Journal of Chromatography, 240 (1982) 475–487
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,708

USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO QUANTITATE THYMINE-CONTAINING PYRIMIDINE DIMERS IN DNA

JACK D. LOVE and ERROL C. FRIEDBERG*

Laboratory of Experimental Oncology, Department of Pathology, Stanford University, Stanford, CA 94305 (U.S.A.)

(Received December 24th, 1981)

SUMMARY

We have developed two high-performance liquid chromatographic systems for the measurement of pyrimidine dimers in hydrolysates of DNA. Normal-phase chromatography on an NH_2 column in methanol–ethyl acetate (3:97) at an elution rate of 2.0 ml/min allowed quantitation of thymine-containing (thymine–thymine plus thymine–uracil) pyrimidine dimers at levels as low as 0.1% of the total radioactivity as thymine in DNA. This system was unaffected by the presence of up to 1 mg of contaminating protein (bovine serum albumin) or 40 μg of DNA in hydrolysates prepared for chromatography. Reversed-phase chromatography on a μBondapak C_{18} column allowed measurement of thymine–thymine dimers at concentrations as low as 0.02% of the total radioactivity. With 0.1% tetrahydrofuran in water as the solvent at a flow-rate of up to 0.6 ml/min, thymine–thymine, thymine–uracil, and uracil–uracil dimers were completely resolved. We were not able to quantitate the latter two dimeric forms, however, owing to the presence of other radioactive components of undefined origin that eluted concomitantly with the uracil-containing dimers.

INTRODUCTION

The accurate measurement of thymine-containing pyrimidine dimers is an important and widely-used technique, since these photoproducts serve as the lesion in one of the most extensively studied models for the repair of damaged DNA. Various procedures have been employed for the quantitative separation of thymine-containing dimers from thymine monomer in hydrolysates of radiolabeled ultraviolet (UV)-irradiated DNA. These include thin-layer chromatography (TLC)[1,2], paper chromatography[3,4], ion-exchange chromatography,[5,6] high-performance liquid chromatography (HPLC)[7] and others[8,9]. Most published procedures are somewhat cumbersome and time-consuming, and, except for the use of HPLC, are limited because of the extreme sensitivity required for accurate measurement of very low levels of thymine dimers (*i.e.*, in the range 0.01–0.02% of total thymine in DNA).

Breter *et al.*[7] reported the measurement of thymine-containing pyrimidine

0021-9673/82/0000–0000/$02.75

dimers down to 0.01% of the total thymine content of DNA by cation-exchange HPLC. However, their technique requires the use of a column (300 × 0.18 cm) that is not commercially available. In addition, the back pressure generated by the use of such a long and narrow column requires heating the column to 75°C to reduce the viscosity of the solvent to a level that facilitates elution at less than the 6000 p.s.i. that most available pumps can generate. A recent report by Cadet *et al.*[10] demonstrated that the four isomers of cyclobutyl thymine-containing pyrimidine dimers could be separated by reversed-phase HPLC. However, these investigators did not report the measurement of thymine-containing pyrimidine dimers in hydrolysates of UV-irradiated DNA.

We have previously reported the use of TLC techniques that reliably measure thymine-dimer contents of *ca.* 0.2% or higher[2]. We have recently sought to develop methods that are both faster and more sensitive than TLC. In this paper, we describe two HPLC procedures for the measurement of thymine-containing pyrimidine dimers in hydrolysates of radiolabeled DNA. Both procedures use commercially available equipment and each has its own merit for particular applications.

MATERIALS AND METHODS

Instrumentation and supplies

We used a Waters Associates liquid chromatograph (Model 6000 A pump and U6K injector) attached to a variable-wavelength UV-detector (Model 450) and the following HPLC columns from Waters Associates: μBondapak C_{18} (30 cm × 3.9 mm), and μStyragel (100 Å column, 30 cm × 7.8 mm). We also used an NH_2 column (25 cm × 4.6 mm) and a Nucleosil C_{18} column (25 cm × 2.1 mm) from Alltech Associates. All solvents were Burdick and Jackson UV-grade. Thymine, thymidine and uracil were from Sigma (St. Louis, MO, U.S.A.). [2-^{14}C]Uracil and [methyl-^{3}H]-thymine were from Amersham. [Methyl-^{3}H]thymidine was from ICN.

The solvent output was connected to a Gilson Mini-Escargot or Micro-frac (for high flow-rates) fraction collector. Aqueous samples were collected and transferred to liquid scintillation vials containing 1.0 ml of water and 10.0 ml of aqueous counting fluor [1 part of Triton X-100 plus 2 parts of toluene-based Omnifluor (New England Nuclear)] for radioactivity determinations. Organic solvents were added directly to 5.0 ml of toluene-based Omnifluor for liquid scintillation counting. The quenching by 2.0 ml of ethyl acetate–methanol was approximately 25%; as it was uniform in all fractions, no quench corrections were made.

Preparation of pyrimidine dimer standards

Authentic pyrimidine dimers were prepared for use as chromatographic standard markers by UV-irradiating the free base (thymine, uracil or both) in the frozen state with 12 kJ/m^2 at 254 nm, essentially as described by Beukers and Berends[11]. Dimers were separated from monomer bases by TLC as described by Cook and Friedberg[1] or by Reynolds *et al.*[2], and were harvested by elution from the silica layer matrix by soaking the relevant regions of the chromatograms overnight in distilled water. Each water fraction was dried down, and the dimer residue was redissolved in the appropriate solvent.

