acteristics of 0.27 and 0.28 reported earlier (1, 2). Waning of ptotic activity at +24 hr. decreased the slope of the dose–response curves, causing a wide range in the 95% confidence limits of the potency and an increase in the calculated λ value (0.96) for this assay. From these results, it can be seen that the potency of the reserpine–deoxycholic acid combination was equivalent to 1.0 over the entire time-course spectra of activity.

To detect if there was any acceleration of activity, ptotic readings were also collected at the +1-hr. interval. However, because of the characteristic slowness of the reserpine response, ptotic activity was seen only with the top doses of both the test and standard treatments. The mean responses were equivalent. Controls receiving a 20-sec. intravenous injection of 0.029% acetic acid, with and without orally administered deoxycholic acid, were without ptotic activity.

The absence of reserpine potentiation with the concomitant administration of reserpine acetate and deoxycholic acid by different routes lends further support to the theory that the mechanism of potentiation with orally administered reserpine–bile acid coprecipitates is by physicochemical rather than by pharmacological means. A physical mixture of 1:16 *M* reserpine and deoxycholic acid was shown to cause a slight but significant rise in potency (maximum of 1.8×) when given orally (1). Although such a mixture does not allow the intimate combination of ingredients found in a coprecipitate and does preclude reduction in particle size during precipitation, there still exists the possibility of local surface-tension lowering by the bile acids. As both drugs enter the circulation in the present experiment, actual physical contact in the gastrointestinal tract is eliminated, yet any true pharmacologic potentiation should occur. The only chance for physical interaction in the gastrointestinal tract is the possibility of enterohepatic excretion of the intravenous reserpine into the intestine. The absence of potentiation, therefore, also precludes an enterohepatic recirculation phenomenon as a potentiation mechanism for the reserpine–deoxycholic acid coprecipitates.

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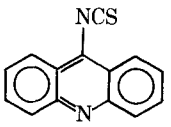
Fluorescent Analysis of Primary Aliphatic Amines by Reaction with 9-Isothiocyanatoacridine

J. E. SINSHEIMER, D. D. HONG*, J. T. STEWART†, M. L. FINK‡, and J. H. BURCKHALTER

Abstract □ Primary aliphatic amines are reacted with 9-isothiocyanatoacridine to yield the corresponding thiourea derivatives. These derivatives, after reaction in base, yield new compounds of intense fluorescence. Conditions have been developed so that it is possible to relate the fluorescence of the final reaction mixture to the concentration of original amine or amine salts without separation of products, even in the presence of excess fluorescent reagent. This method of analysis is successful at the microgram level and can be extended to the nanogram level by TLC separation and direct TLC fluorimetry of the reaction product.

Keyphrases □ 9-Isothiocyanatoacridine reagent—fluorescent analysis, primary aliphatic amines □ Amines, primary aliphatic—fluorescent analysis using 9-isothiocyanatoacridine □ TLC fluorimetry—analysis

These laboratories are interested in the development and evaluation of fluorescent isothiocyanate compounds as new fluorescent protein-labeling agents (1). Such compounds are also being evaluated as functional group reagents for the analysis of trace amounts of amines by formation of fluorescent thiourea derivatives. For example, 9-isothiocyanatoacridine (I) has been found useful for the detection of traces of penicillin (2).



I

In the course of developing assays based upon fluorescent thiourea compounds from 9-isothiocyanatoacridine, it was observed that base treatment of the initial reaction product resulted in an increase in fluorescence of the reaction mixture. This increase was also accompanied by a change in the activation and fluorescent wavelengths of the mixture. These conditions are advantageous for assay of traces of amines based upon the measurement of a reaction product, even in the presence of excess original fluorescent reagent. For example, when the procedure is applied to *n*-butylamine, activation for the final reaction mixture is at 300 nm. with fluorescence at 490 nm. These are compared to an activation wavelength of 410 nm. and fluorescence at 440 and 460 nm. for the butylthiourea derivative of 9-isothiocyanatoacridine and also for the reagent *per se*. The purpose of this paper is to report the development and results of such an assay.

