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Derivatization of thymine and thymine photodimers with 4-bromomethyl-7-methoxycoumarin for fluorescence detection in high-performance liquid chromatography

J.K. Robinson, W.G. Scott[1], K.L. Rowlen, J.W. Birks*

Department of Chemistry and Biochemistry, University of Colorado, Campus Box 215, Boulder, CO 80309, USA

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Abstract

Exposure of DNA to ultraviolet radiation results in the formation of a number of photoproducts, including thymine photodimers. A sensitive and selective analytical method based on high-performance liquid chromatography (HPLC) and fluorescent labeling with 4-bromomethyl-7-methoxycoumarin has been developed to quantify both thymine and thymine photodimers. The identity of the thymine and thymine dimer derivatives were determined by HPLC–electrospray ionization mass spectrometry. The derivatization reaction yield was maximized by optimizing several reaction variables. The limit of detection for HPLC method was 1.0 pmol thymine and 0.4 pmol thymine dimer for $S/N=3$.

Keywords: Derivatization, LC; Thymine; 4-Bromomethyl-7-methoxycoumarin

1. Introduction

In recent years decreases in stratospheric ozone have been linked to increases in UV-B radiation (280–320 nm) at the Earth's surface [1]. UV-B radiation generates a number of lesions in DNA which have been implicated in cell lethality, mutagenesis and carcinogenesis [2]. Furthermore, clinical studies have shown UV-B radiation as the primary etiological agent in human skin cancer [3]. Sensitive methods are needed for detection of UV-induced DNA lesions in order to understand their role in biological processes. The major photoproducts resulting from UV-B radiation are cyclobutane pyrimidine dimers (CPDs), generated by a (2+2) photochemical cycloaddition between adjacent pyrimidine bases. Thymine photodimers are the most prevalent of the CPDs, while smaller amounts of cytosine–thymine, and cytosine–cytosine dimers are formed [4]. Therefore, the thymine photodimer is often used as a biological marker for UV-B exposure [5,6].

Several methods have been used to quantify thymine photodimers in DNA. One approach utilizes immunological methods in which specific antibodies for the thymine photodimer have been prepared and isolated [7–10]. The antibodies are applied to intact irradiated DNA and are detected by radioactive labeling, fluorescence, or most recently immuno-

*Corresponding author. Tel.: +1-303-4926-531; fax: +1-303-4925-894.

[1]Present address: Department of Chemistry, University of California, Santa Cruz, CA 95064, USA.

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electron microscopy [6]. Although these assays have excellent selectivity and sensitivity, the procedures for isolating and purifying antibodies are laborious. In another method the thymine photodimer repair enzyme T4 endonuclease has been used to induce single-strand breaks which are subsequently quantified by alkaline sucrose sedimentation with radioactive labeling or fluorescence [11,12]. Excision repair assays are limited by enzyme selectivity for dimer type as well as DNA coiling preventing all lesions from being available for repair. Other methods involve base release by acidic hydrolysis, followed by separation with chromatography or capillary electrophoresis, and detection by mass spectrometry, fluorescence, radioactivity, amperometry or absorbance [2,5,13,14].

Here we report a sensitive and selective high-performance liquid chromatographic assay for the analysis of thymine (T) and thymine photodimers (TT) based on fluorescent labeling with 4-bromomethyl-7-methoxycoumarin (BMC), a reagent used in the past for derivatizing a wide range of analytes [15–25]. Since the TT/T ratio is a good index of DNA photodamage, the method has the advantage of not requiring an absolute calibration so long as the relative sensitivities to T and TT are known and their responses are linear. The BMC tag has been used to derivatize thymine and other pyrimdine bases in the past [26], but it has not been applied to the thymine photodimer.

2. Experimental

2.1. Reagents

All the chemicals purchased were of the highest purity available. The fluorescent derivatizing agent, BMC, was obtained from Molecular Probes (Eugene, OR, USA). Anhydrous potassium carbonate (>99%), 18-crown-6 ether (1,4,7,13,16-hexaoxacyclooctadecane), dimethyl sulfoxide (DMSO), thymine (>99%), adenine (>99%), guanine (>99%) and cytosine (>99%) were purchased from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade water and acetonitrile were from Fisher Scientific (St. Louis, MO, USA).

