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Determination of laser-induced thymine–thymine dimer in DNA by LC

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Abstract

A new simple and selective high-performance liquid chromatography (HPLC) method has been developed for the determination of thymine–thymine (T–T) dimer in deoxyribonucleic acid (DNA) for the study of relationship between the yield of T–T dimer and laser irradiation conditions. The HPLC method is not disturbed by other hydrolysate formed in DNA with the good stability and reproducility. The detection of limit of the method is about 8.8 ng of T–T dimer in DNA blank. Three lever of laser intensity to irradiate DNA were selected for calf thymine to test the influence of the pulse number. The mechanism of DNA lesion and repair caused by laser irradiating was studied.

Keywords: Laser irradiation; DNA; Pyrimidine cyclobutane; HPLC

1. Introduction

Deoxyribonucleic acid (DNA) is the foundation of the biological heredity and variation since it carries genetic information. However, DNA is easily damaged by ultraviolet (UV)–Vis irradiation, and the main photoproducts after irradiation lesion are pyrimidine cyclobutane dimers [1–3]. Laser technology has developed to the point where it is possible as a more strong and sophisticated tool than original equipment of UV–Vis irradiation [4–6]. Laser irradiation is widely used in mutagenic breeding in the field of industrial microorganism and has shown large economic value. Some researches show that the mutagenic effects of laser irradiation on the several producing strains, wheat distant hybridization and so on. It confirmed that mutation effect of laser on DNA and showed certain economic value [7–9].

However, the mutagenic mechanism is not exactly clear while different dimers was produced by laser irradiation. Therefore, some researches are still focused on the exploratory study of mechanism of DNA lesion and repair caused by laser irradiation. Wilson et al. [10] reported 193 nm laser-induced chromosome aberration and gene mutation; Sage [11] found that 193 nm laser-induced production thymine–thymine (T–T), and the yield was only 1/10 of that produced by 254 nm UV light. Green et al. [12] indicated that

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dimers were found when human skin fibroblast cells were exposed to 385 and 405 nm laser light, but the mechanism was not clear. It was reported [13] that T–T dimer was found when DNA was exposed to 532 nm laser light. Because biological tissue absorbs long wavelength light more easily, this visible laser interests us for the probability of inducing biological variation efficiently. Recently, Cao and Wang [14] reported the exposure of thymine and DNA to high-intensity 532 nm pulsed radiation from a Nd:YAG laser resulted in the cyclobutyl pyrimidine dimers and analyzed the non-linear biological effects of high-intensity visible laser radiation on DNA. Kuluncsics et al. [15] also reported involvement of direct or indirect mechanisms and possible artifacts to study the wavelength dependence of UV-induced DNA damage distribution.

From the above studies, working on the technique of determining these laser-induced dimers in DNA is necessary and meaningful. Numerous chromatographic methods have been described for the determination of these dimers, including TLC, ion-exchange liquid chromatography (LC), high-performance liquid chromatography (HPLC) (both normal and reversed phase) and HPLC–GC/ECD [16–23]. Some published procedures such as TLC and cation-exchange LC are limited because of high background noise due primarily to tailing of thymine into dimer-containing regions [16,17]. Love and Fredberg [18] showed good separation of pyrimidine dimers from monomers using HPLC method with a μBondapak C18 column. The method was unable, however, to quantitative uracil–uracil (U–U) or thymine–uracil (T–U) in DNA due to poor separation of several species of products. Cadet at el. [19] also separated these three dimers (T–T, T–U, U–U) on an ODS-2 reversed-phase column, but the chromatographic process takes 40 min for the last compound (thymine) to elute out in the system. Ramsey and Ho [20] developed a sensitive method based upon HPLC fractionation and electrophore labeling followed by GC/electron capture detection to quantitative all dimers.

Susan and Phyllis [21] summarizes the methods used for the optimization of nucleotides by CE and includes the most recent techniques to improve selectivity, reproducility and sensitivity. However, CE techniques seem to have poor sensitivity. Robinson et al. [22] reported the derivatization of thymine and thymine photodimers with 4-bromomethyl-7-methoxycoumarin for fluorescence detection in HPLC. Douki et al. [23] proposed an electrospray-mass spectrometry for the characterization and measurement of thymine photoproducts.

In this paper, we investigated the effect of 532 nm laser radiation on DNA based on measurements of the yield of T–T dimers. We set up a sensitive HPLC method to measure T–T. The relations between parameters such as laser intensity, pulse number and the yield of T–T were discussed.

