Fluorometric Determination of Primary and Secondary Aliphatic Amines by Reaction with 9-Isothiocyanatoacridine

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Abstract □ An improved procedure was developed for the analysis of primary and secondary aliphatic amines based upon the fluorescence of a cyclized product derived from isothiourea derivatives of amines and 9-isothiocyanatoacridine. Initial isothiourea formation as well as its cyclization is carried out in toluene, which results in the formation of highly fluorescent 2-alkylamino-1,3-thiazino-[4,5,6-*kl*]acridine with a minimum of by-products. The fluorescence of these thiazinoacridine compounds is determined either directly in solution after dilution with acidified alcohol or after TLC separation and direct scanning of the TLC plate. For example, amphetamine could be determined with a sensitivity of 0.12 nl./ml. in solution or at a 3-pl. level by the TLC procedure.

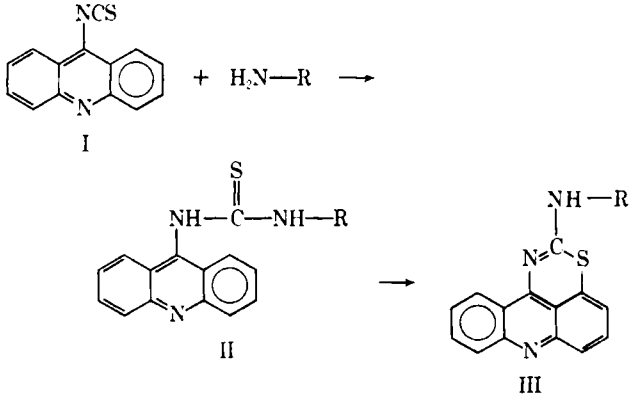
Keyphrases □ Amines, primary and secondary aliphatic—fluorescent analysis with 9-isothiocyanatoacridine □ Fluorescent analysis—primary and secondary aliphatic amines using 9-isothiocyanatoacridine, TLC fluorometry □ Amphetamine—fluorescent analysis using 9-isothiocyanatoacridine □ 9-Isothiocyanatoacridine—used as a reagent in fluorometic determination of primary and secondary aliphatic amines

The use of 9-isothiocyanatoacridine (I) as a reagent for the fluorometric determination of primary amines was previously reported from these laboratories (1). In that reaction (Scheme I), the thiourea (II) that formed was found to undergo a photochemical oxidation to yield a cyclized product (III), whose fluorescence can be related to the concentration of the original amine present (2). Knowledge of this cyclization has led to modification of the original analytical procedure (1), which has increased its sensitivity as well as permitted the analysis of secondary aliphatic amines.

This paper describes the modified procedure and its application to primary and secondary amines, with special emphasis on amphetamine analysis.

EXPERIMENTAL

Fluorometric Procedure—Solutions of 10–50 nl. (nanoliters) of liquid amines or of 10–50 mcg. of *p*-anisidine in 5.00 ml. of toluene were allowed to react with 2 ml. of 9-isothiocyanatoacridine reagent (80.0 mg. of 9-isothiocyanatoacridine in 100.0 ml. of toluene). Liquid amines were distilled over zinc dust, and *p*-anisidine was recrystallized from *n*-hexane prior to use. These solutions were allowed to react in 15-ml. glass-stoppered centrifuge tubes overnight (15 hr.) on the laboratory desk. These conditions permitted exposure to indirect morning sunlight after an initial reaction period in the dark. Fluorometric measurements[1] were made directly on the toluene solutions as well as on dilutions of 0.5 ml. of the toluene solution with 4.5 ml. of alcohol and of 0.5 ml. of the toluene solution with 4.5 ml. of alcohol and 0.1 ml. of concentrated hydrochloric acid. Reagent solutions without amine present were allowed to stand under the same conditions and were measured as controls.



Scheme I

TLC Separation and Fluorometry—Aliquots (15 μl.) of the toluene reaction mixtures from butylamine, amphetamine, and the reagent control described under *Fluorometric Procedure* were chromatographed on commercial[2] 250-μ precoated silica gel G plates (20 × 20 cm.), which were scored in 0.5-cm. channels prior to use. Development was in benzene–methanol (140:40) with detection under UV light. Quantitative fluorometric measurements were made directly from thin-layer plates with the aid of a scanner[3] as previously described (2).

RESULTS AND DISCUSSION

Reaction of 9-isothiocyanatoacridine with amines in alcoholic solutions leads to a mixture of fluorescent compounds (1). The fluorescence of one of these products could be related to the amount of amine present. It was subsequently recognized (2) that this fluorescent product was a cyclized compound (III) produced by photooxidation of thiourea II. Therefore, a study was undertaken to develop an analytical procedure with an increased yield of cyclized product in a minimal background of fluorescent by-products. The use of toluene in place of alcohol produced a more stable reagent solution and eliminated the interaction of reagent and solvent. Experiments to establish a maximum yield of cyclized product by exposure of the initial reaction mixture of amine and excess reagent to UV light in a photochemical reactor[4] were unsuccessful. While individual thioureas such as *N*-butyl-*N'*-(9-acridinyl)thiourea could be cyclized under these conditions, the procedure could not be applied to the analytical reaction mixture. Under these conditions, the required excess of the reagent, 9-isothiocyanatoacridine, was readily converted into acridone [λ (activation) 320 nm.; λ (emission) 440 nm.] by exposure to UV radiation. This resulted in excessive background fluorescence.

