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Note

Separation of radiation and photo-induced 5,6-dihydrothymine derivatives by reversed-phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC), particularly in the reversed-phase mode, has been successfully applied to the separation of DNA and RNA nucleobases and related nucleosides[1–4]. This method was shown to be very efficient for the qualitative and quantitative determination of chemically modified bases and nucleosides resulting from alkylation[5–7] or covalent binding reactions[8–10] with carcinogenic agents.

To the best of our knowledge, the HPLC separation of 5,6 saturated pyrimidines which constitute a major class of radiation or photo-induced DNA lesions has received less attention. One major exception is the recent report on HPLC analysis of the various isomers of cyclobutane dimers of thymine (Thy<>Thy) and thymidine (dThd<>dThd)[11]. This approach may be used as a sensitive method for the quantitation of cyclobutadipyrimidines within the deoxyribonucleic acids (DNA) of living cells exposed to ultraviolet light[12].

We now report the reversed-phase (RP) HPLC separation of various stable 5,6-dihydrothymine derivatives which may be produced by gamma irradiation[13–15] and/or ultraviolet photolysis[16] of aqueous solutions of thymine. Three types of octadecylsilyl (ODS) silica gel, *i.e.*, bulk, monolayer and capped forms, were used throughout this study. Flow-rate gradients appear to be more efficient and more convenient than solvent gradients in a complete separation of the modified pyrimidines which possess a wide range of polarities.

MATERIALS AND METHODS

HPLC instrument

The chromatographic system includes the following Waters Assoc. (Milford, MA, U.S.A.) equipment: two Model M 6000 dual-piston pumps, a Model 660 solvent programmer and a Model U6K universal loop injector. The column was monitored at 220 nm by using a Cecil Model 212 variable-wavelength detector (Cecil Instruments, Cambridge, Great Britain) equipped with a 8-μl flow cell. The chromatographic runs were recorded on a Model Servotrace PE recorder (Sefram, Paris, France) at a chart speed of 0.5 cm/min.

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Columns

Prepacked octadecylsilyl silica gel ODS-2 and ODS-3 columns (25 × 0.46 cm I.D., mean particle size 10 μm) were obtained from Whatman (Maidstone, Great Britain). A third reversed-phase column (25 × 0.47 cm I.D.) was packed with 10-μm ODS Nucleosil C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) by a slurry packing technique[17] using a Haskell pump system (Chromatem, Paris, France). The packing material was homogenized for 3 min by sonication using butanol as the dispersing agent.

Chromatographic procedures

The buffer used either in the flow-rate gradient or in the gradient elution experiments consisted of 0.01 *M* KH_2PO_4 aqueous solutions adjusted to pH 5.5 with phosphoric acid. Aqueous solutions and methanol were filtered using respectively Millipore HA 0.45-μm and cellulose FH 0.5-μm filters (Millipore, Bedford, MA, U.S.A.) prior to use.

The flow-rate gradient experiments were made following the programmed curve 10 from the M 660 programmer. The final flow-rate value, 2.8 ml/min, was reached over a period of 20 min from a starting flow-rate of 0.8 ml/min. The high permeability of the packing materials chosen made possible the use of a relatively high flow-rate. The volume of eluent was estimated using the following equation

$$V\,(\text{ml}) = 0.8\,t + at^6/6$$

where t is expressed in min for $0 < t < 20$ min and $a = 6.25 \cdot 10^{-7}$.

Chemicals

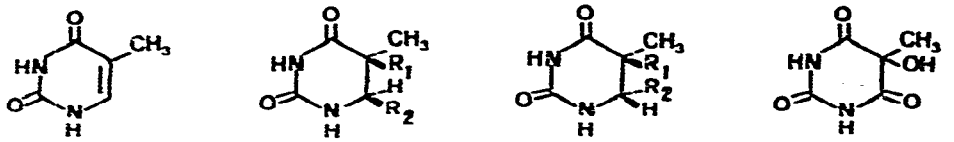
Thymine (8) and 5,6-dihydrothymine (7) were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. *trans*- and *cis*-5,6-dihydroxy-5,6-dihydrothymine (1,2)[18], 5-hydroxy-5-methylbarbituric acid (3)[19], 5-hydroxy-5,6-dihydrothymine (4)[20], *trans*-6-hydroxy-5,6-dihydrothymine (5)[21], *cis*-6-hydroxy-5,6-dihydrothymine (6)[22] and *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (9)[23] were prepared according to literature procedures.

