Journal of Chromatography, 200 (1980) 324–329
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,078

Note

Fluorescence detection of secondary amines on thin-layer plates using a fluorogenic reaction with fluorescamine

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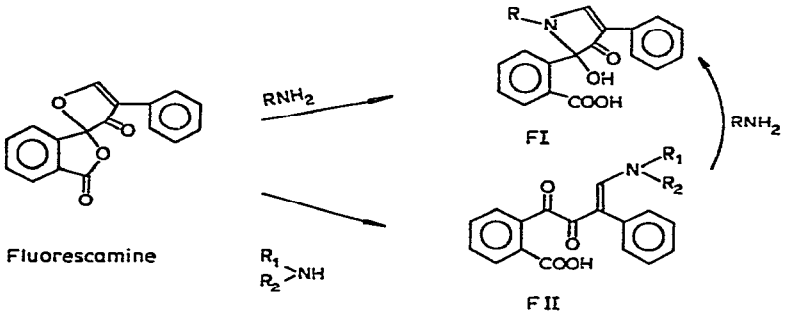
(Received June 2nd, 1980)

Fluorescamine reacts with primary amines to give fluorescent pyrrolinones (FI)[1]. Adaptation of this fluorogenic reagent to the analysis of primary amino groups by thin-layer chromatography (TLC) has been reported by several authors[2–9]. On the other hand, fluorescamine gives no fluorescent products with secondary amines, but it produces aminoenone chromophores (FII) having an absorption maximum at 300–330 nm (ε 16,000–18,000)[10,11]. By utilizing the UV-absorbing property of FII, Ranieri and McLaughlin[12,13] detected alkaloid secondary amines as quenched spots after spraying with fluorescamine.

Recently, we found that FII was converted into FI by primary amines as shown in Scheme 1, leading to the development of the fluorometric assay of secondary amines[14]. In this paper, a TLC method for the detection of secondary amines based on the fluorogenic reaction is reported.



Scheme 1. The reactions of fluorescamine with primary and secondary amines and the conversion of FII into FI by primary amines.

EXPERIMENTAL

Reagents and solvents

The following were purchased from commercial sources: fluorescamine (Nippon Roche, Tokyo, Japan); L-proline, skatole (grade I), DL-metanephrine

0021-9673/80/0000–0000/$02.25

hydrochloride, DL-epinephrine, N-methyltryptamine, N,N-dimethyltryptamine, melatonin (Sigma, St. Louis, MO, U.S.A.); imidazole (guaranteed reagent, GR), diphenylamine (GR), N-methyltaurine sodium salt, N-methylaniline (GR), sarcosine (GR), N-acetyl-D-β-phenylalanine (GR), N-methyl-D-glucamine (GR), diethanolamine hydrochloride (GR), benzimidazole (GR), carbazole (extra pure, EP), N-ethylethanolamine (GR), N-methyl-benzylamine (EP), pyrrole (GR), morpholine (Ninshyo Shiyaku) and hippuric acid sodium salt (GR) (Tokyo Kasei, Tokyo, Japan); monoethanolamine (GR) and ammonia solution (28% as NH_3) (Kanto Chemical, Tokyo, Japan). Other chemicals and solvents used were of the highest purity commercially available. Glass-distilled water was used throughout this work.

Preparation of stock solutions

N-Methyl-D-glucamine, diethanolamine hydrochloride, sarcosine, N-ethylethanolamine, morpholine, N-methyltaurine sodium salt, L-proline and imidazole were dissolved in water to give 10 m*M* stock solutions. DL-Metanephrine hydrochloride and DL-epinephrine were dissolved in 0.01 *N* HCl to give 10 m*M* and 5 m*M* solutions respectively just before use. Other secondary amino compounds were dissolved in methanol to give 10 m*M* stock solutions. The same solvents also were used to prepare dilute solutions.

Solvent systems for separation of secondary amines

(A) Acetone–methanol–water (3:3:4).
(B) Benzene–dioxane–methanol–water (1:3:4:3).
(C) *n*-Butanol–methanol–acetic acid–water (4:1:1:5).
(D) Ethanol–ammonia solution (8:2).
(E) Isopropanol–ammonia solution (8:2).

