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CAPILLARY GAS CHROMATOGRAPHY OF PYRIMIDINES AND PURINES: N,O-PERALKYL AND TRIFLUOROACETYL-N,O-ALKYL DERIVATIVES

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SUMMARY

Preparation and capillary gas chromatographic properties of volatile derivatives of eighteen pyrimidine and purine nucleic acid bases are described. N,O-peralkylation using methylsulfinyl carbanion, methyl or ethyl iodide reagent, and alkylation preceded by N-trifluoroacetylation produced derivatives having minimal adsorption and tailing compared with trimethylsilyl derivatives. Relative retention times and linearity of flame ionization or nitrogen—phosphorus detector response were measured using polar (Superox-FA) and apolar (SE-30) liquid phases. Application of gas chromatography—mass spectrometry to derivatives of DNA hydrolysates using mass chromatography is demonstrated.

INTRODUCTION

The nucleic acid bases play major roles in the chemical and biological characteristics of nucleic acids. The detection and quantification of chemically or biologically modified bases are of importance in a number of areas, including studies of carcinogenesis and the structure and function of transfer RNA. Analytical approaches based on gas chromatography (GC) [1,2] are often appropriate due to favorable sensitivity, structural selectivity, and the complementary use of gas chromatography—mass spectrometry (GC—MS) [3,4].

The most common procedure for analysis of bases using GC requires conversion of the free base to the corresponding trimethylsilyl (TMS) derivative [2], and detailed studies aimed at defining optimum conditions for these reactions have been reported [5—7]. Procedures for methylation of bases using sodium methoxide—methyl iodide [8], diazomethane or on-column methods [9,10], methylsulfinyl carbanion—methyl iodide [11], or other techniques [12] have been reported, some of which were used for assays of 5-fluorouracil [10, 11,

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13]. Most GC analyses have been made using short packed glass columns, while limited use of glass capillary columns have been reported more recently [4,14—17].

Increased demands for improved detection limits and selectivity in analysis of nucleic acid hydrolysates prompted us to use glass capillary columns for chromatographing the TMS derivatives of a variety of bases. Initial attempts using commercially prepared, wall-coated and support-coated open tubular glass columns produced poor results because of high levels of sample adsorption by the column. Several column deactivation techniques reported in the literature [18—20] were used to prepare in our laboratory columns of exceptionally low activity. Although these columns were an improvement over those available commercially, they still exhibit substantial reactivity toward the derivatized bases. Commercially prepared fused silica columns have now been tested and show the least adsorption. These columns are, however, still unable to satisfactorily elute very small quantities of TMS derivatives of purine bases. Fig. 1 shows a chromatogram of the TMS derivatives of approximately 100 ng each of the common nucleic acid bases, separated on a 12-m fused silica column coated with SE-30. The quality of this chromatogram is high, based on our experience with this particular separation, yet it shows tailing and baseline humps which are typically observed at the base of peaks from cytosine, adenine and guanine derivatives.

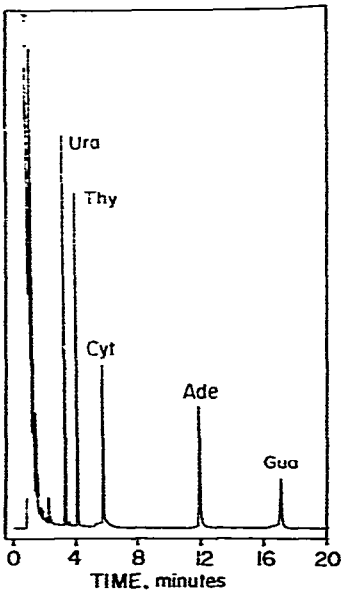

Fig. 1. Chromatogram of ca. 100 ng each of TMS derivatives of uracil (Ura), thymine (Thy), cytosine (Cyt), adenine (Ade), and guanine (Gua), using 12-m SE-30 fused silica column and flame ionization detection.

Another possible limitation of the use of TMS derivatives for analysis of DNA hydrolysates is inadequate resolution of cytosine from 5-methylcytosine [21], the principal modified base in DNA.

Since we were unable to find capillary columns (either commercial or home made; glass or fused silica) which satisfactorily elute small quantities of silylated bases, we have developed a procedure for preparation of N,O-peralkyl or trifluoroacetyl-alkyl derivatives. It will be shown that these derivatives exhibit excellent chromatographic properties on both polar and apolar capillary columns, and provide linear detector response over the range 0.2—20 ng per injection. Flame ionization, nitrogen—phosphorus and MS detection were used.

