Journal of Chromatography, 259 (1983) 111–119
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,569

SEPARATION OF NUCLEIC ACID COMPONENTS AND THEIR RADIATION-INDUCED DEGRADATION PRODUCTS ON CHEMICALLY BONDED C_{12} REVERSED-PHASE THIN-LAYER PLATES

J. CADET*, L. VOITURIEZ and M. BERGER
Laboratoires de Chimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble 85 X, F.38041 Grenoble Cedex (France)

(First received November 15th, 1982; revised manuscript received November 27th, 1982)

SUMMARY

Monodimensional separation of the main DNA nucleobases, nucleosides and related 5'-nucleotides have been accomplished on dodecyltrichlorosilyl silicagel precoated thin-layer chromatography (TLC) plates using three buffered aqueous systems. This analytical procedure provides also an efficient and fast separation of the mixtures of radiation-induced derivatives of thymine and 2'-deoxyguanosine. A good correlation has been observed between the TLC migration pattern and the elution profile on octadecylsilyl silicagel high-performance liquid chromatography column for these various natural and modified DNA constituents.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been shown to be a powerful and efficient analytical method for the separation of nucleic acid components[1–4]. However, very few thin-layer chromatographic (TLC) separations of these compounds on hydrophobic bonded silica gel supports have been reported. This concerns mostly alkylated[5] nucleobases or derivatized nucleotides[6]. The reversed-phase separation of hydrophilic DNA and RNA components requires a high percentage of water in the mobile phase, and until recently commercially available RP-TLC plates were extremely fragile under these conditions. The low wettability of these supports was also a limitation for the application of water soluble compounds. Most of these difficulties have recently been overcome by the development of dodecyltrichlorosilyl silica gel pre-coated TLC plates[7,8], available under the trade-name OPTI-UP C_{12} from Antec (Bennwil, Switzerland). The recently reported separation of two purine ribonucleosides constitutes to our knowledge the first successful application of this new support for the RP-TLC analysis of nucleic acid components[9].

This paper describes a more comprehensive study of the TLC separation of the major purine and pyrimidine DNA nucleobases, nucleosides and their related 5'-phosphomonoesters using this support. Three buffered aqueous solutions which

0021-9673/83/$03.00

were reported to give excellent results in RP-HPLC[10–12] have been used in this work. Comparison between RP-TLC and HPLC analyses have been extended to radiation-induced degradation products of thymine (2) and 2'-deoxyguanosine (7)[13]. The relative merits of this new support and of conventional cellulose, ion exchange or silica gel TLC plates for the separation of DNA components are briefly discussed.

MATERIALS AND METHODS

Chemicals

Adenine (Ade, 1). guanine (Gua, 3) and cytosine (Cyt, 4) were obtained from Fluka (Buchs, Switzerland). Thymine (Thy, 2), 2'-deoxyadenosine (dAdo, 5), thymidine (dThd, 6), 2'-deoxyguanosine (dGuo, 7), 2'-deoxycytidine (dCyd, 8), 2'-deoxyadenosine-5'-monophosphate (5'-dAMP, 9), thymidine-5'-monophosphate (5'-dTMP, 10), 2'-deoxyguanosine-5'-monophosphate (5'-dGMP, 11), 2'-deoxycytidine-5'-monophosphate (5'-dCMP, 12) and 5,6-dihydrothymine (18) were purchased from Sigma (St. Louis, MO, U.S.A.). These compounds were used without further purification either as standards and/or as intermediates for the preparation of radiation-induced thymine (2) or 2'-deoxyguanosine (7) derivatives. *trans*- and *cis*-5,6-dihydroxy-5,6-dihydrothymine (13, 14)[14], 5-hydroxy-5-methylhydantoin (15) (ref. 15), 5-hydroxy-5,6-dihydrothymine (16) (ref. 16), *cis*-6-hydroxy-5,6-dihydrothymine (17) (ref. 17) and *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (19) (ref. 18) were prepared and purified according to literature procedures. [methyl-^{14}C]Thymine was obtained from Departement des Radioéléments, C.E.A. Saclay, France. The five modified 2'-deoxyguanosine nucleoside derivatives (20–24) were prepared by γ-radiolysis of oxygen-free aqueous solutions of dGuo (7). The bulk of non-degraded dGuo (7) was removed by preparative RP-HPLC on a Prep LC 500 chromatograph (Waters Assoc., Milford, MA, U.S.A.) using a prepacked octadecylsilyl silica gel cartridge with water–methanol (9:1) as the mobile phase. The radiation-induced degradation products (20–24) which were collected before and after the major peak of dGuo (7) were further purified by analytical HPLC. For this purpose a Model M 6000 dual-piston pump was used along with a Model U6K universal loop injector (Waters Assoc.) and an Isco Model 1850 variable-wavelength detector (Isco, Lincoln, NE, U.S.A.). The separations were carried out on a capped octadecylsilyl silica gel ODS-3 column (25 × 0.46 cm I.D., mean particle size 10 μm) from Whatman (Maidstone, Great Britain) using twice distilled water (pH 6.0) as the mobile phase.