EXPERIMENTAL

Development of Reaction Conditions—Fluorescence intensity as a function of the period of heating was studied. Samples containing 1.5012 mg. of 9-isothiocyanatoacridine (I) and 0.0339 mg. of *n*-butylamine in 10 ml. of absolute alcohol were shaken in a 60°

Table I—Analysis of Amines and Amine Salts

Compound	Range,[a] mcg./10 ml.	Fluorescence: Activation, nm.	Fluorescence: Fluorescent, nm.	Intensity/ Concentration[b]
1. *n*-Propylamine	2–13	300	490	5.6
2. *n*-Butylamine	9–18	300	510	4.7
3. *iso*-Butylamine	26–60	300	510	1.28
4. *tert*-Butylamine	30–60	300	490	1.01
5. 2-Ethylhexylamine	6–20	295	490	1.0
6. Allylamine	7–10	295	490	3.46
7. *n*-Butylamine HCl	10–20	295	490	1.76
8. Adamantamine HCl	5–20	295	490	0.8
9. Cyclohexylamine HCl	5–20	295	490	1.08
10. *n*-Butylamine HCl (in 0.5 ml. of water)	20–30	295	510	0.75

[a] Range observed for a linear response extending through the origin. [b] Slope of linear range: given as fluorescence (calculated for a meter multiplier reading of 1) and divided by concentration (in mcg. per ml.).

water bath.[1] After reaction periods from 0 to 30 min., 1 ml. of 0.2 *N* sodium ethoxide was added to each sample and its corresponding reagent control; heating in a bath was continued for 2 hr. Samples and controls were transferred to 25-ml. volumetric flasks, which contained 1 ml. of 10% HCl and were made to volume with absolute alcohol. Fluorescence was read on a spectrophotofluorometer,[2] with an activation wavelength of 340 nm. and emission wavelength of 490 nm., and compared to the corresponding control solution. From this study, 0.5 hr. was selected as the initial reaction period.

In a similar manner, the optimal period of heating with base, after the 0.5-hr. initial reaction, was found to be 1 hr. The effect of temperature during the initial 0.5-hr. reaction period was examined by reacting samples at different temperatures in the 30–80° range. The optimal temperature was found to be 60°. To study the effect of acid quenching, the fluorescences of samples with and without the addition of 1 ml. of 10% HCl were compared.

General Analytical Procedure—The quantities of amines indicated in Table I, dissolved in 4 ml. of absolute alcohol and 2 ml. of an absolute alcohol solution of 9-isothiocyanatoacridine (0.8 mg./ml.), were placed in 10-ml. volumetric flasks. Samples, together with control solutions of reagent, were heated for 0.5 hr. at 60° before 1 ml. of 0.2 *N* sodium ethoxide was added; heating was continued for an additional hour at 60°. After the flasks were cooled, 1 ml. of 10% HCl was added and solutions were diluted to 10 ml. with absolute alcohol. The increase in fluorescence of the reaction mixtures compared to their corresponding reagent blanks was measured. Activation and fluorescent wavelengths for each compound were selected so that there was a maximum difference between the control and sample.

The analysis of amine salts was conducted in the same manner, except that 1 ml. of absolute alcohol in the original amine solution was replaced by 1 ml. of 0.5% absolute alcohol solution of sodium acetate before reaction with the reagent solution.

TLC Fluorimetry—Cellulose plates, scored into 0.5-cm. channels, were spotted in alternate channels with reaction mixture material and developed for a distance of 13 cm. The cellulose plates, after air drying, were turned upside down and scanned with a thin-layer scanner[3] connected to the spectrophotofluorometer[2] and a recorder[4] with expanded range attenuation (2).

The following conditions were used in all analyses: (*a*) spectrophotofluorometer—sensitivity, 45; meter multiplier, 0.003; scanner motor, 1 r.p.m.; (*b*) recorder—recorder speed, 36 in./hr.; damping, 1/4, and (*c*) expanded range attenuation—zero offset to chart reading of 10%; sensitivity of 0.4 of maximum sensitivity.

The assay of *n*-butylamine serves as a typical example of the procedure; it was performed in the following manner. To each of a series of 5-ml., glass-stoppered, pear-shaped boiling flasks were added 50 μl. of an absolute alcohol solution of Reagent I (9 mcg.) and 10–250 μl. of an absolute alcohol solution of amine (0.12–3.06 ng.). The mixture was shaken in a 60 ± 1° water bath for 30 min., 50 μl. of 0.2 *N* sodium ethoxide was added, and the mixture was heated for an additional hour. The mixtures were cooled, 50 μl. of 10% HCl (aqueous) was added to each flask, and the contents were lyophilized. A control containing no amine was prepared in a similar manner. The lyophilized products and control were dissolved in 100 μl. of absolute alcohol, and 1 μl. of each was spotted on a cellulose thin-layer plate. Development was with a DMF–$CHCl_3$–28% NH_4OH (5:13:3) system with measurement at $\lambda_{act.}$ 270 and $\lambda_{fluor.}$ 485 nm.

