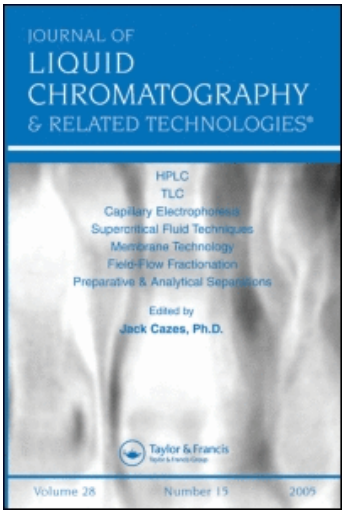

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Y. N. Vaishnav[a]; L. -S. Kan[b]; C. E. Swenberg[b]
[a] Radiation Biochemistry Department, Armed Forces Radiobiology Research Institute, Bethesda, Maryland [b] Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR MONITORING KINETICS OF INTERCONVERSIONS OF STEREOISOMERS OF THYMIDINE GLYCOL

Y. N. VAISHNAV[1], L. -S. KAN[2], AND C. E. SWENBERG[1]

[1]*Radiation Biochemistry Department*
Armed Forces Radiobiology Research Institute
Bethesda, Maryland 20889-5145
[2]*Department of Biochemistry*
School of Hygiene and Public Health
The Johns Hopkins University
Baltimore, Maryland 21205

ABSTRACT

A reverse-phase high-performance liquid chromatography methodology was validated for rapid, sensitive and simultaneous analysis of all stereoisomers of thymidine glycol. The procedure involved direct injection of the samples on a microbore C-18 reverse-phase column with ultraviolet detection at 220 nm wavelength. The lower limits of detection for all thymidine glycol stereoisomers were close to 2.5 pmole under optimized conditions of detection and were linear up to at least 5000 pmole. The mobile phase consisted of 3% acetonitrile in water (v/v). The procedure allowed qualitative as well as quantitative measurements of pH and temperature -dependent interconversions of isomers of thymidine glycol directly (underivatized) from aqueous solution.

INTRODUCTION

Certain chemicals and ionizing radiation induce formation of a wide variety of DNA lesions, including strand breaks, modified base residues, crosslinkings, and abasic sites (1,2). One of the major forms of DNA damage caused by radiation and chemicals is modification of the thymine base to form ringsaturated derivatives. The nucleobase thymine in DNA may convert either to **cis-** or **trans**-5,6dihydro-5,6-dihydroxy -thymidine, otherwise known as thymidine glycol (TG). Formation of TG lesion is known to cause distortion in DNA local conformation, disrupting base pairing with complementary nucleic acid strands (1,3), which can affect normal functioning of polymerases. A TG lesion has been shown to strongly inhibit DNA elongation **in vitro**, and the lethal effects of putative TG in M13mp8-phage DNA have been determined (3). It has been demonstrated experimentally that modified thymines are removed from cellular DNA by active enzymatic repair processes (4,5). Support for this hypothesis stems from reports that DNA exposed to ionizing radiation **in vitro** was attacked by DNA endonuclease activities that did not attack DNA (6,7,8). It is possible that some but not all of the ring-saturated thymines are substrates for the enzymatic initiation of DNA excision repair. In spite of extensive studies, the identity of the particular Modeling studies do however suggest that the active isomer is (-)-**cis-5R,6S-** (9). The pioneering studies of Cadet and others (10-13) on isomerization of TG, and other hydrates of pyrimidine derivatives suggest that, under specific conditions, this class of compounds do isomerize and, furthermore, different isomers are likely to have different chemical and

biological activities. In this paper we report the complete and rapid resolution of the four stereoisomers of TG simultaneously from an isomeric mixture by reverse-phase high-performance liquid chromatography (HPLC). This system is relatively easy to assemble, requires a conventional HPLC instrument, and reaches pmole levels of sensitivities. In particular, emphasis has been placed on using this methodology to study pH and temperature-dependent interconversions of TG isomers.

MATERIALS AND METHODS

Materials:

Thymidine and osmium tetroxide were obtained from Chemical Corps. (S. Plainfield, NJ) and Colonial Metals, Inc. (Elkton, MD), respectively. Potassium chlorate was purchased from Mallinkodt, Inc. (St. Louis, MO), and all solvents used were acquired from Fisher Co. (Pittsburg, PA) and used without purification.