TLC

Thin-layer plates (20 × 20 cm) precoated with a dodecyltrichlorosilyl silica gel containing a fluorescent indicator (Antec, Bennwil, Switzerland) were used as supplied (Siccap-Emmop, Marseille, France). About 5 μl of an aqueous solution of each DNA component (5 μg) were applied with an Hamilton syringe as a round spot or a narrow band at a distance 1.5 cm from one side of the plate. The diameter of the spot or the width of the band was limited to 2 mm in order to achieve higher resolution[19]. The TLC separations were carried out at room temperature (20–22°C) in chromatographic tanks (21 × 11 × 9 cm) supplied by Desaga (Heidelberg, G.F.R.). The tanks were filled with various solvents to a height of 0.5 cm. The plates were developed for 15 cm without saturation of the atmosphere. The development

time varied from 40 to 50 min depending on the eluent system used. The following mobile phases were prepared from reagent grade salts, analytical grade solvents and twice distilled water: I, 5 m*M* tetrabutylammonium phosphate aqueous solution adjusted to pH 2.5 with formic acid–5% methanol[10]; II, 0.4 *M* ammonium phosphate aqueous solution (pH 3.5)[11]; III, 0.1 *M* ammonium acetate aqueous solution (pH 6.9)–5% acetonitrile[12]; IV, twice distilled water (pH 6.0).

The plates were dried in a stream of cold air after the chromatographic development. Compounds absorbing within the UV range 250–260 nm were visualized by fluorescence quenching of the TLC plates under short-wave UV light emitted by an UVIS lamp (Desaga). 2′-Deoxyribonucleosides were detected as pink spots after spraying the chromatoplate with cystein–sulphuric acid reagent and subsequent heating at 105°C for 2 min[20]. ^{14}C-labelled compounds were visualized by autoradiography. Overnight exposure of Kodak NS-2T X-ray film allowed detection of spots with radioactivity as low as 0.01 μCi.

RESULTS AND DISCUSSION

Separation of nucleic acid components on RP-TLC plates

Fig. 1 illustrates a typical TLC separation of authentic samples of the major DNA nucleobases (1 and 4), nucleosides (5–8) and related 2′-deoxyribonucleoside-5′-monophosphates (9–12) on an OPTI-UP C_{12} plate with solvent I. The corresponding R_F values and those obtained on the same support using the eluents II and III are listed in the Table I. In most cases, the R_F values are the averages from four determinations obtained from independent runs.