TLC

Precoated silica gel 60 HPTLC plates (10 × 10 cm, without fluorescent indicator) from E. Merck (Darmstadt, G.F.R.) were used without any treatment.

A 0.2–0.5-μl aliquot of the stock solution was applied 0.8 cm from the lower edge of a plate with a 1-μl Hamilton syringe and air-dried. Ascending chromatography was performed in a glass chromatographic tank (12 × 5 × 12 cm; Yazawa Scientific, Tokyo, Japan) at room temperature until the solvent front travelled 7 cm from the origin. The developed plate was heated at 110°C for 10 min in an electric oven. If the plate still smelled of acetic acid (solvent C) or ammonia (solvents D and E), it was dried further under a hot stream from a hair-dryer until free from smell. The plate was sprayed with 0.05 *M* sodium borate buffer (pH 10.5) and heated at 110°C for 15 min. In the case of plates developed with solvent C, the spraying and heating were repeated once more. After cooling, the plate was sprayed with an acetone solution of fluorescamine (20 mg/100 ml) and allowed to stand at room temperature for 15 min in the dark. Then the plate was sprayed with 0.2 *M* taurine in 0.2 *M* sodium phosphate buffer (pH 7.5) and heated at 60°C for 5 min. The fluorescence was observed in the dark with a Pan UV lamp (Type PUV-1A; Tokyo Kogaku Kikai, Tokyo, Japan) which provided continuous light at 250–400 nm.

RESULTS

Reaction of secondary amines with fluorescamine on TLC plates

In preliminary spray experiments it was found that the optimal pH for the reaction of secondary amines with fluorescamine differed depending on the nature of the amines. Nevertheless, FII was generally formed in high yield under slightly alkaline conditions, while no substantial amounts of FII were formed below pH 5 or above pH 13. As in solution[14], FII seemed to be most stable on plates buffered at around pH 9.5–11. Therefore, the formation of FII was performed at pH 10.5 by prespraying with 0.05 *M* sodium borate buffer prior to a fluorescamine spray. Allowing the plate sprayed with fluorescamine to stand at room temperature for 15 min led to almost complete hydrolysis of the fluorogenic reagent, which was confirmed by the absence of fluorescence after spraying a primary amine solution.

Conversion of FII into FI by primary amines on TLC plates

Of many primary amines, taurine was chosen as the converting agent since it was cheap and non-volatile and gave relatively intense fluorescence with FII. When various concentrations of taurine were sprayed on FII derived from sarcosine, it wan found that higher concentrations of taurine permitted more sensitive detection (Table I). Therefore, taking into account its solubility, the concentration of taurine sprayed was chosen to be 0.2 *M* in 0.2 *M* sodium phosphate buffer, pH 7.5, where the conversion of FII into FI by taurine was optimal[14].

TABLE I

EFFECT OF TAURINE CONCENTRATION ON THE DEVELOPMENT OF FLUORESCENCE FROM THE AMINOENONE DERIVATIVE OF SARCOSINE

Fifty microlitres each of various concentrations of secondary amine and 0.1 *M* sodium borate buffer (pH 9.0) were mixed and rapidly added to 100 μl of an acetone solution of fluorescamine (20 mg/100 ml) during vigorous stirring on a vortex mixer. A 1-μl aliquot of the reaction mixture was spotted onto a plate and sprayed with various concentrations of aqueous taurine solution. The fluorescence intensity was designated as follows: −, negative; ±, trace to negative; +, weak; ++, intense.

Taurine (M)	*Sarcosine spotted (pmole)*					
	0	*10*	*25*	*50*	*100*	*250*
0.05	−	−	−	−	−	−
0.075	−	−	−	−	−	+
0.10	−	−	±	+	+	+
0.15	−	−	±	+	+	+
0.20	−	±	±	+	+	++

As shown in Table II, the heating of plates after the taurine spray accelerated the induction of fluorescence from FII. However, the heating also induced delayed fluorescence from the background giving a colour indistinguishable from that of secondary amines. Heating at 60°C for 5 min gave the highest sensitivity.

Separation of secondary amines

Table III summarizes the R_F values of ten secondary amines obtained with five solvent systems. By use of these solvents, the identification of these authentic secondary amines was possible.

