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Note

Analysis of thymidine hydroperoxides by post-column reaction high-performance liquid chromatography

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Hydroperoxides are common products or intermediates of oxidative processes[1]. Thymidine hydroperoxides result from the action of hydroxyl radicals on DNA in oxygenated solutions[2,3]. In fact, initially formed hydroperoxides are estimated to make up about 70% of the total DNA base damage induced by the action of ionizing radiation on DNA in aqueous solution[4]. Since exposure of 2′-deoxycytidine, 2′-deoxyguanosine or 2′-deoxyadenosine to ionizing radiation in aqueous oxygenated solutions does not yield detectable amounts of hydroperoxides, the majority of DNA hydroperoxides is expected to be made up of thymidine hydroperoxides. Similar pathways involving hydroxyl radicals and the formation of thymidine hydroperoxides can also be implicated in the action of therapeutic drugs[5] and in the process of aging[6].

We report the complete separation of the eight possible 5(6)-hydroxy-6(5)-hydroperoxides of thymidine by reversed-phase high-performance liquid chromatography (HPLC). The hydroperoxides are selectively detected by mixing the HPLC eluent with Fe^{2+}–xylenol orange reagent. This system is easy to assemble, uses conventional HPLC equipment and reaches pmol levels of detection. Previously, this method was reported in a qualitative study of several hydroperoxides formed from $OH^{\cdot}$ induced oxidation and one-electron oxidation of thymidine[7]. In this article, emphasis has been placed on the methodology and quantitative aspects of the post-column reaction for the detection of hydroperoxides.

EXPERIMENTAL

Chemicals

Thymidine and the reagents for post-column reaction were purchased from Sigma and used as received. Solvents were of the highest purity available from

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Anachimia (Montreal, Canada). Water in all experiments was distilled twice in quartz. [^{14}C(2)]Thymidine was obtained from Amersham and was purified immediately before use by HPLC. Thymidine hydroperoxides were obtained by two methods. The four diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine were obtained from peroxidation of thymidine using trifluoroperacetic acid[8]. The four 6-hydroxy-5-hydroperoxy-5,6-dihydrothymidine diastereomers and 5-hydroperoxymethyl-2'-deoxyuridine were prepared by near-ultraviolet photolysis of thymidine solutions in the presence of 2-methyl-1,4-naphthoquinone[9]. The stereochemistry of each hydroperoxide was determined by comparing the corresponding 5,6-diol of thymidine, obtained after treatment of the hydroperoxide with zinc powder in 3% aqueous acetic acid, to authentic reference samples and assigned structures were confirmed by ^{1}H NMR and ^{13}C NMR[10].

Chromatography

Thin-layer chromatography (TLC) was carried out on SIL-G UV 0.25 mm thick silica plates (Machery-Nagel, Düren, FRG) by two-dimensional analysis, first the plates were developed in solvent I [chloroform–methanol–water (4:2:1, v/v/v); methanol was added (5%, v/v) to the organic phase] and then after drying, at right angles in solvent II [methyl propionate–2-propanol–water (75:16:9, v/v/v)][11]. Hydroperoxides were detected directly on the TLC plates by spraying with N,N-dimethyl-1,4-phenylenediamine in 50% aqueous methanol[12]. R_F values are reported relative to those of thymidine which migrates with an absolute R_F of 0.67 in both solvent systems. HPLC equipment consisted of a dual piston pump (Waters No. M6000; Mississauga, Canada), a fixed-volume injector (Rheodyne; Berkeley, CA, U.S.A.), a fixed-wavelength detector (Waters No. 441) and an analog–digital integrator (Varian No. 4270; Georgetown, Canada). Two C_{18} reversed-phase columns were used: a semi-preparative column, 25 cm × 7.5 mm I.D., packed with 5-μm Spherisorb (Chrom. Specialities, Brockville, Canada) and an analytical column, 25 cm × 4.6 mm I.D., packed with 5-μm Ultrasphere (Beckman; Berkeley, CA, U.S.A.). Water (pH $\simeq$ 6) was used as the mobile phase for both columns.

Post-column reaction

The basic flow system described above was also used in the post-column reaction HPLC system to detect thymidine hydroperoxides, except that a second pump was used to mix a peroxide reagent into the first HPLC line (Fig. 1). In order to assure a stable baseline, it was essential that the pumps were equiped with pulse dampeners. All compounds, including hydroperoxides, were detected prior to the post-column reaction by an UV absorption detector set at 229 nm. The eluent and reagent were mixed in a right angle tee before entering the reactor coil. The reactor coil consisted of 6 m × 0.52 mm I.D. stainless-steel tubing, shaped in 80 coils of 2 cm diameter submerged in a water bath thermostated at 60°C. An additional 2 m of tubing shaped in 20 coils was submerged in a water bath kept at room temperature. Absorbance of the eluent-reagent mixture was measured at 546 nm. The peroxide reagent consisted of $5.8 \cdot 10^{-4}$ *M* xylenol orange (tetrasodium salt) and $2.3 \cdot 10^{-4}$ *M* ammonium ferrous sulfate in 0.035 *M* H_2SO_4. The final concentration of the reactants after mixing with the HPLC eluent is identical to that used in the conventional colorimetric test for hydroperoxides[13]. The post-columns reactions are presented by eqns. 1 and 2.

