Pharmacol., **7**, 706(1955).
(5) V. D. Kartseva and B. P. Bruns, *Antibiotiki*, **4**, 45(1959).
(6) B. A. Newton, *Biochem. J.*, **55**, 10(1953).
(7) W. Troll and R. K. Cannon, *J. Biol. Chem.*, **200**, 803(1953).
(8) E. Ivashkiv, *J. Pharm. Sci.*, **57**, 642(1968).
(9) B. C. Lippold, *Pharmazie*, **28**, 713(1973).
(10) E. Ivashkiv, *Biotechnol. Bioeng.*, **15**, 821(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 21, 1974, from the *Squibb Institute for Medical Research, New Brunswick, NJ 08903*

Accepted for publication January 16, 1975.

The author thanks Dr. T. B. Platt for the microbiological assays of broth samples.

Colorimetric Determination of Isoniazid with 9-Chloroacridine

JAMES T. STEWART ˣ and DAVID A. SETTLE

Abstract □ A colorimetric method based on the interaction between isoniazid and 9-chloroacridine was developed. Analytical solutions are shaken for 30 min at 50°, and the absorbance is measured at 500 nm. The procedure is sensitive for isoniazid in the 10^{-5} *M* range. The method was applied to the analysis of isoniazid in pharmaceutical dosage forms and found to be comparable to the USP XVIII assay. Preliminary investigations suggested that the procedure is useful for the determination of free isoniazid in urine and plasma samples containing isoniazid metabolites.

Keyphrases □ Isoniazid—colorimetric analysis with 9-chloroacridine □ 9-Chloroacridine—colorimetric reagent for determination of isoniazid □ Colorimetry—analysis, isoniazid with 9-chloroacridine

The interaction of isoniazid (isonicotinic acid hydrazide) with 9-chloroacridine to give highly colored solutions has been observed in this laboratory. This observation led to the development of a new colorimetric method for determining isoniazid with 9-chloroacridine. The reagent was shown previously to be applicable to the colorimetric analysis of primary aromatic amines and aromatic hydroxylamines (1–4).

Existing analytical procedures for the assay of isoniazid include visible and UV spectrophotometry, polarography, and titrimetry. The colorimetric methods involve the interaction of the drug with 1-chloro- or 1-fluoro-2,4-dinitrobenzene (5, 6) and/or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (7). Although highly sensitive and specific, these procedures have the disadvantages of instability of color formation and preparation of multireagent solutions. Vanillin has also been used as a chromogenic reagent in the analysis of isoniazid (8).

A procedure involving the hydrolysis of isoniazid to hydrazine, with subsequent color development with *p*-dimethylaminobenzaldehyde, was reported (6). This method is subject to interference by aldehydes and/or ketones. UV spectrophotometry (6) and polarography (6) also have been utilized in the analysis of microgram quantities of isoniazid. The USP XVIII method (9) involves an iodometric titration and is laborious and time consuming.

This paper presents a new colorimetric method for determining microgram quantities of isoniazid with 9-chloroacridine. The method has been successfully applied to the analysis of the drug in pharmaceutical dosage forms. In addition, preliminary investigations suggest that the procedure is useful for the determination of free isoniazid in urine and plasma samples.

Table I—Analysis of Known Isoniazid Mixtures for Isoniazid

Mixture	Components[b]	Isoniazid[a] Found, $M \times 10^{-5}$	Isoniazid[a] % of Theory
1	Isoniazid Pyridoxine hydrochloride	4.00 ± 0.04[c]	100.00
2	Isoniazid Chlorobutanol	3.96 ± 0.04	99.00
3	Isoniazid Aminosalicylic acid	3.95 ± 0.06	98.75
4	Isoniazid Acetylisoniazid Isonicotinic acid Isonicotinuric acid	3.98 ± 0.02	99.50
5	Isoniazid 1,2-Diacetylhydrazine	3.98 ± 0.02	99.50
6	Isoniazid *N*-Acetylhydrazine	3.95 ± 0.03	98.75

[a] Based upon three replicate determinations of each solution. [b] Final concentration of isoniazid and all other components in the mixture, except aminosalicylic acid, was 4×10^{-5} *M*; aminosalicylic acid was present in a final concentration of 2×10^{-5} *M*. [c] Confidence limits at $p = 0.05$.

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers[1,2]. Matched cells with a 1-cm optical path were used.

Reagents and Chemicals—9-Chloroacridine[3] was used as the chromogenic reagent. It was recrystallized before use by the method of Albert (10). Isoniazid[4] was purified by recrystallization from 80% methanol and dried under reduced pressure, mp 172–173°. Isonicotinic acid[5] was used as received. Acetylisoniazid, α-ketoglutaric acid isonicotinoylhydrazone, pyruvic acid isonicotinoylhydra-

[1] Perkin-Elmer model 450.
[2] Bausch and Lomb Spectronic 20.
[3] Eastman Organic Chemicals, Rochester, N.Y.
[4] Matheson, Coleman and Bell, East Rutherford, N.J.
[5] Aldrich Chemical Co., Milwaukee, Wis.

