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SEPARATION OF SYNTHETIC CYCLOALKYLATED BASES, NUCLEOSIDES AND NUCLEOTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography was used to determine the elution profiles of a series of synthetic cycloalkylated bases, nucleosides, and their corresponding 5′-monophosphates. A 70% aqueous methanol solution proved to be the most efficient solvent system for the separation of a mixture of the bases, all of which were eluted in times ranging from 3.3 to 4.8 min at a flow-rate of 0.8 ml/min. Subsequently, the same percentage of methanol solvent, at 0.8 ml/min, eluted the nucleoside mixture as well, with retention times ranging from 3.3 to 5.0 min. Optimum separation and resolution were achieved with 70% methanol at a flow-rate of 0.6 ml/min for a mixture of the base and nucleoside series. A phosphate buffer, containing acetonitrile–tetrabutylammonium ion, was used to analyze the 5′-monophosphate derivatives. Elution times ranged from 2.6 to 6.1 min at a flow-rate of 1.0 ml/min. Three variables were considered in order to determine optimum conditions for separation and resolution: (a) the percentage of methanol in the solvent; (b) flow-rate of solvent; and (c) the size of the cycloalkylated group of each synthetic analogue. The procedures and conditions described herein have potential use as a monitoring system to detect modified nucleic acid derivatives which are prevalent in the body fluids of patients with certain metabolic disorders.

INTRODUCTION

Accurate determination of the effects of modified bases, nucleosides, and nucleotides upon biological systems is of major importance. Modified nucleic acid derivatives are present in both DNA and RNA (transfer, ribosomal, and messenger). Particular interest has been given to modification(s) of the major nucleosides of tRNAs, since it is known that the proportion of modified nucleosides is much higher in tRNA molecules than in any of the other nucleic acid species.

Extensive studies have been carried out, utilizing chromatographic techniques, in efforts to separate specific modified nucleosides. Modified nucleosides are found

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in the urine of both normal and cancerous laboratory animals and humans. Since there is no apparent mechanism for the reincorporation of these modified nucleosides into tRNA molecules, their levels in urine may reflect the extent of tRNA modification, as well as the turnover rate of specific tRNA molecules. The quantitation of these nucleosides could indicate changes in the tRNA profile during differentiation or tumor induction. High-performance liquid chromatography (HPLC) has been utilized as a reliable method, for the quantitative analysis of nucleosides, which aids in the search for biological cancer markers [1].

Studies involving purine salvage enzymes and de novo purine biosynthesis have demonstrated that hypoxanthine–guanine phosphoribosyltransferase (hg-prt) cells may have altered regulation of the de novo purine pathway. Individuals suffering from hg-prt deficiency (Lesch-Nyhan syndrome), have elevated levels of uric acid. HPLC has been utilized to quantitatively determine changes that might occur in purine nucleotide pools in cells grown under different conditions [2]. Other studies used HPLC to study levels of oxypurines (hypoxanthine and xanthine) in biological fluids such as plasma, urine, and erythrocytes of xanthinuria and hyperuricemic patients [3–5]. In addition, many other studies involved determining optimum conditions for the separation of nucleic acid derivatives utilizing HPLC [6–10].

The alteration of bases, as well as damage of DNA due to deletorious agents, is also an area of concern. Recent studies, employing analysis methods other than chromatography, have shown that synthetic oligo- and polynucleotides containing a modified nucleoside may be utilized to study point mutation in the translation of messenger RNAs, measurement of codon–anticodon interaction and hybridization of polynucleotides [11,12].

The focus of this investigation was to develop a method for separating synthetic cycloalkylated bases, nucleosides, and nucleotides, that may be utilized as a model for the separation of naturally occurring modified derivatives found in tRNA molecules, as well as in other nucleic acid species. This method of separation may aid in sequencing derivatives of DNA and RNA. It also has potential use as a monitoring system for markers to detect diseased states, in which degraded nucleic acid derivatives are present in various body fluids.

EXPERIMENTAL

Apparatus

A Beckman Model 331 isocratic HPLC system (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with a UV 160 absorbance detector with a fixed wavelength at 254 nm was used.

Chromatographic conditions

A Beckman Ultrasphere ODS pre-packed, reversed-phase column (25 cm × 4.6 mm I.D.) equipped with a guard column (4.5 cm × 4.6 mm I.D.) was used (Beckman Instruments). The operating pressure varied between 13.8 MPa and 20.7 MPa and the elution profiles were recorded at a chart speed of 2.51 cm/min, providing retention times with a standard deviation of 0.02.

