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Chemiluminescent detection of thymine hydroperoxides by high-performance liquid chromatography☆

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ABSTRACT

High-performance liquid chromatography (HPLC) with chemiluminescence detection was used to detect thymine hydroperoxides, which are expected to occur in biological tissues as primary products in free radical-mediated DNA damage. The thymine hydroperoxides were chemically prepared by the acid-catalysed hydrogen peroxide oxidation of thymine and 5-hydroxymethyluracil and their hydroperoxide products were identified as *trans*- or *cis*-5-chloro-6-hydroperoxy-5,6-dihydrothymine and 5-hydroperoxymethyluracil, respectively, by means of NMR, IR and mass spectrometry. The results confirmed that these thymine hydroperoxides are specifically detectable at picomole levels by the described HPLC method with high reproducibility.

INTRODUCTION

It is well known that ionizing radiation or chemical oxidation causes DNA damage [1–7], and thus results in strand breakage in cellular DNA and modification of DNA bases. Among the DNA bases, thymine is the most susceptible to these modifying effects [8], and the detection and characterization of the modified thymine may be of considerable importance in studying the mechanism of DNA damage.

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Ionizing and near-ultraviolet irradiation of DNA causes modification of thymine with the formation of saturated ring compounds [1,8–12]. The *cis*- or *trans*-5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) was found to be formed in aqueous thymine medium by permanganate oxidation [13,14] and irradiation [15,16], and also formed in DNA by the action of ionizing and near-ultraviolet irradiation [9] and by chemical oxidation with potassium permanganate [1,8,17] and osmium tetraoxide [9]. These compounds are suggested to be removed from irradiated cellular DNA in repairing processes and yielded from the oxidized DNA by endonucleases. It has been reported that *Escherichia coli* endonuclease III contains N-glycosylase activity which can release oxidized thymine [11,18–22].

On the other hand, irradiation of DNA solutions

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in the presence of oxygen was shown to lead to the formation of stable hydroperoxides on the pyrimidine base moieties of the nucleic acid molecule [23–25] and the formation of several thymine hydroperoxides by irradiation or chemical oxidation has been suggested (Fig. 1). It is known that 5- or 6-hydroperoxy-6- or -5-hydroxy-5,6-dihydrothymine (Fig. 1a–d) are formed by X-irradiation, gamma-irradiation [26–30], photo-excitation at 1849 Å [16] and chemical oxidation with OsO_4 in aerated aqueous solutions of nucleic acids, pyrimidine nucleotides and pyrimidine bases [31,32], and these are also known to have mutagenic activity [33,34]. 5-Hydroperoxymethyluracil (Fig. 1e) was formed from 5-hydroxymethyluracil by acid-catalysed oxidation with H_2O_2–HCl [35] and also has mutagenic activity [34]. 5,6-Dihydroperoxydihydrothymine (Fig. 1f) was formed from 6-hydroxy-5-bromothymine by chemical oxidation with H_2O_2–H^+–Ag_2O [32].