Table II—Determination of Isoniazid in Tablets by the 9-Chloroacridine Method and the USP XVIII Method

Tablet	Claim, mg/Tablet	Found[a], mg/Tablet		Percent of Claim	
		9-Chloroacridine Method	USP XVIII Method	9-Chloroacridine Method	USP XVIII Method
A[b]	100	101.15 ± 0.35[c]	101.46 ± 1.69	101.15	101.46
B[d]	100	100.00 ± 0.93	100.61 ± 0.84	100.00	100.61

[a] Based upon two replicate determinations of each solution. [b] Dosage form contained only isoniazid as active ingredient. [c] Confidence limits at $p = 0.05$. [d] Dosage form also contained pyridoxine hydrochloride (10 mg).

zone, acetylhydrazine, and 1,2-diacetylhydrazine were synthesized by procedures listed by Boxenbaum and Riegelman (8). Isonicotinuric acid was prepared by the method of Gardner *et al.* (11). The melting points of the synthesized compounds were in agreement with literature values. All other chemicals were commercially available and were utilized as received.

Solutions were prepared by dissolving weighed amounts of isoniazid in water or ethanol and the remaining compounds in ethanol. The reagent solution of 9-chloroacridine was prepared by dissolving a weighed amount in tetrahydrofuran[6] and storing in a light-resistant volumetric flask[7].

General Procedure—A quantity of an aqueous or ethanolic solution containing isoniazid was placed in an appropriate volumetric flask. A tetrahydrofuran solution containing an approximate twofold excess of 9-chloroacridine was added to the flask. The solution was acidified with 2 drops of a 5% (v/v) aqueous hydrochloric acid solution and shaken in a constant-temperature water bath–shaker[8] at 50 ± 1° for 30 min. Ethanol was added to volume so that the final concentration of isoniazid in the flask was equal to or greater than 4×10^{-6} *M*. The absorbance was measured at 500 nm, and measurements were corrected for reagent blanks in the procedure.

Analysis of Solid Dosage Form—Tablets were weighed and powdered, and a weighed aliquot was dissolved in distilled water by shaking vigorously for 5–10 min and filtering, if necessary, to give a 10^{-3} *M* solution. Then 1 ml was removed and assayed according to the *General Procedure*.

RESULTS AND DISCUSSION

9-Chloroacridine interacts with isoniazid in the analytical procedure to yield a highly colored orange solution. The absorption curve in the visible spectrum for an analytical solution of isoniazid shows an absorption maximum at 500 nm (Fig. 1).

The interaction between 9-chloroacridine and isoniazid is affected by the temperature and time of heating and the quantity of acridine reagent utilized. Adherence to the Beer–Lambert law was not observed for various concentrations of isoniazid at room temperature for 30–45 min (Fig. 2). However, when the analytical solutions were heated at 40, 50, and/or 60° for 30–45 min, linearity was observed. The results showed that 50° represented the lowest temperature at which both maximum sensitivity and linearity could be obtained. Thus, it was chosen as the desired temperature for the analytical procedure.

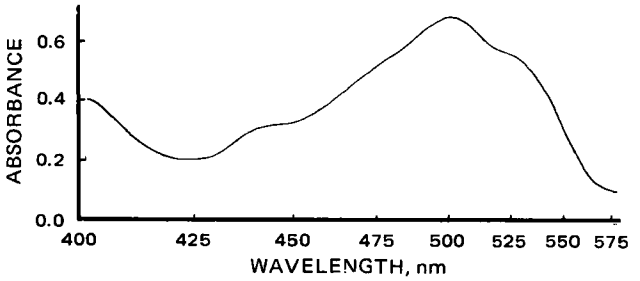


Figure 1—*Absorption spectrum of reaction product at isoniazid concentration of* 4×10^{-5} M.

The minimum heating time was then determined by following the color development. Absorbance values increased through 30 min and then stabilized. A heating period of 30 min at 50 ± 1° was satisfactory for reproducible readings. The color produced appeared to be stable for at least 1 day after the heating. Under these conditions, the reference blank was colorless.

The intensity of the color produced was directly affected by the concentration of 9-chloroacridine employed. Higher absorbance values were obtained up to a twofold excess of the reagent. Quantities of the acridine beyond the twofold excess did not effectively increase the absorbance readings for isoniazid. The stability of the 9-chloroacridine reagent solution was discussed previously (3, 4).

Standard curves can be prepared by plotting observed absorbance readings *versus* volumes taken of equimolar concentrations of isoniazid.