A study of the mass spectra of these derivatives is reported in a separate publication [22].

EXPERIMENTAL

Materials

Purine and pyrimidine bases were obtained from commercial sources: adenine, 5-fluorouracil, N^6,N^6-dimethyladenine, and uracil (Calbiochem-Behring, La Jolla, CA, U.S.A.); guanine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 1-methyladenine, N^6-methyladenine, 5-methylcytosine, orotic acid, 2-thiouracil, and thymine (Sigma, St. Louis, MO, U.S.A.); cytosine, N^2,N^2-dimethylguanine, 5-fluorocytosine, N^2-methylguanine, and 7-methylguanine (Vega Biochemicals, Tucson, AZ, U.S.A.). Analytical grade dimethyl sulfoxide, cyclohexane, hydrochloric acid, formic acid, sodium hydride (50% oil suspension), ethyl acetate, and trifluoroacetic anhydride were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.); ethyl iodide, methylene chloride, diethyl ether, methanol, and sulfuric acid were obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.). Methylsulfinyl carbanion solution, 0.5 *M*, was prepared as reported previously [23]; the sodium hydride oil suspension was extracted several times with anhydrous diethyl ether just prior to use. The reagent was filtered and 0.4-ml portions were stored at −18°C.

N,O-Perethyl derivatives for group I compounds (1—12)

Up to 100 μg of purine or pyrimidine base, dissolved in 100 μl of dry dimethyl sulfoxide, were mixed with 20 μl of 0.5 *M* methylsulfinyl carbanion reagent; after 10 sec, 40 μl of ethyl iodide were added and the solution allowed to react for 1 h. Then, 3 ml of water were added and the reaction mixture extracted twice with 1.5 ml of cyclohexane—methylene chloride (9:1); the combined extracts were evaporated to dryness at 50°C under a stream of nitrogen.

N-Trifluoroacetyl-N,O-ethyl derivatives for group II compounds (13—18)

Stock solutions of these bases were made up in 0.06 *N* hydrochloric acid in methanol; aliquots containing up to 100 μg of product were dried in vacuo prior to derivatization. The residue was mixed with 100 μl of ethyl acetate and 70 μl of trifluoroacetic anhydride and heated for 3 min at 90°C. The reaction mixture was dried at 50°C under a nitrogen stream. The acylated base was subsequently ethylated, as described above. The reaction was terminated using 0.05 *N* sulfuric acid instead of water. The dry residues were in each case dissolved in ethyl acetate, and 20 ng or less of each derivative injected onto the capillary column.

Calf thymus DNA (Sigma) was hydrolyzed by heating with 88% formic acid for 2 h at 150°C in a sealed glass tube. Separate samples containing 10 μg of hydrolyzed DNA were dried and derivatized using both procedures described above. Aliquots representing 2% of the total hydrolysate were injected into the gas chromatograph, using the falling needle injector.

Gas—liquid chromatography

A Varian Model 3700 gas chromatograph, equipped for operation with capillary columns and with flame ionization and nitrogen—phosphorus-selective detectors was used for most of the chromatographic analyses. A fused silica column (12 m × 0.22 mm I.D.) coated with SE-30 was supplied by Hewlett-Packard (Avondale, PA, U.S.A.) and a whisker-type [24] glass column (10 m × 0.25 mm I.D.) coated with Superox-FA (*m*-nitroterephthalic acid-modified Superox-4), was obtained from Alltech Assoc. (Deerfield, IL, U.S.A.). Operating conditions were as follows: injector and detector temperatures 260 and 280°C, respectively; helium was used as a carrier gas at a linear velocity, $\bar{u}_0$ of 20 cm/sec. The thermionic detector was operated with air and hydrogen flow-rates of 175 and 4.5 ml/min, respectively; the bead current was set to obtain a background current of 10^{-10} A. Samples were injected through an inlet splitter (1:60 ratio) or with an all-glass falling needle injector (Alltech Assoc.). The fused silica columns were coupled to this device by means of a 1/4 to 1/16 in. reducing union, with the column end just below the resting point of the needle. A small glass insert (1.0 mm O.D., 0.5 mm I.D.) was used to center the column inside the injector.

