Journal of Chromatography, 512 (1990) 409–414
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1838

Preparation of ethylenediaminephosphoramidates of nucleotides and derivatization with fluorescein isothiocyanate

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ABSTRACT

Fluorescence post-labeling of nucleotides is a potentially useful technique for the detection of trace amounts of damaged DNA (DNA adducts). Towards this goal, we have studied a derivatization procedure starting with the four major 5′-deoxynucleotides as model compounds. The 5′-phosphate group was first labeled with ethylenediamine via a phosphorimidazolide intermediate in methanol with an organic-soluble carbodiimide. The resulting ethylenediaminephosphoramidate products were reacted in turn with fluorescein isothiocyanate. The reaction sequence has been characterised at all stages by high-performance liquid chromatography.

INTRODUCTION

In order to label a nucleic acid with a reporter group, the nucleic acid often is first prepared in a form which contains one or more aliphatic amine substituents. For example, Chu *et al.*[1] labeled nucleotides and polynucleotides at the terminal 5′-phosphate group with ethylenediamine.The reaction took place in imidazole buffer using a water-soluble carbodiimide. In turn, the amino-DNA product has been labeled with biotin[2,3], peroxidase and amplifiable reporter RNA[4]. Kelman *et al.*[5] utilized the same chemistry for the fluorescent labeling and high-performance liquid chromatography (HPLC) detection of damaged nucleotides (DNA adducts) derived from irradiated calf thymus DNA. As the authors pointed out, this approach could potentially be used for the assay in general of DNA adducts produced *in vivo*.

We are similarly interested in fluorophore labeling of DNA adduct nucleotides.

0021-9673/90/$03.50

Our goal is to use this technique to facilitate the detection and isolation of DNA adducts for subsequent structural elucidation by mass spectrometry. In the present work, we have investigated the conditions for fluorophore labeling. Some improvements are presented especially for the initial coupling of ethylenediamine to nucleotides.

MATERIALS AND METHODS

Chemicals and reagents

2′-Deoxyadenosine 5′-monophosphate (5′-dAMP), 2′-deoxycytosine 5′-monophosphate (5′-dCMP), 2′-deoxyguanosine 5′-monophosphate (5′-dGMP), thymidine 5′-monophosphate (5′-TMP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, ethylenediamine and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO, U.S.A.). Imidazole, 2,6-lutidine, N,N′-dicyclohexylcarbodiimide and acetic acid were from Aldrich (Milwaukee, WI, U.S.A.). Silylation-grade dimethyl sulfoxide (DMSO), triethylamine (sequanal grade) and ninhydrin were from Pierce (Rockford, IL, U.S.A.). Sodium carbonate, sodium bicarbonate, HPLC-grade monobasic potassium phospate, ammonium hydroxide, 0.22-μm MSI Cameo, 3-mm diameter filters and HPLC solvents were from Fisher (Bedford, MA, U.S.A.).

HPLC

The analytical HPLC comprised a Series 4 LC system from Perkin-Elmer (Norwalk, CT, U.S.A.), a Brownlee RP C_{18}, Spheri-5, 150 × 4.6 mm I.D. cartridge column (Rainin, Woburn, MA, U.S.A.), a 9060 Polychrom diode array detector (Varian, Walnut Creek, CA, U.S.A.), a FS970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.), a Cl-10B Integrator (LDC/Milton Roy, Bloomfield, CT, U.S.A.) and a Timberline column oven (Boulder, CO, U.S.A.). The fluorescence and diode array detector were connected in series. Ultraviolet spectra were saved on an IBM personal computer using the Polysoc program from Varian. Solvent A was 0.1 *M* potassium dihydrogen phosphate (pH 4.6) and solvent B was acetonitrile. The flow-rate was 1 ml/min. The column was equilibrated for at least 15 min with the starting solvent composition for each gradient prior to sample injection. At the end of each gradient elution, the solvents were returned to their starting compositions over a 2-min period and then the column was equilibrated for 15 min prior to the next injection. The deoxynucleotide-5′-monophosphates were separated with a 20-min gradient from 0-20% B. A 20-min, 0–30% B gradient was used for the nucleotide-phosphorimidazolide reaction mixtures. The nucleotide–ethylenediaminephosphoramidate reaction mixtures were separated with a 0–10% B gradient in 15 min. For all of the above, the column temperature was maintained at 30°C. The nucleotide-fluorescein derivatives were subjected to HPLC with a 20-min, 0–70% B gradient at 30°C, or with a 40-min, 10–20% B gradient at a column temperature of 80°C. Preparative HPLC was carried out (as described below) on the same equipment using a Rainin 25 × 1.0 cm RP-18, 5-μm Microsorb cartridge column (Rainin).