TABLE II

EFFECTS OF TEMPERATURE AND TIME ON THE DEVELOPMENT OF FLUORESCENCE AFTER THE TAURINE SPRAY

Various secondary amines were tested by the standard procedure described in Experimental, except for the temperature after the taurine spray.

Temperature (°C)	*Time (min) required for the fluorescence development after the taurine spray from*	
	FII	*Background*
20–30	30–120	120
60	5–10	30
80	3–6	10
110	3	5

TABLE III

R_F VALUES OF VARIOUS SECONDARY AMINES

5 nmole were spotted.

Compound	R_F *value in solvent system*				
	A	*B*	*C*	*D*	*E*
DL-Metanephrine	0.04	0.03	0.82	0.62	0.61
DL-Epinephrine	0.03	0.04	0.69	0–0.71*	0–0.60*
N-Methyltryptamine	0.04	0.06	0.76	0.74	0.77
Melatonin	0.96	0.94	0.93	0.98	0.98
Sarcosine	0.69	0.52	0.54	0.39	0.22
Benzimidazole	0.87	0.90	0.78	0.93	0.93
Carbazole	0.95	0.94	0.98	0.98	0.96
N-Methylbenzylamine	0.03	0.05	0.81	0.92	0.89
Morpholine	0.01	0.04	0.50	0.71	0.71
N-Methyltaurine	0.84	0.71	0.60	0.60	0.39

* Tailing.

Sensitivity of the method

Table IV summarizes the detection limits of various secondary amines obtained with three kinds of solvents. Generally, the neutral solvent system (A) gave higher sensitivity of detection, due probably to the simplicity of the procedure. In the case of the solvent containing acetic acid (C), incomplete removal of the acid sometimes resulted in interference with the reaction of secondary amines with fluorescamine. With this procedure, volatile secondary amines such as skatole, pyrrole and N-methylaniline, which could be determined in the manual assay[14], were not detected even in quantities of 5 nmole. The present spray method was not applicable to the detection of amides and compounds having a peptide bond, such as hippuric acid.

DISCUSSION

In this investigation, the utilization of fluorescamine as a fluorogenic spray reagent for secondary amines is first reported. The method permits the detection of 20–30 pmole of secondary amines in some cases. However, it is not adequate for the

TABLE IV

LIMITS OF DETECTION OF VARIOUS SECONDARY AMINES IN TLC

Compound	*Detection limit (nmole) obtained with solvent system*		
	A	*C*	*E*
DL-Metanephrine	0.05	0.9	0.3
DL-Epinephrine	0.1	4	0.3
N-Methyltryptamine	0.02	0.09	0.1
N,N-Dimethyltryptamine	0.3	5	>5
Melatonin	0.5	0.8	2
Skatole	>5	>5	>5
Pyrrole	>5	>5	>5
N-Methyl-D-glucamine	0.3	5	0.5
Diethanolamine	0.2	>5	4
Sarcosine	0.2	>5	0.08
N-Ethylethanolamine	0.3	4	4
Benzimidazole	3	4	3
Carbazole	0.5	0.9	0.4
N-Methylbenzylamine	0.03	0.3	0.4
Morpholine	0.02	1	0.5
N-Methyltaurine	0.07	3	0.05
N-Acetyl-D-β-phenylalanine	>5	>5	>5
L-Proline	>5	>5	>5
Imidazole	>5	>5	>5
N-Methylaniline	>5	>5	>5
Diphenylamine	>5	5	>5

detection of volatile amines. The fluorescence produced in the background reduces the sensitivity of the method. In this context, the background fluorescence is reasonably considered to originate from the hydrolysis product of fluorescamine in the reaction with taurine. The following results support the above assumption that the hydrolysis product reacts with primary amine to give a fluorescent product, probably FI: (i) fluorescamine hydrolyzed in sodium hydroxide solution (pH 13) gave a single fluorescent spot (R_F 0.63), different from that of fluorescamine (R_F 0.94), after development with ethyl acetate–*n*-hexane–methanol–water (60:20:25:10) followed by spraying a primary amine; and (ii) the colour of the fluorescence of the background was always identical to that of the corresponding FI derived from fluorescamine and the primary amine sprayed.

As the present method also permits the detection of primary amines as fluorescent spots before the taurine spray, it will be useful for the stepwise detection of primary and secondary amines.

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