2.2. Preparation of cyclobutane thymine photodimers

Cyclobutane thymine photodimers were prepared by the method of Wang [27]. An aqueous solution of thymine with a concentration of 2 mg/ml was placed in a petri dish. The solution was then frozen on dry ice and irradiated for 1 h with a 254-nm Mineralight lamp from Ultraviolet Products (San Gabriel, CA, USA). The petri dish was placed 10 cm from the lamp and the intensity was 0.01 W/cm^2. The solution was then thawed and the freezing and irradiating cycle repeated five times. After a final thawing, a white precipitate was isolated by filtration on Whatman No. 32 filter paper. The precipitate was then rinsed with ethanol in order to remove excess thymine. The purity of the thymine dimer was checked by HPLC–electrospray ionization mass spectrometry (ESI-MS). The HPLC column used was a Vydac protein and peptide C_{18} column (250 mm×4.6 mm I.D.) and the mobile phase was 100% water. The amount of thymine in the prepared TT dimer sample was below the detection limit of the mass spectrometer, and the dimer was determined to be at least 95% pure.

2.3. Preparation of 4-bromomethyl-7-methoxycoumarin derivatives

Aqueous standard solutions of thymine (300 μM) and thymine photodimer (100 μM) were prepared in acetonitrile due to their low solubilities and stored in the dark at 4°C. Standard solutions of BMC (5 mM), and 18-crown-6 ether (10 mM) were prepared in acetonitrile. The acetonitrile was dried over molecular sieves (4-8 mesh beads, Fisher Scientific). The derivatization reaction was performed in 5.0-ml conical vials (Pierce, Rockford, IL, USA). Aliquots (0.5 ml) of the aqueous thymine or thymine dimer standard were added to the conical vial and evaporated overnight at 25°C. BMC standard solution (1 ml), 18-crown-6 ether (1 ml), and 15 mg of anhydrous K_2CO_3 were then added to the vial containing thymine or thymine dimer and heated at 90°C by refluxing in a sand bath. The K_2CO_3 served as a base catalyst, and the function of the crown ether was to increase its solubility by complexing with K^+. The

thymine derivatization reaction was complete in 1 h and the dimer derivatization in 24 h.

2.4. Calibration plots

The calibration standards were prepared by diluting the thymine and thymine photodimer stock solutions and adding T or TT in the range of 0.1–10 pmol into the reaction vials. The aqueous solutions were evaporated, and the derivatization mixture was added as described above. Each point on the working curve was generated from the average of three injections.

2.5. HPLC analysis

A Hewlett-Packard 1050 Series liquid chromatograph with a UV absorbance detector and Schoeffel FS970 Model fluorometer were used for the analysis. A 20-μl sample was injected on a Vydac Protein and Peptide C_{18} column (250×4.6 mm I.D.). The solvent mobile phase was acetonitrile–water (10:90, v/v). The solvent gradient was to 10% to 80% acetonitrile in 20 min, followed by isocratic elution for 5 min. Both the UV absorbance and fluorescence chromatograms were recorded for confirmation of derivative peak identity. A wavelength of 325 nm was used to excite the fluorescence of the derivative peak and a 389 nm cut-off filter was used to decrease the level of excitation radiation reaching the photomultiplier tube. The fluorescence chromatograms were recorded with a Shimadzu C-R3A chart recorder, and the UV absorbance chromatograms were recorded by a computer.

2.6. Electrospray ionization liquid chromatography–mass spectrometry

The analysis was performed with a Hewlett-Packard 1050 liquid chromatograph interfaced to a VG Platform mass spectrometer. The column used was a Phenonemex IB-Sil C_{18} (5 μm, 250×4.6 mm). The solvent mobile phase was acetonitrile–water (10:90, v/v). The solvent gradient was to 10% to 80% acetonitrile in 20 min, followed by isocratic elution for 5 min. A 50-μl sample was injected and the column effluent was split 1:50 between the mass spectrometer and UV detector. The electrospray ionization was performed with a needle voltage of 3500 V and a cone voltage of 20 V. Mass spectra were continuously acquired in the positive ion mode over a mass range of 100 to 1200 at the rate of 10 s/scan. A solution of ammonium acetate and trifluoroacetic acid in acetonitrile was added post-column to enhance ionization.

2.7. Fluorescence spectra

A Hitachi F-2000 fluorescence spectrophotometer was used to acquire excitation and emission spectra for the derivatizing agent (BMC) and the thymine and thymine photodimer derivatives. The excitation and emission bandwidths were 5 nm. Thymine and thymine photodimer derivatives were collected after separation on the column for fluorescence spectral analysis.

