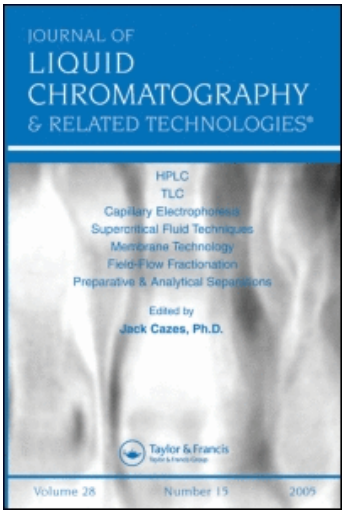

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HPLC SEPARATIONS OF NORMAL AND MODIFIED NUCLEOBASES AND NUCLEOSIDES ON AN AMINO SILICA GEL COLUMN

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ABSTRACT

An amino substituted silica gel column was used for the normal phase HPLC separation of nucleic acid components, including free bases and the corresponding 2'-deoxyribo- and ribonucleosides. The chromatographic system was also found to be particularly convenient for the separation of various modified nucleosides, including oxidation products of 2'-deoxyguanosine and pyrimidine components, thymidine cyclobutane dimers, dinucleoside monophosphate photoproducts and pyrimidine (6-4) pyrimidone adducts.

INTRODUCTION

High performance liquid chromatography (HPLC) is one of the most convenient tool currently used in analytical chemistry. For example, one of the most important difficulties associated with the determination and quantification of modified nucleosides is their separation from normal

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DNA and RNA nucleosides. Reverse phase HPLC, which is mainly used for this purpose, was found to be particularly appropriate for the resolution of mixture of hydrophobic nucleic acid derivatives. On the other hand, oxidized products, which are more polar than the starting nucleosides, exhibit usually shorter retention times on octadecylsilyl (ODS) silica gel columns, and thus may be eluted too rapidly, in the close vicinity of the void volume.

As an alternative and complementary approach, we have developed a new chromatographic assay for the separation of nucleobases and nucleosides. This is based on the use of an amino (NH_2) substituted silica gel column in a normal phase HPLC mode. This approach was already used for the thin layer chormatography separation of synthetic nucleosides [1]. It should also be added that its application to HPLC separation of DNA components was restricted to the resolution of mixtures of nucleotides in the ion exchange mode, by using phosphate buffer at either acidic [2,3] or neutral pH [4,5].

The advantage of the HPLC amino silica gel column used in normal phase lies in the increase of the retention time of the products with their polarity, in contrast to reverse phase HPLC in which hydrophobic interactions play a predominant role. This difference in the mechanism of separation allows the resolution of mixtures which was not possible to achieve on an ODS silica gel column. We report here results dealing with the HPLC separation of normal and modified nucleic acid derivatives (including nucleobases, 2'-deoxyribo- and ribonucleosides) by using an amino substituted silica gel column.

MATERIAL AND METHODS

HPLC system

The HPLC system consisted of two model 302 HPLC pumps (Gilson, Middleton, WI, USA), a Sil-9A Shimadzu automatic injector (Touzard & Matignon, Paris, France) and a L-4000 UV spectrophotometer (Hitachi, Tokyo, Japan). These instruments were connected to an Apple IIe computer which controls the mobile phase composition (pump A : acetonitrile, pump B : 25 mM ammonium formate in H_2O), and a Data

Master model 621 (Gilson) which analyses the signal coming from the variable wavelength UV detector.

The normal phase amino substituted silica gel Hypersil NH_2 column (mean pores size = 5 µm, 250x4.6 mm I.D., Ref : $H5NH_2$-25F) was purchased from Interchim (Montluçon, France). The mobile phase used for the purification of the nucleosides consisted of a mixture of 25 mM ammonium formate (Eastman Kodak Company, N.Y., USA) in milli-Q water and acetonitrile type RS HPLC (Carlo Erba, Milano, Italy). Conditions A [90:10], B [75:25] and C [93:7] were used, the flow rate being 1 ml/min.