Gas chromatography—mass spectrometry

Mass spectra were obtained with an LKB 9000S instrument interfaced to a DEC PDP-11/40 computer. The samples were introduced through the gas chromatographic inlet, fitted with a 1.8 m × 2.0 mm I.D. silanized glass column, packed with 1% OV-17 on 100—200 mesh Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.); helium was used as a carrier gas at a flow-rate of 30 ml/min. Injector, separator and ion source temperatures were 250, 270, and 290°C, respectively. Electron-impact mass spectra were recorded at an electron energy of 70 eV. For the analysis of DNA hydrolysates, an SE-30 coated fused silica column (12 m × 0.22 mm I.D.) was used. The separator was provided with make-up gas (helium, flow-rate 26 ml/min) [25] and samples were introduced with the falling needle injector. Data were acquired in the repetitive scanning mode (50—350 mass units) at a rate of 4 sec per scan.

RESULTS AND DISCUSSION

As a means of derivatization for GC, peralkylation provides a useful means of maximizing favorable chromatographic characteristics by complete replacement of active hydrogens. The use of a strong base such as methylsulfinyl carbanion assures hydrogen replacement for a wide structural variety of bases. This reagent has found use for a range of compound classes, including carbohydrates [26], peptides [27], and nucleosides [28]. When applied to bases in the present study, those listed in group I (see Fig. 2) formed satisfactory

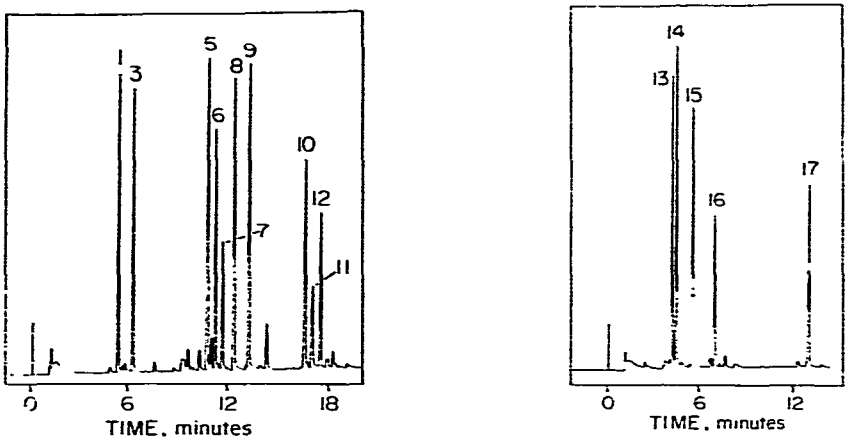

Fig. 2. N,O-Perethyl derivatives (group I) of uracil (1), thymine (3), 5-hydroxymethyluracil (5), N^6,N^6-dimethyladenine (6), orotic acid (7), N^6-methyladenine (8), adenine (9), N^2,N^2-dimethylguanine (10), 1-methyladenine (11), and N^2-methylguanine (12). Fused silica column, coated with SE-30; oven temperature 130°C, linearly programmed at 5°C/min, nitrogen-selective detection.

Fig. 3. N-Trifluoroacetyl-ethyl derivatives (group II) of 5-fluorocytosine (13), cytosine (14), 5-methylcytosine (15), 5-hydroxymethylcytosine (16), and guanine (17). Fused silica column, coated with SE-30; oven temperature 170°C, linearly programmed at 5°C/min, nitrogen-selective detection.

derivatives, while those in group II (see Fig. 3) required N-acylation prior to alkylation. Of the bases studied, adenine is unique among the members of group I because it cannot be derivatized using the group II procedure. The other members of group I remain unreacted during the acetylation step. It has been suggested that methylation of free amino groups may produce quaternized methyl iodide salts [23], a problem which is circumvented by acylating the amino group prior to alkylation. Although methylation can also be used, ethyl derivatives are preferred in order to distinguish bases which contain natural methyl groups. In addition, mass spectra of the ethyl derivatives [22] offer more detail for structural characterization and more variety for selection of masses for selected ion monitoring.

Typical chromatograms from group I and group II compounds obtained using a 12-m apolar fused silica capillary column are given in Figs. 2 and 3. It is evident that this procedure yields principally a single product for each component base and that these derivatives do not undergo significant reversible adsorption under these chromatographic conditions. This is in contrast to trimethylsilyl derivatives of the same compounds (cf. Fig. 1) which, except for uracil and thymine, show tailing and adsorption on the same column under similar conditions. The superior chromatographic properties of the peralkyl derivatives are probably because alkylation replaces both hydrogens on the exocyclic amino groups while silylation replaces only one under usual reaction conditions.