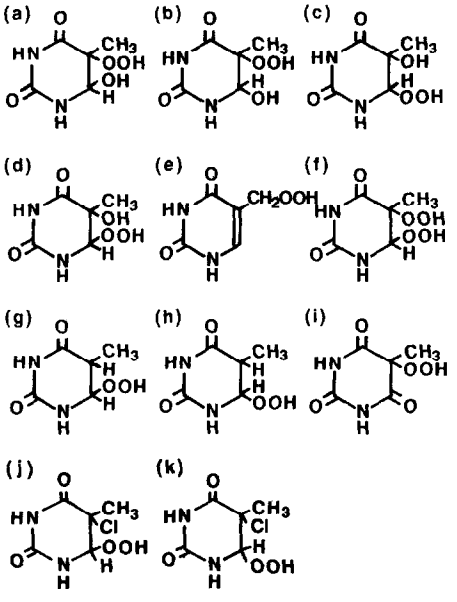

Fig. 1. Structures of thymine hydroperoxides. a = *trans*-5-hydroperoxy-6-hydroxy-5,6-dihydrothymine; b = *cis*-5-hydroperoxy-6-hydroxy-5,6-dihydrothymine; c = *cis*-6-hydroperoxy-5-hydroxy-5,6-dihydrothymine; d = *trans*-6-hydroperoxy-5-hydroxy-5,6-dihydrothymine; e = 5-hydroperoxymethyluracil; f = *trans*-5,6-dihydroperoxy-5,6-dihydrothymine; g = *cis*-6-hydroperoxy-5,6-dihydrothymine; h = *trans*-6-hydroperoxy-5,6-dihydrothymine; i = 5-hydroperoxy-5-methylbarbituric acid; j = *trans*-5-chloro-6-hydroperoxy-5,6-dihydrothymine; k = *cis*-5-chloro-6-hydroperoxy-5,6-dihydrothymine.

6-Hydroperoxy-5,6-dihydrothymine (Fig. 1g and h) and 5-hydroperoxy-5-methylbarbituric acid (Fig. 1i) were also formed from 6-hydroxy-5-bromothymine by H_2O_2–H^+–Zn and H_2O_2–H_2O–Ag_2O–Br_2 oxidation, respectively [32]. *trans*-5-Chloro-6-hydroperoxy-5,6-dihydrothymine (Fig. 1j) was formed from thymine by acid-catalysed oxidation with H_2O_2–HCl [36].

Thin-layer chromatography, paper chromatography, gas chromatography–mass spectrometry with selected-ion monitoring [5–7] and high-performance liquid chromatography (HPLC) with UV detection have been used for the detection of thymine glycols and hydroperoxides. However, the hydroperoxide group-specific determination of thymine hydroperoxide has not been accomplished. Chromatographic determination of thymine hydroperoxides as primary intermediates in the oxidation reaction would be the most direct and useful approach in the study of DNA damage, in both *in vitro* and *in vivo* systems.

In this study, we used chemiluminescence (CL) detection for the hydroperoxide group-specific determination of thymine hydroperoxides by HPLC. The chemiluminescent nature of thymine hydroperoxides is not generally known. The CL assay based on detecting photon emission in the oxidation of luminol during the interaction of hydroperoxides and cytochrome *c*-haeme has high sensitivity and specificity for determining hydroperoxide groups [37,38].

EXPERIMENTAL

Oxidation of thymine

Ten milligrams of thymine (Wako, Osaka, Japan) were dissolved in 1.5 ml of 30% hydrogen peroxide containing 25 μl of concentrated hydrochloric acid and oxidized for 96 h at room temperature [35,36]. After oxidation, the reaction mixture was diluted twentyfold with distilled water and a 10-μl portion was injected directly into the HPLC system.

Luminescence reagent

The post-column luminescence reagent for the assay of hydroperoxide groups was prepared by dissolving 10 μg/ml of cytochrome *c* (from horse heart, type IV) (Sigma, St. Louis, MO, USA) and 1 μg/ml of luminol (3-aminophthaloyl hydrazine) (Wako) in

50 m*M* borate buffer (pH 9.3), as described by Miyazawa *et al.* [37].

Instruments and chromatographic conditions

A schematic diagram of the HPLC system used for determining thymine hydroperoxides is shown in Fig. 2. It consisted of a JASCO HPLC instrument and reversed-phase column (Finepak SIL C_{18}, 10 μm, 250 × 4.6 mm I.D.) (Japan Spectroscopic, Tokyo, Japan). The flow-rate of the mobile phase (distilled water) was 1.0 ml/min with a JASCO 880-PU pump. The column eluate was introduced into a JASCO 875-UV detector set at 210 nm, and the eluate was then mixed with the luminescence reagent at a mixing joint attached close to the entrance to the flow cell of the CL detector. The CL generated by the reaction of hydroperoxide and the luminescence reagent was monitored with a CLD-100 single-photon counting CL detector (Tohoku Electronic, Sendai, Japan) equipped with a PTFE flow cell (200 μl) [38]. The chromatograms detected by

