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Fluorometric Determination of Catecholamines on a Three-Phase Thin-Layer Plate

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Catecholamines (dopamine, norepinephrine and epinephrine) in solutions were spotted on an alumina thin-layer, dansylated (5-N, N-dimethylaminonaphthalene-1-sulfonylation), separated by adjacent cellulose and silica gel thin-layer and determined fluorometrically. The determination range was between 10^{-10} mole and 10^{-8} mole. The method was applied to the determination of norepinephrine and epinephrine in injections.

Introduction

In the proceeding paper[2)] we reported on the detection of catecholamines (CA) by N-mono-dansylation (5-N,N-dimethylamino naphthalene-1-sulfonylation) of the amines on a surface of alumina. However the losses of the amines and their dansyl derivatives occurred at the time of their extraction and transfer, prevented their quantitative estimation. Therefore we tried to improve the method by performing all the procedure on a single plate. Fluorophores obtained after dansylation of the amines on a surface of alumina were not separable on the alumina layer by any acidic developing solvent system. They were separable on a silica gel layer, however, tailing occurred when the layer was combined with the alumina layer. By a three-phase of thin-layers (alumina, cellulose and silica gel as shown in Fig.1) the difficulties were overcomed.

The method was applied to the determination of ÇA in injections.

Experimental

Materials and Reagents——*l*-Epinephrine was purchased from Merck Co., Ltd. *dl*-Norepinephrine, dopamine hydrochloride and 5-N,N-dimethylaminonaphthalene-1-sulfonic acid were purchased from Tokyo Kasei Co., Ltd.

The injections of 0.1% *l*-epinephrine (J.P.) and that of 0.1% norepinephrine were purchased from Sankyo Co., Ltd. 5-N,N-dimethylaminonaphthalene-1-suflonyl chloride was synthesized from 5-N,N-dimethylaminonaphthalene-1-sulfonic acid with phosphorous pentachloride according to Laurence.[3)]

Silica gel (Silica Gel H, Merck Co., Ltd.), alumina (Aluminium oxide G, Merck Co., Ltd.) and cellulose (Avicel S.F. Asahi Kasei Co., Ltd. Tokyo) for thin layer chromatography were used. The galss plate for thin layer chromatography was 1 mm thickness and 20×20 cm width.

All the solvents were reagent grade and redistilled.

Apparatus——A multiphase applicator with slit width of 0.25 mm and 0.3 mm for thin layer chromatography (Mitamura Riken Kogyo Co., Ltd. Tokyo) and a scanning fluorometer (Model SFR-11, Yamato Kagaku Kikai Co., Ltd.) were used.

Preparation of Three-Phase Thin-Layer Plate——Suspensions of alumina, cellulose and silica gel were prepared in an ordinary way, separately poured into three chambers of the applicator, and applied on the plate as shown in Fig. 1. The plate was activated at 90° for 20 min in a drying oven.

Procedure for Dansylation, Separation and Determination of Catecholamines——1) A solution of CA was spotted on the layer of alumina with a 2 μl micro-pipet (Microcaps, Drummond Scientific Co., Broomall, Pa., U.S.A.) and dried with a hair dryer.

2) To the position of the amines was applied a solution of dansyl chloride in dioxane (0.5%) with a 5 μl micro-pipet (Microcaps).

3) The plate was left standing for a period at room temperature in the air.

1) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo.*

2) K. Kitani, K. Imai, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **18**, 1495 (1970).

3) D. J. R. Laurence, "Method in Enzymology," IV, Academic Press Inc., New York, 1957, p. 209.

4) The reaction was stopped by adding one drop of pyridine or by exposing to a vapor of ammonia for 5 min, and the plate was dried with a hair dryer. In another way the reaction was stopped by putting the plate in a desiccator containing calcium chloride for 30 min under a reduced pressure.

5) The plate was connected with a Toyo Roshi No. 514 paper (20×40 cm) at one side and developed with methanol in the direction A in Fig. 1 over periods of 7 or 24 hours, removed off the paper, then dried with a hair dryer or in a desiccator under a reduced pressure for 30, 60 or 90 min.