Diazomethane with the catalyst boron trifluoride etherate [29] was also examined as an alternative alkylation procedure to derivatize N^4-trifluoroacetyl-

cytosine. This method was rejected because it gave many structurally different products and because reagents such as diazoethane are not readily available.

Fig. 3 shows that the trifluoroacetyl-ethyl derivative of the modified base 5-methylcytosine elutes much later than cytosine. This is in contrast to the trimethylsilyl derivatives where 5-methylcytosine follows very closely after cytosine on the same apolar column. This feature should prove advantageous in the analysis of DNA hydrolysates where 5-methylcytosine may be present at levels below 1% relative to cytosine.

Since methylation of the base is the most common form of biological modification occurring in nucleic acids, the ethyl derivatives are generally more useful than their methyl analogues. The ability of the present analytical system to unambiguously deal with the problem may be illustrated with the series N^6,N^6-dimethyladenine (6), N^6-methyladenine (8), and adenine (9), in which ethyl groups are substituted for methyl groups on the exocyclic nitrogen. Fig. 2 shows that this relatively short, apolar capillary column easily separates these three substances.

Methylene unit (MU) values [30] of these derivatives were measured by co-chromatography with even-numbered *n*-alkanes on capillary columns coated with polar (Superox-FA) or apolar (SE-30) liquid phases. These data are given in Table I and represent the average of three replicate measurements, with a standard deviation of approximately 0.02 MU. In general, the SE-30 column elutes the alkylated bases between the temperatures of 130 and 220°C within

TABLE I

RELATIVE RETENTION TIME DATA FOR N,O-PERETHYL AND N-TRIFLUOROACETYL-ETHYL DERIVATIVES OF PYRIMIDINE AND PURINE BASES

Parent base	Mol. wt.	No. of C_2H_5 groups	No. of CF_3CO groups	MU values	
				SE-30	Superox-FA
Group I					
Uracil (1)	168	2	–	14.34	23.53
5-Fluorouracil (2)	186	2	–	14.40	24.00
Thymine (3)	182	2	–	14.75	23.22
2-Thiouracil (4)	184	2	–	14.80	22.92
5-Hydroxymethyluracil (5)	226	3	–	17.01	24.99
N^6,N^6-Dimethyladenine (6)	191	1	–	17.25	26.94
Orotic acid (7)	240	3	–	17.46	25.34
N^6-Methyladenine (8)	205	2	–	17.78	26.92
Adenine (9)	219	3	–	18.24	26.82
N^2,N^2-Dimethylguanine (10)	235	2	–	19.88	30.73
1-Methyladenine (11)	205	2	–	20.08	34.74
N^2-Methylguanine (12)	249	3	–	20.31	30.71
Group II					
5-Fluorocytosine (13)	281	2	1	16.97	27.33
Cytosine (14)	263	2	1	17.13	27.17
5-Methylcytosine (15)	277	2	1	17.94	28.29
5-Hydroxymethylcytosine (16)	321	3	1	18.96	28.81
Guanine (17)	331	3	1	22.33	35.72
7-Methylguanine (18)	317	2	1	22.30	37.75

20 min, while the Superox-FA column elutes the same compounds 8—13 MU higher. The change in MU values for all of these bases studied on these two liquid phases is very large and is useful in the identification of unknowns. For most analyses we prefer to use the SE-30 column because of the lower elution temperatures. As is evident from Table I, the reversal of elution order between the two columns may be useful for qualitative identification of bases from their relative retention times.

The number of functional groups in each case was determined from mass spectrometric data and is presented in Table I. These data also show that alkylation occurs only on nitrogen atoms except for those bases which contain free hydroxyl (5, 16) or carboxylic acid (7) groups. Acylation attaches one trifluoroacetyl group to the exocyclic nitrogen in the cytosine and guanine series of bases. Attempts to substitute a trifluoroacetyl group at the exocyclic nitrogen of adenine were unsuccessful. A description of structural and MS fragmentation properties of these derivatives will be published elsewhere [22].