Chemicals

HPLC-grade methanol, water, and acetonitrile, used to prepare the solvent systems, were obtained from Fisher Scientific (Norcross, GA, U.S.A.) and were filtered through a 0.2-μm membrane before use.

The percentages of the methanol–water solutions used were 60 and 70%. The phosphate buffer contained 19% acetonitrile, 0.03 *M* potassium dihydrogenphosphate, and 0.01 *M* tetrabutylammonium ion (pH 2.7).

The compounds used as standards, adenine, adenosine, and adenosine-5′-monophosphate, were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Standard procedures were utilized to synthesize the modified bases, nucleosides [13] and ribonucleoside-5′-monophosphates [14] (Table I).

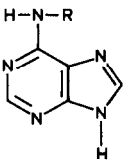
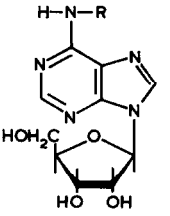
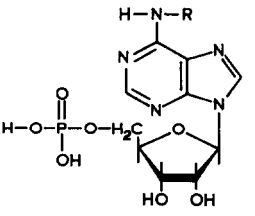
Cell line

A subclone (HEPA-2) of BW-7756 mouse hepatoma cells was used in these studies. The cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with 3.5 g/l glucose, 3.7 g/l bicarbonate, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified

TABLE I

STRUCTURES OF SYNTHETIC N^6-CYCLOALKYLATED NUCLEIC ACID DERIVATIVES

+ = Derivatives used in the study; − = derivatives not used in the study.

Parent compound	Structure	Cycloalkyl group (R)			
					2-NH_2, 
Base		+	+	+	−
Nucleoside		+	+	+	−
Nucleotide		+	+	+	+

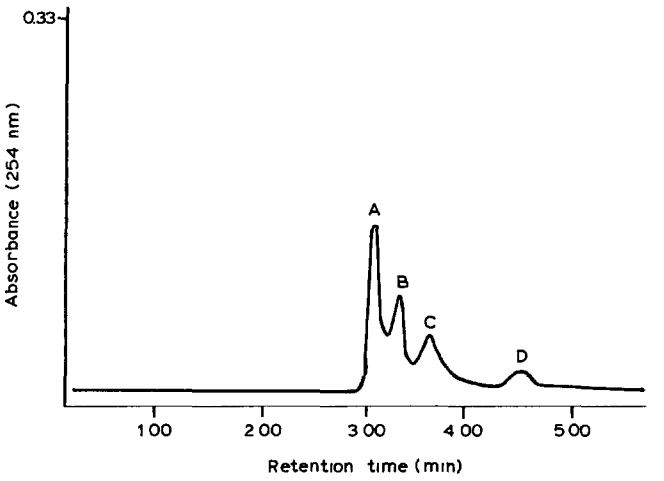


Fig. 1. Elution profile of a mixture of N^6-cycloalkylated bases. Peaks: A = adenine; B = N^6-cyclopropyladenine; C = N^6-cyclobutyladenine; D = N^6-cyclopentyladenine. Solvent: 70% methanol–water. Flow-rate: 0.8 ml/min.

atmosphere of 5% carbon dioxide. Subsequently, the cells were inoculated with the cycloalkylated derivatives at concentrations varying from 100 to 500 μg in 3 ml of culture medium. The treated cells were incubated, along with a control, at 37°C from 0 to 48 h, after which they were collected by centrifugation (2930 *g*, 10 min) and lysed with distilled water or a buffer solution containing 0.5% Triton X-100, 0.13 *M* sodium chloride, 5 m*M* potassium chloride and 7.4 m*M* magnesium chloride. After removing the cell debris by centrifugation (2930 *g*, 10 min), the supernatant was centrifuged, as above, through an ultrafiltration cone (Type CF-25, Amicon, Lexington, MA, U.S.A.) to remove proteins and other macromolecules. Aliquots of the ultrafiltrate (10 μl) were analyzed by HPLC to determine if the cycloalkylated derivatives were present in the intracellular fluid. A cycloalkylated derivative was occasionally added to the 0-h control sample as an internal standard.

Peak identification

Initially, standard solutions (1 m*M*, 10 μl) of the bases, nucleosides, and nucleotides were analyzed to determine the general area of elution. Each derivative was analyzed individually to determine its retention time. Mixtures of each (10 μl) base, nucleoside, and 5′-monophosphate (0.7 μg/μl) series were analyzed in an effort to determine the order of elution, as well as the retention times and resolution of each component of each series. Subsequently, a combined mixture of the base and nucleoside series (10 μl, 0.35 μg/μl) was analyzed.

RESULTS AND DISCUSSION

Reversed-phase HPLC was used to determine the elution profiles of the series of synthetic bases, nucleosides, and their corresponding 5′-monophosphates.