An identical elution order is observed for each set of purine or pyrimidine DNA components irrespective of the eluents used: nucleotide > nucleobase > nucleoside. This behaviour is in agreement with reported HPLC data obtained on an

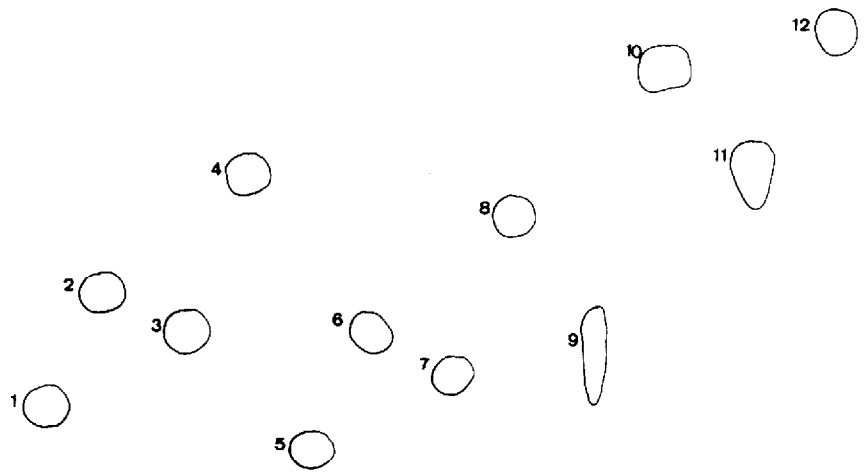

Fig. 1. Reversed-phase TLC separation of purine and pyrimidine DNA components on a dodecyltrichlorosilyl silica gel OPTI-UP C_{12} plate. Eluent: 5 m*M* tetrabutylammonium phosphate aqueous solution adjusted to pH 2.5 with formic acid–5% methanol.

TABLE I

R_F VALUES OF PURINE AND PYRIMIDINE DNA COMPONENTS ON REVERSED-PHASE TLC PLATES (OPTI-UP C_{12})

Solvents: I, 5 m*M* tetrabutylammonium phosphate aqueous solution adjusted to pH 2.5 with formic acid–5% methanol[10]; II, 0.4 *M* ammonium phosphate aqueous solution (pH 3.5)[11]; III, 0.1 *M* ammonium acetate aqueous solution (pH 6.9)–5% acetonitrile[12].

Compound	*I*	*II*	*III*
1 Adenine	0.06	0.08	0.07
2 Thymine	0.21	0.17	0.34
3 Guanine	0.16	0.15	0.33
4 Cytosine	0.35	0.61	0.35
5 2′-Deoxyadenosine	0.11	0.03	0.05
6 Thymidine	0.26	0.08	0.24
7 2′-Deoxyguanosine	0.21	0.07	0.18
8 2′-Deoxycytidine	0.40	0.31	0.37
9 2′-Deoxyadenosine-5′-monophosphate	0.19	0.15	0.29
10 Thymidine-5′-monophosphate	0.53	0.34	0.62
11 2′-Deoxyguanosine-5′-monophosphate	0.40	0.52	0.68
12 2′-Deoxycytidine-5′-monophosphate	0.57	0.59	0.67

octadecylsilyl silica gel column with various aqueous buffered solutions[3,21] and particularly with eluents II (ref. 11) and III (ref. 12). The chromatographic retention of solutes on a reversed-phase packing is generally explained in terms of solvophobic interactions[22,23]. Nucleotides are expected to be less retained than the less hydrophilic corresponding nucleobases and nucleosides. Nucleosides show higher vertical stacking ability than nucleobases. This has been suggested to explain the higher retention of the latter compounds on ODS columns with respect to the corresponding purine and pyrimidine bases[3]. The rôle of the hydrocarbonaceous part of the osidic moiety of nucleosides 5–8 in the retention mechanism should also be taken in consideration. It has recently been reported that diastereoisomers of cyclobutidithymidine (dThd< >dThd) which are not able to self-associate are more retained on reversed-phase columns than the corresponding pyrimidine derivatives[24]. The large observed differences in the capacity factors of the various diastereoisomers of dThd< >dThd may be at least partly accounted for in terms of the configuration of the sugar unit.

