*Journal of Chromatography*, 195 (1980) 139–145
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,778

## Note

# Separation of cyclobutyl dimers of thymine and thymidine by high-performance liquid chromatography and thin-layer chromatography

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(Received February 28th, 1980)

Cyclobutadipyrimidines (pyrimidine dimers, Pyr <> Pyr) (the abbreviation system used is that proposed in ref. 1) represent the major class of DNA lesions induced by the action of far and near ultraviolet (UV) light on biological systems[2–4]. The four stereoisomeric thymine dimers (Thy <> Thy) produced by UV irradiation of Thy have been separated by thin-layer chromatography (TLC)[5] and by gas–liquid chromatography (GLC) after N-methylation[6]. Surprisingly, in view of the large number of separations of mixtures of nucleic acid components[7–10] and modified bases or nucleosides[11–15] by high-performance liquid chromatography (HPLC) using chemically bonded reversed-phase packings, to our knowledge the only reported separation of pyrimidine dimers (*cis-syn* Thy <> Thy and Ura <> Thy) by HPLC[16] was by ion exchange.

This paper reports the development of an HPLC method for rapid and complete separation of the four Thy <> Thy dimers on a conventional octadecyl reversed-phase column and on the new radial-PAK A cartridge with the Waters radial compression separation system (RC 55). Separations of the optically active stereoisomers as well as the *meso* pairs of cyclobutadithymidine (thymidine dimers, Thd <> Thd) by reversed-phase HPLC and two-dimensional TLC are also described.

## MATERIALS AND METHODS

### *High-performance liquid chromatography*

A Model 6000A dual-piston pump, a Model U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength detector, either a Cecil Model CE 212 (Cecil, Cambridge, Great Britain) equipped with a 8-$\mu$l flow cell or a Schoeffel Model 770 (Kratos Corporation, Schoeffel Instrument Division, Westwood, NJ, U.S.A.), were used. The detector was operated at 230 nm. Although photoreversal is known to occur with Pyr <> Pyr at this wavelength resulting in the formation of Pyr monomers[17] absorbing at $\approx$ 260 nm, the very low light intensity used in these

0021-9673/80/0000–0000/$02.25 

experiments did not produce any detectable absorbancy changes in the 260-nm region. All these chromatographic separations were performed under isocratic conditions at ambient temperature.

The prepacked columns used were 25 × 0.46 cm I.D. of Partisil ODS-2 (Whatman, Clifton, NJ, U.S.A.) with 10-$\mu$m, totally porous silica particles to which octadecyl groups were bonded. In addition, I/4 stainless-steel columns (25 × 0.47 cm I.D.) were packed under pressure with 10-$\mu$m ODS bonded phases Nucleosil $C_{18}$ (Macherey, Nagel & Co., Düren, G.F.R.) or LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) by a "non-balanced" slurry procedure[18] using a Haskell pump system (Chromatem, Paris, France). The packing material was homogenized by sonication using propan-1-ol as the dispersing agent. The stainless-steel Swagelok fittings used were adjusted for minimum dead volume. The outlets and inlets were terminated respectively with 2-$\mu$m metallic frits and PTFE frits (Chromatem).

The radial compression separation system comprised of a RCM-100 module and radial reversed-phase PAK A cartridge (10 × 0.8 cm I.D.) was a generous loan from Mr. C. Weiss of Waters Assoc., France and is gratefully acknowledged.

*Thin-layer chromatography*

Chromatographic separations were carried out on 20 × 20 cm pre-coated silica gel plates $F_{254}$ (E. Merck). Eluent A was the lower layer of chloroform–methanol–water (4:2:1, v/v/v) to which 5 ml of absolute methanol had been added for each 100 ml of organic phase, and eluent B was ethyl acetate–propan-2-ol–water (75:16:9, v/v/v)[19].

Dimers were detected by fluorescence with 254-nm light emitted by Pyr monomers resulting from photoreversal at that wavelength. 2'-Deoxyribonucleoside derivatives were visualized by heating for 10 min after the plates had been sprayed with a cysteine–sulfuric acid reagent[20].

Radioisotopically labeled compounds were detected by autoradiography using X Kodirex Kodak film.

*Chemicals*

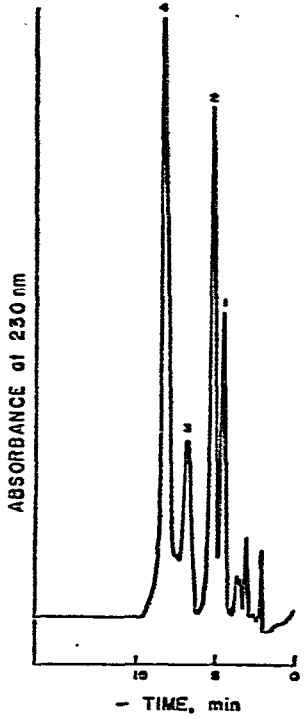
Thymine (Thy) and thymidine (Thd) (Sigma, St. Louis, MO, U.S.A.) were used without further purification.

