Analysis by the USP assay for methimazole indicated a purity of better than 98%. Analysis by the BP assay for methylthiouracil and propylthiouracil indicated a purity of better than 98%.

Borax (0.025 M)—Dissolve 9.535 g. of $Na_2B_4O_7 \cdot 10H_2O$ (reagent grade) in sufficient water to make 1000 ml.

Phosphate Buffer (pH 7.2)—Dissolve 17.337 g. of $Na_2HPO_4 \cdot 12H_2O$ (reagent grade) and 2.485 g. of KH_2PO_4 (reagent grade) in sufficient water to make 1000 ml.

Acetate Buffer (pH 4.6)—Dissolve 3.28 g. of CH_3COONa (reagent

[1] Metrohm model E 148c.
[2] Metrohm EA 406.