6) The plate was developed with benzene–dioxane–glacial acetic acid (90:25:4) for 3 hours in the direction B.

7) The plate was immediately covered with a glass plate (20×20 cm, 1 mm thickness).

8) The fluorescence intensities of spots on a thin layer chromatogram were estimated with a scanning fluorometer at periods of following 10, 20, 30, 60, 90 and 150 min.

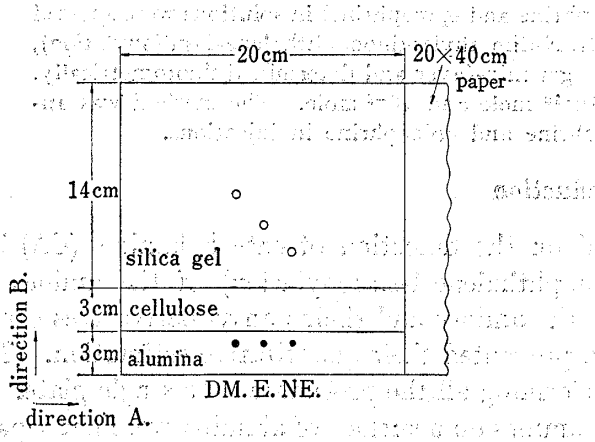

Fig. 1. A Typical Three-Phase Thin-Layer Plate and a Chromatogram of Catecholamines

DM: dopamine, E: epinephrine, NE: norepinephrine

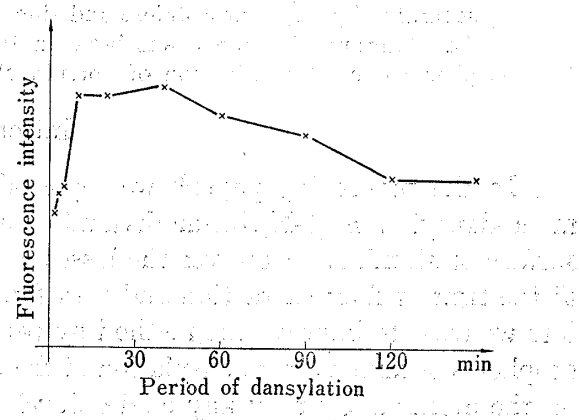

Fig. 2. Effect of Dansylation Periods in the Air

sample: dopamine 1 0 μg

Result and Discussion

Preliminary Investigation

For the sake of the rigidity of the thin-layer and good separation of three CA, the slit width of the applicator was decided to be 0.3 mm rather than 0.25 mm, and the width of the phases of alumina and cellulose were 3 cm and that of silica gel was 14 cm for 20×20 cm plate. For one plate, there required 2 g of alumina in 2.5 ml of water, 1 g of cellulose in 2.5 ml of water and 5 g of silica gel in 10 ml of water. For 10^{-8} mole of CA, 10^{-7} mole of dansyl chloride was proved sufficient to produce maximum fluorescence.

The reaction period was decided to be 10 min since the longer reaction in the air made a few fluorescence by-products and reduced the fluorescence intensity of CA (Fig. 2). The excess reagent was decomposed smoothly in a desiccator under a reduced pressure (4 mm Hg) for more than 20 min. On the other hand pyridine disturbed the following development and ammonia produced much quantity of undesirable dansyl amide.

By the first development with methanol in direction A, dansyl amide and dansyl sulfonic acid which disturbed in the final estimation were removed almost completely leaving dansyl CA at the original positions, although the developing period required was more than 7 hr.

As the presence of methanol reduced the fluorescence intensity on the thin-layer, the solvent was required to be removed under a reduced pressure (4 mmHg) for one or two hours.

The development in the direction B in Fig. 1 was achieved with a solvent system of benzene–dioxane–glacial acetic acid (90: 25: 4).[2)]

Each CA gave one spot on a chromatogram (Apparent *Rf* values were 0.46, 0.38, and 0.23 for dopamine, epinephrine, and norepinephrine respectively when the border line be-

tween silica gel and cellulose was taken as the original point.) which was identified with an authentic N-mono-dansyl CA.[4]

Since the fluorophore of the amine on a thin layer declined in a few minutes as the solvent vaporized off from the plate (Fig. 3, dotted line), it was difficult to determine exactly the fluorescence intensity. Seiler, *et al.*[5] also found the declinement and recommended to spray triethanolamine on a chromatogram to strengthen and retain the fluorescence. However, it was not successful in our experience. The difficulty was overcomed by covering the plate with a glass plate (1 mm × 20 cm × 20 cm) when the development was ceased. The fluorescence intensity was retained for a few hours as shown in Fig. 3 (solid line).