During the hydrolysis of DNA before chromatographic resolution of dimers

from monomeric bases, cytosine is deaminated to form uracil[12]. Therefore, we prepared markers representing cytosine–cytosine or thymine–cytosine dimers by UV-irradiating uracil or a thymine–uracil mixture, respectively. As regards the latter, we irradiated [2-^{14}C]uracil in the presence of a 100-fold excess of thymine to ensure that the majority of radioactively labeled dimers were of the mixed (U< >T) form.

Preparation of hydrolysates of radiolabeled DNA from Escherichia coli

E. coli B was labeled with [methyl-^{3}H]thymidine or thymine in L-broth (5 g/l of yeast extract, 10 g/l of tryptone, 5 g/l of NaCl) for at least two generations. Cells were harvested by centrifugation, washed free of medium, resuspended in 50 m*M* Tris–HCl (pH 7.6), 10 m*M* EDTA, 100 μg/ml of lysozyme and 200 μg/ml of RNase A, and incubated at 37°C for 60 min or until the suspension became very viscous. Cell debris was removed by centrifugation at 6000 *g* for 10 min. DNA in the aqueous phase was extracted extensively with buffer-saturated phenol, chloroform–isoamyl alcohol (20:1), and diethyl ether, successively, until the ratio of absorbance at 260 nm to that at 280 nm was >1.85. The final specific radioactivity was between 14,000 and 28,000 cpm/μg of DNA.

DNA was irradiated under UV light with constant stirring. The UV-light source was a standard 15-W germicidal bulb (General Electric G8T5); the UV fluence was determined with a calibrated photometer (International Light Model IL254), and detector (Model PT100), and was corrected for absorbance at 254 nm by the method of Morowitz[13].

Irradiated DNA (not more than 100 μg of DNA per tube) was dried in a vacuum centrifuge (Speed Vac, Savant, Inc.) and solubilized in 0.2 ml of 97% formic acid. Solutions were placed in 75 × 10 mm ignition tubes, and the ends were sealed as described[2]. Hydrolysis was typically for 1 h at 175°C, but was for up to 4 h at 220°C in certain experiments. Following heating, the tubes were submerged in liquid nitrogen until frozen. Frozen tubes were opened by breaking the tips with a hemostat clamp. Hydrolysates were then dried in a vacuum centrifuge and redissolved in the appropriate solvent.

Calculations and measurements by HPLC

Since the advent of the widespread use of HPLC technology, a number of specifically defined terms have become accepted parameters that allow for quantitative comparisons between different chromatographic systems; the interested reader is referred to references 14–16 for detailed explanations of these terms.

The retention volume (V) of a given component during HPLC was measured as the distance (mm) from the point of injection to the eluted peak of the component. The width (W) of eluted peaks was determined by drawing tangents to the linear portion of the sides of the peaks to the baseline and measuring the distance (mm) between the intercepts. The total volume of each column was calculated from the known dimensions. Void volumes (V_0) were determined by injection of water into the column and observing the position of the peak caused by the Schlieren effect. The capacity factor (k') was calculated for each component as the ratio $(V - V_0)/V_0$. The selectivity factor (α) was calculated as the ratio k'_2/k'_1, where k'_1 is for thymine–thymine dimers and k'_2 is for thymine. Resolution $(R_s) = 2(V_2 - V_1)/(W_1 + W_2)$, where V_1 is the retention volume and W_1 is the peak width for thymine–

thymine dimers, and V_2 is the retention volume and W_2 is the peak width for thymine. Column efficiency (N) was calculated by the formula $N = 16(V/W)^2$.

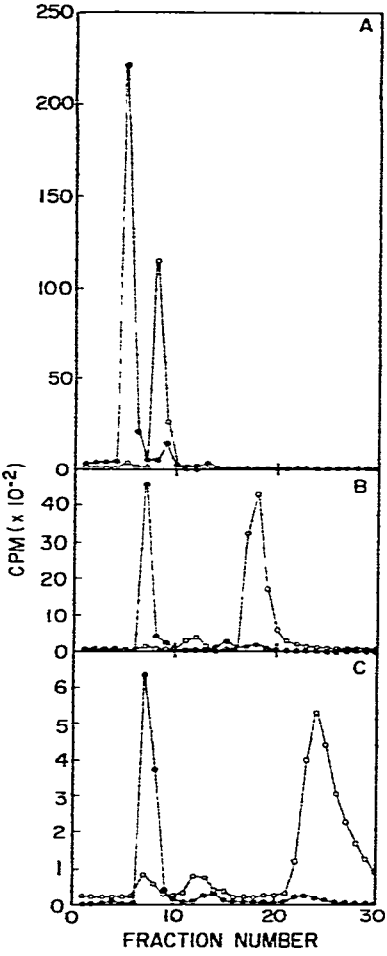
RESULTS

Normal-phase chromatography (NH_2 column)