Chemicals

Purine bases and the corresponding ribo- and 2'-deoxyribonucleosides were purchased from Sigma (St Louis, MO, USA). Pyrimidine components were obtained from Pharma Waldhorf (Mannheim, Germany), with the exception of thymine and thymidine which were provided by Genofit (Geneva, Switzerland). 5-Methylcytosine (m^5Cyt) and 5-methyl-2'-deoxycytidine were obtained from Fluka (Fluka Chemie, Buchs, Switzerland). Cysteine was from Merck (Merck, Darmstadt, Germany). $NaBH_4$, methylene blue and acetophenone were obtained from Fluka (Fluka Chemie, Buchs, Switzerland) whereas calcium carbonate and the 70% solution of hydrogen fluoride in pyridine (HF/pyr) were from Aldrich (Aldrich, Steinheim, Germany).

The 5R and 5S diastereomers of 5-hydroxy-5,6-dihydrothymidine and 5,6-dihydrothymidine were prepared by gamma radiolysis of a deaerated aqueous solution of thymidine [6] containing cysteine. The preparation of 5-formyl-2'-deoxyuridine was achieved by menadione mediated photooxidation of thymidine. 5-Hydroxymethyl-2'-deoxyuridine was prepared by $NaBH_4$ reduction of 5-formyl-2'-deoxyuridine [7]. 5-Formyluracil was obtained by acidic hydrolysis of the corresponding nucleoside.

Photosensitization of thymidine

20 mg of thymidine were dissolved in 30 ml of a 0.01 M aqueous solution of acetophenone. Then, the solution was irradiated for 6 hours in quartz vessels (Φ=6cm) in a Rayonet photoreactor (The Southern New

England Ultraviolet Company, Handem, MA, USA) equipped with sixteen 15 W lamps emitting at 300 nm. After irradiation, the solution was concentrated *in vacuo* to 2 ml. Then, 500 µl of the resulting solution were injected on a home packed (300x7.5 mm I.D.) ODS column with a C_{18} (10 µm mean pores size) Nucleosil phase (Macherey-Nagel, Düren, Germany). The separation was achieved by using 10 % of methanol in 0.025 M ammonium formate. 5 Fractions were collected as detected by UV absorption at 230 nm. Fractions 1, 2 and 3 were analyzed on the NH_2 amino silica gel column. The isocratic eluent was a [93:7] mixture of acetonitrile and 25 mM ammonium formate solution (conditions C). The detection was provided by a UV spectrophotometer set at 230 nm.

Hydrolysis of (6-4) photoproducts

The TpT, dCpT and TpdC (6-4) photoproducts were prepared by 254 nm irradiation of the corresponding dinucleoside monophosphates. They were purified by reverse phase HPLC on an octadecylsilyl silica gel column in the ion suppression mode [19]. Each of the (6-4) photoproducts (1 mg) was freeze-dried and dissolved in 1 ml of HF/pyr. The resulting solution was heated at 37°C for one hour. Then, the hydrolyzed samples were freeze-dried after neutralization of the acidic solution by addition of approximatly 2 g of calcium carbonate in 1 ml of water.

Calf thymus DNA (50 µg) in aqueous solution was irradiated for 3 minutes with a 15 W VL 15G germicidal lamp (Bioblock, Illkirch, France) emitting at 254 nm. After irradiation, the DNA solution was freeze-dried and hydrolyzed by HF/pyr as previously described for the (6-4) photoproducts (*vide supra*).

The freeze-dried samples of hydrolyzed dinucleoside monophosphates (6-4) adduct and far-UV irradiated DNA were dissolved in a [90 : 10] mixture of acetonitrile and water prior to HPLC purification on the amino silica gel column. The isocratic eluent was a mixture of acetonitrile and 0.025 M ammonium formate aqueous solution [90 : 10] (conditions A). The detection was achieved by using a F1050 spectrofluorimeter (Hitachi, Tokyo, Japan) with the excitation and emission wavelengths set at 310 and 380 nm, respectively.