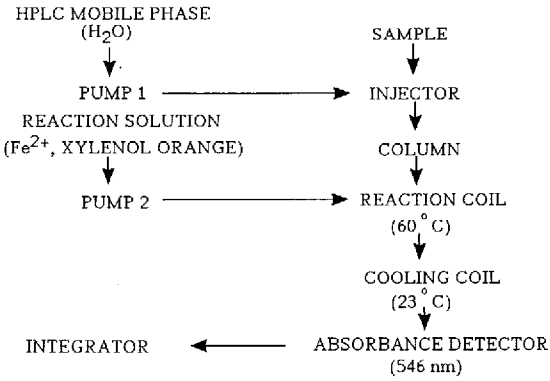


Fig. 1. Flow diagram of post-column reaction HPLC for the detection of thymidine hydroperoxides.

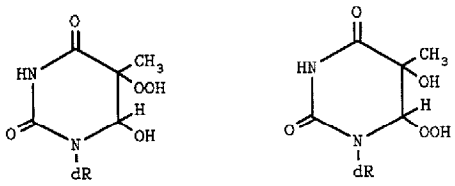
$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + ROH \quad (1)$$

$$Fe^{3+} + \text{xylenol orange} \rightarrow Fe^{3+}\text{–xylenol orange} \quad (2)$$
$$(\varepsilon_{540nm} = 26\,800\ M^{-1}cm^{-1})$$

(where ROOH is hydroperoxide). Under our experimental conditions, the background absorption measured at 546 mm was 0.08 absorbance units with water as a reference. The flow-rates provided by the primary and reagent pumps were adjusted to 0.7 and 1.5 ml/min respectively which gave reasonable head pressures of 28 and 86 atm. This flow-rate combination gave the best response. Lowering the flow-rate of the reagent pump, while keeping the final concentration of reagent the same, resulted in greater baseline fluctuations likely due to poor mixing. At these flow-rates, the reagent and eluent are in contact for less than 1 min. The reaction was considered to be complete at this time since neither increasing the temperature of the bath nor diminishing the flow-rate gave higher signals for the hydroperoxides. The reaction time is considerable faster than the 10 min required in the standard colorimetric test at room temperature[14]. The response of the detector was found to be linear with the amount of hydroperoxide injected from 100 nmol, at which point the detector is saturated, down to 10 pmol. A signal representing 10 pmol is about 20 times greater than the background absorption. Comparison of the bandwidths of eluting compounds using the 229 nm UV detector *versus* the 546 nm post-column reaction detector indicates that the reaction coil introduces about 20% diffusion of the elution peaks. This diffusion was independent of the injection volume over the range of 20–100 μl.

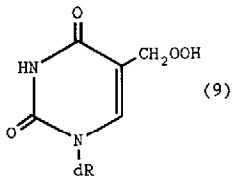
ANALYSIS OF THYMIDINE HYDROPEROXIDES

There are eight possible isomers of 5(6)-hydroxy-6(5)-hydroperoxides of thymidine (Fig. 2). They are not completely resolved by two-dimensional TLC on silica gel (Table I) and the diastereomers **3** and **4** are indistinguishable under the conditions used. Furthermore, the quantitation of thymidine hydroperoxides by TLC is difficult because they tail and partly decompose under these conditions. The R_F values were



(1) *trans*(5S,6S)
(2) *trans*(5R,6R)
(7) *cis*(5R,6S)
(8) *cis*(5S,6R)

(3) *trans*(5R,6R)
(4) *trans*(5S,6S)
(5) *cis*(5S,6R)
(6) *cis*(5R,6S)



(9)

Fig. 2. Structures of thymidine hydroperoxides. dR = 2-β-D-*erythro*-Pentofuranosyl moiety.

obtained from analysis of pure hydroperoxides and agree with previous analysis of these compounds from complex mixtures of the hydroxyl radical-induced thymidine oxidation products[2,10]. All eight isomeric 5(6)-hydroxy-6(5)-hydroperoxides of thymidine are resolved by reversed-phase HPLC (Table I and Fig. 3). Unlike TLC