The nucleosides are eluted in the same order C > T > G > A, on the RP-TLC plates irrespective of the developing solvent used (Table I). A similar migration pattern was observed for the corresponding 2′-deoxyribonucleosides. This is in agreement with most previous HPLC data[1,3,11,25,26] which indicate that Ade (1) is the nucleobase most strongly retained on an octadecylsilyl silica gel support whereas Cyt (4) exhibits the lowest capacity factors. The use of solvent II led to a significant increase in the mobility of Cyt (4) and dCyd (8) with respect to the other bases and related nucleosides. This could be correlated with the high basic p*K* (ref. 27) of these two compounds (p$K \approx 4.5$) since ionization is expected to decrease retention in RP chromatography.

The four nucleotides 9–12 were poorly separated when the TLC plates were eluted with solvent III. Excessive migration and severe tailing were observed for three

nucleotides (10–12) under these conditions. A notable improvement in the resolution of the fast eluting nucleotides (10–12) was obtained by using either solvent I (Fig. 1) or solvent II (Table I). This may be partly accounted for by the lowering of the pH through an ion suppression mechanism. An inversion of the order of migration is observed between 5′-dTMP (10) and 5′-dGMP (11) in these two eluting systems. It should also be mentioned that the relative order of migration of the four nucleotides, 5′-dCMP > 5′-dTMP > 5′-dGMP > 5′-dAMP, is identical to that of the related nucleobases and nucleosides with eluent I. Of the three solvents used throughout this study, the eluent containing the ion-pair reagent (solvent I)[10] provides the best separation of the various purine and pyrimidine DNA constituents (1–12).

RP-TLC separation of radiation-induced degradation products of thymine (2) and 2′-deoxyguanosine (7)

Two other examples of separation of DNA components are the RP-TLC analyses of 5,6-dihydrothymine derivatives (13–19) and chemically modified dGuo products within the purine ring or/and the osidic moiety (20–24).

Thymine degradation products. The RP-TLC separation of various radiation-induced degradation products of thymine (13–18) and of the *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (19) using water (pH 6.0) as the eluent is illustrated in Fig. 2. The R_F values and the related capacity factors obtained previously on an octadecylsilyl silica gel column[28] are listed in Table II. These compounds which exhibit

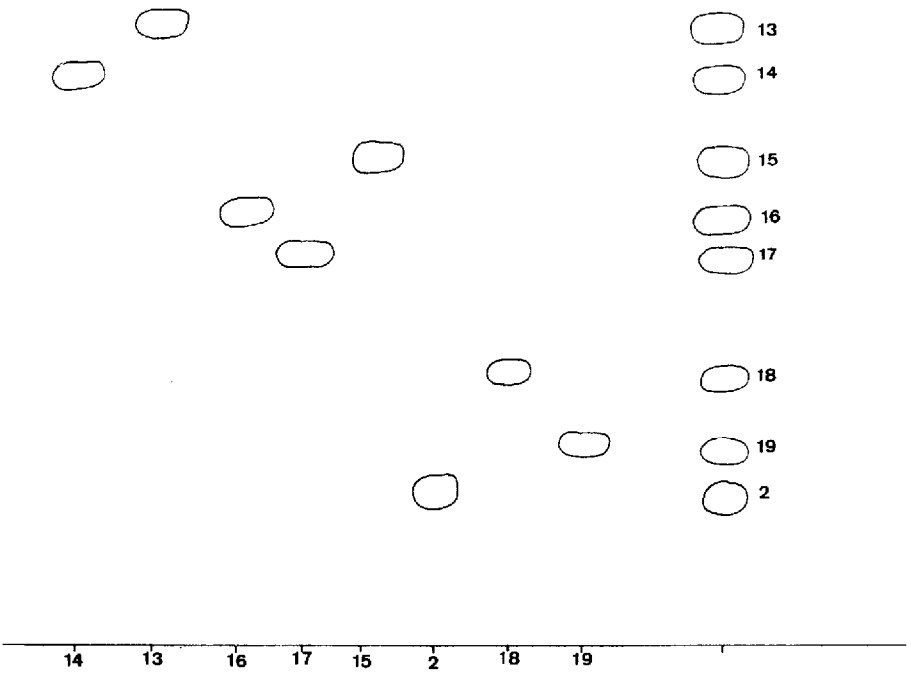

Fig. 2. Reversed-phase TLC separation of 5-hydroxy-5-methylhydantoin (15) and various 5,6-dihydrothymine derivatives (13, 14, 16–19) on an OPTI-UP C_{12} precoated plate. Eluent: twice distilled water (pH 6.0). Spot numbers refer to Table II.