Both thymine and thymine-containing pyrimidine dimers contain several polar groups. In particular, the N-1 and N-3 groups of pyrimidines carry lone-pair electrons that have potential for hydrogen-bonding. In addition, the covalent hydrogens of these secondary amines can form hydrogen-bonds. The double-bonded oxygens at C-2 and C-4 are negatively polarized and thus should be able to form bonds with amine hydrogens. Consequently, the choice of a column containing bound amine groups (*i.e.*, NH_2) for the chromatographic resolution of thymine from thymine dimers seemed logical. When either 100% methanol or pure water was used as the exclusive solvent, both thymine and dimers eluted at $k' = 0$ (*i.e.*, at the solvent front). Fig. 1 shows the elution profiles of thymine and thymine–thymine dimer standards at several solvent strengths. In every instance, thymine eluted ahead of the dimer. We settled on a compromise between reduced separation time and increased separation distance by establishing our routine elution solvent as 3.0% of methanol in ethyl acetate, and this solvent was used for the remainder of the studies described. Pure ethyl acetate is a solvent with an intermediate polarity index[17], in which neither thymine nor dimers have much solubility[18]. Both components were somewhat soluble in 100% methanol, 100% *n*-propanol or 100% tetrahydrofuran, and all of these solvents are miscible with ethyl acetate. We noted that 3% of methanol in ethyl acetate absorbed air very readily and therefore we degassed the solvent by filtration through a sintered-glass filtration apparatus (Millipore) fitted with a PTFE filter (pore size 0.2 μm) at least once a day. We also noted that different lots of ethyl acetate had different properties. Therefore, when changing lots, it was often necessary to readjust the exact percentage of methanol required for reproducible elution profiles.

By using the system just described, we carried out a series of experiments with hydrolysates of UV-irradiated *E. coli* DNA labeled as described in Materials and methods. For these experiments, we prepared markers of pyrimidine dimers containing thymine only, thymine and uracil, or uracil only, as described in the Materials and methods section. The uracil-containing dimers are representative of cytosine-containing dimers that occur naturally in DNA exposed to UV radiation, because, as previously indicated, cytosine is deaminated to uracil during hydrolysis at 175°C in formic acid[12]. The uracil-containing dimers (U<>U and U<>T) eluted just behind the thymine–thymine dimers at about 34 to 40 min (Fig. 2), and the U<>T dimers were included in the calculation of total thymine-containing pyrimidine dimers. In practice, one ordinarily labels only thymine in DNA, and hence would detect only thymine-containing dimers (*i.e.*, not U<>U dimers).

Table I shows a comparison of the results obtained by HPLC and by TLC. Replicate samples at a given dimer content gave reasonably comparable results by either method, but the accuracy of the measurement decreased progressively as the dimer content was lowered. The measurement of thymine-containing pyrimidine dimers by normal-phase HPLC was relatively unaffected by the presence of protein during hydrolysis and elution (Table II). Table III shows that the total DNA content



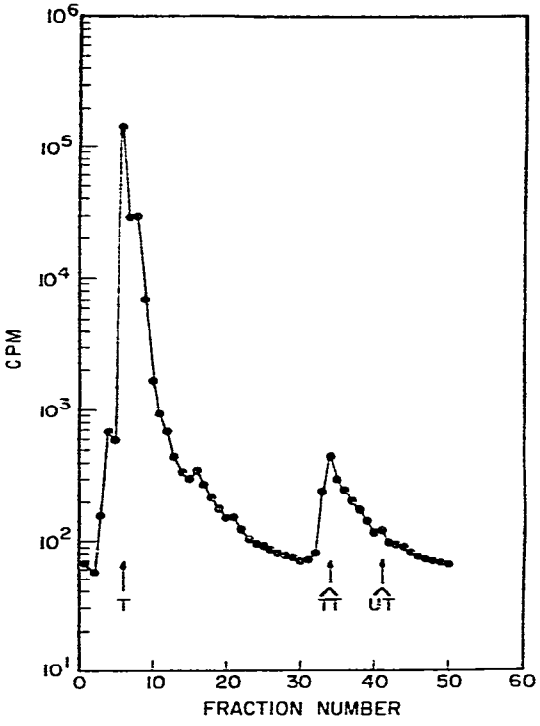


Fig. 1. Separation of thymine and thymine–thymine dimers by normal-phase chromatography. The ^{14}C-labeled thymine–thymine dimers (○) and ^{3}H-labeled thymine (●) were prepared and purified as described in Materials and methods. Chromatography on an NH_2 column (0.46 × 25 cm) was at 2.0 ml/min, and 2.0-ml fractions were collected and measured for radioactivity as described in the text. The elution solvents contained the following amounts (%) of methanol in ethyl acetate: A, 10; B, 8; C, 3.

Fig. 2. Chromatography of a hydrolysate of *E. coli* DNA that had been UV-irradiated as described in the text and contained 0.58% of thymine-containing pyrimidine dimers as total radioactivity. Hydrolysis of the DNA was at 170°C for 1 h. Chromatography and measurement of radioactivity were under the conditions described in Fig. 1, with methanol–ethyl acetate (3:97) as elution solvent. The arrows indicate the peak positions of the standard markers shown.

can be varied over at least a 150-fold range with only a small effect on the measured dimer content.