TABLE 1

Retention time (t_1) and capacity factor (k') of the normal nucleobases and the corresponding 2'-deoxy- and ribonucleosides on the NH2 column (conditions A)

t_1/k'*	bases	2'-deoxyribonucleosides	ribonucleoside
Thymine	4.8 / **0.6**	5.6 / **0.9**	6.6 / **1.2**
Adenine	8.0 / **1.7**	7.2 / **1.4**	8.2 / **1.7**
Cytosine	12.2 / **3.1**	13.8 / **3.6**	18.2 / **5.1**
Guanine	n.d.	15.4 / **4.2**	20.2 / **5.7**
Uracil	4.9 / **0.6**	5.8 / **0.9**	7.3 / **1.4**
Hypoxanthine	9.2 / **2.0**	10.4 / **2.5**	13.4 / **3.5**
m^5Cyt	12.1 / **3.0**	13.2 / **3.4**	n.d.

* $k' = (t_1 - t_0)/t_0$ with t_0 = 3 min

Photosensitization of 2'-deoxyguanosine

10 ml of a 1 mM 2'-deoxyguanosine aqueous solution were irradiated for one hour in the presence of 0.5 mM methylene blue. The irradiation system consisted of a 100 W tungsten lamp fitted with a heat filter (10 mm, circulating water) and a 590 nm cut-off filter no. 23 A (Eastman Kodak Company, N.Y., USA). After irradiation, the solutions were evaporated to dryness and the resulting residue was dissolved in 200 µl of HPLC buffer prior to chromatographic analysis. The UV detector was set at 230 nm and the mobile phase was constituted by 25 % of an aqueous solution of ammonium formate and 75 % of acetonitrile (conditions B).

RESULTS AND DISCUSSION

Separation of the normal nucleosides

The retention times and the capacity factors of a series of normal nucleobases and the corresponding (ribo- and 2'-deoxyribo-)

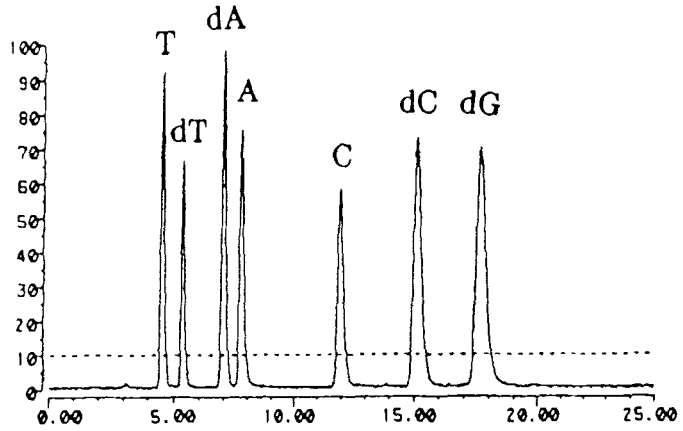

FIGURE 1 : Separation of the 2'-deoxyribonucleosides dT, dA, dC, dG and the corresponding bases T, A, C (G is not enough soluble) on a NH_2 silica gel column (conditions A).

nucleosides on the NH_2 column eluted with 10 % of ammonium formate in acetonitrile (conditions A) are listed in Table 1. The elution profile of the normal nucleobases (with the exception of guanine) and the corresponding 2'-deoxyribonucleosides obtained in the same conditions (UV detector set at 260 nm) is shown in Figure 1.

The nucleobases were found to be eluted in the following decreasing order : thymine > uracil > adenine >> 5-methylcytosine > cytosine. Similar order of elution is observed for the corresponding ribo- and 2'-deoxyribonucleosides (Table 1). This contrasts with the chromatographic behaviour of these nucleic acids components on the ODS column. Under the latter conditions, the pyrimidine nucleosides (dC and dT) are more rapidly eluted than the purines (dG and dA).