Method:

The synthesis of TG by osmium tetroxide methodology and full characterization of all stereoisomers of TG have been reported (2). However, for the quantitative analysis of stereoisomers of TG, the HPLC system was modified for rapid and baseline resolution of the components of the isomeric mixture. Hypersil ODS (250mm x 10mm) semipreparatory column was used, with 3:97 acetonitrile-water as mobile phase

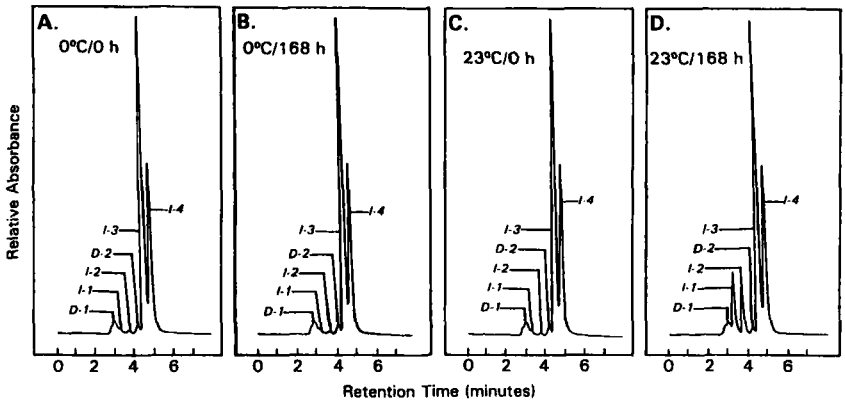

Figure 1. HPLC product profiles of the reaction product mixture from osmium tetroxide-induced oxidation of thymidine after temperature treatments. I-1 to I-4 and D-1 and D-2 refer to TG isomers 1 to 4 and dimers 1 and 2, respectively.

in an isocratic manner. All HPLC analysis were performed using Kratos Analytical Spectroflow 400 Solvent Delivery System on-line with SP 4100 Computing Integrator and Applied Science Variable Wavelength Detector.

RESULTS AND DISCUSSION

Each component from the reaction product mixture from osmium tetroxide- induced oxidation of thymidine was characterized according to the procedure reported previously (2). A modified HPLC system was used for the present studies. An HPLC product profile of the reaction product mixture is shown in Figure 1 A. The four stereoisomers I-1, I-2, I-3, and I-4 of

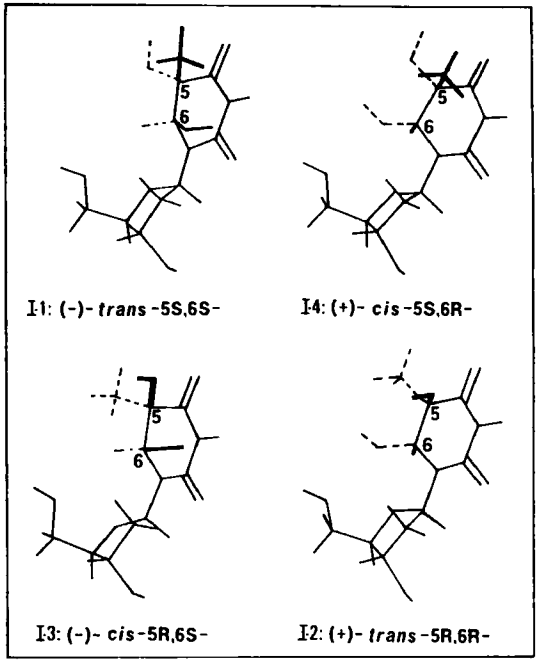

Figure 2. Molecular structure of all four stereo isomers of thymidine glycol produced by Alkemy II program (Tripos Associates, St. Louis, MO) on an IBM P/S personal computer (see Reference 2).

TG(see Figure 2) refer to (-)-**trans-5S,6S-**, (+)-**trans-5R,6R-**, (-)-**cis-5R,6S-** and (+)-**cis-5S,6R**, respectively; D-1 and D-2 refer to dimer-1 and dimer-2. It is interesting to note that under the modified HPLC conditions, D-1 eluted before I-1, while in the earlier studies (2) the order of elution was in reversed, and that there were no other differences observed in the order of elution for the modified HPLC system.

Optimum separation conditions for TG I-1 to I-4 selected under our present protocol conditions were as follows: Solution containing all four isomers of TG or HPLC-purified individual isomers were eluted under our standard conditions. The mobile phase was a mixture of acetonitrile and water (3:97 v/v) in an isocratic manner with the flow rate 2 ml/min. The TG isomer profiles were monitored by optical detection at 220 nm wavelength. The retention times were 3.15, 3.76, 4.40, and 4.90 min for I-1 to I-4, respectively. Only minor variations (± 0.06 min) were observed during the course of these experiments. Solution containing all four isomers or only one of the four isomers gave the same retention times. Once the optimal conditions for our protocol were worked out, calibration curves for each of the four TG isomers were plotted against peak areas from the corresponding peak integration. The lowest concentration of an isomer detected was close to 2.5 pmole of the injected isomers. The calibration curves were linear up to at least 5 nmole. These plots were based on at least 5-6 different isomer concentrations (not shown). Linear regression was used to test the calibration curves: in all four cases the observed correlation coefficients were less than 0.997, indicative of an acceptable relationship for the data ranges of concentrations of I-1 to I-4 examined.