Synthesis

Deoxynucleotide-5′-ethylenediaminephosphoramidates (5′-dNMP-EDP). Three μmol of 5′-dAMP, 5′dGMP or 5′-TMP were dissolved in 20 μl of DMSO and added to

300 μl of a methanol solution of N,N-dicyclohexylcarbodiimide (DCC) and imidazole (1 *M* each). A 3-μmol amount of 5′-dCMP was dissolved in 32 μl of water and added to 600 μl of the same DCC–imidazole solution. (The 5′-dCMP was not soluble in DMSO, and the larger volume of the DCC–imidazole solution was necessary to prevent precipitation of the DCC in the presence of water.) In parallel, 20 μl of each of the four nucleotide solutions were combined and added to 600 μl of the DCC–imidazole solution. After 1 h at room temperature, one-half of each sample was added to an equal volume (200 or 300 μl) of 0.63 *M* aqueous ethylenediamine that had been adjusted to pH 7.5 with hydrochloric acid. The precipitate which formed (from DCC) was removed by centrifugation (2000 *g*) or by filtration through a 0.22-μm nylon filter. Each reaction mixture was heated to 55°C for 2 h and then kept at −20°C until further use.

The samples were evaporated to dryness with a Speed-Vac concentrator (Savant, Farmingdale, NY, U.S.A.), redissolved in 200 μl of water and purified by preparative HPLC. The mobile phase buffer was 0.005 *M* acetic acid, adjusted to pH 4.6 with triethylamine. The 5′-dCMP-EDP was purified isocratically with 100% buffer, 5′-TMP-EDP with a 10-min linear gradient from 0 to 5% acetonitrile and 5′-dGMP-EDP as well as 5′-dAMP-EDP with a 10-min gradient from 0 to 10% acetonitrile at a flow-rate of 5 ml/min. The purity of each compound was then determined by analytical HPLC. The 5′-dCMP-EDP required a second purification, which was done on the 22 × 0.46 cm I.D. Brownlee RP C_{18} column using 100% buffer. The pure products were evaporated to dryness in a Speed-Vac concentrator and stored at −20°C until further use.

FITC conjugates

Each 5′-dNMP-EDP (0.2 μmol) was dissolved in 0.1 ml of sodium carbonate–bicarbonate buffer, 0.1 *M*, pH 9.5. FITC (0.8 mg, 2 μmol) was added in 4 μl of dimethylformamide and the reaction was kept for 18 h at room temperature in the dark, followed by freezing at −20°C until further use.

The 5′-dNMP-FITC derivatives were purified by preparative gradient HPLC, 10 to 70% acetonitrile over 30 min in 0.01 *M* acetic acid–triethylamine (pH 4.6) at 5 ml/min.

RESULTS AND DISCUSSION

Deoxynucleotide-5′-ethylenediaminephosphoramidates (5′-dNMP-EDP)

In order to label nucleotides, we initially followed the procedure of Chu *et al.*[1] developed for labeling the 5′-terminus of oligodeoxynucleotides. The 5′-dNMP-phosphorimidazolides (5′-dNMP-PIs) were prepared in an aqueous solution (pH 6) of imidazole and water-soluble carbodiimide. These products were then treated with an excess of ethylenediamine buffered with lutidine–HCl (pH 7.5) and the reactions were monitored by HPLC. The UV spectra of the 5′-dNMPs and their corresponding PI and EDP products were identical as expected (Fig. 1). Based on measurements of the relative peak areas of the products and starting materials for each reaction, the yields of the 5′-dNMP-EDPs were 80–90%.

We intend to use this reaction to facilitate the detection and isolation of nucleotide adducts obtained from enzymatic hydrolysates of DNA. These nucleotide

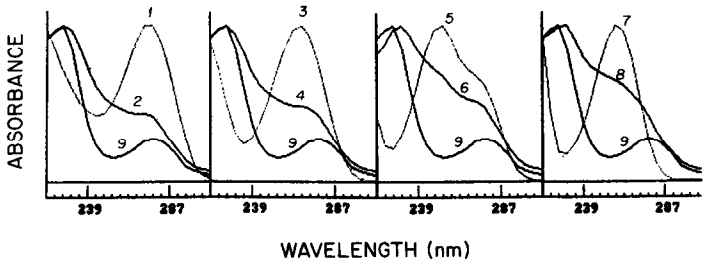

Fig. 1. Diode array UV spectra of HPLC peaks of the 5′-dNMPs and their corresponding FITC derivatives. 1 = 5′-dCMP; 2 = 5′-dCMP-FITC; 3 = 5′-TMP; 4 = 5′-TMP-FITC; 5 = 5′-dGMP; 6 = 5′-dGMP-FITC; 7 = 5′-dAMP; 8 = 5′-dAMP-FITC; 9 = FITC. The spectra were normalized to give full-scale absorbance at the UV maximum.