[1] Research Specialties No. 2156.
[2] Aminco-Bowman 4-8106 with slit arrangement 4.
[3] Aminco No. 4-8221A.
[4] Heath No. EU-20B.

RESULTS AND DISCUSSION

The general procedure for the synthesis of isothiocyanates has been to react the corresponding amine with thiophosgene (1). However, in the case of 9-isothiocyanatoacridine, this approach was unsatisfactory in yield and purity. That is, the HCl liberated during this synthesis appeared to promote the well-recognized (3) attack upon 9-aminoacridine derivatives. Therefore, the procedure of Kristan (4) employing an S_N2 displacement of the chloro group in 9-chloroacridine by the thiocyanato group from silver thiocyanate was used.

The analytical procedure involves essentially three steps: (*a*) reaction of amine and 9-isothiocyanatoacridine to form a thiourea; (*b*) reaction of the thiourea in sodium ethoxide to form compounds which change the fluorescence activation and emission wavelengths of mixture; and (*c*) quenching of this reaction with acid. Optimal conditions were sought for the amine–reagent reaction and the subsequent treatment with base. As indicated in Fig. 1, a temperature of 60° was most satisfactory for the reaction of an absolute alcohol solution of amine and excess 9-isothiocyanatoacridine. The evaluation of this part of the procedure also depends upon the subsequent addition of base to convert any thiourea formed into the fluorescent compounds capable of detection in the presence of excess reagent.

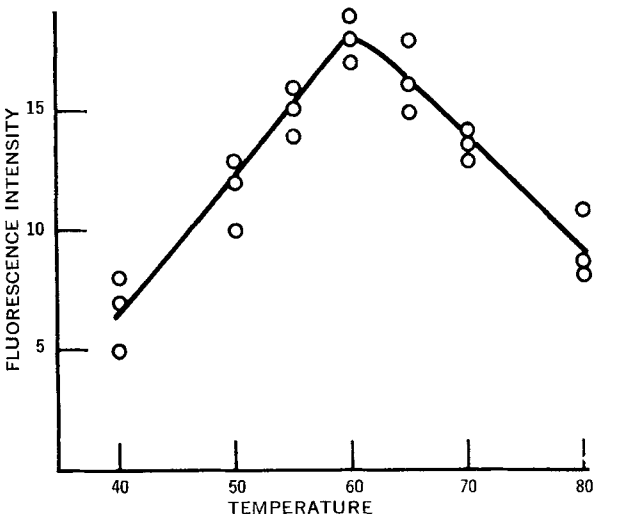


Figure 1—*Fluorescence of the reaction of 9-isothiocyanatoacridine with butylamine as a function of temperature.*

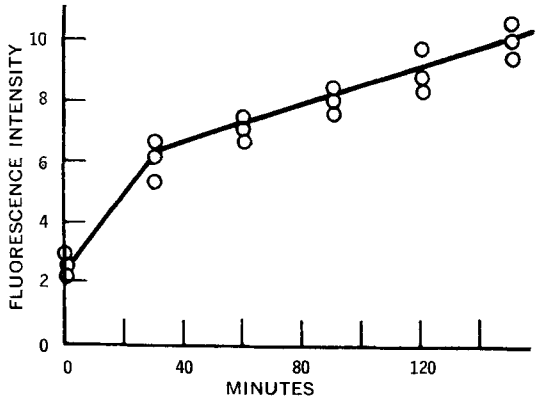


Figure 2—*Fluorescence of the reaction mixture of 9-isothiocyanatoacridine and* n*-butylamine as a function of the time of reaction in sodium ethoxide solution.*