It is interesting to note that complementary bases exhibit closely retention times on the NH_2 column. dT and dA which may undergo base pairing through two hydrogen bonds in DNA are eluted more rapidly than dC and dG which are involved in three hydrogen bonds. Therefore, it is reasonable to suggest that the capacity factor of a compound on the NH_2 column (under the present conditions) depends on its ability to

TABLE 2

Influence of the increasing aqueous buffer content of the eluent on the retention time and capacity factor of 2'-deoxyguanosine (dG) on the NH_2 silica gel column

%	10	15	20	25
t_1 - dG	15.4	8.8	6.7	5.6
k' - dG	4.2	1.9	1.2	0.9

induce hydrogen bonds. Further support for this hypothesis was provided by the observed retention times of the ribonucleosides which are longer than those of the corresponding 2'-deoxyribonucleosides. Moreover, retention times of the free bases are generally shorter than those of the corresponding nucleosides (Figure 1, Table 1).

It should also be added that the presence of an apolar methyl group leads to a decrease in the retention time of the pyrimidine derivatives. Thus uracil derivatives are more retained than thymine derivatives, and 5-methylcytosine is eluted faster than cytosine. In addition, it was shown that adenine is well separated from its less polar deamination product, hypoxanthine (Table 1).

Influence of the proportion of aqueous buffer

Attempts were made to determine the effect of water content of the eluent on the chromatographic behaviour of 2'-deoxyguanosine. The retention times (t_1) and the capacity factors (k'), obtained for a percentage of water comprised between 10 and 25%, are listed in Table 2. As expected, the retention time of the nucleoside decreases when the percentage of the aqueous buffer of the mobile phase increases. In addition, a linear relationship between log(k') *versus* the ammonium formate percentage was obtained with a correlation coefficient close to 0.98. It is worth mentioning that the chromatographic system is rapidly equilibrated after modifying the ammonium formate percentage. In addition, it should be noted that a gradient can be easily used in order to separate mixtures of compounds with large differences in polarity.

TABLE 3

Retention times of the photooxidation products of 2'-deoxyguanosine (dG) and 3',5'-di-O-acetyl-2'-deoxyguanosine (diOAc-dG) on the NH_2 column (conditions B)

	nucleoside	type I (1)	type II (2, 3)
dG	5.6	9.3	17.9 - 19.2
diOAc-dG	3.9	4.9	7.6 - 8.1

2'-deoxyguanosine photooxidation products

The NH_2 substituted silica gel column was successfully used in order to separate the photooxidation products of 2'-deoxyguanosine (**1**) as well as its 3',5'-di-O-acetylated derivative (**1a**). The retention times of type I (**1** or **1a**) and type II photooxidation products (**2**, **3** or **2a**, **3a**) of the nucleoside, acetylated or not, are listed in Table 3. As expected from polarity consideration, retention times of the acetylated products are shorter than those of the corresponding nucleosides. Protection of the hydroxyl groups of the 2-deoxy-ß-D-erythropentofuranosyl moiety makes the compound less polar and thus leads to a decrease in its retention time. This applies as well to the photooxidation products of 2'-deoxyguanosine (dG).

The separation of the three main photooxidation products of 2'-deoxyguanosine is illustrated in Figure 2 [8,9]. Products **1**, **2** and **3** are more polar and consequently are more retained than the starting nucleoside dG (Table 3). It is important to note that the two $4R^*$ and $4S^*$ diastereoisomeric photoproducts **2** and **3**, arising from type II mechanism (singlet oxygen oxidation) are separated under these conditions. On the other hand, the two type II photoproducts are eluted together in the void volume of the ODS column, followed by the main type I photoproduct and 2'-deoxyguanosine.

The use of the NH_2 substituted silica gel column with 25 % of ammonium formate in acetonitrile as the eluent, allowed us to isolate both the three main oxidation products of 2'-deoxyguanosine and their