2. Experimental

2.1. Preparation of pyrimidine dimer standards

2.1.1. Instrumentation and chemicals

Two UV lamps (15 W) installed in a wood box made by ourselves ($60 \times 40 \times 80$ cm^3), Enamel pan ($40 \times 30 \times 1.5$ cm^3), revolving evaporator. Thymine and uracil were obtained from Sigma Chemical Co. and Sino-American Biotechnology Co.

2.1.2. Method of preparation of pyrimidine dimer standards

The pyrimidine dimers were prepared using a modified procedure proposed originally by Wang [24]. That is, for the T–T dimer, 1 l of 2 mmol/l aqueous thymine solution was frozen and exposed to 254 nm UV light from two germicidal lamps for 30 min, the distance between the light and the solution for irradiation was kept at about 30 cm. Following irradiation, the frozen sample was thawed in a water bath at 40 °C. This 'freeze-irradiation-thaw' process was repeated five times. The solution was then concentrated to approximately 100 ml. A white precipitate was obtained which was recrystallized three times from boiling water, yielding a product with 99.8% purity as determined by HPLC described later.

The U–U and T–U dimers prepared in the same manner except that the initial reacting solution were 0.2 mmol uracil and 0.5 mmol thymine–0.5 mmol uracil, respectively.

2.2. Chromatography

2.2.1. Instrumentation and chemicals

A LC (LC-10AT, SHIMADZU Co.) with a variable wavelength UV-detector (mode SPD-10A) was used, the column used was Kromasil RP18 (15×0.46 cm^2, 5 μm). Data were acquired with the aid of a chromatographic workstation from Dalian Chemical–Physical Institute of China. Pure water was used as the mobile phase, and the flow-rate was 0.6 ml/min. The column effluent was monitored at 220 nm by a UV detector.

2.2.2. Preparation of working standard solution

A series of T–T standard solution with the concentration from 0.002 to 0.2 mg/ml were prepared by dissolving the purified T–T in water.

2.3. DNA irradiation and hydrolysis

2.3.1. Instrumentation and chemicals

A mode-locked Nd:YAG laser with single-pulse selection (Quantel YAG 501-10), a KDP crystal used to double frequency, a Joulemeter (Molectron J-25) to measure energy. A series of lens and prisms was from Shanghai Instrumental Factory.

Calf thymus DNA was obtained from Sigma Co. (St. Louis, MO), all other regents were AR grade.

2.3.2. Sample preparation

Twenty-five milligrams of calf thymus DNA was dissolved in 4 mmol Tris–HCl, 0.4 mmol EDTA buffer solution (pH 7.4) and diluted to 10 ml. Thirty microliters of the solution was irradiated for each test.

2.3.3. DNA irradiation and hydrolysis

Fig. 1 shows a schematic diagram of the experimental apparatus. Laser light (1.06 μm) from Nd:YAG laser was doubled in frequency by KDP crystal resulting in 15-ns-long full width, 532 nm pulse at 10 Hz. The beam was 0.5 cm in diameter after passing through a beam-reducing telescope. After a prism and a lens, the collimated beam was passed vertically through a 2-mm-diameter, 1.5-cm-long glass tube, which served as a sample holder. An absorption filter placed before the sample ensured that no UV irradiation reached the sample. The energy/pulse incident on the sample was measured with a Joulemeter. Control the laser amplifier to choose different intensity as designed.

The irradiated calf thymus DNA and 1 ml trifluoroacetic acid were combined in a glass vial which was then sealed to the air. The mixture was heated for 1 h at 160 °C, cooled, and dried under reduced pressure. The residue was dissolved in 0.2 ml water prior to applying to HPLC injector.

3. Results and discussions

3.1. Chromatography

The pure water was selected as the mobile phase with flow-rate 0.6 ml/min for this study. HPLC elution profiles are shown for T–T, T–U, U–U, U and T in Fig. 2. It seems to be able to keep excellent chromatographic behavior.

Since our study was focused on the production and determination of T–T, only the results for separation of T and T–T were introduced in the following text.

