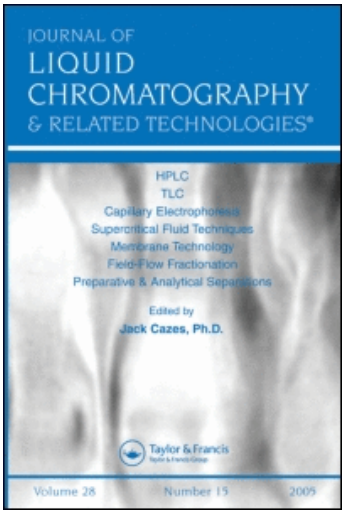

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High-Performance Liquid Chromatographic Analysis of 2'-Deoxynucleoside 5'-Monophosphate Using N-(Dansyl)Ethylenediamine as a Fluorescent Derivatizing Reagent

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF 2'-DEOXYNUCLEOSIDE 5'-MONOPHOSPHATE USING N-(DANSYL)ETHYLENEDIAMINE AS A FLUORESCENT DERIVATIZING REAGENT

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ABSTRACT

A method for quantitative determination of 2'-deoxynucleoside 5'-monophosphate (dXmp) has been developed. This based on the preparation of fluorescent derivatives by the reaction of 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)] sulfonamide (dansylEDA) with the phosphoric acid moiety of dXmp, and then the resolution of fluorescent derivatives by high-performance liquid chromatography with a spectrofluoro monitor. The fluorescent dansylEDA derivatives of dXmp were separated more sharply and symmetrically from each other compared with the separation of non-derivatized dXmp. The detection limits for dansylEDA derivatives of dXmp were 6.4-6.7 pmol per 10-μl injection, and these values indicate that the fluorimetric analyses were approximately 10- to 20-times more sensitive than the UV-monitoring method. The simplicity, sensitivity and selectivity make this method an attractive alternative to the established assay systems of dXmp.

INTRODUCTION

The analysis of components of nucleic acids, purine and pyrimidine bases, nucleosides and nucleotides is of primary importance for the under-

standing of metabolism of nucleic acids. During the last decade, the development of instrumental analyses has made it possible to determine with high resolution the bases, nucleosides and nucleotides. Especially, high-performance liquid chromatography (HPLC) has greatly facilitated the precise quantitative analysis of these compounds (1-6). Notwithstanding this radical development, very little attempt has been made to develop detection procedures with high sensitivity and selectivity. Recently synthetic oligonucleotides labelled with non-radioactive tags such as fluorophores are widely used as probes for the detection and isolation of specific genes and as primers for DNA sequencing (7-11). Labelling of oligonucleotides with fluorescent groups is performed preferably at the 5' termini of oligonucleotides containing aliphatic amino groups. These amino oligonucleotides are highly nucleophilic and hence are readily reacted with electrophilic fluorescent dyes which are commercially available. This strategy has been adopted by Kelman *et al.*(12) for the synthesis of fluorescent 2'-deoxynucleoside 5'-monophosphate (dXmp) and the analysis by HPLC with fluorescence detection.

In this paper, we describe the simple and rapid procedure for the synthesis of fluorescent dXmp by the reaction of 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)]sulfonamide (dansylEDA), and data on the optimization of the fluorescent derivatization of dXmp and their chromatographic separation are presented.

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 × 4.6 mm; Asahikasei, Tokyo, Japan) was used for the separation of dansylEDA derivatives.

Chemicals

2'-Deoxyadenosine 5'-monophosphate (dAmp), 2'-deoxyguanosine 5'-monophosphate (dGmp), 2'-deoxycytidine 5'-monophosphate (dCmp) and thim

dine 5'-monophosphate (Tmp) 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). All other chemicals used were of analytical grade from commercial sources. Standard solutions were freshly prepared by dissolving the each of 2'-deoxynucleoside 5'-monophosphate (dXmp) in distilled water to a concentration of 3 mM.

