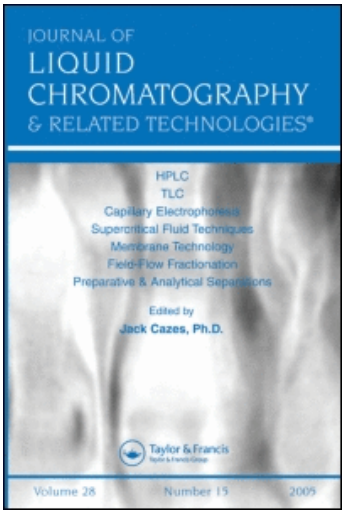

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Simultaneous Determination of Mono-, Di-, and Trinucleotides by High-Performance Liquid Chromatography Using *N*-(Dansyl)Ethylenediamine as a Fluorescent Derivatizing Reagent

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SIMULTANEOUS DETERMINATION OF MONO-, DI-, AND TRINUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING *N*-(DANSYL)ETHYLENEDIAMINE AS A FLUORESCENT DERIVATIZING REAGENT

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ABSTRACT

A method for simultaneous determination of mono-, di- and trinucleotides with high sensitivity has been developed. This based on the fluorescent derivatization of nucleotides by the reaction of 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA) with the phosphoric acid moiety of nucleotide, and then the resolution of fluorescent derivatives by high-performance liquid chromatography with a spectrofluoro monitor. The fluorescent dansylEDA derivatives of nucleotides were separated sharply each other. The detection limits for dansylEDA derivatives of nucleotides were between 4.7 and 20.3 pmol per 10-μl injection. This method was applied to analyze the yeast RNA after the generation of ribonucleoside 5'-monophosphate by nuclease P1 digestion. As a result, no more than 40 ng of RNA was needed for one analysis, and so such sensitivity of this method gives an advantage to analyze the RNA in a small scale.

INTRODUCTION

The analysis of purine and pyrimidine bases, the corresponding nucleosides and nucleotides is an essential step in many areas of biochemical study. The development of instrumental analyses such as gas chromatography - mass spectrometry and high - performance liquid chromatography (HPLC) has made it possible to analyze precisely the bases, nucleosides and nucleotides (1-8). Especially, various HPLC methods have been reported for the determination of these compounds, however, these methods have some disadvantages with respect to the sensitivity and specificity.

Recently we have reported the procedure with which 2'-deoxynucleoside 5'-monophosphate (dXmp) was detected with high sensitivity and selectivity (9). This procedure is based on the fluorescent derivatization by the reaction of 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA) at the phosphoric acid moiety of dXmp, then the resolution of these fluorescent compounds by HPLC. This procedure was successfully applied to the determination of guanine+cytosine content of DNA (10).

In this report, we describe the application of the procedure to analyze ribonucleoside 5'-mono-, di- and triphosphates which are essential in biological metabolism as constituents of RNA and high-energy phosphates.

MATERIALS AND METHODS

Apparatus

A Japan Spectroscopic (JASCO) Model 800-MP-15 high-performance liquid chromatograph with a JASCO FP-210 spectrofluoro monitor was used. Chromatograms were recorded on a JASCO Model 805-GI graphic integrator, while fluorescence spectra were obtained on a JASCO FP-770 spectrofluorometer. Reversed phase octadecyl-bonded polyvinyl alcohol gel column, Finepak ODP-50 (250 mm x 4.6 mm; Asahikasei, Tokyo, Japan) was used for the separation of dansylEDA derivatives.

Chemicals

Adenosine 5'-monophosphate (AMP), Guanosine 5'-monophosphate (GMP), Cytidine 5'-monophoaphate (CMP), Uridine 5'-monophosphate (UMP), Adenosine 5'-diphosphate (ADP), Guanosine 5'-diphosphate (GDP), Adenosine 5'-triphosphate (ATP) and Guanosine 5'-triphosphate (GTP) were obtained from Sigma (St. Louis, MO, U.S.A.). 1-Methylimidazole was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). 5-Dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA) was synthesized by the method described in the previous paper (9). All other chemicals used were of analytical grade from commercial sources. Standard solutions were prepared separately by dissolving each of the ribonucleoside 5'-monophosphate (Xmp), ribonucleoside 5'-di- or triphosphates in distilled water to a concentration of 30 mM. Standard mixture solution was prepared by mixing standard solutions each other to a final concentration of 3 mM.

Preparation of DansylEDA Derivatives of Mono-, Di- and Trinucleotides

Unless specified otherwise, the following procedure was used. A 10 μl of the standard mixture solution or biological sample in 200 μl of 1-methylimidazole buffer (0.1 M, pH 7.5) was reacted with 10 μl of 0.1 M EDAC in 1-methylimidazole buffer and 40 μl of 50 mM dansylEDA in dimethylsulfoxide. The reaction was carried out in dark for 18 hr at 27℃.