Determination of the relative amounts of modified and unmodified forms of base is often an objective in the analysis of nucleic acid hydrolysates. The quantitative performance of the present derivatization procedure was tested using N^6-methyladenine and 5-methylcytosine where these compounds are present over the range from 1—10% of adenine and cytosine in DNA. Adenine and cytosine were present at 1 μg prior to derivatization. These experiments were performed using the SE-30 capillary column, the nitrogen—phosphorus detector, and the falling needle injector system. The GC oven temperature was isothermal at 170°C and the retention times approximately 7 min. Peak height ratios of N^6-methyladenine to adenine and of 5-methylcytosine to cytosine were plotted against the respective quantities of these substances present in the original samples. Linear calibration curves were obtained for both cases: $y = 0.004x + 0.001$, $r = 0.998$ and $y = 0.010x - 0.004$, $r = 0.998$, respectively, when plotted as peak height ratios vs. weight ratios. These results demonstrate satisfactory performance of the entire analytical procedure over the range studied. In particular, sample adsorption by the column is not significant even in the sub-nanogram range. We have been unable to satisfactorily elute trimethylsilyl derivatives of these bases at low levels using the same capillary column.

When using the falling needle injector, the entire sample less the solvent is placed on the column, a factor of potential importance for quantitative measurements [31]. This system is particularly useful where only a small amount of nucleic acid is available for analysis. The nitrogen—phosphorus detector typically has a much lower detection limit than the flame ionization detector because of its selective ionization of compounds containing nitrogen or phosphorus, and because of its inherently higher sensitivity, The detection limit for both N^6-methyladenine and 5-methylcytosine is about 10^{-10} g (signal-to-noise ratio > 10). Similar measurements show that the sensitivity of the nitrogen—phosphorus detector is about 30 times greater than that of the flame ionization detector. The trifluoroacetyl derivatives are potentially useful for high sensitivity measurements using an electron-capture detector or negative ion chemical ionization MS.

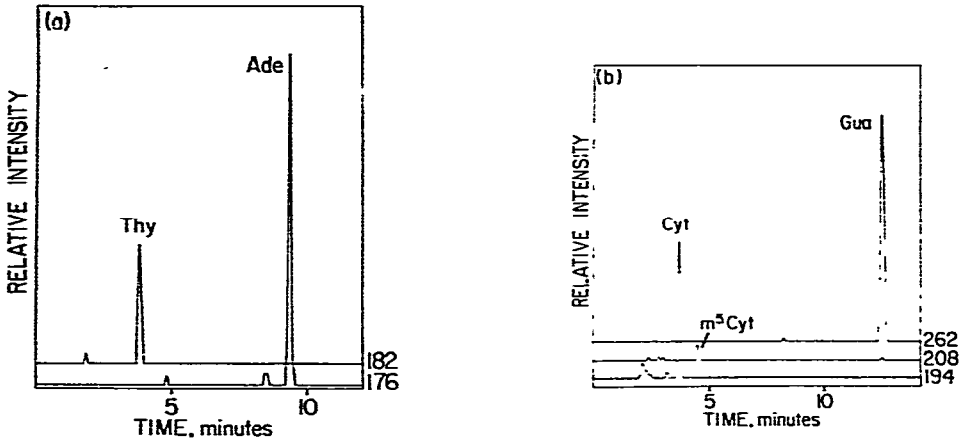

Fig. 4. Selected ion recordings (mass chromatograms) of a calf thymus DNA hydrolysate, analyzed on a fused silica column, coated with SE-30. Oven temperature 100°C, linearly programmed at 4°C/min. (a) N,O-Perethyl derivatives, *m/z* 182 thymine (3), *m/z* 176 adenine (9); (b) N-trifluoroacetyl-ethyl derivatives, *m/z* 262 guanine (17), *m/z* 208 5-methylcytosine (15), *m/z* 194 cytosine (14).

These derivatives are also potentially useful for analysis of nucleic acid hydrolysates by GC—MS. Fig. 4 shows a selected ion recording of a derivatized hydrolysate of calf thymus DNA, with data acquired in the repetitive scanning (mass chromatography) mode. The quantity represented corresponds to 200 ng of DNA, or approximately 80 ng of free bases. The ions chosen for display were selected from mass spectra of each derivative [22]. Although use of a calibration curve is preferable, a crude estimate of quantities can be calculated after correction for percentage of total ion current carried by each selected ion. In this fashion the 5-methylcytosine content is estimated as 11% of cytosine compared to literature reports of 5—9% [32], while guanine/cytosine and thymine/adenine ratios are approximately 0.80 and 0.97, respectively, suggesting a uniform and high yield in hydrolysis and derivatization. Further experiments based on internal calibration techniques and the more sensitive and accurate method of selected ion monitoring are in progress.

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