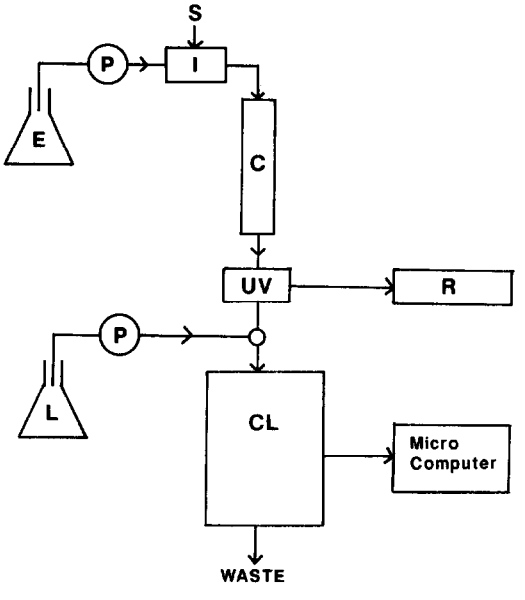

Fig. 2. Schematic diagram of HPLC system. P = Pump (JASCO 880-PU); I = injection valve (Rheodyne 7125); C = column (JASCO Finepak SIL C_{18} or Megapak SIL C_{18}); UV = UV detector (JASCO 875-UV); CL = chemiluminescence detector (Tohoku Electronic, CLD-100); R = recorder (SIC Chromatocoder 12); E = eluate (distilled water, flow-rate 1.0 ml/min); L = luminescence reagent containing cytochrome *c* and luminol in borate buffer (pH 9.3, flow-rate 0.5 ml/min).

CL were recorded by a microcomputer and saved on a diskette. The chromatograms detected at 210 nm were recorded with a SIC Chromatocoder 12.

Structural characterization of thymine hydroperoxides

The experiments were carried out on a larger scale in order to isolate the oxidation products by using a reversed-phase column (Megapak SIL C_{18}, 10 μm, 250 × 7.5 mm I.D.) (JASCO). The isolated fractions were then lyophilized with an EYELA FD-1 lyophilizer (Tokyo Rikakikai, Tokyo, Japan). NMR spectra were determined in tetramethylsilane (TMS) as an internal standard by using a JEOL JNM-FX100 instrument. Infrared analyses were performed on KBr pellets with a JASCO IR Report-100 instrument. Mass spectra were recorded with a JEOL JMS-DX303 mass spectrometer in the fast atom bombardment mode.

Preparation of 5-hydroperoxymethyl uracil

A 114-mg amount of 5-hydroxymethyluracil (Sigma) was dissolved in 20 ml of 15% hydrogen peroxide, then 100 μl of concentrated hydrochloric acid in 10 ml of hydrogen peroxide were added dropwise. After standing at room temperature for 48 h with stirring, the reaction mixture was lyophilized. The residue was washed with cold water and the purified product, 5-hydroperoxymethyluracil, was obtained by recrystallization from 10% methanol solution [35].

RESULTS AND DISCUSSION

Fig. 3 shows a typical HPLC pattern of the H_2O_2–HCl oxidation products of thymine. Oxidized thymine gave two sharp CL peaks, peak 1 (9 min) and peak 2 (21 min), with good reproducibility together with a CL H_2O_2 peak. These two CL peaks were completely eliminated by $NaBH_4$ reduction (OOH → OH), indicating that both the peak 1 and 2 compounds possess a hydroperoxide group in their thymine molecules [38].