RESULTS AND DISCUSSION

Flow-rate gradient separation on an ODS-2 column

The complete separation of a mixture of 5-hydroxy-5-methylbarbituric acid (3), 5,6-dihydrothymine (7) and its five possible 5- and/or 6-hydroxylated derivatives (1, 2, 4–6) (Fig. 1) on a bulky octadecylsilyl silica gel column (ODS-2, Whatman) is illustrated in Fig. 2. Thymine (8) and *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (9), which is a key intermediate in the chemical synthesis of the *trans* and *cis* thymine glycols (1, 2)[18], as well as in the preparation of thymine "photohydrates" (5, 6)[22], were included in this analytical separation. The convex flow-rate gradient appeared to be very useful for achieving a complete resolution of the mixture of compounds 1–9 in a relatively short period of time. This mixture contained both closely related structural compounds and products exhibiting a wide range of polarities.

The mechanisms of RP-HPLC are still open to debate[24,25]; however, it can



8 2 : $R_1 = R_2 = OH$ 1 : $R_1 = R_2 = OH$ 3

4 : $R_1 = OH$, $R_2 = H$ 6 : $R_1 = H$, $R_2 = OH$

5 : $R_1 = H$, $R_2 = OH$ 9 : $R_1 = Br$, $R_2 = OH$

7 : $R_1 = R_2 = H$

Fig. 1. Chemical structure of thymine and its various 5,6-dihydro derivatives. See Table I for names of the compounds.

safely be assumed that solvophobic interactions play a major rôle in the retention of solutes. Accordingly, the observed order of elution, glycols > ketol > 5- or 6-hydroxylated derivative > 5,6-dihydrothymine > bromohydrin (Table I), may be correlated with the increase in the lipophilic character of the molecules. The *trans*

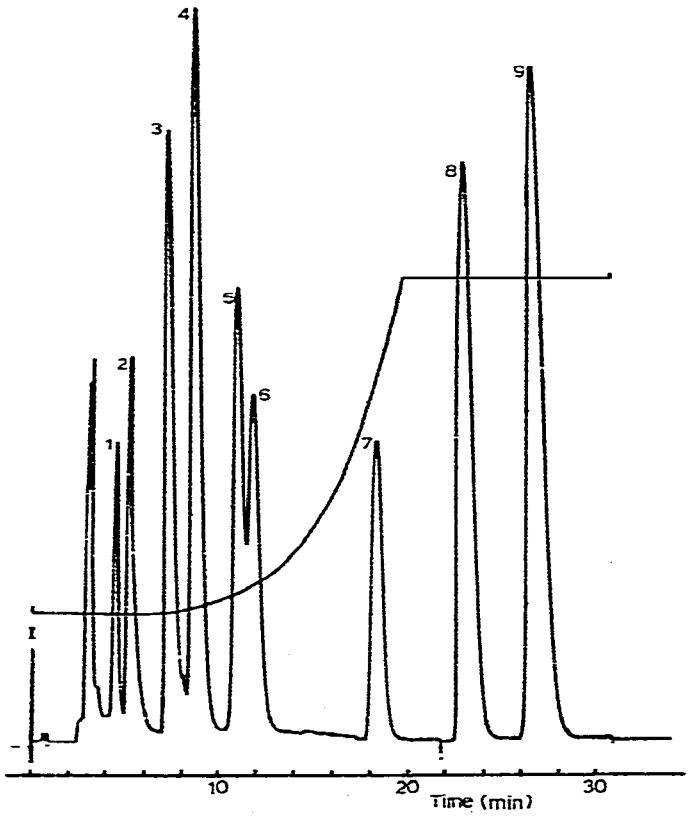


Fig. 2. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Partisil-10 ODS-2 column (25 × 0.46 cm I.D.). Eluent: buffered aqueous solution, pH 5.5; flow-rate gradient, 0.8 to 2.8 ml/min over a period of 20 min.

TABLE I

CAPACITY FACTORS OF 5,6-SATURATED DERIVATIVES OF THYMINE ON THREE ODS COLUMNS USING FLOW-RATE GRADIENT ELUTION

Eluent: phosphate aqueous buffer, pH 5.5; exponential convex flow-rate gradient, 0.8 to 2.8 ml/min over a period of 20 min.

