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Note

Usage of fluorescamine as a spray reagent for thin-layer chromatography

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Fluorescamine, 4-phenylspiro[furan-2(3H),1′-phthalan]-3,3-dione, is a novel reagent which reacts instantaneously with primary amino compounds to give highly fluorescent pyrrolinones[1] and has been used for quantitative analysis of primary amino acids[2–4]. Recent work by Weigele *et al.*[5] has made it possible to include proline[6] and hydroxyproline[7] detection on the single column fluorimetric amino acid analyzer. Fluorescamine has also been utilized for the detection of amino acids and peptides on thin-layer chromatograms[2,3]. We have been using this reagent routinely for development of the plates and have also found the sensitivity to be greater than that obtained with ninhydrin. Unfortunately, by the old procedure after spraying the plates with fluorescamine, the fluorescence rapidly diminished and within several hours generally decreased to less than half of its original intensity. This required that the plates be viewed and photographed immediately in order to take full advantage of the enhanced sensitivity afforded by the reagent. Since it is known that amino acids react optimally with fluorescamine at pH 8–9 to give fluorophors[1], attempts were made to improve the stability and sensitivity of fluorescence by spraying the plates with borate buffer (0.10 *M*, pH 9.0) prior to and/or following the fluorescamine treatment. This buffering treatment gave no significant advantage. We had observed earlier[8] that, when peptide-resin (from solid phase peptide synthesis) was allowed to react with fluorescamine, pre-treatment of the peptide-resin with triethylamine enhanced and stabilized the fluorescence of the resin by preventing the conversion of the fluorophor to the non-fluorescent γ-lactone. We applied this observation to the detection of amino acids and peptides after TLC development and found a significant improvement in both sensitivity and stability of the spots.

EXPERIMENTAL

Materials

Fluorescamine was prepared in the Chemical Research Department of Hoffmann-La Roche Inc. Solutions of 0.05% fluorescamine in acetone (reagent grade) were freshly prepared each week and stored at room temperature in a stoppered vessel. Sequanal grade triethylamine was purchased from Pierce, Rockford, Ill., U.S.A. Solutions of 10% triethylamine in methylene chloride (reagent grade) were prepared each week. Individual amino acid standards and L-alanyl-L-alanine were purchased from Schwarz-Mann, Orangeburg, N.Y., U.S.A.

Thin-layer chromatography

Silica gel plates (10 × 20 cm and 20 × 20 cm) were prepared in the Chemical Research Department of Hoffmann-La Roche Inc. using silica gel G without indicator (E. Merck, Darmstadt, G.F.R.). The plates were spotted with 0.5–1.0 μl of solutions of standard amino acid and peptide solutions ranging from 0.10 nmole/μl to 100 nmole/μl in 0.01 *M* HCl using calibrated 5-μl micropipets (Camag, New Berlin, Wisc., U.S.A.). The plates were developed with butanol–acetic acid–ethyl acetate–water (1:1:1:1), dried at 110° for 10 min, cooled to room temperature and treated by the fluorescamine spray procedure (Table I). The plates were viewed using a long wave (366 nm) ultraviolet light source. Equally satisfactory results were obtained with a variety of developing systems including those without acetic acid.

TABLE I

FLUORESCAMINE SPRAY PROCEDURE

Step	*Treatment*
1	Spray with a solution of 10% triethylamine in methylene chloride; air dry for several seconds.
2	Spray with a solution of 0.05% fluorescamine in acetone; air dry for several seconds.
3	Re-spray with a solution of 10% triethylamine in methylene chloride.

RESULTS AND DISCUSSION

Studies to optimize the visualization of amino acids and peptides in TLC by fluorescamine spray revealed that the use of triethylamine in a variety of organic solvents improved both the stability and sensitivity of fluorescence. Other bases (*e.g.* ammonia, pyridine, dimethylamine and quinuclidine) did not produce comparable results. The intensity of the fluorescence was strongest and most stable when the fluorescamine spray procedure (Table I) was employed.

The stability of the fluorescent spots of 5.0 nmoles each of L-alanine, L-glutamic acid, L-lysine and L-alanyl-L-alanine is shown in Fig. 1. It can be seen that the fluorescence intensities of the amino acids and model peptide were only slightly faded after 24 h. When triethylamine was omitted from the fluorescamine spray procedure the spots were less intense and faded almost completely after 24 h. The presence of triethylamine neutralizes any residual acetic acid from the developing system and stabilizes the fluorophor in the triethylammonium salt form thereby preventing conversion to the non-fluorescent γ-lactone.

Studies to determine the limits of sensitivity using fluorescamine for the visual detection of a model amino acid and peptide were also carried out. Varying amounts of L-alanine and L-alanyl-L-alanine (50 nmoles–50 pmoles) were treated by the new fluorescamine spray procedure (Table I). The results are shown in Fig. 2. As little as 500 picomoles of the model amino acid and peptide were visually detectable. The detection of longer peptides on TLC was achieved with equally satisfactory results. The fluorescamine spray procedure has also been used for the detection of amino acids and peptides on paper following high-voltage electrophoresis.

