Journal of Chromatography, 96 (1974) 250–254

CHROM. 7518

Note

Separation of fluorescamine derivatives of aliphatic diamines and polyamines by high-speed liquid chromatography

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(Received March 29th, 1974)

Fluorescamine[1] is a powerful fluorogenic reagent that was synthesized after elucidation of the structure of fluorophors derived from ninhydrin, phenylacetaldehyde and primary amines[2–4]. The reagent has been used to determine primary amines[5–10], but no information was available on the separation of fluorescamine derivatives. This paper reports a new application of the reagent to aliphatic diamines and polyamines.

EXPERIMENTAL

Materials

Vydac reversed-phase chromatographic separation material (The Separations Group, Hesperia, Calif., U.S.A.) was used as column packing.

Fluorescamine was obtained from Hoffman–La Roche (Nutley, N.J., U.S.A.) (courtesy of Dr. William E. Scott); a stock solution of it was prepared in a special grade of acetone (Sanko Junyaku Co., Tokyo, Japan). Borate buffer was prepared from boric acid cryst. GR (E. Merck, Darmstadt, G.F.R.).

The diamines butane-1,4-diamine (putrescine), pentane-1,5-diamine (cadaverine), hexane-1,6-diamine, heptane-1,7-diamine and octane-1,8-diamine were obtained from Tokyo Kasei Kogyo Co., (Tokyo, Japan), and spermidine and spermine, as hydrochlorides, were purchased from Sigma (St. Louis, Mo., U.S.A.).

N-3-Aminopropylpentane-1,5-diamine and N-3-aminopropylhexane-1,6-diamine were synthesized by a modification of the method of Jackson[11], and N-3-aminopropylheptane-1,7-diamine and N-3-aminopropyloctane-1,8-diamine were synthesized analogously as described by Israel *et al.*[12]. Various chemical and physical parameters were measured to confirm the purity of these compounds.

All other organic solvents and inorganic chemicals used were commercially available highly purified reagents.

Fluorescent derivative formation

To a mixture of one volume of sample solution (less than 1 nmole of amine/μl) and one volume of 0.1 *M* borate buffer of pH 8.0 (prepared by titrating 0.1 *M* boric acid with 6 *N* sodium hydroxide) was added one volume of a solution of fluorescamine (20 mg/10 ml in acetone) at room temperature; rapid addition and mixing were es-

sential to achieve optimal fluorescence. An aliquot of the reaction mixture was directly applied to the column.

Preparation of mobile phase

A master solution was prepared by diluting 60 ml of 0.1 *M* sodium borate buffer of pH 8.0 to 100 ml with methanol and each mobile phase used was prepared by diluting this solution with the sodium borate buffer to give the desired methanol content.

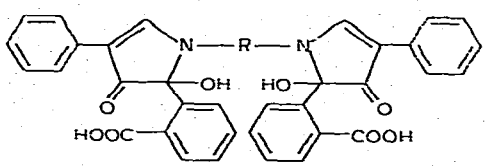
Equipment

The high-speed liquid chromatograph was constructed in our laboratory. A high-pressure piston pump (model WU-1 Minimicro pump, Kyowa Seimitsu Co.) delivered mobile phase at 1 ml/min to a column in a thick-walled glass tube (50 × 0.3 cm) fitted with a head for syringe injection through a rubber septum. The temperature of the jacketed column was kept at 60° by circulating water from a thermo-bath (model CTE-2, Coolnics, Komatsu Solidate Co.). The eluted fluorophors were measured with an Aminco fluoro-microphotometer (American Instrument Co.) equipped with a high-intensity mercury vapour lamp, a Corning No. 7–51 primary filter, a Wratten No. 4 secondary filter and a quartz flow-through cell (2 mm I.D.); the measurements were recorded on a Model EPR-2TC Electronic polyrecorder (TOA Electronics Ltd.) with a chart speed of 18 cm/h. A screw cock was fitted at the outlet of the column to provide a back-pressure, and all lines were connected with Teflon tubing (1 mm I.D., 2 mm O.D.).

The apparatus used to provide a linear gradient for elution was a simple one based on a pair of cylindrical glass vessels of the same size (1.8 cm I.D., 7 cm high). The other experimental details are given in the legends to the figures.