In addition, we chose 220 nm as the wavelength of UV detector to keep the higher sensitivity for both T and TT. It shows the good purity of T–T was obtained after three times of recrystallizing procedure, was performed. If fact, the purity of

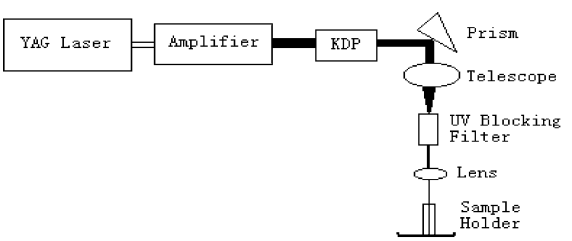

Fig. 1. Schematic diagram of the experimental setup for 532 nm irradiation of DNA.

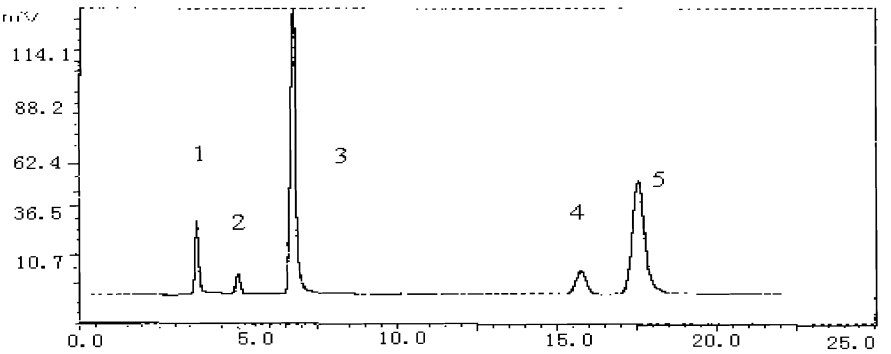

Fig. 2. Separation of pyrimidine dimers by HPLC. (1) U–U; (2) U–T; (3) U; (4) T–T; (5) T.

T–T prepared in our method can reach to 99.8% according to the results obtained by peak area normalization (Fig. 3).

3.2. System suitability

The system suitability tests were carried out using standard solutions of T–T and T. The experimental parameters containing theoretic plates numbers, tailing factor, resolution factor were tested (see Table 1).

The LOD was separately determined in both water and DNA blank for the purpose of comparison. It was observed that the detected lowest concentration is 1.35×10^{-5} mg/ml in pure water, and 4.42×10^{-5} mg/ml in hydrolyzed DNA blank for T–T. Therefore, the limit of detection at a signal-to-noise ratio of 3 for this method is about 2.7 ng in pure water and 8.8 ng in DNA, which is enough for general analysis. For the limit of quantitation at a signal-to-noise ratio of 10, about 0.13 ppm of concentration in DNA blank can be reached. It should be an enough low concentration for this application.

3.3. Recovery test

To evaluate the accuracy of the proposed method, recovery experiments were carried out using standard addition technique by adding a known amount of prepared T–T to a DNA blank solution. The amount of found T–T in recovery test was then determined by the HPLC method. The average recovery was 91.6% with R.S.D. 0.3%. The results and statistical parameters were reported in Table 2.

3.4. Precision test

Intermediate precision was evaluated by testing in different days; three levels of concentrations prepared by the same one stored T–T solution. The results in Table 3 show the good precision and stability of test solution.

3.5. Linearity

The plot of peak areas versus the respective concentration of T–T were found to be linear in the range 0.002–0.2 mg/ml (Table 4). They were presented by the linear regression equation $A = 519.4 + 5.218 \times 10^6 C$ ($r = 0.9996$).

3.6. Amount of T–T in DNA exposed to laser light

HPLC elution profiles are studied for hydrolysates of DNA exposed to laser intensity of three kinds of energy intensities (1.39×10^7, 2.30×10^7 and 3.30×10^7 W/cm^2 s) with different pulse number. The results are listed in Table 5. It lists the peak areas of T–T corresponding to different intensities and pulse number. The results are also shown in Fig. 4.

From Fig. 4, one may observe that as the pulse number increases, the peak area of T–T increases under lower intensity (1.39×10^7 W/cm^2 s) exposure. The experimental dots are almost placed on the straight lines with a slope of 1492 ($r = 0.96$).