Preparation of 5-Dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)] sulfonamide(dansylEDA)

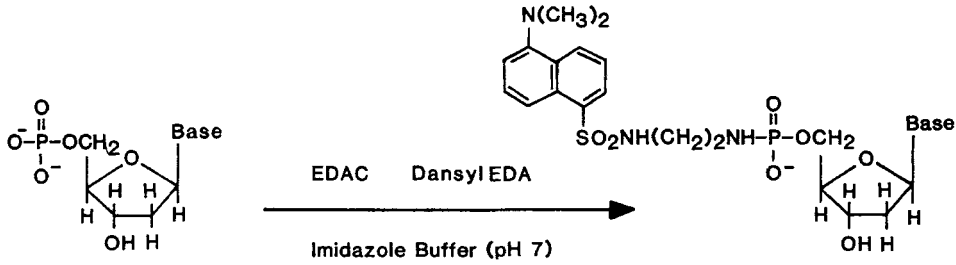
A solution of dansylchloride in dimethylformamide was added slowly to 10% molar excess of ethylenediamine in dimethylformamide. The dimethylformamide was removed by evaporation and the residue was dissolved in sodium carbonate buffer(pH 10). DansylEDA was separated on the TLC plate of silica gel from the unreacted ethylenediamine, dansylchloride and didansylethylenediamine. The dansylEDA fraction was scraped off the TLC plate, then dansylEDA was extracted a few times with methanol.

Preparation of DansylEDA Derivatives of 2'-Deoxynucleoside 5'-monophosphate

A 50mM dansylEDA was prepared by dissolving a synthesized dansylEDA in dimethylsulfoxide. Unless specified otherwise, the following procedure was used. A 10 μl each of the standard solution of dXmp or biological sample in 200 μl 1-methylimidazole buffer (0.1 M, pH 7.0) was reacted with 10 μl of 0.1 M EDAC in 1-methylimidazole buffer and 40 μl of 50 mM dansylEDA. The reaction mixture was kept in dark for 18 hr at room temperature.

Chromatographic Conditions

Separations of dansylEDA derivatives were performed at a flow rate of 0.6 ml/min at 40°C. The eluting solvents were: A, 10 mM phosphate buffer(pH 10.3)-acetonitrile (85:15, v/v); B, 10 mM phosphate buffer(pH 10.3)-acetonitrile (70:30, 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 30 min. The column effluent was monitored fluorometrically at an excitation



Scheme I Synthesis of proposed dansylEDA derivative of 2'-deoxynucleoside 5'-monophosphate

wavelength of 270 nm and at an emission wavelength of 546 nm. Separations of non-derivatized dXmp were performed at a flow rate of 0.6 ml/min at 25°C with 10 mM phosphate buffer (pH 3.5) as the eluting solvent.

RESULTS

Fluorescence Spectra of DansylEDA Derivatives

After the derivatization of standards containing 0.1 μmol each of the dXmp, the dansylEDA derivatives of dXmp were separated on the TLC plate of silica gel and the fluorescent band corresponding to each derivative and the fluorescent by-product was scraped off the plate, extracted with 70% methanol and analyzed, respectively. Each derivative of dXmp exhibited similar fluorescence excitation and emission spectra patterns, however the by-product had a different fluorescence excitation spectra pattern. To eliminate the disturbance by the fluorescent by-product in HPLC analysis, 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 dXmp is shown in FIGURE 1A, while FIGURE 1B shows the separation of non-derivatized dXmp as monitored by UV absorbance at 260 nm. The dansylEDA derivatives were separated more sharply and symmetrically from each other compared with the separation of the non-derivatized dXmp. The effect of pH of the eluting sol-