The single most significant factor that limited the accurate quantitation of very low levels of thymine-containing pyrimidine dimers by normal-phase chromatography was the consistent tailing of the thymine peak into the dimer area (Fig. 2). This problem was not satisfactorily eliminated, even when the flow-rate was reduced to as little as 0.2 ml per min. When we collected the peak thymine fractions and con-

TABLE I

MEASUREMENT OF THYMINE-CONTAINING PYRIMIDINE DIMERS BY HPLC AND BY TLC

E. coli DNA was UV-irradiated as described in the text to give varying dimer contents. Thymine-containing pyrimidine dimers in each sample were then quantitated both by TLC and by HPLC. Samples were eluted isocratically on an NH_2 column with methanol–ethyl acetate (3:97) at 2.0 ml per min, and 2.0-ml fractions were collected. The TLC was performed as described by Reynolds *et al.*[2]. The figures to the right of each quoted dimer value are the standard deviations and the percentages of the mean encompassed by the standard deviations.

Experiment No.	*Thymine dimers (% of total radioactivity)*	
	HPLC	*TLC*
1	6.36 (10)*–0.44 (6.9%)	6.89 (4)*–0.53 (7.8%)
2	3.29 (7)–0.27 (8.3%)	4.15 (4)–0.51 (12.1%)
3	1.29 (4)–0.15 (11.7%)	1.76 (3)–0.21 (11.8%)
4	0.55 (4)–0.10 (18.2%)	–
5	0.076 (4)–0.023 (30.3%)	–

* Number of replicates.

centrated and re-chromatographed them, the tail reappeared, suggesting poor column efficiency as a factor contributing to the tailing (data not shown). In addition, when we collected the tail region in separate fractions and concentrated and re-chromatographed it, we recovered two peaks, one at the original position of the tail and one at the position of thymine (data not shown). This indicated that the tailing also represented the presence of some non-thymine radiolabeled material, perhaps arising from sample hydrolysis or from breakdown of multiply-labeled thymine by tritium decay. We also observed that [^{3}H]thymidine and thymine stocks delivered by

TABLE II

EFFECT OF ADDED PROTEIN ON THYMINE-DIMER MEASUREMENTS

The effect of adding protein (bovine serum albumin) to DNA samples before hydrolysis is shown. The samples were chromatographed on an NH_2 column as described in Table I. After hydrolysis, samples were dried in a vacuum centrifuge, and 100 μl of 100% methanol were added to each. Samples were vigorously mixed (vortex-type mixer) and precipitates were removed by centrifuging for 2 min in an Eppendorf Microfuge before HPLC. Each value represents the average of two analyses.

Added bovine serum albumin (μg)	*Thymine dimers (% of total radioactivity)*
25	6.8
50	6.2
100	6.3
250	5.5
500	5.9
1000	6.0

TABLE III

EFFECT OF TOTAL RADIOACTIVITY AND MASS OF DNA ON THYMINE-DIMER MEASUREMENTS

The effect of total radioactivity and mass of DNA on the measured dimer content is shown. Samples, after treatment as described in Table II, were chromatographed on an NH_2 column as described in Table I. The volume of the samples varied from 3 to 20 μl. Each value represents the average of two analyses.

Total mass of DNA dimers (μg)	*Total radioactivity as DNA (cpm)*	*Thymine (% of total radioactivity)*
0.26	3600	6.6
0.98	13,500	6.4
2.72	37,500	6.2
5.43	75,000	6.7
41.67	575,000	6.0

the manufacturer eluted with a significant tail before hydrolysis, the severity of which increased with age over a few months.

With samples containing relatively high levels of thymine-containing pyrimidine dimers, as shown in Table III, the tailing problem had little effect on quantitation, even when >500,000 cpm were present as thymine. However, at thymine-dimer levels as low as 0.1%, the presence of more than 200,000 cpm as thymine precluded accurate quantitation of the dimer species (data not shown).

Gel-permeation chromatography

In light of the limitations of normal-phase chromatography using the system described above, we sought a means by which we could reverse the elution order of the two peaks, since, if the dimers could be eluted first, tailing from the thymine peak would not present a problem in their accurate quantitation. We initially experimented with gel-permeation chromatography on a μStyragel column. This matrix is quoted to have a resolving capacity in the molecular mass range of >100 to <700 daltons. Thymine is 125 daltons in mass, whereas the thymine–thymine dimer is twice that value. In gel-permeation chromatography, larger molecules elute first, so that we anticipated the elution of dimers ahead of thymine. Although the expected result was achieved, the actual resolution was poor ($R_s = 0.18$, $\alpha = 1.05$) when only 600 cpm as radioactivity in dimers were chromatographed (data not shown). Resolution was further decreased as the amount of radioactivity in the dimer peak was increased to 6000 cpm ($R_s = 0.13$), due to increased peak width (data not shown). Slower flow-rates somewhat reduced the band width, but not enough to achieve the required resolution.

Reversed-phase chromatography

We successfully reversed the elution order and achieved satisfactory resolution of thymine from thymine-containing pyrimidine dimers by reversed-phase chromatography on a C_{18} column, with a mixture of water and tetrahydrofuran as the elution solvent. We compared the resolving capacity of two C_{18} columns for the standard markers referred to above, over a range of solvent strengths (Fig. 3 and Table IV).

Surprisingly, the Nucleosil C_{18} column, with a particle size averaging 5 μm, had a lower efficiency than the μBondapak C_{18} column, with a particle size of about 10 μm, at the same solvent strength. Essentially, the same elution profile was observed at tenfold lower solvent strength with the former column, but, due to the difference in column dimensions and respective void volumes, the k' values were approximately the same at similar solvent strengths. When we eluted from the Nucleosil C_{18} column using pure water as the solvent, the maximum k' for thymine was 8.2. The same conditions generated a k' of 6.8 for thymine with the μBondapak C_{18} column.

