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FLUORIMETRIC ASSAY OF DOPAMINE, NOREPINEPHRINE AND THEIR 3-O-METHYL METABOLITES BY USING FLUORESCAMINE*

KAZUHIRO IMAI
Faculty of Pharmaceutical Sciences, Tokyo University, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)
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SUMMARY

Fluorescamine was subjected to reaction with dopamine and norepinephrine (catecholamines) and with 3-methoxytyramine and normetanephrine (3-methyl metabolites of catecholamines) in phosphate or borate buffer. Catecholamines gave the highest fluorescent intensity at pH 8.0 in phosphate buffer but lower fluorescence in borate buffer. The fluorophores produced in phosphate or borate buffer were the same but the fluorescence intensities were suppressed in borate buffer. The dopamine and norepinephrine fluorophores were separated by high-pressure liquid chromatography on Hitachi 3011 gel with methanol–0.10 *M* Tris buffer of pH 8.0 (7:3). They were measurable at the 100-pmole level. The metabolites were also measurable by the same chromatography. By using methanol–0.15 *M* borate buffer of pH 8.0, catechol-O-methyltransferase activity might be assayed.

INTRODUCTION

Of the fluorimetric methods for the determination of catecholamines, *e.g.*, dopamine (DM) and norepinephrine (NE), and their 3-O-methyl metabolites, *e.g.*, 3-methoxytyramine (3-MT) and normetanephrine (NM), the trihydroxyindole (THI) method is the most popular[1]. However, the selectivity of the method is not high as the co-existing catecholamine or metabolite causes interferences.

Recently, fluorescamine, which easily reacts with primary amines to yield highly fluorescent products, has become available[2,3]. In a previous paper, we reported that fluorescamine-labelled amines could be separated on thin layers of silica gel and detected at the picomole level[4]. However, it was difficult to quantitate the fluorophores on thin layers. In order to overcome this difficulty, the high-pressure liquid chromatographic determination of the fluorophores was investigated, and the applicability of the method is discussed in this paper.

* Part of this work was presented at the 3rd Annual Meeting of the Society for Neuroscience held in San Diego, U.S.A., November 7–10th, 1973.

EXPERIMENTAL

Materials

Acetone (spectrograde) was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). Dopamine hydrochloride, norepinephrine bitartrate monohydrate, 3-methoxytyramine hydrochloride and normetanephrine hydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). Fluorescamine was purchased from Hoffmann-La Roche (Nutley, N.J., U.S.A.). All other chemicals were of reagent grade.

Manual procedure for the fluorescamine assay of DM and NE

Twenty microliters of a 1 m*M* solution of DM or NE were transferred into a 13 × 100-mm test-tube (Corning) and the solution was diluted with 1.5 ml of 0.15 *M* sodium phosphate or borate solution at 4°. Then 0.5 ml of a 20 mg-% solution of fluorescamine in acetone was rapidly added to the tube, which was held on a vortex mixer. Fluorescence measurements were made with the excitation set at 390 nm and emission at 475 nm using an Aminco-Bowman spectrophotofluorimeter (American Instruments).

High-pressure liquid chromatography of the fluorophores

A few microlitres of a solution of the amines (less than 1 m*M*) were diluted to 20 μl with 0.05 *M* phosphate or borate buffer of pH 8.0. To the solution were added 10 μl of a 200 mg-% solution of fluorescamine in acetone at room temperature with vigorous shaking. An aliquot of the reaction mixture was directly applied on to the column.

Hitachi 3011 and 3010-OH gels (Hitachi Seisakusho, Tokyo, Japan) were used as column packings. A high-speed piston pump Minimicro Model WU-1 (Kyowa Seimitsu) delivered mobile phase at the rate of 0.72 ml/min to a column in a thick-walled glass tube (0.3 × 50 cm) fitted with a head for syringe injection through a rubber septum. While the injection was performed, the flow was stopped. The eluted fluorophores were measured with an Aminco Fluorocolorimeter No. 4-7439 equipped with ultraviolet lamp (No. 4-7125, maximum energy at 360 nm), a Corning No. 7-51 primary filter, a secondary filter (No. 4-7116) and a quartz flow-through cell (2 mm I.D.). The intensities were recorded on an Electronic Polyrecorder, Model SPR-2TC (TOA Electronics). All lines were connected with PTFE tubing (1 mm I.D., 2 mm O.D.). High-pressure liquid chromatography was performed with methanol–0.10 *M* Tris–hydrochloric acid buffer of pH 8.0 (7:3) at room temperature.