Our structural assignment of the peak 1 and 2 compounds yielded by H_2O_2–HCl oxidation of thymine was further corroborated by the spectral data after their isolation. Fig. 4 shows the time-dependent change in the UV spectra of the oxidized thymine reaction mixture. The time-dependent de-

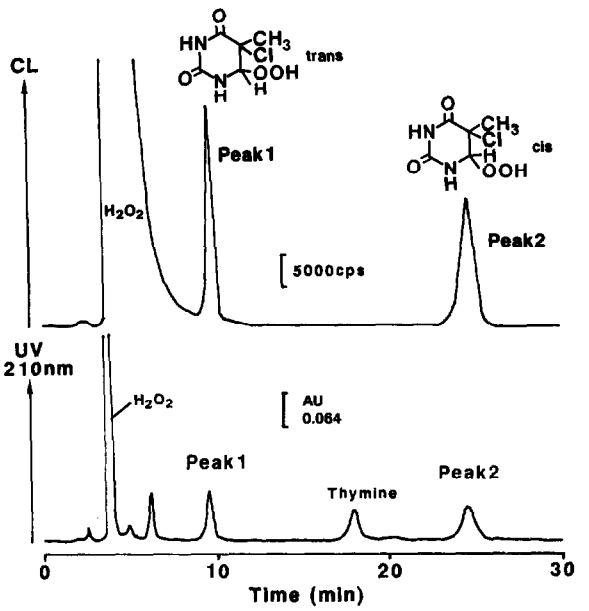

Fig. 3. HPLC of thymine oxidized by H_2O_2–HCl.

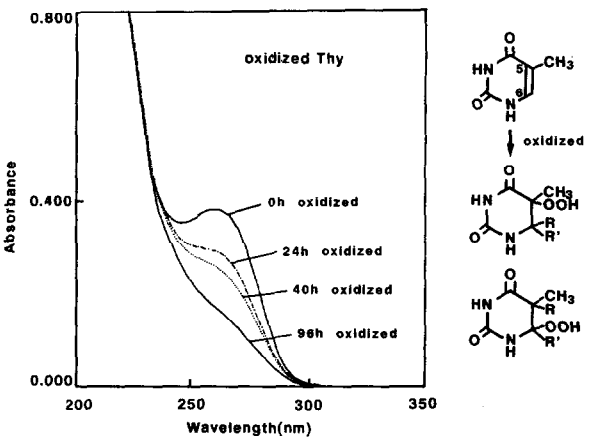

Fig. 4. Changes in UV absorption spectra of thymine (Thy) during H_2O_2–HCl oxidation.

crease in absorption at 265 nm suggests that thymine is oxidized and changed to a ring-saturated compound.

Fig. 5 shows the NMR spectra of the peak 1 and peak 2 compounds in $(CD_3)_2SO$ at 100 MHz with

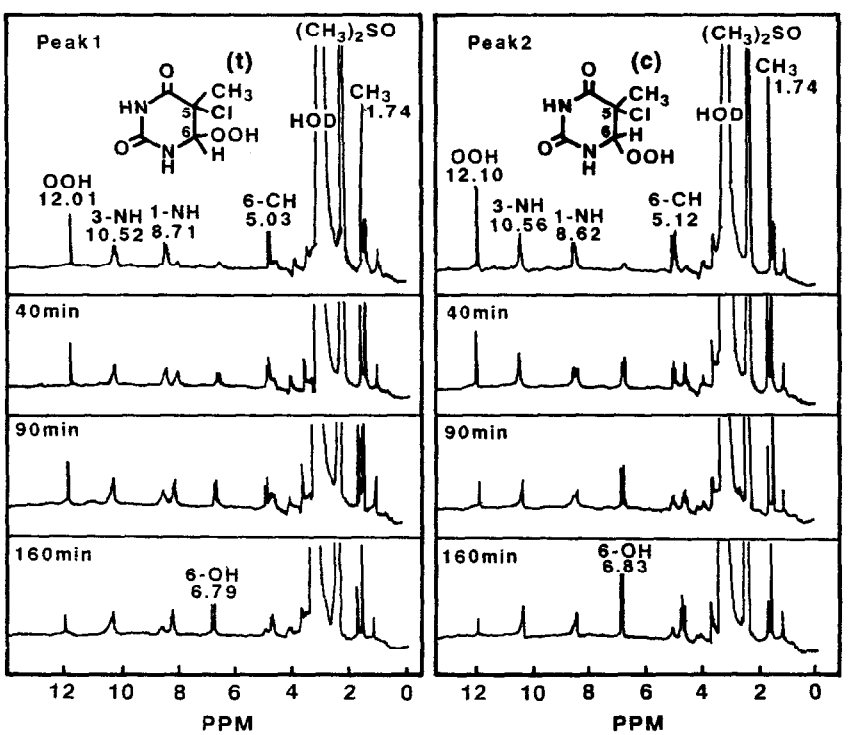

Fig. 5. Sequential changes in NMR signals of *trans*- and *cis*-5-chloro-6-hydroperoxy-5,6-dihydrothymine.