The dimers were prepared[21] by photosensitization of aqueous acetone solutions of Thy and Thd, analogous to a previously described method[22] using an irradiator[23] equipped with seven G.E. BL lamps (> 313 nm). Assignment of the stereoconfigurations was mainly by comparison of infrared spectra with those previously reported[24] for four Thy <> Thy dimers and for Thd <> Thd optical diastereoisomers subsequent to their quantitive acid hydrolysis to the corresponding Thy <> Thy dimers[25]. The two *meso* pairs of Thd <> Thd dimers were further distinguished on the basis of their 250- and 360-MHz $^1$H nuclear magnetic resonance spectra[21].

## RESULTS AND DISCUSSION

*HPLC separation of Thy dimers*

Figs. 1 and 2 show that the four stereoisomers of Thy <> Thy were separated



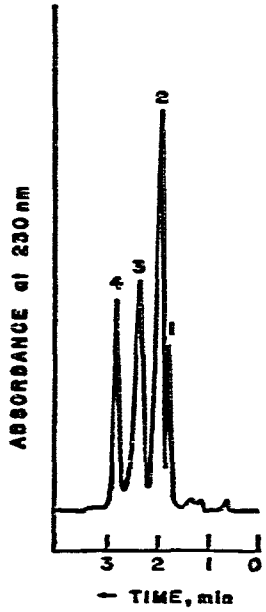




Fig. 1. Chromatogram of thymine dimers by reversed-phase HPLC isocratic separation. Column, ODS-2 (25 × 0.46 cm I.D.). Eluent: water–tetrahydrofuran (99:1); flow-rate, 1.0 ml/min. Peaks: 1 = *cis-anti*; 2 = *cis-syn*; 3 = *trans-anti*; 4 = *trans-syn*.

Fig. 2. Chromatogram of thymine dimers by reversed-phase HPLC isocratic separation. Column, PAK A (10 × 0.8 cm I.D.). Eluent flow-rate, 3.0 ml/min. Other details as in Fig. 1.

in the same elution order, *cis-anti* < *cis-syn* < *trans-anti* < *trans-syn*, under four different chromatographic conditions (Table I). While the resolutions were found to be satisfactory under these experimental conditions, the two *cis* Thy <> Thy dimers ($\alpha$ 1.43) were better separated than the *trans* isomers ($\alpha$ 1.04) using the Nucleosil $C_{18}$ column with only distilled water (eluent C) as the mobile phase. However, use of 1% organic modifier (water–tetrahydrofuran, 99:1, v/v; eluent D)

TABLE I

CAPACITY FACTORS OF THE FOUR ISOMERS OF CYCLOBUTADITHYMINE

Flow-rates were 1 ml/min and 3 ml/min respectively for Nucleosil $C_{18}$ and ODS-2 columns and for the PAK A cartridge. Numbers in parentheses are ratios of capacity factors, $\alpha = k'_{syn}/k'_{anti}$.

| *Thy <> Thy* | *Water* | *Water–tetrahydrofuran (99:1)* | | |
|---|---|---|---|---|
| | *Nucleosil $C_{18}$* | *Nucleosil $C_{18}$* | *ODS-2* | *PAK A* |
| *cis-anti* | 1.84 | 0.64 | 0.60 | 0.96 |
| *cis-syn* | 2.63 (1.43) | 0.77 (1.20) | 0.82 (1.37) | 1.18 (1.23) |
| *trans-anti* | 3.76 | 1.26 | 1.40 | 1.62 |
| *trans-syn* | 3.89 (1.04) | 1.37 (1.09) | 1.91 (1.36) | 2.06 (1.27) |

as the mobile phase improved the separation of the two *trans* Thy <> Thy dimers with the best result of $\alpha = 1.36$ attained by means of a Whatman ODS-2 column. It is worth noting that the longer retention times on the ODS-2 column may be explained in terms of the "bulk-modified" form[26,27] which often results from the use of trichlorosilanes rather than monofunctional silanes in the preparation of the bonded phase. Excellent selectivity and high capacity factor ($k'$) values were also exhibited by the PAK A column, thereby facilitating the separations. Indeed, the complete separation of the four Thy <> Thy dimers can now be achieved in less than 3 min!