RESULTS AND DISCUSSION

Reaction conditions

DM, NE, 3-MT and NM were subjected to reaction with fluorescamine in the borate or phosphate buffer. 3-MT and NM yielded the same curves of fluorescence intensity *versus* pH change as those of other primary amines, as was shown previously[5]. On the other hand, DM and NE gave a little fluorescence by the reaction in the borate buffer between pH 7 and 10, although they gave much more fluorescence in the phosphate buffer, the maximum of which was obtained at pH 8.0 (Fig. 1).

The phenomenon was investigated further by using high-pressure liquid chro-

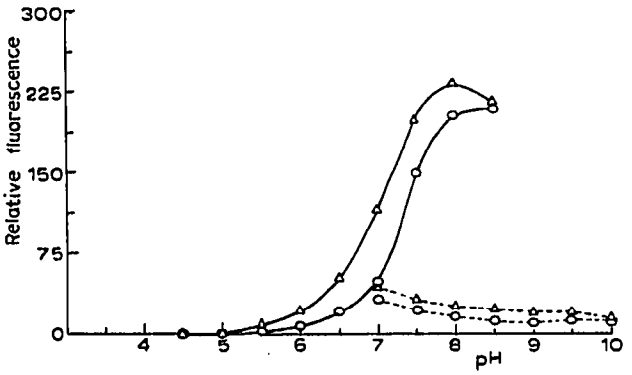

Fig. 1. Influence of pH on the fluorescence attained on interaction of fluorescamine with catecholamines. Solid lines, phosphate buffer; broken lines, borate buffer; ○, dopamine (10 n*M*); △, norepinephrine (10 n*M*).

matography on Hitachi 3011 gel, as indicated later. The reaction products of DM in both the phosphate and the borate buffer were chromatographed and separated. The areas and retention times of each peak corresponding to the DM fluorophores that were produced in the borate and phosphate buffer was the same when methanol–0.10 *M* Tris–hydrochloric acid buffer of pH 8.0 (7:3) was used as the eluting solvent. The same result was also obtained for the NE fluorophores. Therefore, the reaction should occur smoothly even in the borate buffer. Alternatively, when the reaction product of DM with fluorescamine was injected on to the Hitachi 3010-OH gel column with methanol–0.15 *M* potassium borate buffer of pH 8.0 (7:3) as eluting solvent, a small peak appeared (see Fig. 5a). It seems that the fluorophore was chelated with borate, passed through without adsorption on the gel and the fluorescence was suppressed.

Under the most suitable conditions (0.15 *M* phosphate buffer, pH 8.0), the graph of fluorescent intensity *versus* amount of amine was linear between 60 pmole/ml and 10 nmole/ml. The sensitivity was almost the same as that obtained by the THI method[1].

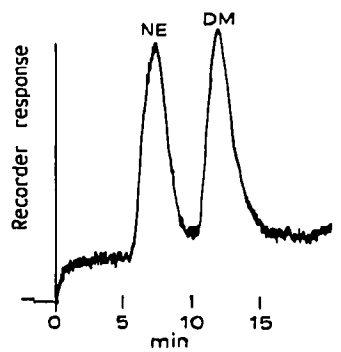

Fig. 2. Chromatogram of fluorescamine derivatives of dopamine (0.11 nmole) and norepinephrine (0.11 nmole). Elution with methanol–0.05 *M* Tris–hydrochloric acid buffer of pH 8.0 (7:3) from a Hitachi 3011 gel column. DM = dopamine; NE = norepinephrine.

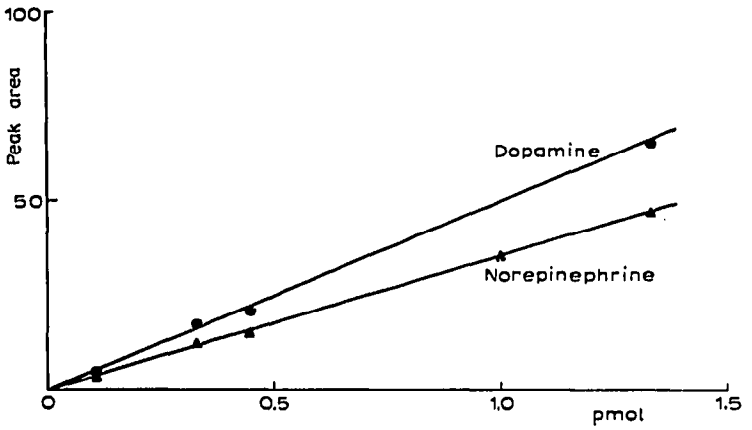

Fig. 3. Calibration graphs for dopamine (●) and norepinephrine (▲).