3. Results and discussion

Fig. 1 shows the products of the thymine and thymine photodimer derivatization reactions. The labeling reaction proceeds by a S_N2 mechanism in which each nitrogen on T and TT dimer reacts with BMC to remove the bromine. If the reaction

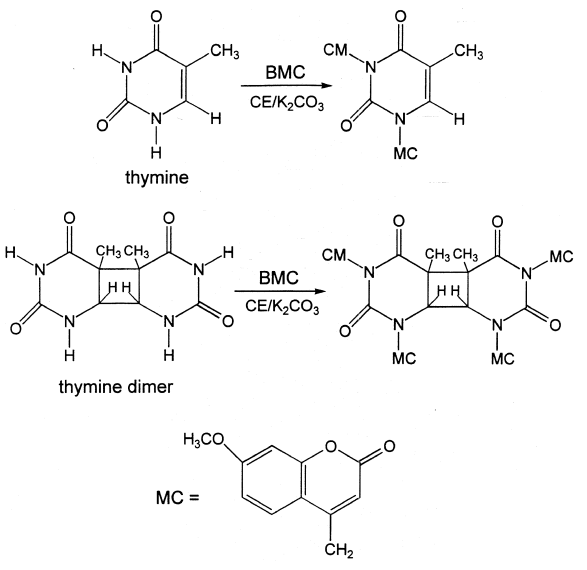

Fig. 1. Derivatization reactions of T and TT with the BMC. Each nitrogen on T and TT is a derivatizable site.

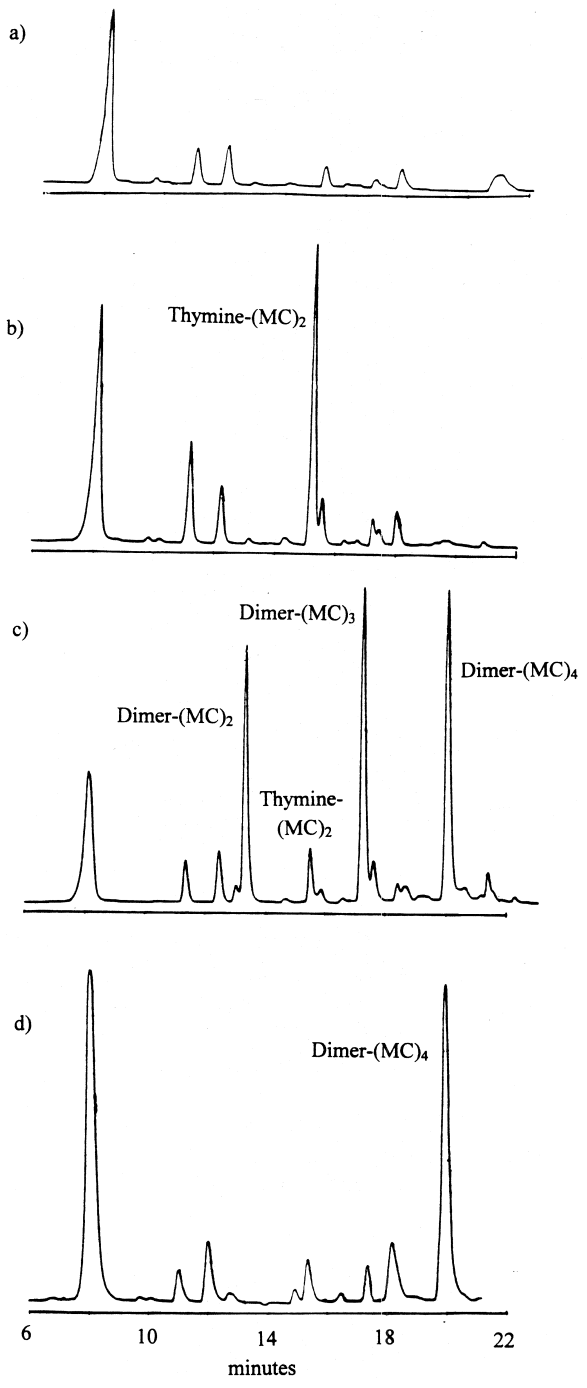

Fig. 2. Fluorescence chromatograms of (a) blank derivatization mixture (BMC, crown ether, K_2CO_3) heated for 24 h, (b) 50 μ*M* thymine heated for 1 h, (c) 50 μ*M* thymine photodimer heated for 6 h, and (d) 50 μ*M* thymine dimer heated for 24 h.

proceeds to completion, the products will contain a fluorescent methyl-7-methoxycoumarin (MC) bonded to each nitrogen. Incomplete reaction of thymine could form a singly substituted product, while derivatization products of the thymine photodimer could range from single to quadruple substitution.