We then used reversed-phase chromatography for the resolution of thymine and thymine-containing dimers present in hydrolysates of UV-irradiated radiolabeled

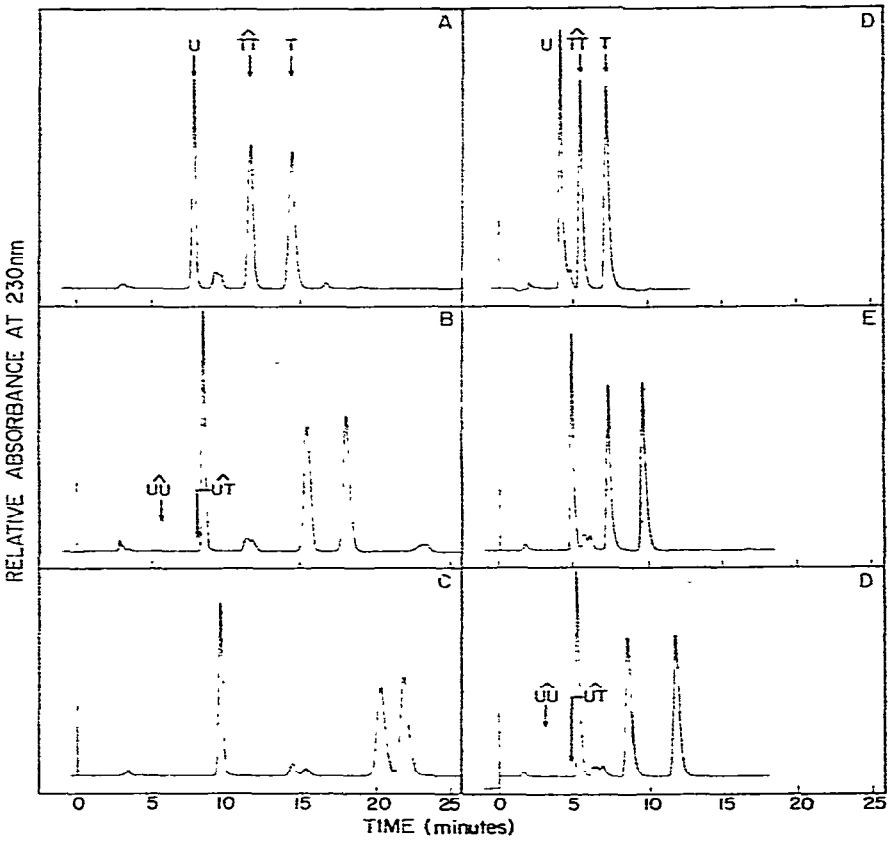


Fig. 3. Reversed-phase chromatography showing the separation of pyrimidine monomers from various pyrimidine-dimer standards. Thymine, thymine–thymine dimers, uracil–thymine dimers and uracil–uracil dimers were prepared as described in Materials and methods. Chromatography on μBondapak C_{18} (panels A–C) and on Nucleosil C_{18} (panels D–F) columns was at 0.3 ml/min, and the effluents were monitored at 230 nm (0.4 a.u.f.s.). The total sample volume injected was 0.05 ml in each instance. In panels B and F, the eluting positions of uracil–uracil (U < > U) and uracil–thymine (U < > T) dimers are indicated with arrows. These positions were determined by collecting 4-drop fractions (*ca.* 0.1 ml) and counting radioactivity. The solvents used contained the following amounts (%) of tetrahydrofuran in water: Panels A and D, 0.4; panels B and E, 0.1; panels C and F, 0.

TABLE IV

QUANTITATIVE PARAMETERS REFLECTING EFFICIENCY OF HPLC BY REVERSED-PHASE CHROMATOGRAPHY

Some useful descriptive values for reversed-phase chromatography at various solvent strengths are shown. Markers were prepared as described in the text. See "*Calculations and measurements by HPLC*" for identification of symbols and for calculation procedures. Values for all markers were obtained from strip-chart recordings of the absorbance at 230 nm. Samples were eluted in the solvent indicated at 0.3 ml per min. Retention volume (V) and peak width (W) are presented in mm as described in Materials and methods; 1 mm = 0.06 ml.

Component	*For μBondapak C_{18} column*						*For Nucleosil C_{18} column*					
	V	k'	W	α	N	R_s	V	k'	W	α	N	R_s
With pure water as mobile phase												
T	223	6.8	11	1.1	6600	1.5	138	7.4	9	1.5	3800	4.5
T< >T	207	6.3	10		6900		100	5.1	8		2500	
Thymidine	>600*						319	18.3	25		2600	
U	94	2.3	6		3900		52	2.2	5		1700	
With aqueous 0.1% tetrahydrofuran as mobile phase												
T	178	5.2	9	1.2	6300	3.0	95	4.8	8	1.4	2300	3.1
T< >T	152	4.3	8.5		5100		72	3.4	7.0		1700	
Thymidine	554	18.5	31		5100		240	13.5	17		3200	
U	84	1.9	6		3100		48	1.9	4.5		1400	
*With aqueous 0.4%** tetrahydrofuran as mobile phase*												
T	141	4.0	9	1.3	3900	3.4	70	3.2	7	1.5	1600	2.6
T< >T	114	3.0	7		4200		53	2.2	6		1200	
Thymidine	344	11.0	19		5200		146	7.8	10		3400	
U	77	1.7	5		3800		40	1.4	4.5		1200	
With aqueous 1.0% tetrahydrofuran as mobile phase												
T	100	2.5	9	1.7	2000	3.8						
T< >T	72	1.5	6		2300							
Thymidine	174	5.1	10		4900							
U	61	1.1	5		2400							

* Thymidine never came off this column in pure water.