Chromatography of the fluorophores

As the adsorption of the fluorophores on the silica gel was too strong for them to be eluted with the buffer solution of pH 8.0, which gave the highest intensity, Hitachi 3011 or 3010-OH gel was used as column packing. Tris–hydrochloric acid buffer containing methanol was used as the eluting solvent because Tris has strong buffer action at about pH 8.0. The best separation was obtained by using a system of Hitachi 3011 gel and methanol–0.05 *M* Tris buffer of pH 8.0 (7:3), as shown in Fig. 2. If the ratio was increased to 8:2, the fluorophores were eluted without adsorption on the column, while at a ratio of 6:4, they were retained too strongly. Linearity of the recorder response with the amount injected was obtained between 0.11 and 1.3 nmole, as shown in Fig. 3. As DM and NE may be isolated simultaneously from biological samples by adsorption on alumina, this assay method would be applicable to the simultaneous measurement of DM and NE in the brain[1].

The same conditions were also useful for the separation and the simultaneous measurement of 3-MT and NM fluorophores (Fig. 4). The isolation procedure was developed recently[6].

The above fluorophores were not retained on an octadecylsilicone (ODS) column, which was used for the separation of the polyamine fluorophores[7], even if water was used as the eluting solvent.

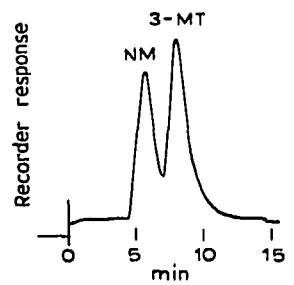

Fig. 4. Chromatogram of fluorescamine derivatives of 3-methoxytyramine (0.33 nmole) and normetanephrine (0.33 nmole). Conditions as in Fig. 2. 3-MT = 3-methoxytyramine; NM = normetanephrine.

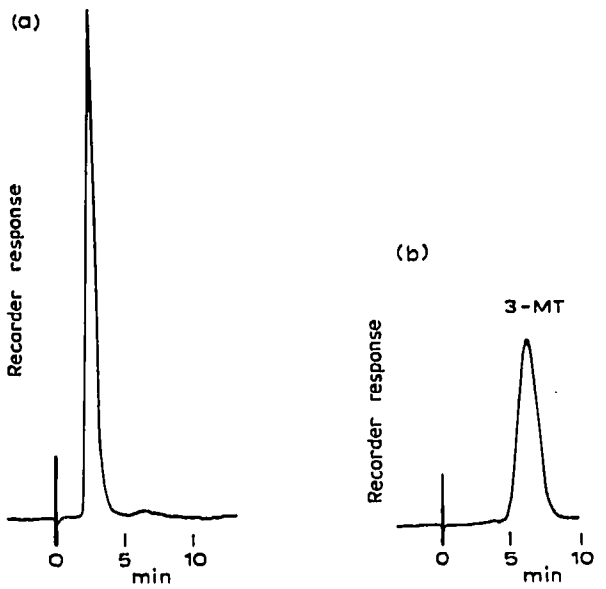

Fig. 5. Chromatograms of fluorescamine derivatives of (a) dopamine (26 nmole) and (b) 3-methoxytyramine (0.33 nmole). Elution with methanol–0.15 *M* potassium borate buffer of pH 8.0 (7:3) from a Hitachi 3010-OH gel column. DM = dopamine; 3-MT = 3-methoxytyramine.

When it is necessary to measure 3-O-methyl metabolites in the presence of DM and/or NE, for example to assay catechol-O-methyltransferase activity the use of borate buffer is recommended. Fig. 5a shows the chromatogram obtained when 26.6 nmole of DM fluorophore were injected. No interfering peaks appeared around the peak of 3-MT fluorophore (0.33 nmole), which appeared about 10 min after injection, as shown in Fig. 5b. It should be noted that the small amount of the product (3-MT) could be measured in the presence of the large amount of the substrate (DM).

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