Compound	*ODS reversed-phase packing material*		
	Nucleosil C-18	*ODS-2*	*ODS-3*
1 *trans*-5,6-Dihydroxy-5,6-dihydrothymine	0.73	0.50	0.19
2 *cis*-5,6-Dihydroxy-5,6-dihydrothymine	0.97	0.80	0.40
3 5-Hydroxy-5-methylbarbituric acid	1.44	1.48	0.94
4 5-Hydroxy-5,6-dihydrothymine	1.44	1.95	1.03
5 *trans*-6-Hydroxy-5,6-dihydrothymine	1.99	2.82	1.80
6 *cis*-6-Hydroxy-5,6-dihydrothymine	2.09	3.13	1.94
7 5,6-Dihydrothymine	4.39	6.82	4.58
8 Thymine	5.51	9.44	5.48
9 *trans*-5-Bromo-6-hydroxy-5,6-dihydrothymine	8.51	11.44	8.89

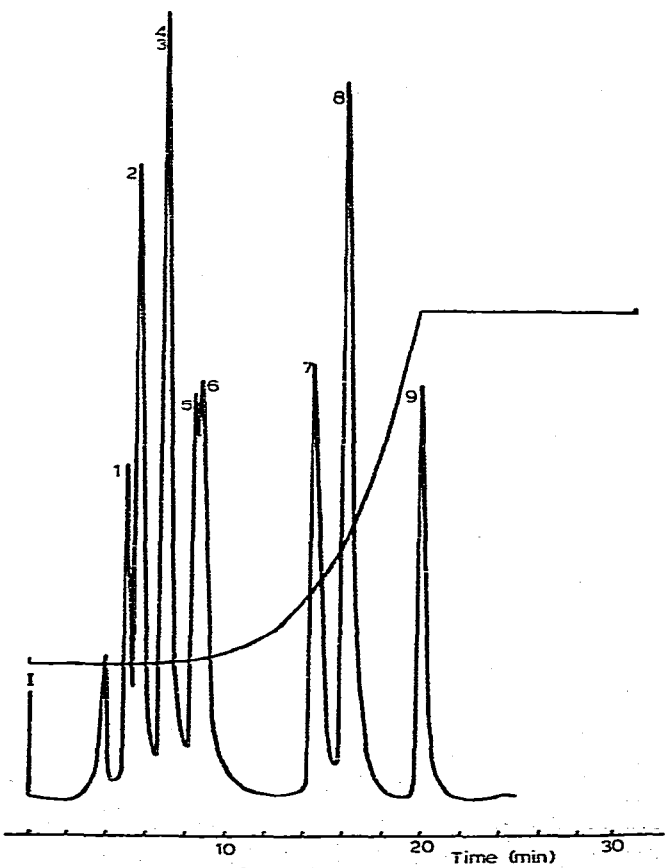


Fig. 3. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Nucleosil-10 C_{18} column (25 × 0.47 cm I.D.); experimental conditions as in Fig. 2.

thymine glycol (1) is eluted faster than the *cis* isomer (2). This may be at least partly explained in terms of lower accessibility for the methyl group of isomer 1 to the hydrocarbon part of the stationary phase. A *gauche* relationship between the methyl substituent and the vicinal hydroxyl group of 1 was deduced from the observation of a pronounced upfield shift (γ effect) of the ^{13}C nuclear magnetic resonance signal of the methyl group[26]. Furthermore, a preferential pseudo equatorial orientation is expected for the methyl substituent of 1, whereas the methyl group of 2 which shows a pseudo axial conformation[27] is less crowded.

The *cis* and *trans* diastereoisomers of 6-hydroxy-5,6-dihydrothymine (5, 6) exhibit higher capacity factors than 5-hydroxy-5,6-dihydrothymine (4) (Table I). The lower retention of 4 may be accounted for by the higher steric hindrance of its methyl group due to the presence of a geminal hydroxyl substituent.

Comparison with other ODS stationary phases

The results of the separation of the various 5,6-dihydrothymine derivatives on a monosubstituted octadecylsilyl silica gel column (Nucleosil C_{18}) and a completely silanized polymeric stationary phase (ODS-3) are shown respectively in Figs. 3 and 4. The elution profiles are similar to those given by the ODS-2 column using identical flow-rate gradients. However, most of the pyrimidines except the hydrophilic *trans* and *cis* thymine glycols (1, 2) are retained longer on the bulky ODS material than on the monomeric substituted ODS column, in agreement with previous findings[28]. This effect is less pronounced for the various compounds 1–9 on the capped ODS-3 column, particularly for thymine (8) (Table I). The complete silanization of the residual silanol groups, which behave as weak acidic cation exchangers[29,30] in neutral aqueous solution, prevents additional interaction between the