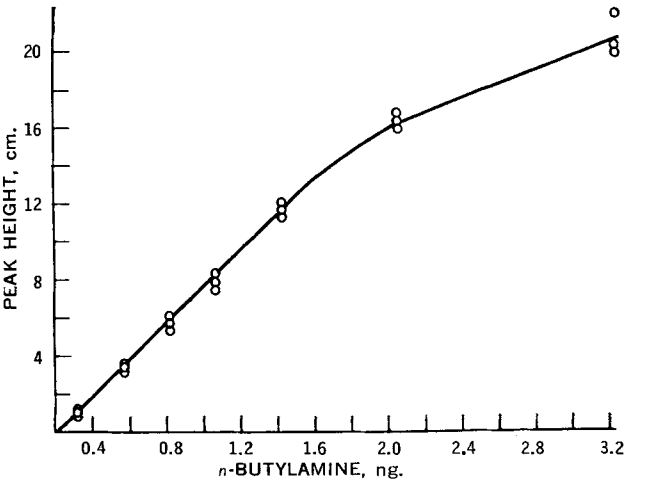


Figure 3—*TLC fluorimetry procedure applied to* n*-butylamine.*

The initial reaction proceeds rapidly at 60°, reaching a maximum plateau of fluorescence within 15 min. so that a reaction time of 0.5 hr. proved to be more than adequate. The increase in fluorescence of the thiourea product over that of its corresponding reagent control upon reaction with sodium ethoxide solution, however, proceeds more slowly and does not plateau (Fig. 2). Therefore, a reaction period of 1 hr. was selected for this step in the procedure. Acid was then added to quench the sodium ethoxide-activated reaction and, thus, to make the procedure less dependent upon the time required in determining the fluorescence of samples. The addition of acids increased the sensitivity of the procedure. That is, the quenched samples showed a difference in fluorescence between the final reaction mixture and a reagent blank about double that of a corresponding nonquenched set of samples.

These parameters were incorporated into a general procedure as described in the *Experimental* section. In the order of 7×10^{-4} moles of 9-isothiocyanatoacridine was employed in this procedure, which amounts to an excess of dye to sample varying from 10- to 70-fold, depending upon the concentration of amine involved. The procedure permits detection of fluorescence of the final reaction product and correlation of fluorescence to concentration of amine, even in the presence of the large excess of fluorescent reagent and its reaction products.

The general procedure is also suitable for salts of aliphatic primary amines. However, amine salt analysis requires addition of 1 ml. of 0.5% solution of sodium acetate in absolute alcohol to yield free amine for reaction with the isothiocyanate reagent.

Analytical results for amines and amine salts successfully examined by this procedure are summarized in Table I. The assay of an unknown concentration of amine by this procedure requires a direct comparison of fluorescence of the unknown to that of a known concentration under the same experimental conditions. The linear fluorescence to concentration range and slope of this response indicates the range over which the sample could be compared and the sensitivity of the procedure. Reproducibility of the technique is indicated from fluorescent data for nine sets of three duplicate samples of propylamine. The average maximum range for these sets was a fluorescent reading equivalent to 2 mcg. of amine.

While water interferes with the method, limited concentrations of water can be tolerated to permit analysis of aqueous solutions of amine. It was found that 0.5 ml. of water could be present if the total final reaction volume was increased to 25 ml. and if a 2-hr. heating period after base addition (instead of the usual 1-hr. period) was employed. The reagent blank must also contain an equivalent amount of water. Results for *n*-butylamine HCl in 0.5 ml. of water under these conditions are listed in Table I.

The general procedure is limited to aliphatic primary amines and their salts. An increase in fluorescence over the corresponding reagent control was not detected when dibutylamine, diethylamine, or aniline was employed in the general procedure. While alcoholic ammonia solutions did yield an increase in fluorescence, results were difficult to duplicate, probably because of a loss of ammonia during the initial heating periods. Also, conditions were not established for an analysis of ammonium chloride or benzylamine. Ammonium chloride showed no indication of reaction when either conditions used successfully for amine salts were tried or when stronger bases were employed. The procedure with benzylamine showed only a limited increase in fluorescence and was not suitable for its assay.

Sensitivity of the procedure was extended by separation of the final fluorescent product from excess reagent and by-products using TLC and measurement of fluorescence directly from the plate. A solvent system of DMF–$CHCl_3$–28% NH_4OH (5:13:3) was employed for separation of the reaction product on cellulose plates. Analogous to the solution procedure, a thiourea was first formed and then reacted with base, followed by quenching with acid as described in the *Experimental* section. Minimal quantities of reaction solutions were employed so that it was possible to assay amines at nanogram levels. Figure 3 depicts the results obtained for the TLC fluorimetry of *n*-butylamine.

The same TLC system developed for TLC fluorimetry was also applicable for investigating compounds present in the reaction mixture. As expected, spots corresponding in R_f value and fluorescence characteristics to the usual hydrolysis products of 9-aminoacridine derivatives (3), *i.e.*, acridone and 9-aminoacridine, were observed. Also, as anticipated, a spot consistent with the presence of *N*-(9-acridinyl)thiourethan was detected. These spots were obtained for both reagent control and reaction mixtures and did not bear a linear relationship to the amount of amine originally present. However, an additional spot (R_f 0.25 for the butylamine reaction mixture) was observed for the reaction mixtures only and could be related to the concentration of amine under investigation. Work is in progress concerning the identification of this highly fluorescent compound.

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* Present address: Sterling-Winthrop Research Institute, Rensselaer, NY 12044

† Present address: School of Pharmacy, University of Georgia, Athens, GA 30601

‡ National Science Foundation Undergraduate Research Participant under Grant GY-6105.