TABLE II

R_F VALUES AND CAPACITY FACTORS, k', OF RADIATION-INDUCED THYMINE DERIVATIVES OBTAINED RESPECTIVELY ON C_{12} RP-TLC PLATES AND A NUCLEOSIL C_{18} COLUMN

Eluents: RP-TLC, twice distilled water, pH 6.0; Nucleosil C_{18}, phosphate aqueous buffer, pH 5.5. R_T is the R_F relative to thymine.

	Compound	R_F	R_T	k'
13	*trans*-5,6-Dihydroxy-5,6-dihydrothymine	0.77	4.05	0.73
14	*cis*-5,6-Dihydroxy-5,6-dihydrothymine	0.70	3.68	0.97
15	5-Hydroxy-5-methylhydantoin	0.61	3.21	
16	5-Hydroxy-5,6-dihydrothymine	0.53	2.79	1.44
17	*cis*-6-Hydroxy-5,6-dihydrothymine	0.48	2.53	2.09
18	5,6-Dihydrothymine	0.34	1.79	4.39
19	*trans*-5-Bromo-6-hydroxy-5,6-dihydrothymine	0.24	1.26	8.51
2	Thymine	0.19	1	5.51

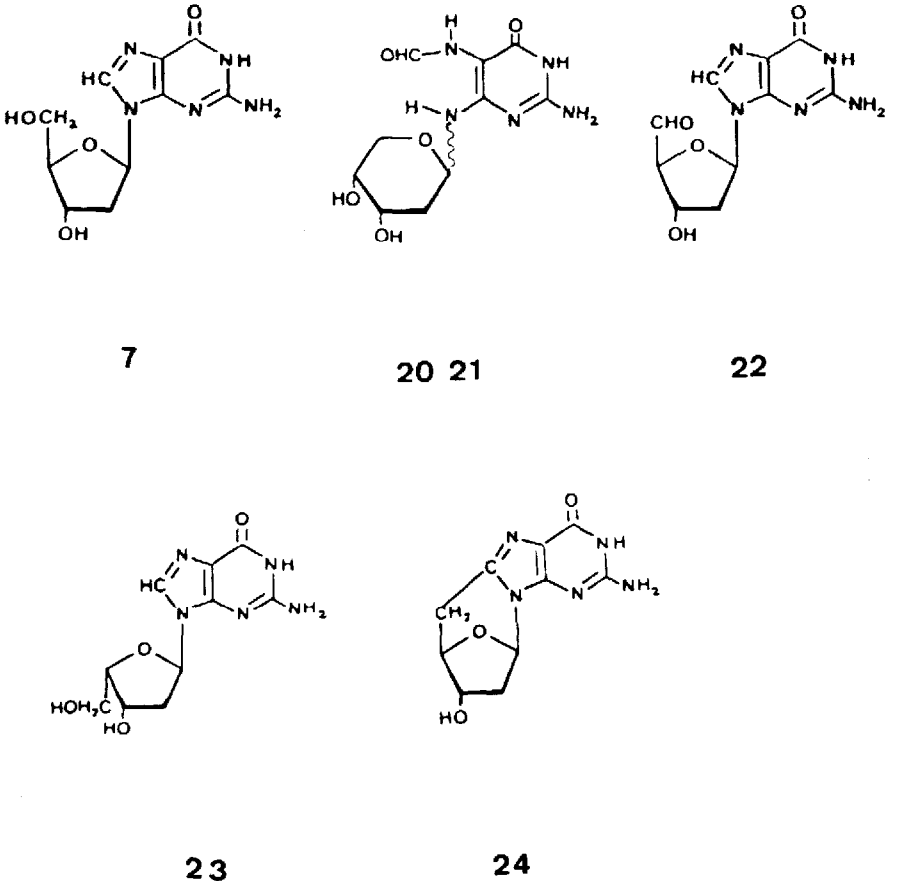

Fig. 3. Chemical structure of 2'-deoxyguanosine (7) and its radiation-induced degradation products (20–24). See Table III for the names of the compounds.