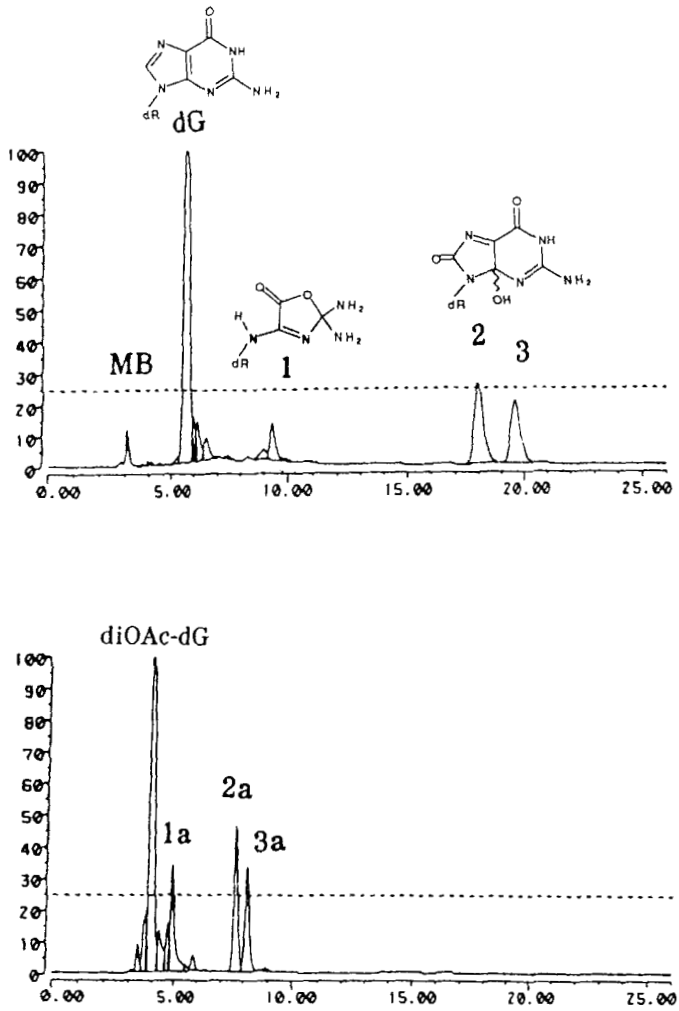

FIGURE 2 : HPLC separation of 2'-deoxyguanosine (dG) and 3',5'-di-O-acetyl-2'-deoxyguanosine (diOAc-dG) photooxidation products on an amino silica gel column (Conditions B, UV detection set at 230 nm 0.01 mv F.S.).

TABLE 4

Retention times and capacity factors of modified products of uracil, thymine and thymidine (conditions A)

Compound	t_1 (min)	k'
5-hydroxyuracil	5.58	0.86
5-formyluracil	6.54	1.18
5-hydroxy-5,6-dihydrothymine	4.22	0.41
5-formyl-2'-deoxyuridine	6.54	1.18
5-hydroxymethyl-2'-deoxyuridine	6.57	1.19
(5S) 5-hydroxy-5,6-dihydrothymidine	5.96	0.99
(5R) 5-hydroxy-5,6-dihydrothymidine	5.82	0.94

corresponding 3',5'-di-O-acetylated derivatives. It is interesting to note that under these chromatographic conditions, the sensitizer is eluted in the void volume of the column. This makes possible the analysis of the photooxidized solution without preliminary purification of the mixture. In contrast, analysis of the photooxidized solution of dG on the ODS column requires the removal of the dye prior to the HPLC separation. Futhermore, the HPLC separation on the amino substituted silica gel column allows the use of a volatile buffer, which could be easily eliminated by lyophilisation.

Radiation-induced decomposition products of pyrimidine bases and nucleosides

Several radiation-induced decomposition products of thymine, uracil and thymidine were separated on the amino silica gel column. Their retention times and capacity factors are listed in Table 4.

All oxidation products were more retained than the corresponding normal bases and nucleosides (Table 1 and 4). This can be explained by the increase in the polarity of the modified compound due either to the

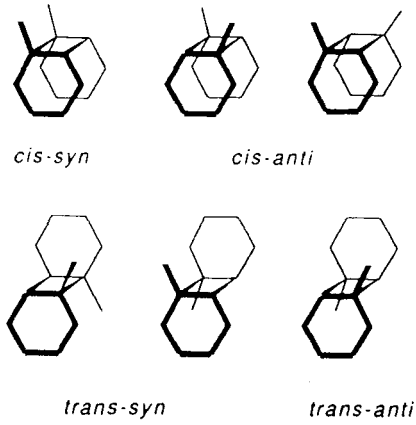

FIGURE 3 : The structure of the thymidine cyclobutane dimers is first related to the position of the two bases moieties relatively to the cyclobutane ring. If they are on a same side, the compound is cis. If they are on opposite sides, the isomer is trans. The relative orientation of the pyrimidine rings must also be taken into account. If the two C5 atoms (and the two C6) are linked to each other, the compound is syn. In the other case, it is anti. It is worth mentioning that the cis-syn and trans-anti isomers are meso diastereomers, whereas both trans-syn and cis-anti are resolved into two pairs of (+) and (-) diastereomers.