To determine the temperature dependence of the interconversions of isomers of TG samples (100 microgram/ml in water, 500 microliter each) from the isomeric mixture of TG as well as HPLC- purified individual isomers, isomers in triplicate were allowed to stand at 0, 23, 50, 70, and 90°C at constant pH=7.0. At various specific time intervals (0, 4,

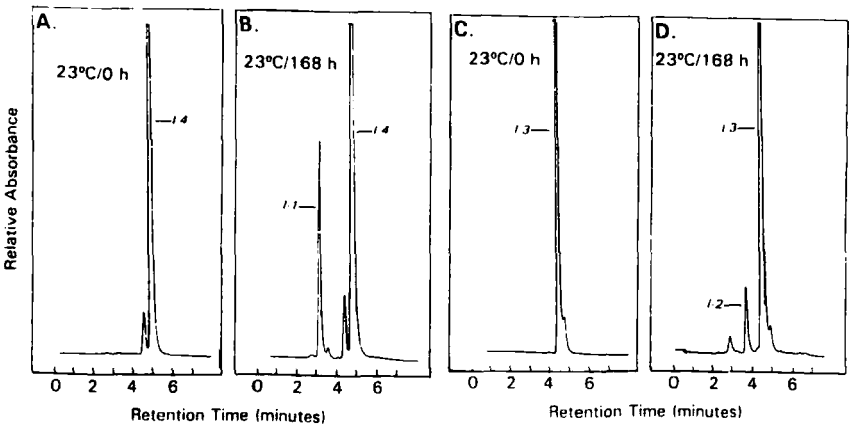

Figure 3. HPLC profiles of the purified thymidine glycol stereoisomers before and after temperature treatments. I-1 to I-4 refer to TG isomers 1 to 4.

8, 24, 48, and 168 hours), 10 microliter aliquots of each sample were withdrawn and injected onto the HPLC column. The concentration of each TG isomer was calculated by triangulation method (% peak areas). Both the isomeric mixtures and the HPLC-purified individual isomers (Figures 1 and 3) showed similar temperature dependence interconversion at pH=7.0. Figure 4 summarizes the measured temperature dependence of the isomerization. During the course of the experiment, representative examples of the time course studies indicated that no detectable amount of isomeric interconversions took place at pH=7.0, whereas raising the temperature to 23°C or higher resulted in partial isomeric conversions as shown in Figure 4 until the mixture reached steady-state equilibrium concentrations. Additional data (not shown) showed

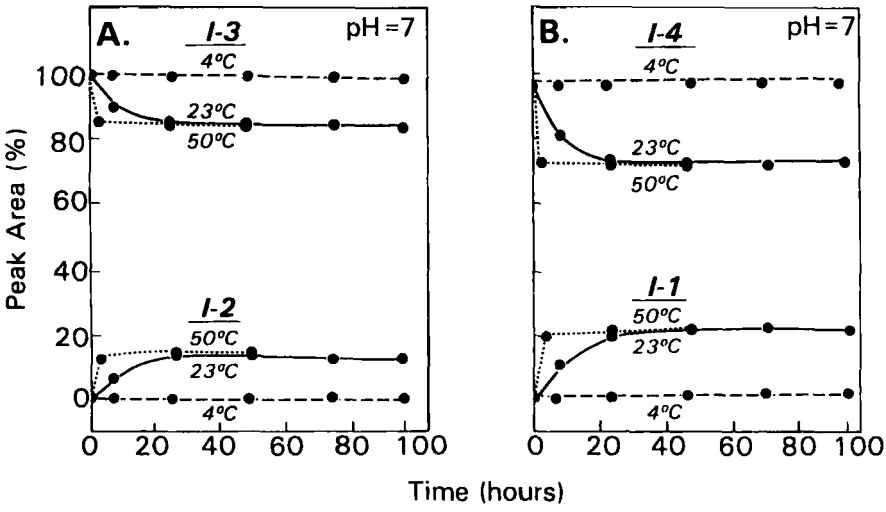

Figure 4. Temperature dependence of the interconversions of aqueous solutions of TG isomers at pH=7.0, as a function of time. (A) Conversion of TG isomer 3 into isomer 2 and (B) conversion of Tg isomer 4 into isomer 1.

that the rate of the initial phase of isomerization was directly proportional to the temperature, whereas once the isomeric mixture achieved steady-state concentration the temperature had no additional effect. Identical results were obtained when isolated HPLC-purified individual isomers were subjected to different temperature conditions. For example, it is clear from Figure 3 that I-3 partially isomerized to I-2 and I-4 to I-1 until the mixture reached steady-state concentration, and that the presence of other enantiomeric isomers during isomeric interconversions of a particular isomer does not alter the overall rate of interconversion.