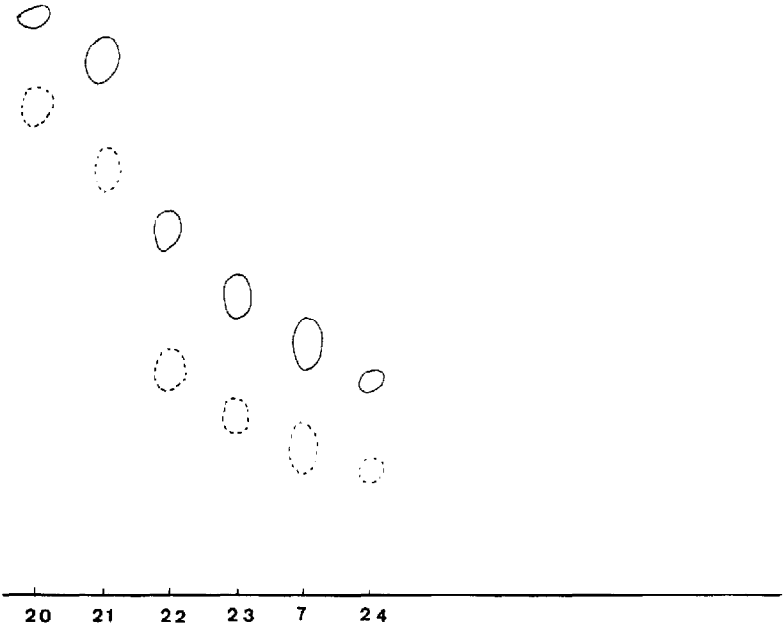

Fig. 4. Reversed-phase TLC separation of 2′-deoxyguanosine (7) and its radiation-induced degradation nucleoside derivatives on a chemically bonded C_{12} precoated plate. Eluent: water–methanol (95:5). Spot numbers refer to Fig. 3 and Table III. · · · · · · · , First run; ———, two successive runs.

a wide range of polarity show almost the same order of migration as in HPLC separations. The only exception involves the reversal of the migration pattern of thymine (2) and *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine (19). We note an improvement in the resolution of the TLC separation of the hydrophilic *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymine (13, 14) with respect to the HPLC sep-

TABLE III

R_F VALUES AND CAPACITY FACTORS, k', FOR RADIATION-INDUCED DEGRADATION PRODUCTS OF 2′-DEOXYGUANOSINE USING RESPECTIVELY WATER (pH 6.0) AND WATER–METHANOL (9:1) AS THE ELUENTS

	Compound	R_F*	R_F**	k'
20	N-6-(2-Deoxy-β-D-*erythro*-pentopyranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine	0.77	0.89	3.81
21	N-6-(2-Deoxy-α-D-*erythro*-pentopyranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine	0.88	0.95	3.22
22	9-(2-Deoxy-β-D-*erythro*-pento-1,5-dialdo-1,4-furanosyl)guanine	0.41	0.60	20.62
23	9-(2-Deoxy-α-L-*threo*-pentofuranosyl)guanine	0.33	0.49	22.14
7	2′-Deoxyguanosine	0.27	0.42	33.03
24	8,5′-Cyclo-2′,5′-dideoxyguanosine	0.23	0.35	38.54

* One TLC development.
** Two successive runs.

aration. On the other hand, the lipophilic compounds (2, 19) which are eluted on the ODS column in a flow-rate gradient or a solvent gradient mode[28] are not very strongly retained on the dodecyltrichlorosilyl silica gel TLC support.