TABLE II

ELUTION PATTERNS OF THE N^6-CYCLOALKYLATED BASE AND NUCLEOSIDE SERIES

A, A′ etc. represent the bases and nucleosides, respectively.

Flow-rate (ml/min)	Concentration methanol (%)	Retention time (min)							
		A	B	C	D	A′	B′	C′	D′
0.6	70	4.24	4.68	5.13	6.37	4.43	4.75	5.94	6.87
	60	5.00	5.32	5.87	6.38	5.83	6.65	7.45	9.53
0.8	70	3.33	3.53	3.88	4.77	3.34	3.70	4.33	5.03
	60	3.65	4.80	5.33	5.77	3.98	4.45	6.90	9.02
1.0	70	2.62	2.72	3.26	3.93	2.67	3.14	3.60	4.17
	60	2.82	3.45	3.78	5.57	3.40	3.75	4.46	6.20

Three variables were considered in order to determine optimum conditions for separation and resolution: (a) the percentage of methanol in the solvent; (b) flow-rate of solvent; and (c) the size of the cycloalkylated group of each synthetic analogue.

N^6-Cycloalkylated bases

The cycloalkylated bases were eluted with 60 and 70% methanol–water solutions, at flow-rates of 0.6, 0.8, and 1.0 ml/min. Although satisfactory separation of a mixture of the bases was successful with both methanol percentages and flow-rates, optimum conditions were achieved with the 70% methanolic solution at 0.8 ml/min, with all components of the mixture eluting in times ranging from 3.3 to 4.8 min. Resolution as well as separation were optimal and retention times were reproducible with a standard deviation of 0.02. The elution profile of the mixture of the synthetic bases, with 70% methanol at 0.8 ml/min, is shown in Fig. 1. Shown in Table II is a summary of the results, including retention times at all flow-rates and methanol percentages considered.

N^6-Cycloalkylated nucleosides

The cycloalkylated nucleosides were eluted with the same methanol percentages and flow-rates previously cited. Subsequently, the 70% methanol solution, at 0.8 ml/min, eluted the nucleoside mixture as well, with retention times ranging from 3.3 to 5.0 min. A complete list of retention times and flow-rates at each methanol percentage is shown in Table II.

A pattern developed which revealed that when the flow-rate and methanol percentage were increased, the retention time decreased, accompanied by improved resolution. A chromatogram of the mixture of synthetic nucleosides, eluted with 70% methanol at 0.8 ml/min, is shown in Fig. 2. Since the retention times of the base and nucleoside series were within the same range, one might not expect complete resolution of the individual peak when the two series were combined and analyzed. However, this was not observed during our investigations, as discussed below.

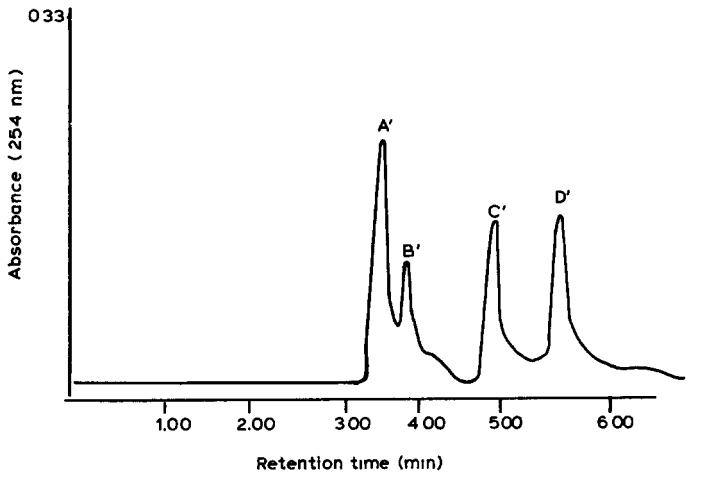


Fig. 2. Elution profile of a mixture of N^6-cycloalkylated nucleosides. Peaks: A' = adenosine; B' = N^6-cyclopropyladenosine; C' = N^6-cyclobutyladenosine; D' = N^6-cyclopentyladenosine. Solvent: 70% methanol–water. Flow-rate: 0.8 ml/min.

TABLE III

ELUTION PATTERNS OF A MIXTURE OF THE N^6-CYCLOALKYLATED BASE AND NUCLEOSIDE SERIES

A, A', etc. represent the bases and nucleosides, respectively.