** Aqueous 0.5% tetrahydrofuran was used with the Nucleosil column.

DNA (Fig. 3 and Table V). We obtained the most satisfactory results using the μBondapak C_{18} column with 0.1% of tetrahydrofuran in water as the solvent at a flow-rate of 0.3 ml/min. We have also performed these analyses at 0.6 ml per min (the upper limit for collecting 0.1-ml fractions by the Gilson Mini-Escargot) with no discernible effects on peak width or resolution. When we used hydrolysates of DNA, we could still resolve uracil–uracil, uracil–thymine, and thymine–thymine dimers. However, quantitation of the former two species was consistently precluded by the presence of unidentified contaminating peaks of radioactivity, the source of which

TABLE V

MEASUREMENT OF THYMINE–THYMINE DIMERS IN DNA BY REVERSED-PHASE CHROMATOGRAPHY

Precision and lower limit of detection of thymine–thymine dimers by reversed-phase chromatography. The samples were chromatographed as described in the text. Samples A–E were prepared by irradiating *E. coli* DNA with the following UV fluences (J m^{-2}) corrected for the absorbance at 254 nm of the sample as described in Materials and methods: A, 30; B, 15; C, 6; D, 3; E, 1. Samples F and G were prepared by mixing ^{3}H-labeled thymine–thymine dimers, prepared by irradiating frozen thymine solutions and purified by TLC (see Materials and methods), with unirradiated hydrolyzed [^{3}H]thymine-labeled *E. coli* DNA.

Sample	*T< >T (%)*	*Number of replicates*	*Standard deviation*
A	1.02	3	0.06
B	0.46	3	0.01
C	0.31	3	0.03
D	0.21	3	0.01
E	0.13	3	0.02
F	0.033	4	0.003
G	0.022	4	0.005

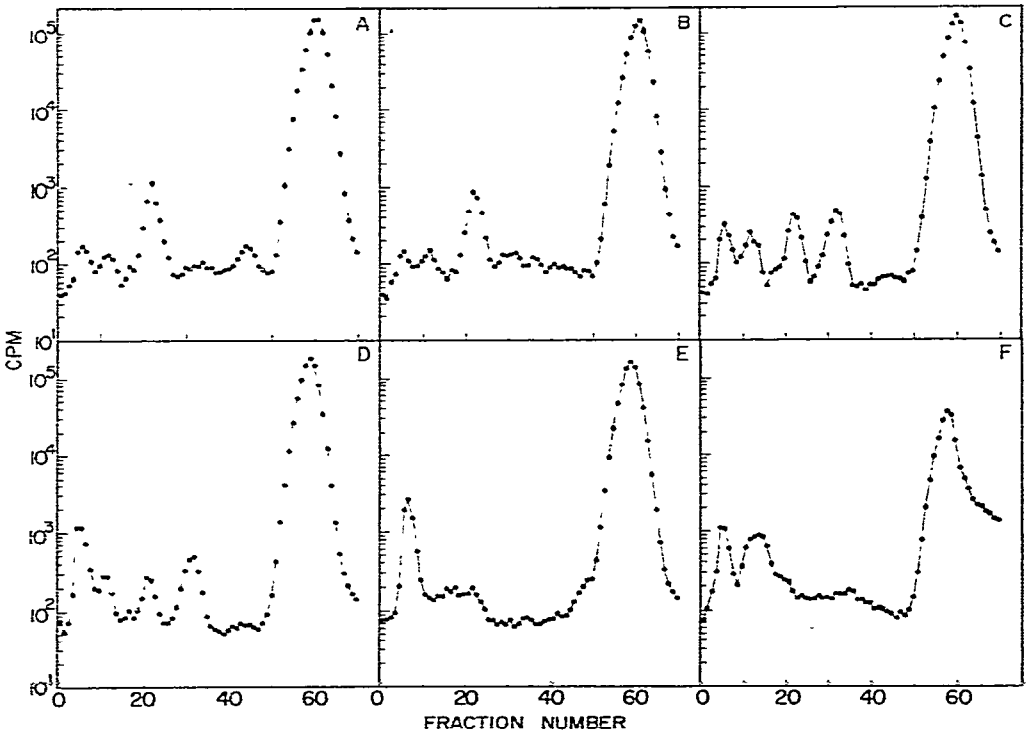


Fig. 4. Reversed-phase chromatography on hydrosylates of unirradiated *E. coli* DNA. The [^{3}H]thymine-labeled unirradiated *E. coli* DNA was prepared and hydrolyzed as described in the text. Hydrolysis was at 170°C (panels A–C) or 220°C (panels D–F) for the following times: A, 30 min; B, 1 h; C, 4 h; D, 1 h; E, 2 h; F, 20 h. Chromatography on μBondapak C_{18} was at 0.3 ml/min, and 5-drop (0.12-ml) fractions were collected; only fractions 11–80 were counted. The elution solvent was 0.1% of tetrahydrofuran in water.