adducts occur at extremely low levels[6]. It is therefore desirable to optimize the reaction in every respect. In order to improve the reaction yields, we varied the conditions of the first step of the reaction sequence, the formation of the phosphorimidazolide. However, under aqueous conditions we could not achieve greater than 90–95% conversion of starting nucleotides to the corresonding 5′-dNMP-PI intermediate products based on HPLC analysis. The remaining 5–10% could be seen by HPLC as

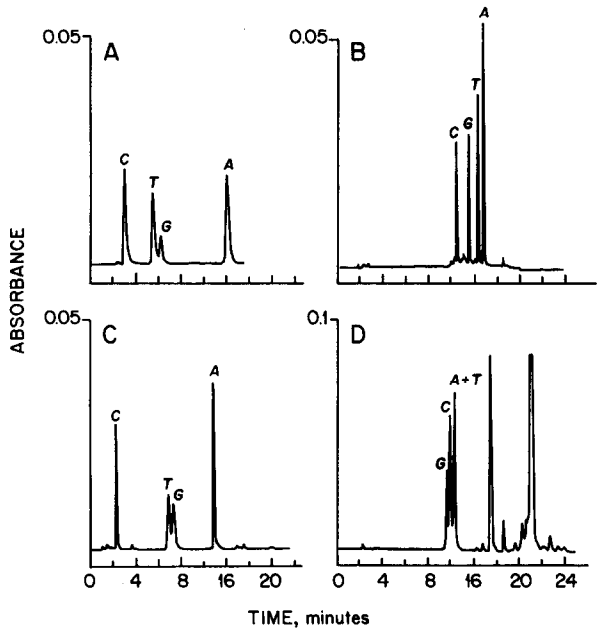

Fig. 2. Reversed-phase HPLC chromatograms of 5′-dNMP starting materials and their subsequent PI, EDP and FITC reaction mixtures. From each reaction mixture, a 2–15-μl sample was injected without prior purification containing *ca.* 1 nmol of each nucleotide or modified nucleotide. The HPLC solvent was a gradient of acetonitrile in 0.1 *M* potassium phosphate, pH 4.5. (A) 5′-dNMPs, 0–20% acetonitrile in 20 min, (B) 5′-dNMP-PIs, 0–30% acetonitrile in 20 min, (C) 5′-dNMP-EDPs, 0–10% acetonitrile in 15 min, (D) 5′-dNMP-FITCs, 0–70% acetonitrile in 20 min. Detection was at 263 nm.

the 5′-dNMP starting materials (data not shown). At pH 6, it is known that 5′-dNMP-PIs slowly hydrolyze back to the 5′-dNMPs[1]. This could account for the presence of the residual starting nucleotides. Since our interest is in the detection of nucleotides rather than oligonucleotides it was not necessary to maintain aqueous conditions. By changing the starting solvent to methanol along with a change to an organic-soluble carbodiimide, we were able to form the PI intermediates in a quantitative yield based on HPLC (Fig. 2B). Under these conditions, the 5′-dNMP-PIs were significantly stabilized: the reaction mixtures could be stored for weeks at 4°C with no change in the amount of 5′-dNMP-PIs. The use of the organic-soluble carbodiimide may also have been advantageous since side reactions are known to occur when a positively charged, water-soluble carbodiimide is reacted with a nucleotide[7].

The PI intermediates were then reacted directly with an aqueous solution of ethylenediamine adjusted to pH 7.5 with hydrochloric acid. The lutidine buffer was eliminated since it seemed to be unnecessary and tended to overload the column during preparative HPLC of the 5′-dNMP-EDPs. With these modifications, a quantitative conversion of the 5′-dNMPs to the corresponding 5′-dNMP-EDPs was obtained (Fig. 2C).