Flow-rate (ml/min)	Concentration methanol (%)	Retention time (min)							
		A	A'	B	B'	C	C'	D	D'
0.6	70	4.32	4.42	4.62	4.82	5.08	5.80	6.33	6.68
	60	6.40	6.62	7.10	7.47	7.75	8.43	10.9	14.3
0.8	70	3.30	3.36	3.52	3.58	3.92	4.40	4.70	5.00
	60	4.82	4.97	5.23	5.40	5.87	6.22	8.15	10.3
1.0	70	2.55	2.63	2.80	2.88	3.07	3.45	3.77	4.10
	60	3.55	3.62	3.92	3.97	4.62	6.10	6.63	7.62

N^6-Cycloalkylated base and nucleoside mixture

The base and nucleoside mixture (0.35 $\mu g/\mu l$) was eluted with both the 60 and 70% methanol–water solutions at the respective flow-rates as indicated in Table III. Although retention times were relatively close for the base and nucleoside mixtures, separation was possible with both solvent systems, with the 70% solution at a flow-rate of 0.6 ml/min giving the better resolution. The retention times of the standards, adenine and adenosine, were almost identical, differing by less than 4 s and as a consequence adenine appears as a shoulder beside the adenosine peak. All of the other components eluted with well resolved peaks.

An important finding was that the patterns of elution showed pairing of the adenine and adenosine derivatives containing the same cycloalkyl group, with

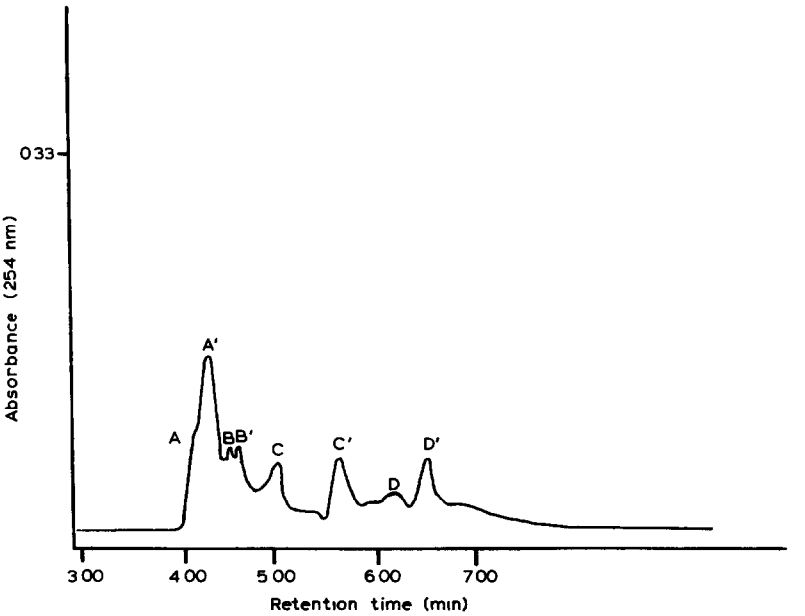


Fig. 3. Elution profile of a mixture of the N^6-cycloalkylated base and nucleoside series. A, A', etc. represent the bases and nucleosides, respectively. Adenine (A) appears as a shoulder on the adenosine (A') peak. Solvent: 70% methanol–water. Flow-rate: 0.6 ml/min.

the adenine derivative being eluted first and the adenosine derivative second. The elution profile is shown in Fig. 3.

N^6-Cycloalkylated adenosine-5'-monophosphates

The ribose moiety of the nucleoside does not alter the elution pattern of the cycloalkylated derivatives; however, a buffered solvent system is required to successfully elute the modified derivatives when a phosphate residue is incorporated into their structures. The cycloalkylated adenosine-5'-monophosphates were eluted with an acetonitrile–tetrabutylammonium ion buffer at flow-rates of 0.5, 0.7, 1.0, and 1.2 ml/min. The elution profile of the nucleotide mixture at 1.0 ml/min is shown in Fig. 4. This flow-rate provided optimum conditions, with retention times ranging from 2.6 to 6.1 min and a standard deviation of 0.017. All of the modified analogues in the nucleotide mixture eluted in their respective order, relative to the molecular mass of the cycloalkyl groups, with the derivatives containing the smallest cycloalkyl group eluting first and those containing the largest, last. A summary of the results is shown in Table IV. Both separation and resolution were independent of flow-rate. Although retention times decreased with an increase in flow-rate, any flow-rate may be utilized if time is not a factor. This method utilizes a simple ion-pair reagent to elute compounds whose differences in molecular mass are relatively small and can be performed in a matter of minutes. Studies are currently in progress to establish optimum conditions for the separation of a mixture of cycloalkylated bases, nucleosides, and nucleotides.