saturation of the C5-C6 double bond or to the presence of polar groups such as formyl or hydroxymethyl. It is worth mentioning that 5-hydroxymethyluracil was separated from uracil on the amino silica gel column whereas these two bases exhibit similar retention times on an ODS column. It should also be noted that the 5R and 5S diastereomers of 5-hydroxy-5,6-dihydrothymidine were separated by using the former chromatographic system.

Separation of pyrimidine adducts

The study of the physical and chemical properties of dipyrimidine photoproducts [10], including cyclobutadipyrimidines and pyrimidine

TABLE 5

Retention times of diastereomeric thymidine cyclobutane dimers on the C_{18} and NH_2 silica gel column (conditions C).

isomer	t_1 C_{18} (min)	t_1 NH_2 (min)
cis-syn	11.2	12.8
(-) trans-syn	12.4	12.0
(-) cis-anti	12.4	17.2
(+) trans-syn	14.6	12.0
(+) cis-anti	14.6	15.8
trans-anti	23.6	not determined

(6-4) pyrimidone adducts, has required the use of model compounds, including bases [11], nucleosides [12] and dinucleoside monophosphates [13]. In this respect, HPLC was found to be a major tool for the isolation of the different photoproducts generated by UV irradiation of these molecules. In particular, the amino column was successfully used for the separation of the diastereomers of thymidine cyclobutane dimers and for the resolution of the mixture of (6-4) adducts obtained by mild acidic hydrolysis of far UV irradiated DNA and dinucleoside monophosphates.

Isolation of thymidine cyclobutane dimers

Far-UV irradiation of thymidine was reported to predominantly generate cyclobutane type dimers [14] as the result of a [2+2] cycloaddition involving a triplet excited pyrimidine base. Similarly, photosensitization of thymidine by acetophenone upon exposure to 300 nm light [15] provided the six possible diastereomers (Figure 3). The resolution of the crude irradiated solution, which contained a mixture of these six photoproducts together with unmodified thymidine, was achieved by using two successive chromatographic systems, including the use of the ODS and an amino silica gel columns, respectively (Table 5).

The HPLC elution profile of the mixture obtained on the ODS column exhibited five major peaks (Figure 4a). The corresponding product(s) of the collected fractions were analyzed by 400 MHz ^{1}H NMR. The products corresponding to peaks **1**, **4** and **5** were identified as the cis-syn dimer, thymidine and the trans-anti cyclobutadithymidine respectively, by comparison of their ^{1}H NMR features with those of authentic compounds [14]. Fractions **2** and **3** were found to be a mixture of two compounds as inferred from ^{1}H NMR measurements.

A complete separation of the two compounds of each of the HPLC fractions **2** and **3** was achieved on the amino silica gel column (Figures 4b and 4c). The (-) trans-syn and (+) cis-anti cyclobutane dimers were isolated from fraction **2**, whereas fraction **3** provided the (+) trans-syn and (+) cis-anti diastereomers. It is worth mentioning that the elution profile of compound **1** only exhibited one peak, which was shown to be homogeneous (Figure 4d).

The analysis of the retention times listed in Table 5, provides evidence that the nucleic acid dimers behave differently on the two HPLC columns. The (-) (and the (+)) diastereomers are eluted together on the ODS column irrespective of the configuration of the compounds. On the other hand, the two trans-syn (and the two cis-anti) diastereomers exhibited a similar chromatographic behaviour on the amino silica gel column. It may be concluded that the latter analytical system is less sensitive to diastereoisomeric factors than the ODS column separation.