The analysis method is a microprocedure, and sensitivity is in the 0.6–5-μg/ml range (10^{-5} *M*) of isoniazid. Reagent preparation is simple and rapid since 9-chloroacridine is commercially available and easily purified. The time involved in the actual analysis procedure is about 45 min; some existing colorimetric and titrimetric methods involve longer heating and sample preparation times (5–7, 9).

Data from several systems (Table I) reveal that this procedure permits the determination of isoniazid in mixtures containing drugs, preservatives, and isoniazid metabolites which might be present in commercial dosage forms or biological samples containing the drug. Aminosalicylic acid interferes with the analysis method if it is present in final concentrations greater than 2×10^{-5} *M*. Since aminosalicylic acid is normally present in greater quantities than isoniazid in dosage forms and biological fluids where the drugs are given concurrently, it would be desirable before analyzing for isoniazid by this method to separate the two drugs *via* some type of separation technique or to employ the technique of simultaneous spectrophotometric assay. The latter technique should be possible since both aminosalicylic acid and isoniazid interact with 9-chloroacridine to produce maximum absorption at 435 and 500 nm, respectively.

Results obtained by applying the assay procedure to commercially available isoniazid tablets are shown in Table II. The method is comparable to the USP XVIII procedure (9). Extraction of the drug from tablets by shaking the tablet mass with distilled water for 5–10 min at room temperature appears to be as efficient as the USP extraction procedure utilizing heat and methanol. In addition, the colorimetric method is quicker and requires the preparation of only one reagent solution.

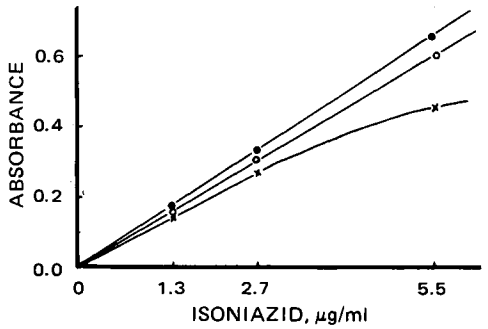


Figure 2—*Effect of temperature on color development and linearity of various isoniazid concentrations. Key:* ×, *30–45 min at room temperature;* ○, *30–45 min at 40°; and* ●, *30–45 min at 50° and also at 60°.*

[6] J. T. Baker Chemical Co., Phillipsburg, N.J.
[7] Low actinic volumetric flask (Corning No. 55640).
[8] Model 2156-1, Warner-Chilcott Instruments Division.

Table III—Analysis of Isoniazid Mixtures in Urine for Isoniazid

Mixture	Components, 4×10^{-5} M	Isoniazid[a] Found, $M \times 10^{-5}$	Isoniazid[a] % of Theory
1	Isoniazid Acetylisoniazid Isonicotinic acid Isonicotinuric acid	4.00 ± 0.04[b]	100.00
2	α-Ketoglutaric acid isonicotinoylhydrazone[c]	4.03 ± 0.18	100.75
3	Pyruvic acid isonicotinoylhydrazone[c]	3.95 ± 0.21	98.75
4	Isoniazid 1,2-Diacetylhydrazine	3.95 ± 0.01	98.75
5	Isoniazid *N*-Acetylhydrazine[d]	3.93 ± 0.04	98.25

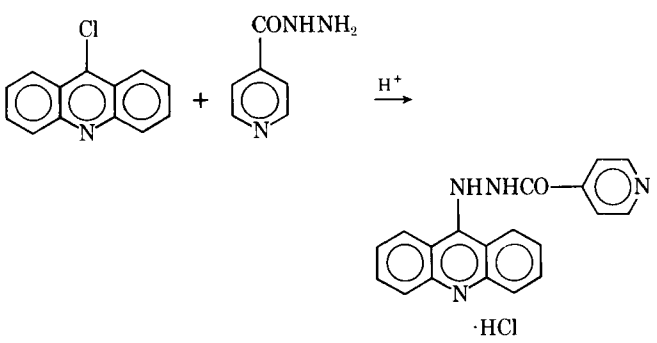
[a] Based upon three replicate determinations of each solution. Percent recovery of isoniazid from urine was 99.38 ± 0.26%. [b] Confidence limits at $p = 0.05$. [c] Hydrazones were hydrolyzed to isoniazid by treatment with 0.2 *N* HCl in a 52° water bath for 60 min; the solution was adjusted to pH 5 with aqueous potassium carbonate followed by the addition of a 9-chloroacridine solution. [d] Final concentration in mixture was 2×10^{-5} *M*.

A preliminary investigation of the analysis of isoniazid in urine and plasma was performed utilizing the 9-chloroacridine method. The biological fluids were spiked with various combinations of isoniazid and its metabolites. For each assay, freshly prepared isoniazid standards in urine and plasma were run concomitantly with the mixtures.