The mechanisms of retention in reversed-phase liquid chromatography have been interpreted in terms of partition, adsorption, or a combination of both processes[28,29]. However, solute retention as an association process governed by the equilibrium of reversible solvophobic interactions between the eluent and the solute can also explain observations in liquid–solid or liquid–liquid chromatography with bonded phases[30,31]. Solvophobic interactions or, more particularly, hydrophobic interactions originating from a net repulsion between the water and the non-polar $C_{18}$-ligand and the non-polar moiety of the solute result in solute retention. Hydrophobic selectivity of solutes is, therefore, determined by the difference between the non-polar surface area of the solutes[30,31]. In contrast, solute adsorption on the chemically bonded stationary phase seems to involve only weak dispersive Van der Waals interactions[32] which do not confer high selectivity.

Accordingly, the difference in the chromatographic behavior of the four stereoisomers of Thy <> Thy reflects mainly the differences in accessibility of the two methyl moieties on the cyclobutane ring. Crystal structure analyses of these dimers have shown, except for the *trans-anti* Thy dimer, a puckered cyclobutane ring[33]. Consequently, the methyl moieties of the *cis-anti* and *trans-syn* dimers will be in pseudo-diequatorial or pseudo-diaxial conformations, whereas the methyl moieties of the *cis-syn* and *trans-anti* isomers adopt an equatorial axial (a,e or e,a) orientation with the possibility of interconversion[34,35]. For the *trans* isomers, the lack of interaction between the methyl groups increases lipophilicity in these molecules and may explain the longer retention times relative to the *cis* dimers. Similarly, considering the two *cis* and *trans* pairs, the orientation of the methyl moieties on opposite sides and on the same side of the cyclobutane ring for the *anti* and *syn* isomers respectively may be responsible for the slight differences in their chromatographic behavior. The low capacity value of *cis-anti* Thy <> Thy is in agreement with a preferential crowded diaxial orientation of the two methyl moieties.

*HPLC separation of Thd dimers*

The HPLC analyses of the six Thd <> Thd dimers, which include four optical diastereoisomers as well as two pairs of *meso* forms, were performed using a LiChrosorb RP-18 column with water–methanol as the mobile phase: eluent E (90:10) and F (95:5, v/v). Figs. 3 and 4 illustrate the HPLC separations of the set of *trans* and the set of *cis* isomers, respectively. Using eluent E, the (—)-*cis-anti* and the (+)-*trans-syn* diastereoisomers showed similar $k'$ values (Table II). The selectivity was improved by the use of eluent F, in which the content of the organic modifier had been decreased. However, eluent F gave poorer resolution ($\alpha$ 1.08) of the two levorotatory diastereoisomers, *cis-anti* and *trans-syn* Thd <> Thd dimers.

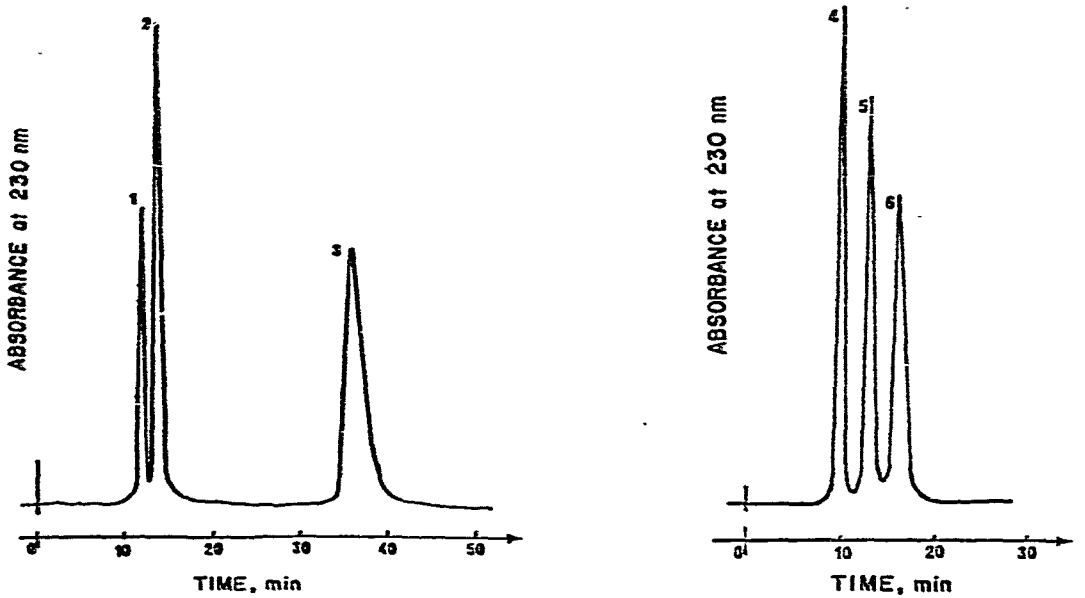



Fig. 3. Chromatogram of *trans*-thymidine dimers by reversed-phase HPLC isocratic separation. Column, RP-18 (25 × 0.47 cm I.D.). Eluent: water–methanol (95:5); flow-rate, 1.0 ml/min. Peaks: 1 = (+)-*trans-syn*; 2 = (−)-*trans-syn*; 3 = *trans-anti*.