Chromatographic Conditions

Chromatographic separations of dansylEDA derivatives were performed at a flow rate of 0.6 ml / min at 40℃. The eluting solvents were: A, 10 mM phosphate buffer (pH 10.3) - acetonitrile (88 : 12, v / v); B, 10 mM phosphate buffer (pH 10.3) - acetonitrile (78 : 22, v / v); C, 10 mM phosphate buffer (pH 10.3) - acetonitrile (60 : 40, v / v). Elution was carried out for 10 min with solvent A, followed by the linear gradient elution system from solvent A to solvent B in 18 min, then solvent C was used for another 10 min. The column effluent was

monitored fluorometrically at an excitation wavelength of 270 nm and at an emission wavelength of 546 nm. The separation of non-derivatized Xmp, ATP, ADP, GTP and GDP was performed at a flow rate of 0.6 mi / min at 25℃ with 10 mM phosphate buffer (pH 3.5) as the eluting solvent.

RESULTS AND DISCUSSION

Fluorescence Spectra of DansylEDA Derivatives

After the derivatization of 10 µl of the standard mixture solution containing 30 nmol each of the Xmp, ATP, ADP, GTP and GDP, each fluorescent derivative, which was separated on the silica TLC plate, was extracted with 70% methanol, then analyzed. Each derivative of Xmp, ATP, ADP, GTP, and GDP exhibited similar fluorescence excitation and emission spectra patterns as shown in FIGURE 1. As a result, the fluorescence intensity was measured using excitation at 270 nm and emission at 546 nm.

Separation of DansylEDA Derivatives by HPLC

The separation of dansylEDA derivatives of Xmp, ATP, ADP, GTP and GDP is shown in FIGURE 2. The dansylEDA derivatives were sharply separated from each other.

Assay Linearity and Detection Limit

The fluorescence intensity of each derivatives was linear over a range of detection limit up to 580 pmol per 10-µl injection. The detection limits for dansylEDA derivatives were between 4.7 pmol of dansylEDA-ATP and 20.3 pmol of dansylEDA-CMP per 10-µl injection at a signal-to-noise ratio of about five..

Assay Precision

Relative standard deviations obtained in 5 measurements for 58 pmol / 10 µl of dansylEDA derivatives were between 0.89 % of dansylEDA-GTP and 8.56 % of dansylEDA-CMP, and between 0.97 % of dansylEDA-GTP and 4.78 % of dansylEDA-CMP for 580 pmol / 10 µl of dansylEDA derivatives.

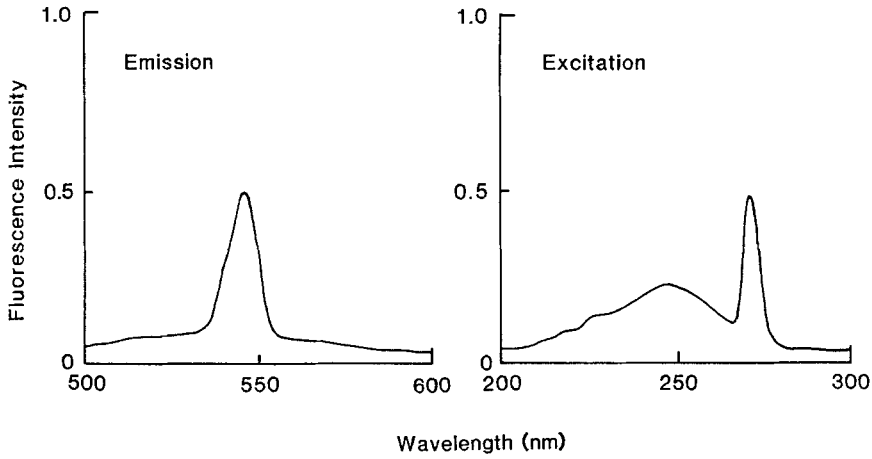

FIGURE 1 Fluorescence spectra of the dansylEDA derivative of AMP. All other derivatives of Xmp, ATP, ADP, GTP and GDP exhibited similar fluorescence spectra patterns. Emission was measured at an excitation wavelength of 270 nm, while excitation was measured at an emission wavelength of 546 nm.