3.1. Identification of BMC derivatives of thymine and thymine photodimer

Fluorescence chromatograms are shown in Fig. 2 for (a) the blank mixture of BMC, crown ether and K_2CO_3 after heating for 24 h, (b) the thymine reaction mixture after heating for 1 h, (c) the thymine dimer reaction mixture after heating for 6 h, and (d) the thymine dimer reaction mixture after heating for 24 h. The peaks were analyzed by HPLC–ESI-MS to confirm the identity of the derivatives. The mass spectral data of the derivative peaks are shown in Fig. 3. In the derivatization mixture containing thymine (Fig. 2b), a peak was found at 15.09 min that was not present in the heated blank (Fig. 2a). The m/z ratio of this peak was 503, which corresponds to the double substitution of MC on thymine. The other peaks present in the chromatogram have the same retention times as peaks present in the blank derivatization mixture. The peak at 8.16 min has a m/z of 207. This is the BMC derivatizing agent where the bromine has been replaced with a hydroxyl group due to nucleophilic attack by trace water. The remaining peaks were below the detection limit of the mass spectrometer and are assumed to be impurities in the BMC. No peak corresponding to single substitution of MC on thymine was found.

Fig. 2c shows the thymine photodimer derivative after heating for 6 h. Four new peaks were found in comparison to the blank mixture. Peaks at 13.68 (m/z=629), 17.85 (m/z=837) and 20.64 min (m/z=1005) correspond to the double, triple and quadruple substitution of MC on TT as shown in Fig. 3. The mass of each peak is the positive ion of the derivative, except for the peak at 17.85 min which is the double substitution of MC on TT plus an adduct of ammonium. As noted in Experimental, ammonium acetate was added post-column to enhance ionization. A small amount of the doubly substituted MC–thymine derivative was found at 15.09 min due to a