The most convenient reaction condition for the present study was to allow the amine and a 10–70-fold molar excess of reagent to react overnight at room temperature in the dark. This was followed by a limited morning exposure to indirect sunlight. An alternative procedure was to allow the amine sample and excess reagent to react in the dark at 60° before exposure to sunlight. In either case, care was taken to expose all sets of reaction mixtures containing increasing concentrations of amines and control reagent solutions to the same conditions.

Progress of the reaction can be monitored by TLC as described in the *Experimental* section. For example, a 15-μl. aliquot of a typical overnight reaction mixture of 0.05 ml. of *n*-butylamine and 1.6 mg. of 9-isothiocyanatoacridine in 7 ml. of toluene showed fluorescent spots corresponding to the R_f values and conditions of the following reference compounds: 0.02, blue, trace (9-aminoacridine);

[1] The Aminco-Bowman spectrophotofluorometer model 4-8106 was employed.
[2] E. Merck AG.
[3] Aminco 4-8221A.
[4] Rayonet, The Southern N. E. Ultraviolet Co.

Table I—Analysis of Amines

	Fluorescence		
	Activation, nm.	Emission, nm.	Intensity/ Concentration[a]
n-Butylamine	300	525	14.0
Cyclohexylamine	300	515	14.4
Benzylamine	300	520	12.2
β-Phenylethylamine	300	520	12.8
Amphetamine	300	520	8.6
Diethylamine	300	520	17.6
N-Methylpiperazine	310	525	12.8

[a] Slope of linear range: given as the difference in fluorescence over the reagent blank (calculated for a meter multiplier reading of 1 at a sensitivity of 25) and divided by concentration of amine (in nanoliters per milliliter). The average value for the acidic, alcoholic dilution of reagent blank was 60 at 465 nm. at a meter multiplier reading of 0.1. This value was reduced to about 12 in the wavelength range of maximum fluorescence of the test compounds.

0.16, yellow-green (cyclized product: 2-butylamino-1,3-thiazino[4,-5,6-*kl*]acridine); and 0.36, blue (excess reagent: 9-isothiocyanatoacridine). While a reagent control solution showed the spot at R_f 0.66 and those corresponding to trace quantities of aminoacridine and acridone, there was no fluorescence in the region of the thiourea or the cyclized compound.

When the final reaction mixture was measured in an alcoholic solution, especially an acidic alcoholic solution, there was a pronounced increase in the difference between fluorescence of the amine reaction solution and the control solution. Thus, while alcoholic solutions were avoided in original reaction mixtures, they could be employed to advantage just before determination of fluorescence. However, there is a decrease in stability of such solutions, with a change in fluorescence appearing in about 6 hr., as opposed to no appreciable change in fluorescence of the more concentrated reaction mixtures in toluene after 3 days.

The final analytical procedure was performed as outlined in the *Experimental* section. This included the acidic, alcoholic dilution of the original toluene reaction mixture just prior to measurement of fluorescence. Of particular importance in this procedure are the facts that the final reaction product has fluorescent characteristics that permit its direct determination in the presence of excess reagent and that this fluorescence is proportional to the amount of amine originally present.

Table I is a summary of compounds that were successfully analyzed by the modified technique. Included are the wavelengths of activation and emission used to obtain a maximum difference in fluorescence between the reaction mixture and its corresponding reagent blank. Table I also includes a summary of the intensity of this difference in fluorescence.

In addition to the compounds listed in Table I, aniline, *p*-toluidine, *p*-anisidine, and *N*-ethylaniline were subjected to the procedure but did not yield satisfactory results. Failure to achieve positive results with these compounds, which is consistent with the nucleophilicity of the amines involved, suggests that the rate of thiourea formation (Compound II) is a critical consideration. Thus, by using the basicity of amines as a measure of nucleophilicity, it was found that all amines that were successfully determined have a pKa $\geq$ 9.33 (benzylamine), while no compound with a pKa $\leq$ 5.34 (*p*-anisidine) (3) could be measured.

The procedure is applicable to amines of biological significance such as amphetamine. A detection limit down to a concentration of 0.12 nl./ml. was established for quantitative results for amphetamine solutions. The results for six samples in the range from 0.2 to 0.5 nl./ml. gave a mean recovery of 97% with a standard deviation of 3.3%. The sensitivity of the solution procedure could be greatly enhanced by combination with TLC followed by direct fluorometric scanning of the plates. The fluorescence scanning procedure was conducted as previously described (2) after chromatography on silica gel G with benzene–methanol (140:40). Measurement of the cyclized product derived from amphetamine was at an activation of 355 nm. and an emission of 518 nm. The recorder response was linear to the original volume of amphetamine over a range of samples of 3, 6, 9, 20, and 25 pl. (picoliters). The results for eight samples over this range gave a mean recovery of 98.9% with a standard deviation of 9.9%. The TLC scanning procedure thus results in a loss in precision but a great increase in sensitivity and it eliminates the interfering fluorescence of products and excess reagent.

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