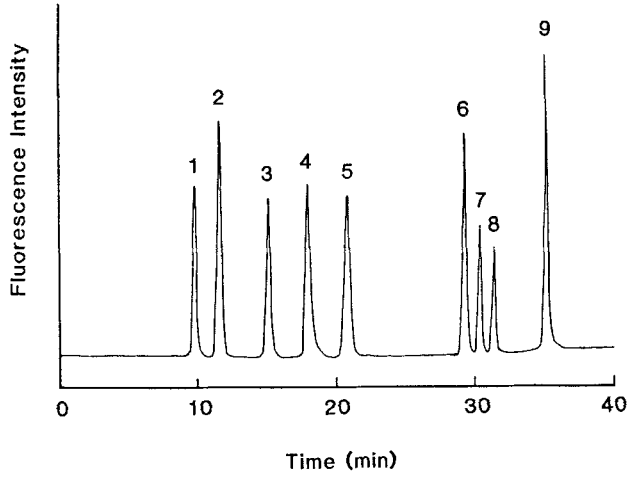

FIGURE 2 Chromatogram of dansylEDA derivatives of mono-, di- and trinucleotides. Peaks: 1 = dansylEDA-GTP; 2 = dansylEDA-ATP; 3 = dansylEDA-GDP; 4 = reaction by-product; 5 = dansylEDA-ADP; 6 = dansylEDA-GMP; 7 = dansylEDA-UMP; 8 = dansylEDA-CMP; 9 = dansylEDA-AMP.

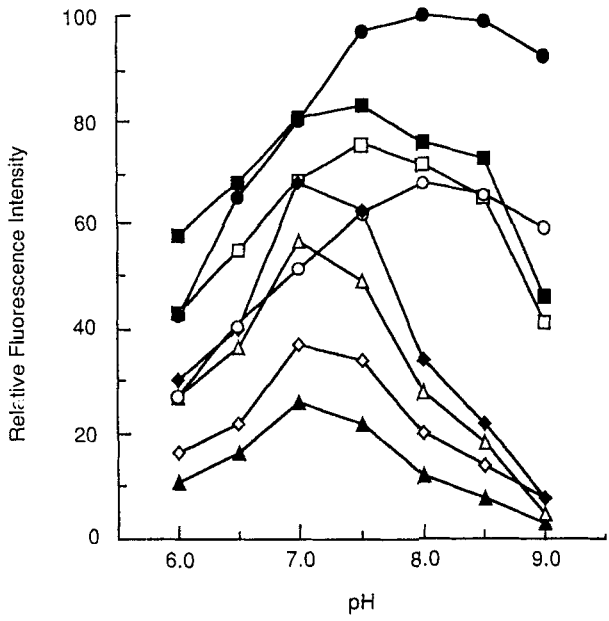

FIGURE 3 Effect of the pH in the reaction mixture on dansylEDA derivatization of mono-, di- and trinucleotides. O, dansylEDA-GTP; ●, dansylEDA-ATP; □, dansylEDA-GDP; ■ dansylEDA-ADP; Δ, dansylEDA-GMP; ▲, dansylEDA-UMP; ◇, dansylEDA-CMP; ◆, dansylEDA-AMP. Each point represents the mean of triplicate determinations of the fluorescence intensity of each peak of the dansylEDA derivative separated on the Asahipak ODP-50 column.

Effect of pH on the Derivatization

The derivatization with dansylEDA was carried out using the procedure described in the MATERIALS AND METHODS, except that the pH was varied from 6 to 9. As shown in FIGURE 3, pH 7 in the reaction mixture was found to be most effective on the derivatization of mononucleotides, on the other hand, pH 7.5 and pH 8 were most effective for dinucleotides and trinucleotides, respectively. Following an increase of the number of phosphoric acid moiety in the nucleotide, an increasing of the most effective pH values on the derivatization was noted, therefore a value of 7.5 was selected as the pH on the derivatization for the simultaneous analysis of all nucleotides.

Effect of Reaction Time on the Derivatization Yield

The effect of the reaction time on the derivatization at 27℃ was examined over a period of 8 days. The result shows that the fluorescence intensities of dansylEDA derivatives increased with the reaction time, then reached the plateau in one day. Every dansylEDA derivatives were stable throughout the investigation period without any decomposition.

Application to the analysis of RNA base composition

Yeast RNA in 10 μl of distilled water was heated in a boiling bath for 5 min, then rapidly cooled on ice. To this was added 10 μl of nuclease P1 (Yamasa, EC 3.1.30.1.) solution prepared by dissolving 2 units of nuclease P1 in 1 ml of sodium acetate buffer (40 mM, pH 5.3) containing 0.2 mM $ZnCl_2$. After incubation at 50 ℃ for 1h, Xmp, which was generated by nuclease P1 digestion, was derivatized directly and analyzed by HPLC. As a result, no more than 40 ng of RNA was enough for one analysis. This means that this method gives an advantage to the analysis of RNA which is hard to be prepared from some biological materials.

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