Fig. 4. Chromatogram of *cis*-thymidine dimers by reversed-phase HPLC isocratic separation. Column details as in Fig. 3. Peaks: 4 = *cis-syn*; 5 = (−)-*cis-anti*; 6 = (+)-*cis-anti*.

TABLE II

CAPACITY FACTORS AND $R_{dTh}$ VALUES OF CYCLOBUTADITHYMIDINES

Eluents: A = the lower layer of $CHCl_3$–$CH_3OH$–$H_2O$ (4:2:1, v/v/v) to which 5 ml of $CH_3OH$ had been added for each 100 ml of organic phase; B = $CH_3COOC_2H_5$–$CH_3CHOHCH_3$–$H_2O$ (75:16:9); E = $H_2O$–$CH_3OH$ (90:10); F = $H_2O$–$CH_3OH$ (95:5).

| *dThd <> dThd* | *Capacity factor (k')* | | $R_{dTh}$ | |
|---|---|---|---|---|
| | *E* | *F* | *A* | *B* |
| *cis-syn* | 1.82 | 4.01 | 0.37 | 0.22 |
| (−) *cis-anti* | 2.21 | 5.39 | 0.24 | 0.16 |
| (+) *cis-anti* | 2.59 | 6.98 | 0.26 | 0.29 |
| (−) *trans-syn* | 2.83 | 5.81 | 0.58 | 0.44 |
| (+) *trans-syn* | 2.23 | 5.02 | 0.54 | 0.54 |
| *trans-anti* | 5.84 | 17.03 | 0.56 | 0.42 |

Retention time increases for Thd <> Thd as compared to Thy <> Thy dimers are in conformity with the findings in reversed-phase liquid chromatographic analyses of nucleic acid components[8–16], which are in accord with the general rule that an increase in the retention time denotes an increase in the lipophilic character of the solute. In the present case, the hydrocarbonaceous nature of the deoxyribofuranosyl (dRib) moiety of the nucleosides is responsible. The observed differences in elution order between Thy <> Thy and Thd <> Thd, *i.e.*, *cis-anti* > *cis-syn*

> *trans-anti* > *trans-syn* and *cis-syn* > *cis-anti* ≈ *trans-syn* >> *trans-anti*, respectively, are also in agreement with this concept. The high retention values exhibited by the *anti* Thd <> Thd dimers may be related to the easy accessibility of the two dRib moieties, most particularly in the *trans-anti* isomer. On the other hand, the low *k'* values of the *syn* Thd <> Thd dimers is a reflection of the steric crowding of the two dRib rings, especially in the *cis-syn* isomer.

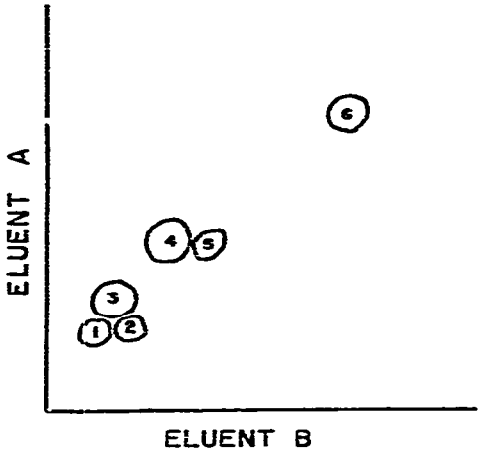



Fig. 5. Chromatogram of thymidine dimers by two-dimensional TLC separation on silica gel $F_{254}$ precoated plates. Eluents: A = the lower layer of $CHCl_3$–$CH_3OH$–$H_2O$ (4:2:1) to which 5 ml of absolute $CH_3OH$ had been added for each 100 ml of the organic phase; B = $CH_3COOC_2H_5$–$CH_3CHOHCH_3$–$H_2O$ (75:16:9). Spots: 1 = (−)-*cis-anti*; 2 = (+)-*cis-anti*; 3 = *cis-syn*; 4 = (−)-*trans-syn* and -*trans-anti*; 5 = (+)-*trans-syn*; 6 = thymidine.

*TLC separation of Thd <> Thd*

The result of two-dimensional TLC analysis of the Thd <> Thd dimers is illustrated in Fig. 5. All diastereoisomers are well separated except for the *trans-anti* and the (−)-*trans-syn* isomers which have similar $R_{dThd}$ values in both eluents A and B.

Since the *trans-anti* isomer can be readily removed from the mixture by HPLC, the TLC and HPLC procedures are complementary. Characterization of the six diastereoisomers of Thd <> Thd will be reported elsewhere[21].

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