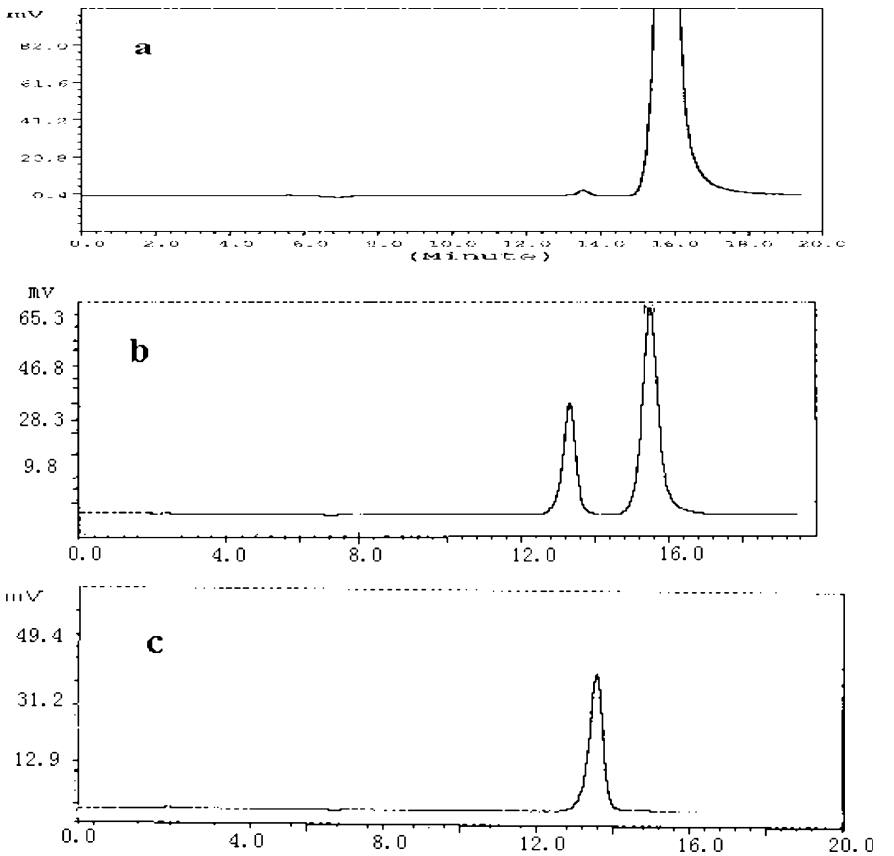

Fig. 3. Chromatography during recrystallizing procedure in preparation of T–T: (a) after the first recrystallizing procedure; (b) after the second recrystallizing procedure; (c) after the third recrystallizing procedure.

Under higher intensity (3.30×10^7 W/cm^2 s) exposure, as the pulse number increases, the peak area of T–T at first increases and then decreases drastically. The maximum is 10 360 corresponding to 2700 pulses. The reason can be explained by the kinetic balance of two reversed affect of laser-lesion and repair during the process of radiation. With small pulses, lesion dominates the reaction direction. At 3600 pulses, the effect of lesion and repair are approximately equal. When surpassing this balance spot, the effect of repair plays the major role.

While under the middle intensity (2.30×10^7 W/cm^2 s) exposure, the peak area of T–T increases with pulses increasing from 900 to 3600 by a slow rate, then by a fast rate with pulses increasing from 3600 to 5400. Among all experimental dots, the yield of T–T dimers with intensity and 2700 pulses is the largest.

Table 1
System suitability and detection limits in T–T and T determination ($n = 3$)

Parameters	T–T	T
Theoretic plates number	7157	6188
R.S.D. (%)	2.7	3.2
Tailing factor	0.98	1.11
R.S.D. (%)	0.02	0.01
Resolution factor	2.9	
LOD (in water)	1.35×10^{-5} mg/ml	
R.S.D. (%)	0.9	
LOD (in DNA blank)	4.42×10^{-5} mg/ml	
R.S.D. (%)	1.5	

Table 2
Recovery test of T–T determination

Amount added (mg)	Amount found (mg)	Recovery (%)	R.S.D. (%)
0.0203	0.0186	91.5	0.3
0.0203	0.0187	91.9	
0.0203	0.0186	91.5	

This result confirms the supposed point from Cao and Wang [14], i.e. there is non-linearity between laser intensity and biological photo damage. The reason may be supposed to come from the kinetic balance of two reversed affects of laser-lesion and repair during the process of radiation. With less pulses, lesion dominates the reaction direction. At some pulses, the effect of lesion and repair are approximately equal. When surpassing this balance spot, the effect of repair plays the major role.