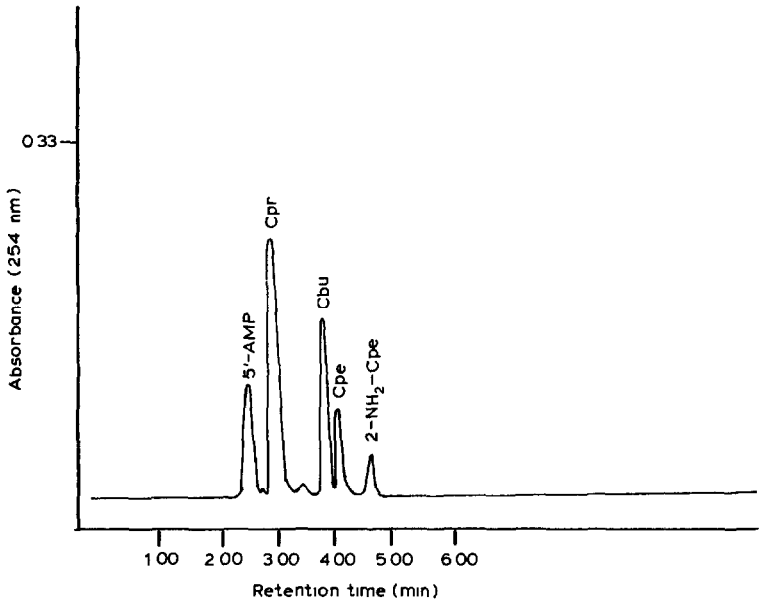


Fig. 4. Elution profile of a mixture of N^6-cycloalkylated adenosine-5′-monophosphates. Peaks: 5′-AMP = adenosine-5′-monophosphate; Cpr = N^6-cyclopropyladenosine-5′-monophosphate; Cbu = N^6-cyclobutyladenosine-5′-monophosphate; Cpe = N^6-cyclopentyladenosine-5′-monophosphate; 2-NH_2-Cpe = 2-amino-N^6-cyclopentyladenosine-5′-monophosphate. Solvent: 19% acetonitrile, 0.03 *M* potassium dihydrogenphosphate, 0.01 *M* tetrabutylammonium ion. Flow-rate: 1.0 ml/min.

TABLE IV

ELUTION PATTERNS OF THE INDIVIDUAL N^6-CYCLOALKYLATED ADENOSINE-5′-MONOPHOSPHATES AND THE CORRESPONDING MIXTURE

The mixture contained 5′-AMP, N^6-cyclopropyl-, N^6-cyclobutyl-, N^6-cyclopentyl-, and 2-amino-N^6-cyclopentyladenosine-5′-monophosphates, respectively.

Flow-rate (ml/min)	Retention time (min)					
	Standard 5′-AMP	N^6-Cyclopropyl 5′-AMP	N^6-Cyclobutyl 5′-AMP	N^6-Cyclopentyl 5′-AMP	2-NH_2-N^6-Cyclopentyl 5′-AMP	Mixture
0.5	5.08	5.50	7.40	8.00	10.0	5.08/5.50/7.73/8.25/9.72
0.7	3.66	4.45	5.53	6.80	9.00	3.56/3.90/5.60/5.90/6.92
1.0	2.57	3.33	4.10	4.93	6.05	2.43/2.67/3.75/4.06/4.78
1.2	2.17	2.80	3.52	4.32	5.03	2.00/2.25/2.87/3.42/4.00

Biological application

N^6-Cycloalkylated bases and nucleosides have varying degrees of effects upon the morphology and viability of mouse hepatoma cells in vitro (unpublished results).

In an effort to obtain some information about the site of action of these derivatives, the intracellular fluid of the treated mouse hepatoma cells was analyzed by HPLC. Although free bases and nucleosides are believed to occur intracellu-

larly only at low levels in healthy well oxygenated cells, the sensitivity of ultraviolet monitors (254 nm) in HPLC systems and the strong chromophores of nucleic acid components allow for the detection of very small quantities (pmol) of these components [15].

When the intracellular fluid, obtained from mouse hepatoma cells that had been treated with N^6-cyclopropyladenine, was examined by HPLC, a peak, corresponding to the internal standard, was detected that increased with the time of exposure of the cells to the cycloalkylated derivative. Furthermore, when a sample of N^6-cyclopropyladenine was added to the intracellular fluid and an aliquot subsequently analyzed, a noticeable increase in the height of the peak, previously detected, was observed.

These results are preliminary and further studies are in progress to ascertain the specific site(s) of action of cycloalkylated nucleic acid derivatives upon mouse hepatoma cells by analyzing their intercellular and intracellular fluids by HPLC.

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