TMS as an internal standard. Peak 1 compound has signals for CH_3 (1.74), C(6)H (4.97), C(6)OH (6.83), N(1)H (8.70), N(3)H (10.51) and OOH (12.00 ppm). Peak 2 compound has signals for CH_3 (1.73), C(6)H (5.06), C(6)OH (6.82), N(1)H (8.62), N(3)H (10.55) and OOH (12.09 ppm). As time progressed, the signals for OOH decreased and the signals for C(6)OH increased. No signals for C(5)OH were present.

In the IR spectra, peak 1 compound had bands for NH (3400), νOH (3300), νCH_3 (3100), $\nu C{=}O$ (1730, 1685), νCH_3 (1480) and $\nu C{-}O$ (1115, 1100, 1065 cm^{-1}), and peak 2 compound had bands for NH (3400), νOH (3300), νCH_3 (3100), $\nu C{=}O$ (1720), νCH_3 (1480, 1385) and $\nu C{-}O$ (1130, 1070 cm^{-1}). A peak at m/z 195 corresponding to $[M+H]^+$, the molecular ion of 5-chloro-6-hydroperoxy-5, 6-dihydrothymine, was confirmed in the mass spectra of both peak 1 and 2 components.

From a comparison of these data with previous findings reported for the spectral characteristics of thymine peroxides [28,36], it is concluded that both the peak 1 and 2 compounds were 5-chloro-6-hydroperoxy-5,6-dihydrothymine, and that the difference between the spectra was solely dependent on their geometrical isomerism. Structurally, *trans* compounds must have a multiple signal for C(6)H and *cis* compounds have a triplet signal for C(6)H [28] in NMR spectra. Peak 1 showed multiple signal patterns for C(6)H and peak 2 showed triplet signals (Fig. 6). From these results, it is concluded that the peak 1 and 2 compounds were *trans*- and *cis*-6-hydroperoxy-5,6-dihydrothymine, respectively.

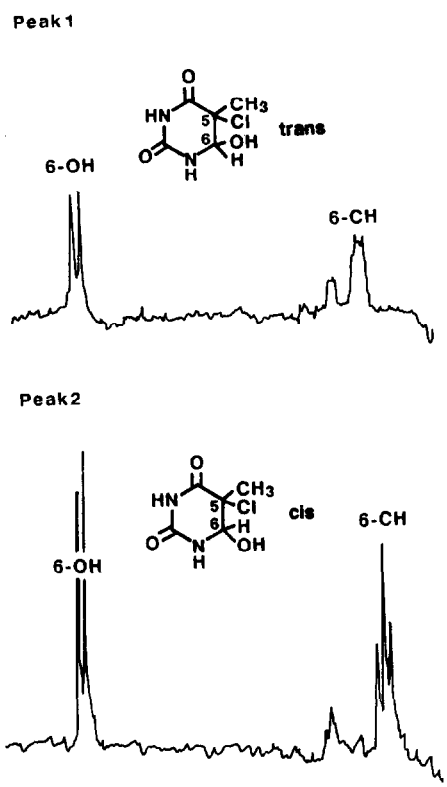

Fig. 6. Expanded NMR signals for 6-CH of reduced derivative of peak 1 and 2 components.

In previous paper [36], it was reported that only a *trans*-compound (Fig. 1j) was produced by the oxidation of thymine with H_2O_2–HCl. However, from the present results, it was confirmed that the *cis* compound (Fig. 1k) was also produced in this oxidation system.