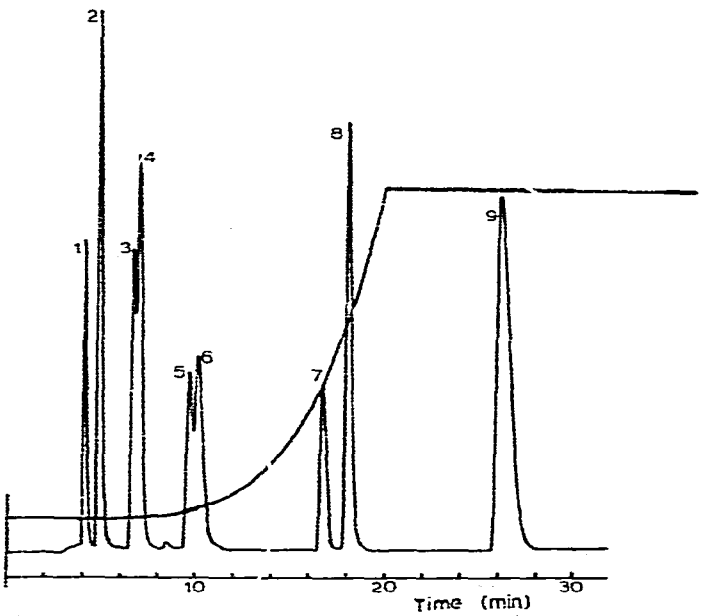


Fig. 4. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Partisil-10 ODS-3 column (25 cm × 0.46 cm I.D.); experimental conditions as in Fig. 2. Peak numbers refer to Fig. 1 and Table I.

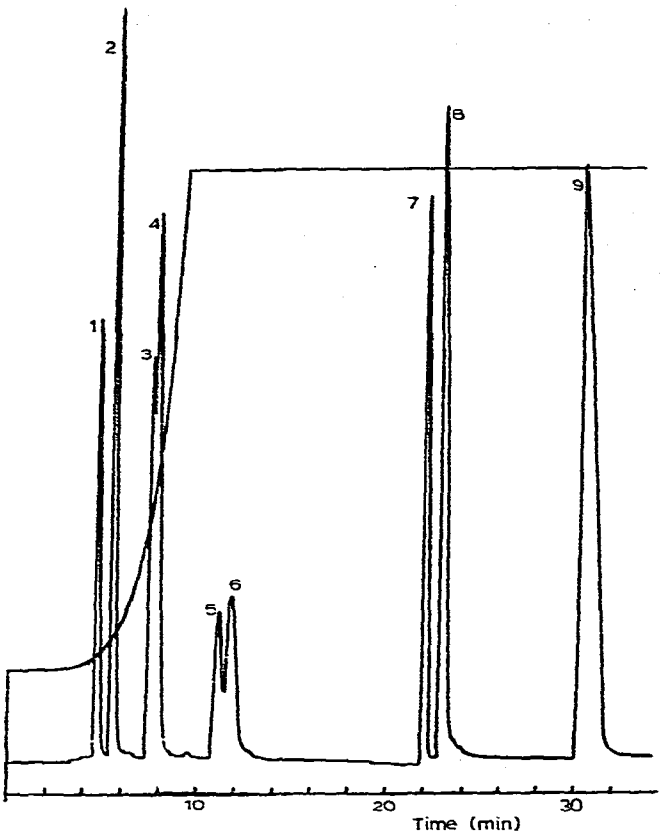


Fig. 5. RP-HPLC separation of 5,6-dihydrothymine derivatives on a Partisil-10 ODS-3 column; solvent gradient of methanol and buffered phosphate buffer (0:100 to 20:80, v/v) over a period of 10 min, followed by isocratic elution. Peak numbers refer to Fig. 1 and Table I.

stationary phase and 5,6-unsaturated 2,4-dioxopyrimidine. Similar adsorption processes are probably involved in the mechanism of retention of heterocyclic compounds which show some aromatic character such as thymine on the slightly negatively charged matrix of dextran gels[31]. This packing appears to be superior to the two other stationary phases used in this study since it presents a higher efficiency (Figs. 2–4) and provides a complete separation of components 1–9 in the shortest period of time.

Solvent gradient elution rate

Fig. 5 depicts a representative separation of compounds 1–9 using a combination of gradient elution and isocratic elution modes. The optimal conditions for the separation of the hydrophilic 5,6-dihydrothymine derivatives 1–4 consist of an exponential convex gradient of methanol and buffered phosphate solution (0:100 to 20:80, v/v) over a period of 10 min. The elution was then pursued in isocratic conditions to resolve the mixture of compounds 6–9. However, this mode of separation requires a longer period of analysis than the flow-rate elution mode to obtain similar

results. Furthermore, the use of isocratic elution is more convenient since it avoids the conditioning of the ODS column after each separation effected in the gradient elution mode.

CONCLUSION

RP-HPLC provides a fast and efficient method of separation of various radiation and photo-induced 5,6-saturated derivatives of thymine (8). This analytical technique can be applied to the quantitation of thymine lesions produced in low yields within nucleic acid chains by exposure to gamma rays[32]. It represents a more efficient and highly sensitive alternative assay for the quantitation of thymine glycol in DNA than liquid chromatographic analysis on a LH-20 column[33].

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