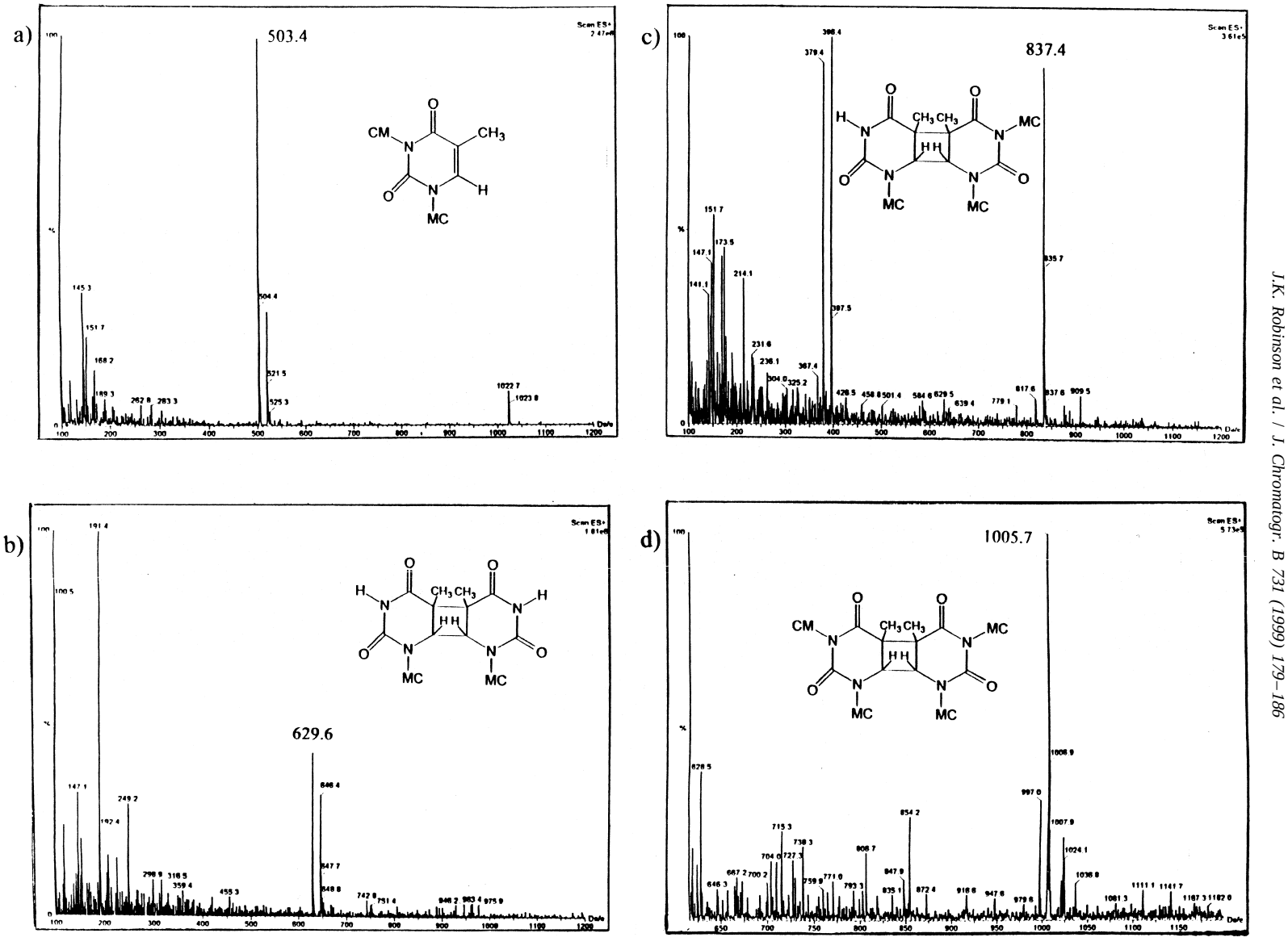

Fig. 3. Mass spectral data for (a) double substitution of MC on T, (b) double substitution of MC on TT, (c) triple substitution of MC on TT, (d) quadruple substitution of MC on TT.

thymine impurity in the dimer. Thus, after 6 h of heating the reaction products resulted in multiple substitutions on TT. However, after 24 h of heating the only peak remaining is the quadruple substitution of BMC on thymine (Fig. 2d). Complete derivatization with quadruple substitution is desirable in order to achieve the most sensitive detection possible without complications arising from multiple derivatization products.

Several methods of decreasing heating time and improving the yield of the quadruple substitution product of TT were explored. The concentration of the derivatization reagent relative to thymine dimer was increased by a factor of 10. However, this resulted in larger impurity peaks which can interfere quantification, and the reaction did not proceed at a significantly faster rate. The reaction temperature was also increased to 150°C. This resulted in additional peaks due to degradation or impurity reaction products. The solvent DMSO was tested in place of acetonitrile. DMSO is a polar aprotic solvent ideal for the S_N2 derivatization reaction of thymine and BMC. The thymine derivatization was complete in 5 min at room temperature, and the dimer reaction was complete after 6 h of heating. However, DMSO was difficult to dry and contains more impurities than acetonitrile. Chromatograms in which DMSO was used as the solvent had many impurity peaks and a large water substitution peak which interfered with the thymine and thymine dimer derivatives.

3.2. Fluorescence spectra

The excitation and emission spectra of the BMC and the T and TT dimer derivatives were obtained in order to determine the maximum excitation and emission wavelengths. BMC was found to have an excitation maximum at 409 nm and an emission maximum at 495 nm in acetonitrile–water (50:50). The excitation and emission wavelengths were shifted in the T and TT derivatives to 325 and 398 nm, respectively. Due to the shift in excitation and emission wavelengths, the excess derivatization reagent BMC could be discriminated against in the fluorescence chromatograms of the derivatives.

3.3. Calibration plots and limits of detection

Fig. 4 shows a calibration plot for the doubly derivatized thymine peak eluting at 15.09 min. The response was linear over the order of magnitude tested, and the relative standard deviation (RSD) averaged 7%. The limit of detection was ≈1.0 pmol