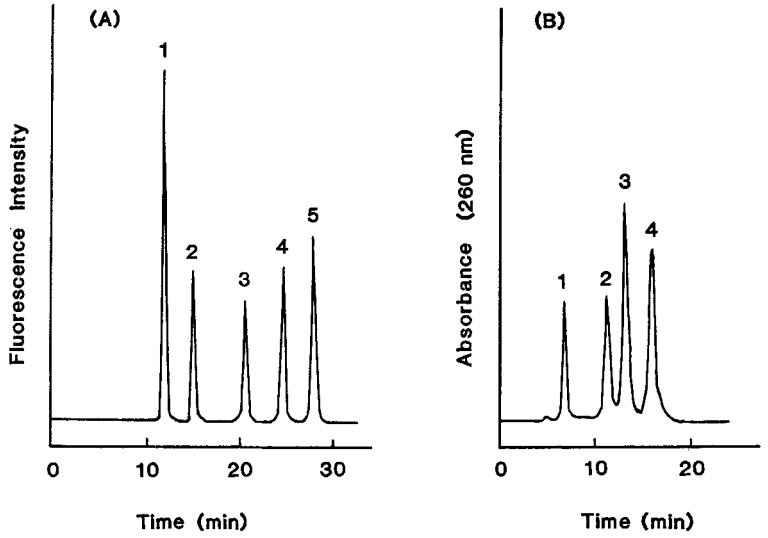


FIGURE 1 Chromatograms of dansylEDA derivatives of 2'-deoxynucleoside 5'-monophosphates (0.5 nmol of each per 10-μl injection)(A) and of non-derivatized 2'-deoxynucleoside 5'-monophosphates (7.5 nmol of each per 10-μl injection) monitored by UV absorbance at 260 nm (B). Peaks: (A) 1 = reaction by-product; 2 = dansylEDA-dGmp; 3 = dansylEDA-dCmp; 4 = dansylEDA-Tmp; 5 = dansylEDA-dAmp; (B) 1 = dCmp; 2 = Tmp; 3 = dGmp; 4 = dAmp.

vents on the separation of dansylEDA derivatives was examined. Following a decrease from pH 11 to 7, a worsening in the separation of each derivative was noted, therefore a value of 10.3 was selected as the pH of the eluting solvents A and B.

Assay Linearity and Detection Limit

The fluorescence intensity of each derivative was linear over a range of detection limit up to 450 pmol per 10-μl injection. The detection limits for dansylEDA-dAmp, dansylEDA-dGmp, dansylEDA-dCmp and dansylEDA-Tmp were 6.5, 6.4, 6.7 and 6.5 pmol per 10-μl injection respectively, at a signal-to-noise ratio of about five. On the other hand, the detection limits for non-derivatized dXmp were 50-130 pmol per 10-μl injection. This is taken

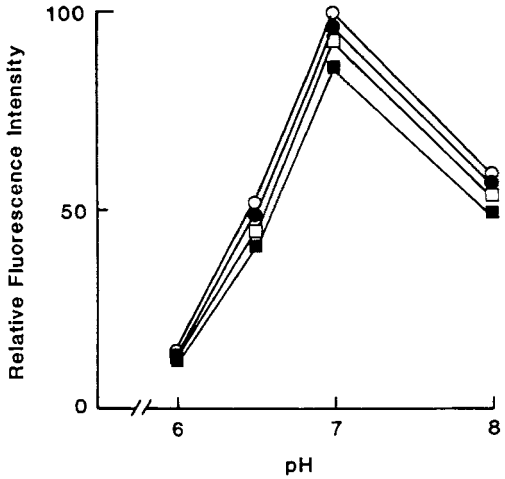


FIGURE 2 Effect of the pH in the reaction mixture on dansylEDA derivatization of 2'-deoxynucleoside 5'-monophosphate. ○, dansylEDA-dAmp; ●, dansylEDA-dGmp; □, dansylEDA-Tmp; ■, dansylEDA-dCmp. Each point represents the mean of triplicate determinations of the fluorescence intensity of each peak of the dansylEDA derivative separated on the Asahipak ODP-50 column.

to indicate that the fluorimetric analyses were approximately 10- to 20-times more sensitive than the UV-monitoring method.

Assay Precision

Relative standard deviation obtained in each of 5 measurements for 56 pmol/10-μl of dansylEDA-dAmp, dansylEDA-dGmp, dansylEDA-dCmp and dansylEDA-Tmp was 2.33%, 2.16%, 3.56% and 4.75% respectively, and 1.28%, 2.13%, 1.85% and 1.02% for 560 pmol/10 μl of dansylEDA-dAmp, dansylEDA-dGmp, dansylEDA-dCmp and dansylEDA-Tmp, respectively.