The drastic conditions required for the ninhydrin test have been reported to

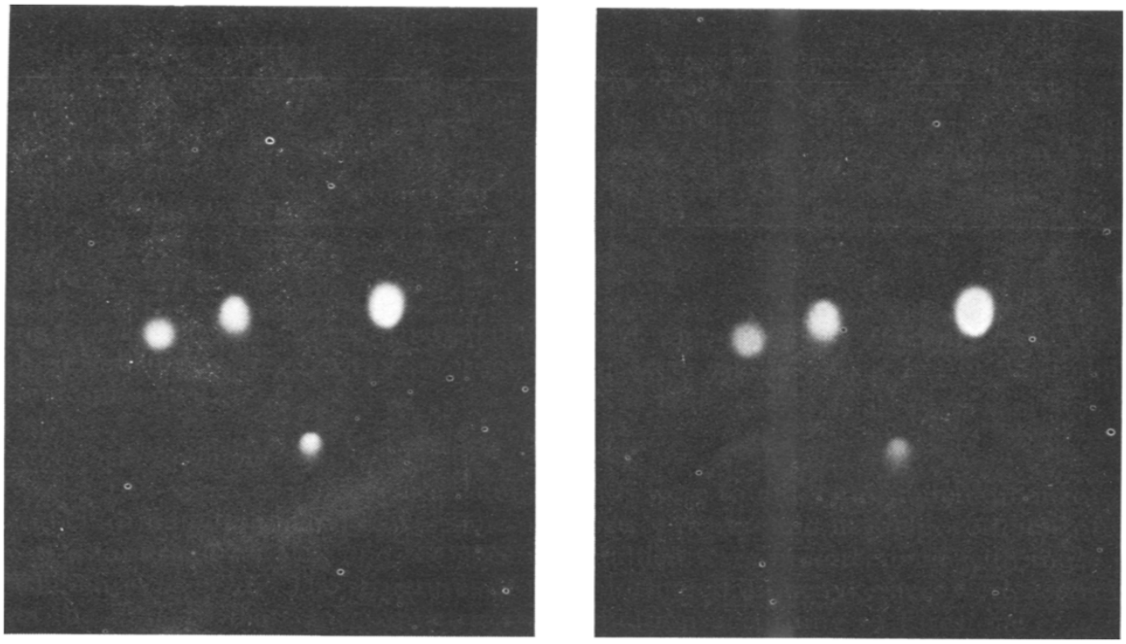

Fig. 1. Thin-layer chromatograms on silica gel G (without indicator). The plates were spotted with 50.0 nmoles each of L-alanine, L-glutamic acid, L-lysine and L-alanyl-L-alanine. The plates were developed with butanol–acetic acid–ethyl acetate–water (1:1:1:1), treated by the fluorescamine spray procedure and viewed under long wave ultraviolet light. Left plate, photographed after 30 min; right plate, photographed after 24 h.

give rise to misleading results[9,10]. For example, Bpoc-amino acids* have been shown to give positive ninhydrin tests[11]. On the other hand the milder conditions of the fluorescamine spray procedure gave the expected negative result. No fluorescence was observed with fluorescamine in the case of peptides containing N-terminal secondary amino acids. However, re-spraying the plate by the chlorine–tolidine procedure[12] gave the expected colored spot, without interference from the fluorescamine spray.

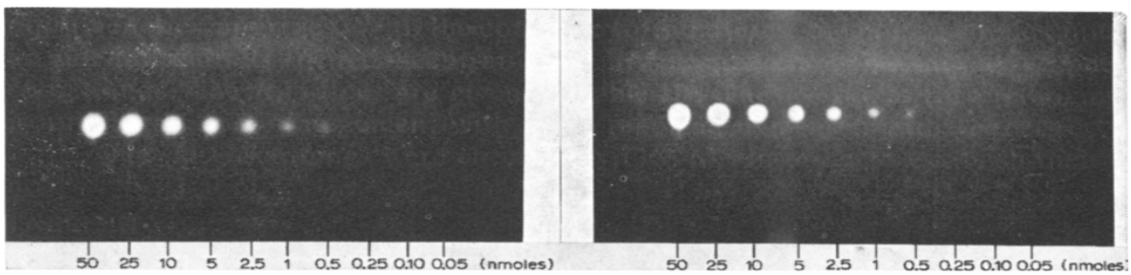

Fig. 2. Thin-layer chromatograms on silica gel G (without indicator). The plates were spotted with (from left to right) 50.0 nmoles, 25.0 nmoles, 10.0 nmoles, 5.0 nmoles, 2.5 nmoles, 1000 pmoles, 500 pmoles, 250 pmoles, 100 pmoles and 50.0 pmoles. The plates were developed with butanol–acetic acid–ethyl acetate–water (1:1:1:1), treated by the fluorescamine spray procedure, viewed under long wave ultraviolet light and photographed after 30 min. Left plate, L-alanine; Right plate, L-alanyl-L-alanine.

* Bpoc = Biphenyldimethylmethyloxycarbonyl.

Therefore, application of the fluorescamine spray procedure followed by the chlorine–tolidine procedure enables one to distinguish between peptides with N-terminal primary amino functions and those containing N-terminal secondary or substituted amino groups. The convenience, sensitivity and stability of products from the fluorescamine spray procedure make it particularly attractive for general usage in peptide chemistry.

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