The calibration lines for *trans*- and *cis*-5-chloro-6-hydroperoxy-5,6-dihydrothymine showed that the CL counts integrated for the peak area corresponding to thymine hydroperoxides after subtraction of the background counts were proportional to the amount present at least in the range 30–500 pmol that was examined.

Fig. 7 shows the HPLC pattern of 5-hydroperoxymethyluracil that was prepared by acid-catalised oxidation with H_2O_2–HCl [35]. A single, sharp CL peak was detected with good reproducibility and was also eliminated after $NaBH_4$ reduction (OOH $\rightarrow$ OH).

The calibration line for 5-hydroperoxymethyluracil (HPMU) showed that the CL counts integrated for the peak area were proportional to the amount present at least in the range 50–250 pmol that was examined.

The present results indicate that thymine hydroperoxides, such as *trans*- and *cis*-5-chloro-6-hydroperoxy-5,6-dihydrothymine and 5-hydroperoxymethyluracil, could be hydroperoxide-specifically determined by the proposed HPLC method. High sensitivity, specificity and reproducibility were confirmed.

HPLC incorporating the luminol reaction has attracted particular interest recently for the detection of trace amounts of biological hydroperoxidic constituents [37–43]. For example, biological lipid hydroperoxides have been determined by this method

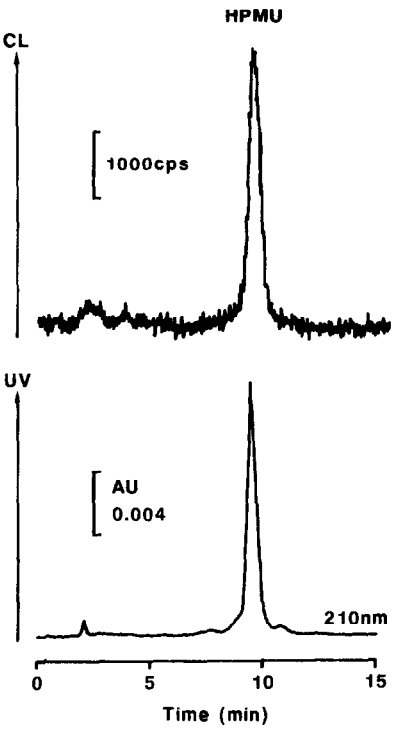

Fig. 7. HPLC of isolated 5-hydroperoxymethyluracil (HPMU).

[38–43]. The present results show that this method is also useful for detecting nucleic acid–base hydroperoxides.

The thymine hydroperoxides that we prepared at low pH were fairly stable under physiological conditions. On preparing thymine hydroperoxide, oxygen and nitrogen did not gave any effect on the hydroperoxide yields, and the yield was solely dependent on the H_2O_2 concentration. Under the present conditions for preparing thymine hydroperoxides, the product possessing a hydroperoxide group in the molecule was only thymine hydroperoxide. Several very minor products were observed with UV detection, but they were not chemiluminescent, thus indicating they were not hydroperoxides.

Thymine hydroperoxides formed by irradiation and chemical oxidation have been characterized in many *in vitro* systems (Fig. 1) and also detected from damaged DNA by irradiation or chemical oxidation [1–12]. The oxidation of denatured DNA with permanganate under certain mild conditions does not result in the selective degradation of its thymine residues [8,13]. Thymine glycol formation in DNA by several oxidizing agents and ionizing and near-UV radiation has also been detected by using radiometric analysis [1,9]. Excision of thymine glycol and thymidine glycols by DNA repair enzymes and their excretion into urine have also been performed by radiometric analysis [44]. However, the hydroperoxide derivatives were not detected especially in biological samples, because of the lack of a suitable method.

The use of sensitive CLD-100 and CLD-110 CL detectors (Tohoku Electronic) that have been improved by Miyazawa's group [38] enables the sensitivity to be increased further down to femtomole levels of hydroperoxides. Thymine hydroperoxide determination would be very important useful for establishing how damage occurs to DNA. Further investigations on the formation of these compounds in various biological systems are needed.

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