In the determination of isoniazid in urine, isoniazid can be quantitated satisfactorily in the presence of its metabolites (Table III). Since the amounts of α-ketoglutaric acid and pyruvic acid isonicotinoylhydrazones present in the urine are usually expressed in terms of *free* isoniazid (8), urine samples containing each hydrazone were quantitatively hydrolyzed to isoniazid using the procedures of Hughes *et al.* (12) and Short (13). After adjusting the solution pH to 5 with aqueous potassium carbonate, 9-chloroacridine was added and the color developed. Results indicate that the 9-chloroacridine method will detect isoniazid released from hydrolysis of the hydrazones. However, the method will not allow the detection of free isoniazid in the presence of the hydrazones since acid present in the analytical solution promotes hydrolysis of the hydrazones during the color development period.

For plasma samples, the results (Table IV) indicate that isoniazid can be successfully analyzed in mixtures containing its metabolites. The spiked plasma samples were denatured with equal volumes of 5% trichloroacetic acid and centrifuged. A portion of the supernatant liquid was transferred to a volumetric flask, and the pH of the sample was then adjusted to approximately 5–6 with aqueous potassium carbonate before addition of the 9-chloroacridine solution. Any cloudiness in the final solutions was clarified by centrifugation.

The color produced from the interaction of isoniazid with 9-chloroacridine suggested that a free amino function in the molecule is necessary for the reaction. This finding is in agreement with previous results from this laboratory concerning the interaction of primary aromatic amines and/or aromatic hydroxylamines with the acridine (1–4) to form highly colored solutions. The latter functional groups produce maximum absorption around 435 nm compared to isoniazid at 500 nm. Therefore, it should be possible to analyze for isoniazid in the presence of any drugs containing primary aromatic amines and/or aromatic hydroxylamine moieties with the use of the simultaneous spectrophotometric assay.



Scheme I

Table IV—Analysis of Isoniazid Mixtures in Plasma for Isoniazid

Mixture	Components, 4×10^{-5} M	Isoniazid[a] Found, $M \times 10^{-5}$	Isoniazid[a] % of Theory
1	Isoniazid Acetylisoniazid Isonicotinic acid Isonicotinuric acid	3.96 ± 0.04[b]	99.00
2	Isoniazid 1,2-Diacetylhydrazine *N*-Acetylhydrazine	3.93 ± 0.04	98.25

[a] Based upon three replicate determinations of each solution. Percent recovery of isoniazid from plasma was 82.70 ± 1.15%. [b] Confidence limits at $p = 0.05$.

Present studies indicated that amides, phenols, heterocyclic amines, alcohols, carboxylic acids, and hydrazines did not produce any color with 9-chloroacridine under the analysis conditions (Table I). Although one cannot exclude the possibility that these other functional groups react with the acridine in typical nucleophilic displacement reactions, only isoniazid produced any colored product under the conditions of the analytical reaction. Therefore, it is concluded that the reaction between isoniazid and 9-chloroacridine under the experimental conditions likely proceeds as depicted in Scheme I.

REFERENCES

(1) J. T. Stewart, T. D. Shaw, and A. B. Ray, *Anal. Chem.*, **41**, 360(1969).

(2) J. T. Stewart, A. B. Ray, and W. B. Fackler, *J. Pharm. Sci.*, **58**, 1261(1969).

(3) J. T. Stewart and D. M. Lotti, *ibid.*, **59**, 838(1970).

(4) R. E. Gammans, J. T. Stewart, and L. A. Sternson, *Anal. Chem.*, **46**, 620(1974).

(5) P. G. W. Scott, *J. Pharm. Pharmacol.*, **4**, 681(1952).

(6) N. F. Poole and A. E. Meyer, *Proc. Soc. Exp. Biol. Med.*, **98**, 375(1958).

(7) H. S. I. Tan, *J. Pharm. Sci.*, **62**, 993(1973).

(8) H. G. Boxenbaum and S. Riegelman, *ibid.*, **63**, 1191(1974).

(9) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 349.

(10) "The Acridines," 2nd ed., A. Albert, Ed., E. Arnold Ltd., London, England, 1966, p. 256.

(11) T. S. Gardner, E. Weins, and J. Lee, *J. Org. Chem.*, **19**, 753(1954).

(12) H. B. Hughes, L. H. Schmidt, and J. P. Biehl, *Trans. Conf. Chemother. Tuberc., 14th*, Veterans Administration, Washington, D.C., 1955, pp. 217–222.

(13) E. I. Short, *Tubercle*, **42**, 218(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 27, 1974, from the *Bioanalytical Laboratory, Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30602*

Accepted for publication January 14, 1975.

Presented at the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, New Orleans meeting, November 1974.

[x] To whom inquiries should be directed.