RESULTS AND DISCUSSION

The reaction between fluorescamine and the primary amines and hydrolysis of the excess of reagent were complete within a few minutes at room temperature at pH 8, and the resulting fluorophors were stable for several hours. A molar ratio of reagent to primary amino-group of at least 3.6:1 was chosen to ensure completion of the reaction; the general structure of the fluorophors is shown in Fig. 1. Under the conditions used, monofluorescamine derivatives of aliphatic diamines and polyamines were not formed, although monofluorescamine derivatives of putrescine,



R: $(CH_2)_n$ (n=4–8); diamines

R: $(CH_2)_3NH(CH_2)_n$ (n=4–8); N-3-aminopropyl-alkyldiamines

R: $(CH_2)_3NH(CH_2)_4NH(CH_2)_3$; spermine

Fig. 1. Chemical structures of fluorescamine derivatives of aliphatic diamines and polyamines.

spermidine and spermine could be separated by elution with a gradient of 0 to 6% of acetone in pH 8.0 borate buffer containing 2% of NaCl: they were eluted at the solvent front under the conditions described below for difluorescamine derivatives.

The best separation was achieved by using a reversed-phase chromatographic system employing a chemically bonded octadecylsilane column, with a gradient of methanol in 0.1 *M* borate buffer of pH 8.0 as mobile phase.

Fig. 2. illustrates a chromatogram of the derivatives of a series of aliphatic diamines. It can be seen that they were clearly separated in order of increasing methylene-chain length: thus, under the elution conditions used, one methylene group was sufficient to produce a distinguishable difference in retention time.

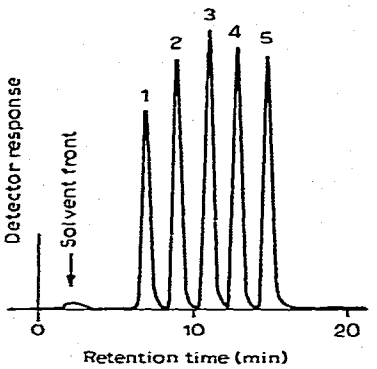


Fig. 2. Chromatogram of fluorescamine derivatives of aliphatic diamines. Gradient elution with 10 ml of 10% methanol in pH 8.0 borate buffer and 10 ml of 40% methanol in pH 8.0 borate buffer. 1 = putrescine; 2 = cadaverine; 3 = hexane-1,6-diamine; 4 = heptane-1,7-diamine; 5 = octane-1,8-diamine.

Before achieving the chromatogram shown in Fig. 2, various factors affecting the elution profile (*e.g.*, the pH and molarity of the eluting buffer and choice of organic solvent) had been studied. A pH of 8.0 was chosen after consideration of the stability of the fluorophors from pH 7 to 9. Both phosphate and borate buffers were examined in combination with various organic solvents. When both buffers were compared under the same conditions of molarity (0.1 *M*), mixing ratio of organic solvent, etc., a marked decrease in retention was observed with the phosphate buffer. This might be explained by the influence of ionic strength, as a similar decrease in retention was produced when sodium chloride was added to the borate system. As it was desirable to increase the retention, borate buffer (0.1 *M*) was preferred. For the organic solvents, retention was decreased in the order methanol, ethanol, acetone: in the same way as with the salt effect, methanol was considered to be more suitable for adjusting the retention of the fluorophors.

Fig. 3 illustrates a chromatogram of a series of N-(3-aminopropyl)alkyldiamine–fluorescamine derivatives in which the elution gradient for the diamines was modified: a difference of one methylene group in a relatively large molecule could be recognized. Another example is shown in Fig. 4, in which the fluorescamine derivatives of the naturally occurring polyamines putrescine, spermidine and sperm-