TABLE I

CHROMATOGRAPHIC PROPERTIES OF THE NINE HYDROPEROXIDES OF THYMIDINE

Thymidine Hydroperoxide	*R_F TLC*[a]		*HPLC*[b] *retention volume (ml)*	*Ratio*[c] *of absorbance 546 nm/229 nm*	*Ratio*[d] *of pmol of hydroperoxide to absorbance at 546 nm*
	solvent I	*II*			
1	0.46	1.15	6.7	3.4	4.5
2	0.50	1.30	8.0	3.1	3.9
3	0.48	1.12	8.8	4.3	–
4	0.48	1.12	9.0	4.3	–
5	0.43	0.77	9.9	7.1	3.7
6	0.43	0.90	13.1	7.7	2.9
7	0.41	0.73	16.1	6.3	3.6
8	0.48	0.78	16.9	5.6	3.8
9	0.56	0.99	10.7[e]	2.9	–

[a] Two dimensional TLC in system I (organic phase of chloroform–methanol–water (4:2:1, v/v/v), to which was added 5% methanol (v/v) and system II methyl propionate–2-propanol–water (75:16:9, v/v/v). R_F values are relative to thymidine.
[b] Analytical reversed-phase column (void volume, 2.2 ml) with water as the mobile phase.
[c] UV detection of hydroperoxides was made before the post-column reaction.
[d] Factor used to convert the integrated 546 nm signal to the quantity of hydroperoxide injected on the HPLC column; 1 corresponds to an absorbance of 0.0002 at the peak maximum at 546 nm.
[e] Mobile phase: 5% aqueous methanol.

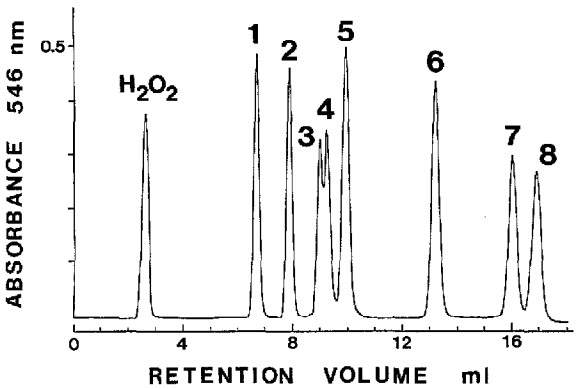


Fig. 3. Analysis of a mixture consisting of 500 pmol each of the eight 5(6)-hydroxy-6(5)-hydroperoxides of thymidine by reversed-phase post-column reaction HPLC. The hydroperoxides were separated on a 5-μm C_{18} column operated with water as the mobile phase. The post-column reaction consisted of mixing the eluent with $5.8 \cdot 10^{-4}$ *M* xylenol orange and $2.3 \cdot 10^{-4}$ *M* NH_4FeSO_4 in 0.035 *M* H_2SO_4 and monitoring the absorption of the Fe^{3+}–xylenol orange complex at 546 nm.

analysis, there is very little tailing of the peaks or decomposition during HPLC analysis and baseline resolution is observed for all hydroperoxides except for **3** and **4**. The isomeric *cis*-hydroperoxides are longer retained on reversed-phase chromatographic columns than the corresponding *trans*-hydroperoxides. This likely reflects intramolecular H-bonding between the vicinal polar hydroxy and hydroperoxy groups of the *cis* isomers. A similar effect has been observed between *cis*- and *trans*-thymidine 5,6-diols[15]. Also, the **6***R* elute faster than the **6***S* diastereomers of 5-hydroxy-6-hydroperoxides whereas the reverse is true for 6-hydroxy-5-hydroperoxides. These differences are likewise attributed to the different orientation of the hydroxy and hydroperoxy groups and to their ability to form intramolecular bonds. In contrast to the 5,6-saturated thymidine hydroperoxides (**1**–**8**), hydroperoxymethyl-2′-deoxyuridine (**9**) is retained much longer during reversed-phase chromatography.

The ratio of absorption at 546 nm (peroxide reagent) to that at 229 nm (direct absorption) varies from 3–8 for the different hydroperoxides (Table I). These variations do not reflect differences in the absorbance at 546 nm induced per mole of hydroperoxide, which appears to be constant for all the hydroperoxides. Instead, this results from variations in absorption by the hydroperoxides at 229 nm, which is an inherent property of these compounds. Thus, *trans*-isomers absorb about twice as much light at 229 nm as *cis*-isomers.

Post-column reaction HPLC using xylenol orange–Fe^{2+} as a colorimetric reagent for hydroperoxides provides a simple and reliable method to selectively detect and quantitate thymidine hydroperoxides in the rang of 1–10 pmol. The sensitivity of this method is comparable to that involving an enzyme-mediated chemiluminescence assay used for the detection of lipid hydroperoxides[16].

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