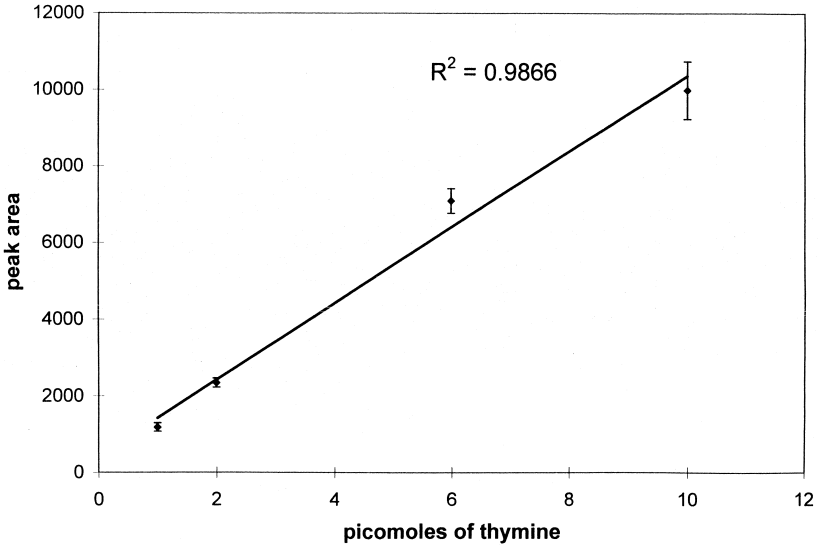

Fig. 4. Calibration plot for thymine derivative. Error bars are one standard deviation.

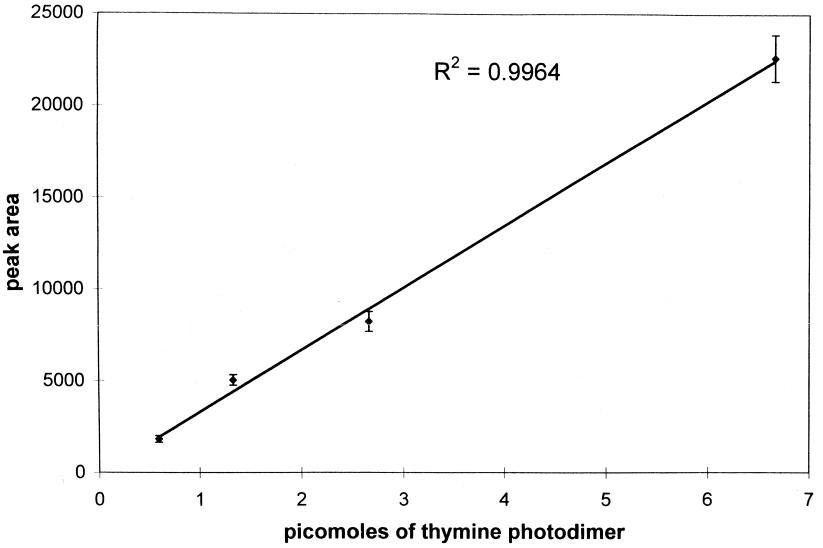

Fig. 5. Calibration plot for thymine photodimer derivative. Error bars are one standard deviation.

of thymine for $S/N=3$ (peak height equal to three-times the standard deviation of the baseline). Fig. 5 is a calibration plot for the peak identified as the quadrupoly derivatized thymine photodimer. The limit of detection for thymine dimer was ≈0.4 pmol for $S/N=3$, and the RSD averaged 8%.

3.4. Derivatization of other DNA bases

The DNA bases guanine, adenine and cytosine were also tested for derivative formation with BMC. Guanine and adenine did not produce derivatization products detectable with 325-nm excitation in combination with the ≥389-nm emission filter. However, cytosine did produce a detectable derivatization product with BMC. This result was expected since cytosine is a also a pyrimidine base and exhibits many of the same chemical properties as thymine. The cytosine derivative peak was well resolved and did not interfere with the T or TT derivatives peaks. Cytosine also forms a photoproduct with an adjacent cytosine (CC) or thymine (CT) upon irradiation with UV-B. Therefore, the derivatizing agent BMC could be useful in quantifying these photoproducts in addition to TT dimers.

4. Conclusions

Increases in UV-B radiation at the Earth's surface have led to an increased interest in the effects of radiation on DNA. Although many of the detrimental effects of UV radiation are known, there continues to be a need to develop sensitive and reliable measurement techniques for DNA photodamage. The HPLC method for the quantification of thymine and thymine photodimers based on fluorescent labeling with BMC meets these requirements. The simplicity and selectivity of the technique make it readily applicable to analysis of cellular DNA through the use of well known cell lysis and DNA hydrolysis procedures. Although the demonstrated limit of detection for the method is not as low as for immunoassays, the sensitivity could be improved through the use of microbore column HPLC coupled with laser-induced fluorescence (LIF). The small dimensions of a microbore column combined with the increase in excitation intensity could improve the limits of detection by several orders of magnitude. Detection limits for highly fluorescent compounds analyzed by LIF are in the range of fmol to amol [28–30]. These improvements could produce limits of detection for thymine and thymine photodimer

that are comparable to or better than those of immunoassays without the disadvantages of raising antibodies or radioactive labeling.

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