5′-dNMP-FITC

The 5′-dNMP-EDPs were purified by preparative HPLC and reacted, both separately and combined, with an excess of FITC at pH 9.5. An HPLC chromatogram of a combined reaction mixture (Fig. 2D) shows complete disappearance of the

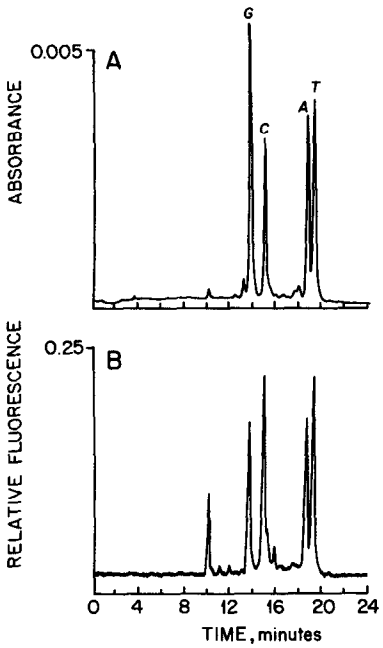

Fig. 3. Reversed-phase HPLC chromatogram of the HPLC-purified 5′-dNMP-FITCs with simultaneous UV (263 nm, A) and fluorescence (480 nm excitation, 520 nm emission filter, B) detection. The separation was performed at 80°C using a 20-min, 10–20% acetonitrile gradient in 0.1 *M* potassium phosphate, pH 4.5.

5'-dNMP-EDPs and the appearance of the 5'-dNMP-FITC derivatives. The later eluting peaks are the hydrolysis products of FITC. These peaks are also present when a reaction blank is tested that contains no 5'-dNMP-EDPs (data not shown).

As seen in Fig. 2D, sharp peaks are obtained for the 5'-dNMP-FITCs, but the resolution is poor. In fact, 5'-dAMP-FITC and 5'-TMP-FITC coelute. This separation was achieved by reversed-phase HPLC using a steep gradient. When a less steep gradient is employed, the latter two peaks remain unresolved, and all 5'-dNMP-FITC peaks tail severely (data not shown).

In order to investigate this separation in more detail, we isolated the 5'-dNMP-FITCs from the separate reaction mixtures by preparative HPLC and prepared a combined sample free of the FITC hydrolysis products. The 5'-dNMP-FITCs could be fully separated by analytical reversed-phase HPLC at 80°C as shown in Fig. 3. Although this elevated temperature dramatically improved the peak shape, it will undoubtedly reduce the column lifetime. Thus, other approaches to this separation need to be investigated. The diode array UV spectra for the 5'-dNMP-FITCs (Fig. 1) correspond exactly to the composite spectra derived from FITC and the 5'-dNMP's. Both UV (Fig. 3A) and fluorescence (Fig. 3B) detection were performed.

The two chromatograms in Fig. 3 show some unidentified, small peaks in addition to the four major peaks for the 5'-dNMP-FITCs. These peaks are either previously unresolved side products formed during the FITC conjugation reaction, or decomposition products generated during the isolation of the 5'-dNMP-FITC.

CONCLUSION

Nucleotide ethylenediaminephosphoramidate derivatives (5'-dNMP-EDPs) can be formed in a quantitative yield, stored without decomposition and labeled efficiently with a fluorophoric reagent such as FITC. In our future work, we will investigate the formation, stability and separation properties of the fluorescein derivatives in more detail and also explore the use of other fluorescent labeling reagents. These studies should lead to a practical method for detecting and isolating DNA adducts.

ACKNOWLEDGEMENTS

The research described in this article was supported by Grant CA 35843 from the National Cancer Institute and by funding from the Channel Program of the Egyptian Government. Contribution No. 415 from the Barnett Institute.

REFERENCES

1 B. C. F. Chu, G. M. Wahl and L. E. Orgel, *Nucleic Acids Res.*, 11 (1983) 6513.
2 A. Chollet and E. H. Kawashima, *Nucleic Acids Res.*, 13 (1985) 1529.
3 B. C. F. Chu and L. E. Orgel, *DNA*, 4 (1985) 327.
4 B. C. F. Chu and L. E. Orgel, *Nucleic Acids Res.*, 16 (1988) 3671.
5 D. J. Kelman, K. T. Lilga and M. Sharma, *Chem.-Biol. Interact.*, 66 (1988) 85.
6 A. M. Jeffery, in D. Grunberger and S. Goff (Editor), *Mechanisms of Cell Transformation by Carcinogenic Agents*, Pergamon Press, New York, 1987, p. 33.
7 P. T. Gilham, *J. Am. Chem. Soc.*, 84 (1962) 687.