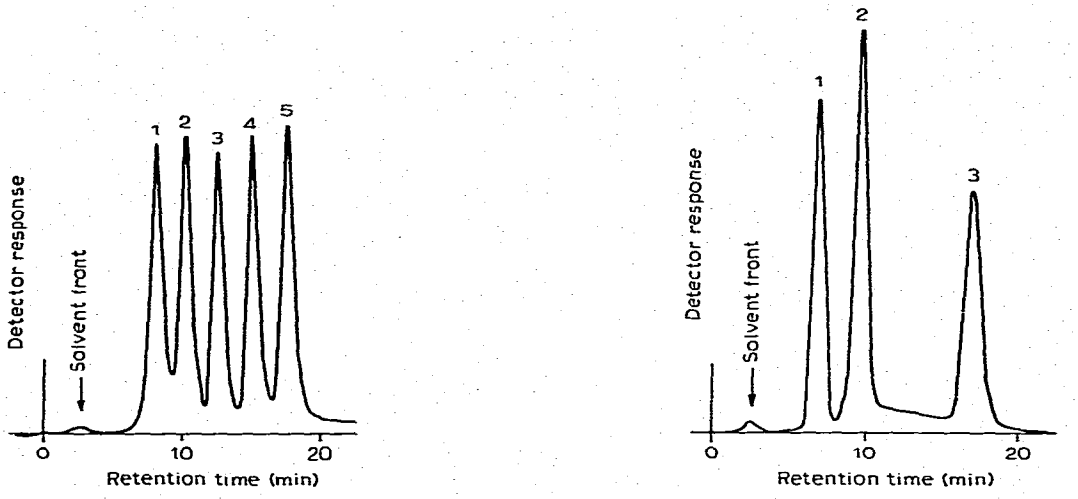


Fig. 3. Chromatogram of fluorescamine derivatives of N-(3-aminopropyl)alkyldiamines. Gradient elution with 10 ml of 18% methanol in pH 8.0 borate buffer and 10 ml of 40% methanol in pH 8.0 borate buffer. 1 = spermidine; 2 = N-3-aminopropylpentane-1,5-diamine; 3 = N-3-aminopropylhexane-1,6-diamine; 4 = N-3-aminopropylheptane-1,7-diamine; 5 = N-3-aminopropyloctane-1,8-diamine.

Fig. 4. Chromatogram of fluorescamine derivatives of putrescine, spermidine and spermine. Gradient elution with 10 ml of 10% methanol in pH 8.0 borate buffer and 10 ml of 25% methanol in pH 8.0 borate buffer. 1 = putrescine; 2 = spermidine; 3 = spermine.

ine were chromatographed by a modified gradient elution different from the two systems mentioned above. Thus it can be seen that various types of modification can be made to the gradient schedule depending upon the objective desired.

The limit of detection was of the picomole order, and determinations could be made from the recorder trace by measuring the peak-height ratio with respect to an internal standard. Fig. 5 shows an example of the calibration curves for some of the

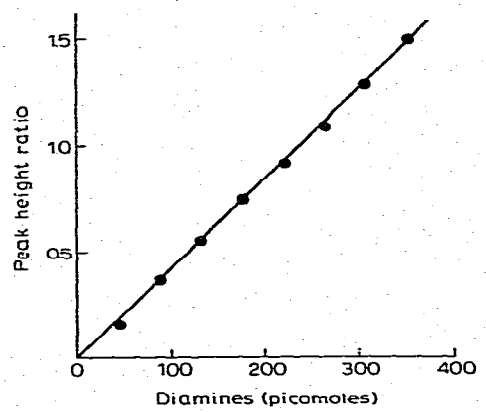


Fig. 5. Calibration graph for aliphatic diamines. Determination was carried out from the peak-height ratio with respect to hexane-1,6-diamine (240 picomoles) as internal standard. This graph is a superimposition of those for putrescine, cadaverine, heptane-1,7-diamine and octane-1,8-diamine.

aliphatic diamines with hexane-1,6-diamine as internal standard: satisfactory linearity is demonstrated, even in the picomole range.

The method described will undoubtedly be a useful tool in research on naturally occurring polyamines, and application of the method to biological material is currently in progress in this laboratory.

ACKNOWLEDGEMENTS

The author expresses his deep gratitude to Dr. Sidney Udenfriend, Roche Institute of Molecular Biology, for his kind suggestions and encouragement throughout this work. He is also indebted to the American Instrument Co., Inc., for supplying an Aminco fluoro-microphotometer.

He wishes to thank Dr. Masashi Okada, Director of this Institute, for his interest and warm support during this work, and he also thanks Dr. T. Tanimura (University of Tokyo) and Dr. H. Takahagi, (Sankyo Co., Ltd.) for their helpful comments and discussions on high-speed liquid chromatography, Dr. I. Matsunaga (Chugai Seiyaku Co., Ltd.), Dr. R. Sakaguchi (Science University of Tokyo) and Miss M. Kawase (of this Institute) for their interest and assistance, and Dr. J. O'D McGee (Royal Infirmary of Glasgow) for advice concerning the preparation of this paper.

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