Pyrimidine (6-4) pyrimidone photoproducts

(6-4) photoproducts may be obtained in good yield by far-UV irradiation of dinucleoside monophosphates in aqueous solution [16-18]. The (6-4) photoproducts of TpT, TpdC and dCpT were hydrolyzed by using hydrogen fluoride stabilized in pyridine [19]. Each of the photoproducts was quantitatively converted into one compound that was isolated on the amino column (Table 6). The three hydrolyzed products were characterized as the corresponding base (6-4) adducts.

Separation of the pyrimidine (6-4) adducts

The mixture of three (6-4) adducts including the thymine-thymine, thymine-cytosine and cytosine-thymine (6-4) photoproducts, was well

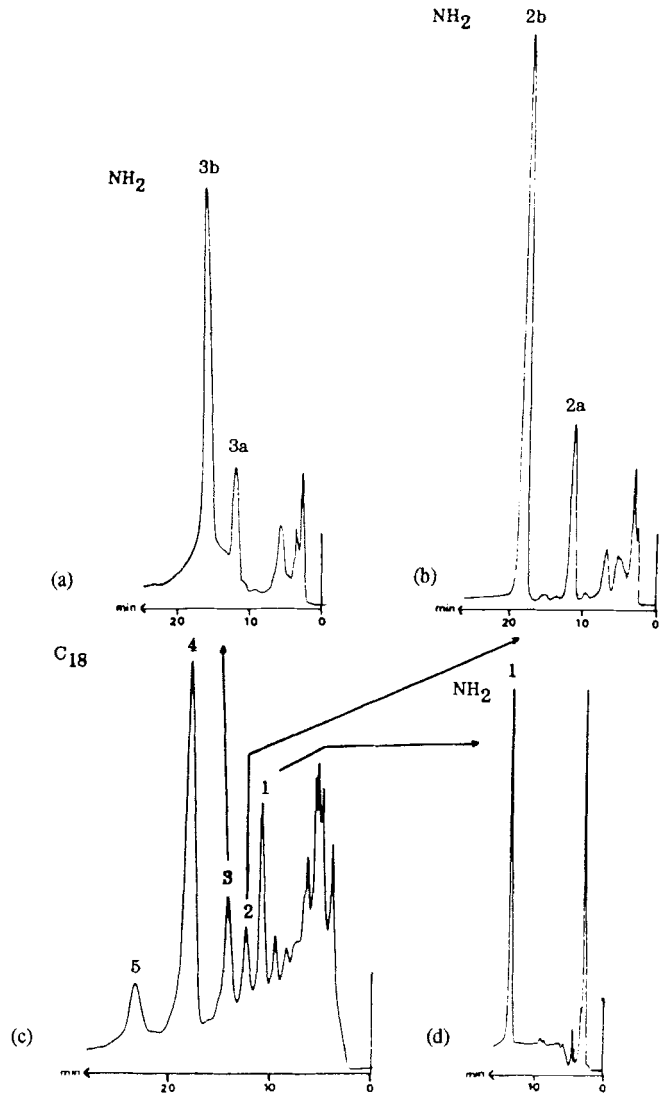

FIGURE 4 : Separations of thymidine cyclobutane dimers. 4a : octadecylsilyl silica gel column (ODS); 4b,c,d : amino silica gel column (HPLC conditions are described in the text).

TABLE 6

Retention times and capacity factors of the base (6-4) photoproducts and partially hydrolyzed thymine-thymine (6-4) adducts on the NH_2 silica gel column (conditions A).