4. Conclusion

This study proposed a HPLC method to estimate the relation between the yield of T–T dimer and the laser irradiation. The chromatographic parameters were studied and optimized. The results are considered to be good enough for a

Table 4
Calibration curve data of T–T determination

C (mg/ml)	Peak area (A)	Average area ($\bar{A}$)	R.S.D. (%)
0.00203	6.390×10^3 6.407×10^3 6.397×10^3	6.398×10^3	0.1
0.01015	4.657×10^4 4.568×10^4 4.582×10^4	4.602×10^4	1.0
0.05075	2.737×10^5 2.749×10^5 2.731×10^5	2.739×10^5	0.3
0.1015	5.341×10^5 5.338×10^5 5.344×10^5	5.341×10^5	0.1
0.15225	8.065×10^5 8.089×10^5 8.104×10^5	8.086×10^5	0.2
0.2030	1.038×10^6 1.058×10^6 1.042×10^6	1.046×10^6	1.0

Table 3
Evaluation test of precision

C (mg/ml)	Within days ($n=5$)		Between days ($n=5$)	
	Peak area (A)	R.S.D. (%)	Peak area (A)	R.S.D. (%)
0.00203	6.408×10^3 6.405×10^3 6.344×10^3 6.390×10^3 6.417×10^3	0.4	6.332×10^3 6.325×10^3 6.397×10^3 6.369×10^3 6.394×10^3	0.5
0.1015	5.603×10^5 5.594×10^5 5.563×10^5 5.497×10^5 5.475×10^5	0.9	5.519×10^5 5.341×10^5 5.344×10^5 5.338×10^5 5.524×10^5	1.6
0.2030	1.038×10^6 1.024×10^6 1.042×10^6 1.067×10^6 1.062×10^6	1.7	1.071×10^6 1.058×10^6 1.057×10^6 1.046×10^6 1.042×10^6	0.8

reasonable accuracy and precision in general requirement. The method is quite simple for the quantitative analysis of T–T dimer.

The physical parameters for laser irradiation were further tested. It is confirmed, the intensity of laser light and number of pulse are main factors to affect the yield of T–T dimer. The study should be helpful to know the mutagenic mechanism of laser irradiation in industrial microorganism field. The further research has been developing continually.

Acknowledgements

The authors are grateful to colleagues of China Pharmaceutical University for providing the technical assistances.

Table 5
Peak area ($A \times 10^{-3}$) of T–T in irradiated DNA under different intensities and pulse times

Intensity times	1.39×10^7 W/cm^2 s	2.30×10^7 W/cm^2 s	3.30×10^7 W/cm^2 s
900	0	1.174	2.523
	0	1.172	2.460
	0	1.186	2.559
Mean	0	1.177	2.514
R.S.D. (%)	0	0.5	1.6
1800	1.084	4.572	6.075
	1.030	4.734	6.216
	1.030	4.654	5.986
Mean	1.048	4.653	6.092
R.S.D. (%)	2.4	1.4	1.6
2700	2.730	6.351	10.46
	2.707	6.359	10.39
	2.751	6.348	10.24
Mean	2.729	6.353	10.36
R.S.D. (%)	0.7	0.1	0.9
3600	3.951	8.450	5.085
	3.615	8.473	5.387
	3.974	8.415	5.380
Mean	3.874	8.466	5.284
R.S.D. (%)	4.3	0.3	2.7
4500	4.752	19.26	4.975
	5.177	19.29	4.922
	4.752	19.26	5.027
Mean	4.885	19.24	4.975
R.S.D. (%)	4.1	0.3	0.9
5400	6.887	27.36	4.889
	6.851	27.66	5.061
	6.877	27.61	5.031
Mean	6.872	27.54	4.994
R.S.D. (%)	0.3	0.5	1.5

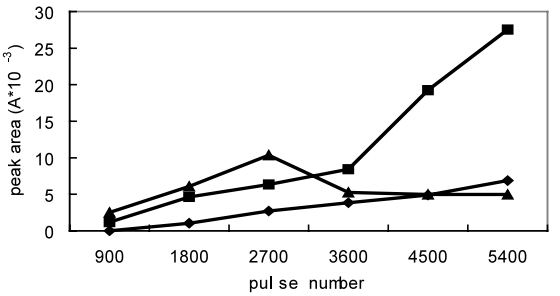

Fig. 4. The peak area of T–T measured by HPLC versus the number of laser pulses under different intensity conditions.

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