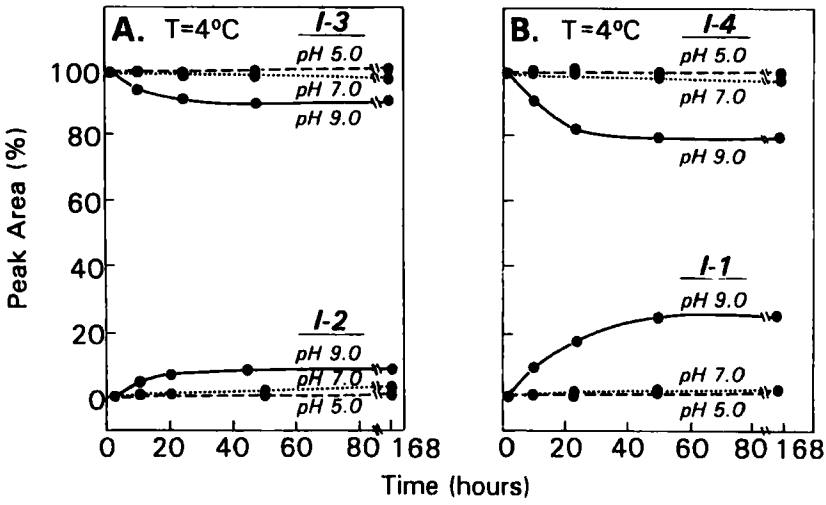

Figure 5. pH dependence of the aqueous solution of TG isomers at 4°C as a function of time. (A) Conversion of TG isomer 3 into isomer 2 and (B) conversion of TG isomer 4 into isomer 1.

In measuring the pH dependence of isomerization, the reaction product mixture or the HPLC-purified individual isomers were used. Samples of 100 microgrm/ml in water (500 microliter) were adjusted to pH 9.0, 7.0, or 5.0 and allowed to stand at 4°C. Aliquots were periodically analyzed using the HPLC system as described above, and the changes in the concentrations of each isomer were measured as a function of time. Figure 5 represents the time course of pH-dependent interconversions of TG isomers. At 4°C and at pH=7.0 and 5.0, no appreciable isomeric conversions were detected in the individual isomers or in the isomeric reaction product mixture (data not shown); however, at pH=9.0, I-3 partially gave

rise to I-2. Likewise, I-4 gave rise to I-1 as shown in Figure 5A and 5B.

Thermal isomerization of TG I-3 into I-2, I-4 into I-1, and vice versa in aqueous solution can best be explained by considerations of ring chain tautomerism of the isomer. The support for this pathway comes from the elegant work of Cadet and coworkers (10-13): isotope incorporation ^{14}C at C-2 and ^{18}O at either -5 or -6 position of 5,6-dihydro-5,6-dihydroxy-thymine. They observed no modifications in isotopic clusters after isomerization of ^{18}O-5-**exo** into **trans**-glycol ^{18}O-5-**endo**. A reduction of label close to 50% was observed when diols ^{18}O-6-**exo** were warmed for 4 hours at 90°C, suggesting the epimerization of C-6 by opening the N-1-C-6 bond, making the isomeric exchange possible. Base-catalyzed isomeric conversions of TG isomers can also be explained best by considering the initial attack of hydroxyl ion on -OH on C-6, followed by N-1-C-6 bond opening and closing. In our opinion, failure to detect any appreciable isomerization at pH=7.0 and 5.0 at 4°C during the course of the studies suggests the requirements of activation energy to initiate such isomerization processes. Support for this hypothesis comes from our own data: at 23°C in aqueous solution (pH=7.0), such isomerization does take place (see Figure 1 A and B).

The HPLC-UV procedure reported in this paper is a sensitive analytical procedure for quantitative measurements of all TG isomers simultaneously and rapidly. Application of this procedure allowed us to study and demonstrate **in vitro** interconversions of one of the major forms of DNA lesions caused by chemicals or ionizing radiation and some of the factors

that influence their rate of interconversions. The sensitivity of this assay can be further increased by use of radiolabeled thymidine precursor, and the procedure may be productively further applied to quantitate TG isomers in cellular DNA. Future research is essential in order to evaluate the importance of each isomer of TG with respect to its neighboring interaction or its individual role in enzymatic initiation of DNA excision repair processes. Such an understanding will be helpful in clarifying the mechanisms of repair of this potentially significant DNA modification.

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