(6-4) photoproduct	t_1 (min)	k'
5-amino-6-4'-(pyrimidin-2'-one)-5,6-dihydrothymine	22.8	4.7
5-hydroxy-6-4'-(5-methylpyrimidin-2'-one)-5,6-dihydrothymine	18.7	3.8
5-hydroxy-6-4'-(pyrimidin-2'-one)-5,6-dihydrothymine	14.9	2.7
5-hydroxy-6-4'-[1-(2-deoxy-α-D-erythropentofuranosyl) 5-methylpyrimidin-2'-one]-5,6-dihydrothymine	22.2	4.6
5-hydroxy-6-4'-[1-(2-deoxy-α-D-erythropentopyranosyl) 5-methylpyrimidin-2'-one]-5,6-dihydrothymine	23.5	4.9

resolved on the amino silica gel column by using fluorescence as a sensitive and specific method of detection. The elution profile of hydrolyzed far-UV irradiated DNA exhibited the presence of the same peaks (Figure 5). It is worth mentioning that the cytosine containing photoadducts are more retained than 5-hydroxy-6-4'-(5-methylpyrimidin-2'-one)-5,6-dihydrothymine. The HPLC-fluorescence detection assay appears to be an excellent method for monitoring this major class of photoproducts in DNA. It should be mentioned that the photoproducts are eluted too rapidly to be efficiently separated on the ODS silica gel column.

- Partially hydrolyzed compounds

Mild HF/pyridine hydrolysis of the TpT (6-4) photoproduct performed at 0°C for 10 minutes allowed the isolation of two main intermediate compounds. The compounds were characterized as thymine-thymine (6-4) adducts with a 2-deoxy-α-D-erythropentosyl unit linked to the 5'-end unit. The deoxyriboside moieties were found to have a α-furanosidic and α-pyranosidic configuration, respectively.

As already observed for the normal bases, the presence of a sugar moiety increases the retention time of the thymine-thymine

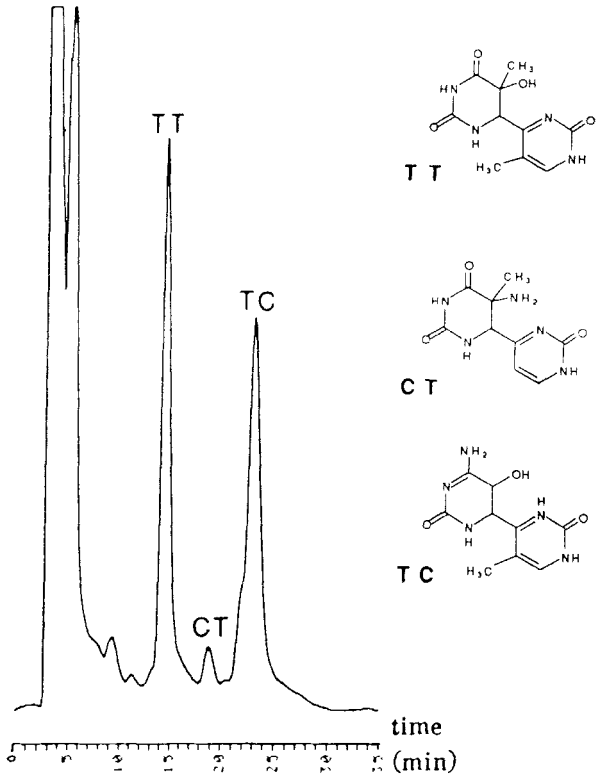

FIGURE 5 : Elution of the acidic hydrolyzalt of far-UV irradiated DNA on an amino silica gel column. (50 µg, UV dose 254 nm : 1 kJ/m^2) (conditions A).

(6-4) photoproduct on the amino silica gel column. In addition, the nucleosidic compounds were separated depending on the configuration of the deoxyribosidic residue. 5-hydroxy-6-4'-[1-(2-deoxy-α-D-erythropentofuranosyl)-5-methylpyrimidin-2'-one]-5,6-dihydrothymine was eluted faster than 5-hydroxy-6-4'-[1-(2-deoxy-α-D-erythropentopyranosyl)-5-methylpyrimidin-2'-one]-5,6-dihydrothymine.

CONCLUSION

The use of the amino substituted silica gel column was found to be an efficient tool for the separation of various normal and modified nucleobases and nucleosides. The method appears to be complementary to more conventional reverse phase HPLC techniques for the resolution of complex mixtures of modified bases, nucleosides and dinucleoside monophosphates. Work is in progress to combine mass spectrometry detection with amino silica gel column HPLC separations for the search of DNA oxidation products in biological fluids [20,21].

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