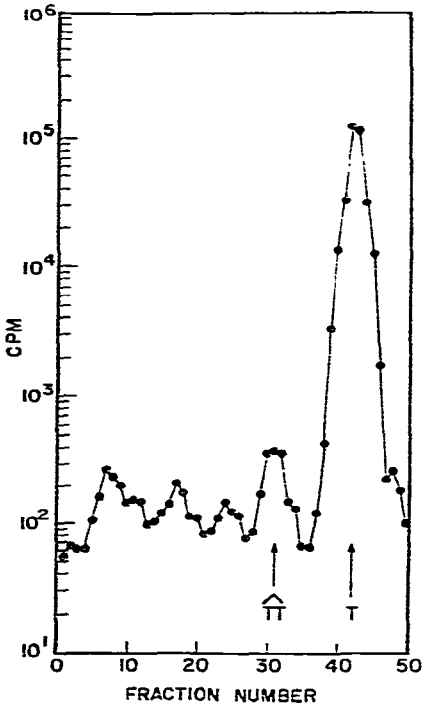


Fig. 5. Reversed-phase chromatography of a hydrolysate of UV-irradiated *E. coli* DNA. The [^{3}H]thymine-labeled *E. coli* DNA was prepared as described in the text and UV-irradiated to yield a thymine–thymine dimer content of 0.34% of the total radioactivity. Hydrolysis was at 220°C for 2 h. Chromatography was as described in Fig. 4. The first 20 fractions eluted were not measured for radioactivity, since none was detected in this region of the chromatogram in numerous previous experiments carried out under identical conditions. The positions of thymine (T) and thymine–thymine dimers (T< >T) are indicated. The exact elution positions of U< >T and U< >U dimers are not shown, but are to the left of the T< >T dimers (see Fig. 3).

was independent of irradiation of the DNA (Figs. 4 and 5). The measurement of thymine–thymine dimers was unaffected by this problem, and this species could be readily quantitated in hydrolysates of UV-irradiated DNA (Table V and Fig. 5).

In order to test the lower limits of the sensitivity for measuring thymine–thymine dimers by reversed-phase chromatography, we mixed ^{3}H-labeled dimers prepared by irradiating frozen thymine and purified by TLC, with hydrolysates of unirradiated labeled *E. coli* DNA. We used this approach to avoid the difficulty of accurately measuring the extremely small UV fluences (< 1 J m^{-2}) required for direct irradiation of the DNA. The results shown in Table V (samples F and G) indicate that this technique can be used to measure thymine–thymine dimers down to at least 0.02% of the total radioactivity. This level of quantitation makes the technique useful for studying thymine–thymine dimer excision at levels of UV radiation well within the biological dose range for both prokaryote and eukaryote cells.

For the measurement of thymine–thymine dimers at levels below 0.1% by reversed-phase chromatography, we found it necessary to adhere to the strictest technical guidelines. We observed that radiolabeled DNA generated thymine degradation products after only a few weeks during storage at 4°C. One of these products had a k'

value of 4.8 that placed it between the dimers and thymine and hence interfered with accurate measurements. We have avoided this potential problem by using freshly prepared radiolabeled DNA. In addition, certain conditions of hydrolysis preclude the formation of some interfering species. We compared the results of hydrolysis of radiolabeled unirradiated DNA in 97% formic acid at 170°C or at 220°C for various times (Fig. 4). Hydrolysis at 220°C consistently generated a lower background with greater separation between thymine and interfering peaks than did hydrolysis at 170°C. Therefore, we routinely hydrolyze DNA samples at 220°C for 2 to 4 h. Under these conditions, sealed ignition tubes build up a great deal of pressure, probably due to liberation of gases. Thus, these tubes must be handled with extreme caution because they are highly explosive. We allow them to stand in liquid nitrogen for at least 15 min, until the gases have liquified, before opening them. Finally, it is noteworthy that we have observed that as much as 20% of the radioactivity in stocks of [methyl-^{3}H]thymine, as shipped to us by the manufacturer, did not elute at the position of authentic thymine (unpublished results). We recommend purifying radiolabeled DNA precursors before labeling cells when thymine-dimer detection is desired at levels lower than 0.1%.

DISCUSSION

We have presented two methods for the separation and quantation of thymine-containing pyrimidine dimers using standard commercially available HPLC equipment. With normal-phase chromatography on a NH_2 column and with 3.0% of methanol in ethyl acetate as eluting solvent, we can clearly separate radiolabeled thymine from uracil–thymine and thymine–thymine pyrimidine dimers present in hydrolysates of UV-irradiated *E. coli* DNA. The two dimer species elute as a single peak of radioactivity and collectively constitute a measure of thymine-containing pyrimidine dimers in DNA. This technique has the significant advantage of being relatively unaffected by the presence of considerable amounts of protein or DNA in the hydrolysates and is recommended for the measurement of thymine-containing pyrimidine dimers at levels of 0.1% or higher. However, since thymine elutes ahead of the dimers during normal-phase chromatography, even slight tailing of the vast excess of radioactivity that constitutes the thymine peak into the relative paucity of radioactivity present in the dimer peak limits accurate measurement of the latter peak at levels below 0.1% of total radioactivity.