Radiation-induced nucleoside derivatives of dGuo (20–24). The radiation-induced degradation products of dGuo (20–24)[29] (Fig. 3) were equally well separated on the OPTI-UP C_{12} TLC plates (Fig. 4) or on the ODS-3 reversed-phase column using respectively water–methanol (95:5) and water as the eluents. Identical elution patterns were observed in both cases for dGuo (7) and its chemically modified derivatives (20–24). The HPLC and TLC data are presented in Table III. The observed elution order is in agreement with the hydrophobic properties. The lack of the hydroxyl group at the osidic carbon C(5) in the cyclonucleoside 24 is expected to increase its lipophilic character. On the other hand, the α and β pyranoid anomers (20, 21) which result from rearrangement of the sugar ring subsequent to radiation-induced splitting of the imidazole ring are more polar due to partial loss of aromaticity and the presence of an additional amino group. These two nucleosides are better resolved by TLC than by HPLC analysis. This appears to be a common behaviour for hydrophilic compounds (*vide supra*).

On the other hand (as observed for the 5,6-dihydrothymine derivatives), the strongly retained nucleosides are less well separated on the TLC plates. However, a second run in the same direction allows a notable improvement in the separation of these compounds. This is particularly true for nucleosides (7, 22–24) having $R_F < 0.5$, the new R_F values being estimated by the following equation[30]:

$$R_F' = 1 - (1 - R_F)^2$$

CONCLUSIONS

RP-TLC appears to be an excellent and a fast analytical method for separating various natural and modified DNA components with mobile phases containing high percentages of water. The results reported show that there is in most cases a good relationship between the RP-TLC migration pattern and the HPLC elution profile of these compounds. As a result many eluents can be tested on these TLC plates in a relatively short time and these analyses may be further adapted to RP-HPLC. Chemical detection which is possible on these pre-coated plates could also rapidly provide useful information on the structure of the analyzed molecules.

Cellulose[31] and various anion-exchange[32–35] TLC supports have been shown to provide excellent separations of nucleic acid bases, nucleosides and related nucleotides. It is likely that the two-dimensional analyses which are used in these separations would be in most cases more efficient than the one-dimensional analysis reported in this paper. This would probably be the case for the separations of various complex mixtures of nucleosides which can be achieved on cellulose[36] or silica gel[37] TLC plates. RP-TLC could be a good alternative to the cellulose and poly(ethyleneimine) cellulose thin-layer analyses of the various 5,6-saturated thymine derivatives[38] and the mixture of nucleobases, nucleosides and nucleotides[39]. This new analytical approach is complementary to the conventional methods and could provide an improvement in the separations when solvophobic factors have to be considered. It should be emphasized that RP-TLC is more likely than other known TLC procedures

to separate the rather polar radiation-induced nucleoside derivatives of 2-deoxyguanosine (7) [29].

The RP-TLC analyses will find an interesting application in the chemical dosage of radiation-induced[28] or photochemical[40,41] lesions within DNA chains in living cells. This would be particularly appropriate for excision repair studies which require the analysis of numerous DNA samples[42].