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 8. As shown in FIGURE 2, pH 7 in the reaction mixture was found to be most effective on the derivatization. Tris(hydroxymethyl)aminomethane,

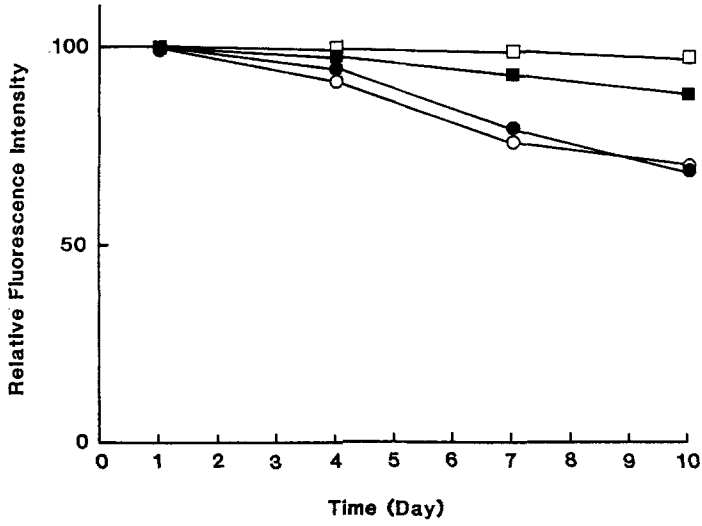


FIGURE 3 Stability of dansylEDA derivatives of 2'-deoxynucleoside 5'-monophosphate. Details as in FIGURE 2.

sodium phosphate, sodium borate or sodium citrate as a constituent of the buffer inhibited the derivatization.

Effect of Concentration of DansylEDA on Quantitative Analysis

The molar ratio of dansylEDA to total amount of dXmp was varied from 1 to 100. The total amount of dXmp present was always 120 nmol. A minimum molar ratio of 20 of dansylEDA to dXmp was required for quantitative analysis.

Stability of DansylEDA Derivatives

The stability of dansylEDA derivatives in the reaction mixture was examined. The results, as shown in FIGURE 3, indicate that each derivatives was stable without any decomposition within a day, however, the fluorescence intensity gradually decreased within 10 days. Especially, dansylEDA derivatives of purine nucleotides, dAmp and dGmp, were less stable than those of pyrimidine nucleotides, dCmp and Tmp.

DISCUSSION

To realize the metabolism of nucleic acids, the microanalysis of components of nucleic acids such as bases, nucleosides and nucleotides with high sensitivity and selectivity is required. Although several instrumental analyses such as gas chromatography and HPLC by monitoring the ultraviolet absorbance have been used for the quantitative estimation of nucleic acids and the related compounds, those procedures have some disadvantages with respect to the sensitivity, selectivity, and simplicity.

In the present study, we attempted to develop the procedure with which 2'-deoxynucleoside 5'-monophosphates as the components of DNA were detected precisely, sensitively, specifically and rapidly. The essential features of this procedure are summarized below. [1] Four 2'-deoxynucleoside 5'-monophosphates (dAmp, dGmp, dCmp, Tmp) were transformed to the fluorescent compounds by the reaction of dansylEDA at the phosphoric acid moiety of dXmp. These derivatives were readily prepared by a single-step method under the mild conditions. [2] The fluorescent derivatives of dXmp were distinctly separated from each other by HPLC and were distinguished from non-phosphorus nucleosides and bases which seemed to be freed from the dansylEDA derivatization.

Kelman *et al.*(12) have reported the two-step derivatization method of dXmp involving 5'-phosphoramidation with ethylenediamine followed by conjugation of the free aliphatic amino group of the phosphoramidate with dansyl chloride, however, each step of this method has different pH dependency on the reaction and reaction time, so this results in the time consumption to complete the whole reaction.

This study was primarily undertaken to develop an analytical method for measuring 2'-deoxynucleoside 5'-monophosphate in biological materials. The precise analysis of components of nucleic acids is required to realize the relationship between the quantitative or qualitative change of nucleic acids and the gene function, therefore the developed method reported in this study should meet this requirements.

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