The order of elution of thymine and thymine dimers can be altered by reversed-phase chromatography. For such work, we have found the μBondapak C_{18} column to be the most satisfactory. For the most sensitive measurements we recommend the use of 0.1–0.4% of tetrahydrofuran in pure water as the eluting solvent. This system separates thymine from thymine–thymine, thymine–uracil, and uracil–uracil dimers and in theory could allow the quantitation of total pyrimidine–dimer contents of UV-irradiated DNA. However, despite all manner of experimental precautions, we have thus far consistently observed that the regions of the chromatogram containing thymine–uracil and uracil–uracil dimers are contaminated by very small amounts of unidentified radiolabeled products, even in unirradiated DNA (Fig. 4). Such products could result from radiation damage associated with the use of DNA of high specific radioactivity, as well as from the use of multiply-labeled thymine or thymidine as a source of radiolabel. With respect to the latter, we have carried out experiments using singly labeled (ring-labeled) thymine for preparing radioactive DNA. However, this problem has not been significantly alleviated. Reversed-phase chromatography does,

however, allow the measurement of thymine–thymine dimers at levels at least as low as 0.02% of the total radioactivity.

The level of sensitivity achieved by reversed-phase chromatography represents a significant improvement over other chromatographic techniques for separating very small amounts of thymine dimers from thymine; thus, we would recommend this particular procedure for biological studies on living cells. In addition, with a total chromatographic time of 20 min, this procedure has the distinct advantage of being much more rapid than most other published techniques. In applying a procedure such as this, in which one is attempting to resolve *ca.* 99.98% of the total radioactivity from the remaining 0.02%, it is mandatory that scrupulous experimental technique be used. We thus recommend pre-purification of thymine or thymidine used for labeling the DNA of living cells, and do not recommend storing radioactively labeled DNA for more than about 2 weeks. Finally, DNA should be extensively purified from biological sources before hydrolysis, since we have observed that protein and large amounts of hydrolyzed nucleic acids had adverse effects on the resolution of peaks in reversed-phase chromatography.

ACKNOWLEDGEMENTS

We gratefully acknowledge LaVonne McConkie and Waters Associates for supplying us with the μStyragel column. We also thank Linda Clogg, of Syntex, Inc., for her helpful advice during these investigations; Eric Radany and Dr. Thomas Bonura for their critical review of the manuscript; and Ms. Gina Johnson for her assistance in its preparation. These studies were supported by research grant CA 12428 from the U.S.P.H.S. and by contract DE AS03 76SF00326 with the U.S. Department of Energy. J.D.L. is supported by U.S.P.H.S. postdoctoral fellowship CA 06441.

REFERENCES

1 K. H. Cook and E. C. Friedberg, *Anal. Biochem.*, 73 (1976) 411.
2 R. J. Reynolds, K. H. Cook and E. C. Friedberg, in E. C. Friedberg and P. C. Hanawalt (Editors), *DNA Repair: A Laboratory Manual of Research Procedures*, Vol. 1, Part A, Marcel Dekker, New York, 1981, pp. 11–21.
3 W. L. Carrier and R. B. Setlow, *Methods Enzymol.*, 21 (1971) 230–237.
4 W. L. Carrier, in E. C. Friedberg and P. C. Hanawalt (Editors), *DNA Repair: A Laboratory Manual of Research Procedures*, Vol. 1, Part A, Marcel Dekker, New York, 1981, pp. 3–10.
5 A. J. Varghese and S. V. Wang, *Science*, 156 (1967) 955.
6 M. Sekiguchi and K. Shimizu, in E. C. Friedberg and P. C. Hanawalt (Editors), *DNA Repair: A Laboratory Manual of Research Procedures*, Vol. 1, Part A, Marcel Dekker, New York, 1981, pp. 23–29.
7 H. Breter, D. Weinblum and R. K. Zahn, *Anal. Biochem.*, 61 (1974) 362.
8 J. T. Cornelis and M. Errera, in E. C. Friedberg and P. C. Hanawalt (Editors), *DNA Repair: A Laboratory Manual of Research Procedures*, Vol. 1, Part A, Marcel Dekker, New York, 1981, pp. 31–44.
9 W. H. Farland and B. M. Sutherland, in E. C. Friedberg and P. C. Hanawalt (Editors), *DNA Repair: A Laboratory Manual of Research Procedures*, Vol. 1, Part A, Marcel Dekker, New York, pp. 45–56.
10 J. Cadet, L. Voituriez, B. S. Hahn and S. Y. Wang, *J. Chromatogr.*, 195 (1980) 139.
11 R. Beukers and W. Berends, *Biochim. Biophys. Acta*, 41 (1960) 550.
12 R. B. Setlow, *Science*, 153 (1966) 379.
13 H. J. Morowitz, *Science*, 111 (1950) 229.
14 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 1974.
15 F. Baumann (Editor), *Basic Liquid Chromatography*, Varian Aerograph, Walnut Creek, CA, 1971.
16 P. R. Brown and A. M. Krstulovic, *Anal. Biochem.*, 99 (1979) 1.
17 L. R. Snyder, *J. Chromatogr.*, 92 (1974) 223.
18 J. D. Love and E. C. Friedberg, unpublished results.