REFERENCES

1 R. A. Hartwick, S. P. Assenza and P. R. Brown, *J. Chromatogr.*, 186 (1979) 647.
2 K. C. Kuo, R. A. McCurie and C. W. Gerhrke, *Nucleic Acids Res.*, 8 (1980) 4763.
3 P. R. Brown and E. Grushka, *Anal. Chem.*, 52 (1980) 1210.
4 H. K. Webster and J. M. Whaun, *J. Chromatogr.*, 209 (1981) 283.
5 M. Gacek and K. Undheim, *Acta Chem. Scand., Ser. B*, 36 (1982) 15.
6 C. Broka, T. Hozumi, R. Arentzen and K. Itakura, *Nucleic Acids Res.*, 8 (1980) 5461.
7 M. Faupel, H. R. Felix and E. von Arx, *J. Chromatogr.*, 193 (1980) 511.
8 M. Faupel and E. von Arx, *J. Chromatogr.*, 211 (1981) 262.
9 W. Meier and J.-F. Conscience, *Anal. Biochem.*, 105 (1980) 3341.
10 T. F. Walseth, G. Graff, M. C. Moos, Jr. and N. D. Goldberg, *Anal. Biochem.*, 107 (1980) 240.
11 A. Wakizaka, K. Kurosaka and E. Okuhara, *J. Chromatogr.*, 162 (1979) 319.
12 H. J. Fritz, R. Belagaje, E. L. Brown, R. H. Fritz, R. A. Jones, R. G. Lees and H. G. Khorana, *Biochemistry*, 17 (1978) 1257.
13 R. Teoule and J. Cadet, in J. Hutterman, W. Kohnlein, R. Teoule and A. J. Bertinchamps (Editors), *Molecular Biology, Biochemistry and Biophysics*, Vol. 23, Springer, Berlin, 1978, p. 171.
14 J. Cadet, J. Ulrich and R. Teoule, *Tetrahedron*, 31 (1975) 2057.
15 H. Murahashi, H. Yuki, K. Kosai and F. Doura, *Bull. Chem. Soc. Jap.*, 39 (1966) 1559.
16 C. Nofre, A. Cier, R. Chapurlat and J. P. Mareschi, *Bull Soc. Chim. Fr.*, (1965) 332.
17 C. Nofre and M. H. Ogier, *C.R. Acad. Sci., Ser. C*, 263 (1966) 1401.
18 O. Baudisch and D. Davidson, *J. Biol. Chem.*, 64 (1925) 233.
19 A. Zlatkis and R. E. Kaiser (Editors), *High Performance Thin-Layer Chromatography*, Elsevier, Amsterdam, 1977.
20 J. G. Buchanan, *Nature (London)*, 168 (1951) 1091.
21 M. W. Taylor, H. V. Hershey, R. A. Levine, K. Coy and S. Olivelle, *J. Chromatogr.*, 219 (1981) 133.
22 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
23 P. Jandera, H. Colin and G. Guiochon, *Anal. Chem.*, 54 (1982) 435.
24 J. Cadet, L. Voituriez, B. S. Hahn and S. Y. Wang, *J. Chromatogr.*, 195 (1980) 139.
25 Y. Rustum, *Anal. Biochem.*, 90 (1978) 289.
26 M. Ehrlich and K. Ehrlich, *J. Chromatogr. Sci.*, 17 (1979) 531.
27 G. D. Fasman (Editor), *Handbook of Biochemistry and Molecular Biology —Nucleic Acids*, Vol. 1, CRC Press, Cleveland, OH, 1975.
28 J. Cadet, M. Berger and L. Voituriez, *J. Chromatogr.*, 238 (1982) 488.
29 J. Cadet and M. Berger, *Radiat. Res.*, 83 (1980) 438.
30 A. Jeanes, C. Wise and R. Dimler, *Anal. Chem.*, 23 (1951) 515.
31 G. Pataki, *J. Chromatogr.*, 29 (1967) 126.
32 E. Randerath and K. Randerath, *J. Chromatogr.*, 16 (1964) 126.
33 B. R Bochner and B. N. Ames, *J. Biol. Chem.*, 257 (1982) 9759.
34 H. P. Raaen and F. E. Kraus, *J. Chromatogr.*, 35 (1968) 531.
35 J. Tomasz, *J. Chromatogr.*, 84 (1973) 208.
36 K. Randerath and E. Randerath, in G. L. Cantoni and D. R. Davis (Editors), *Procedures in Nucleic Acid Research*, Vol. 11, Harper and Row, New York, 1971, p. 188.
37 J. Cadet and R. Téoule, *J. Chromatogr.*, 76 (1973) 407.
38 R. Teoule and J. Cadet, *J. Chromatogr.*, 43 (1969) 368.
39 G. Pataki and A. Niederwieser, *J. Chromatogr.*, 29 (1967) 133.
40 J. Cadet, M. C. Paterson and N. E. Gentner, *9th Annu. Meeting Amer. Soc. Photobiol., Williamsburg, VA, June 1981.*
41 J. P. Leblanc, B. Martin, J. Cadet and J. Laval, *J. Biol. Chem.*, 257 (1982) 3477.
42 J. Cadet, N. E. Gentner, B. Rozga and M. C. Paterson, in preparation.