The limit of detection of each CA was about 3×10^{-10} mole.

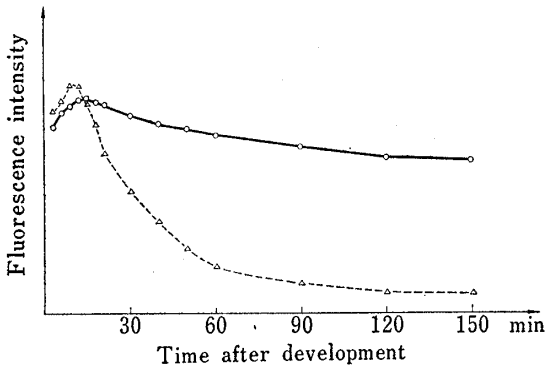

Fig. 3. Stability of a Fluorophore on a Thin-Layer Plate

sample: epinephrine 0.6 μg
—○—: covered with a glass plate
—△—: without covering with a glass plate

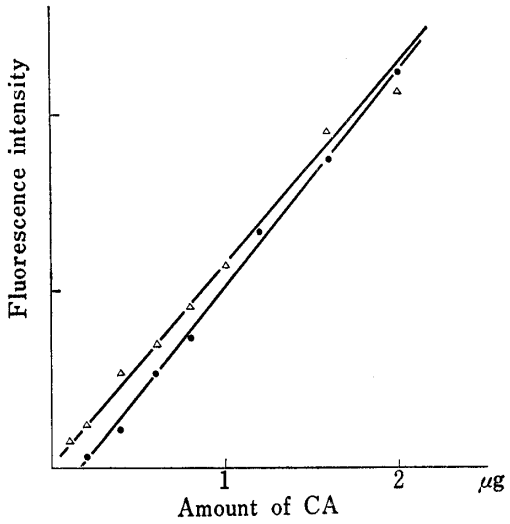

Fig. 4. Relation between Fluorescence Intensities and Amounts of Catechol amines

—△—: dopamine
—●—: norepinephrine and epinephrine

Standard Method for the Determination of Dopamine, Norepinephrine and Epinephrine

Two μl of a sample solution which contained less than 10^{-8} mole of CA is spotted on the plate and 2 μl of two kinds of solution of authentic CA in 0.2N acetic acid, one of which contains less and the other more than the expected content in the sample, are spotted at one and the other side of the sample.

They are dansylated with 5 μl of dansyl chloride in dioxane (0.5%) for 10 min in the air. The plate is transferred into a desiccator and dried at 4 mm Hg for 20 min. The plate is connected with a paper and developed with methanol in direction A for 7 hr.

After drying the plate in a desiccator under the reduced pressure for 60 min, the steps 6) and 7) in the procedure are performed for the plate and the chromatogram thus obtained is traced by a scanning fluorometer. The peak heights for two authentic solutions are plotted against the weight of amine and the content of CA in the sample is estimated from peak height ratio.

The peak height was almost proportional to the amount up to 10^{-8} mole of each CA as shown in Fig. 4.

4) K. Kitani, H. Tsuzuki, K. Imai, and Z. Tamura, in preparation.
5) N. Seiler and M. Wiechmann, *Z. Anal. Chem.*, **220**, 109 (1966).

Determination of Norepinephrine and Epinephrine in Injections

The injection of 0.1% *l*-epinephrine was diluted 4 times with 0.2 N acetic acid. Two μl of the solutions were treated as above and concentration of epinephrine in the injection was found to be 1.07±0.13 mg/ml (n=10).

The injection of 0.1% norepinephrine was treated in the similar way and concentration of norepinephrine was found to be 0.88±0.09 mg/ml (n=10).

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