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RAPID SEPARATION OF DNA CONSTITUENTS, BASES, NUCLEOSIDES AND NUCLEOTIDES, UNDER THE SAME CHROMATOGRAPHIC CONDITIONS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A REVERSED-PHASE COLUMN

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SUMMARY

Four components of three sets of DNA constituents, bases, deoxyribonucleosides and deoxyribonucleoside 5'-monophosphate, were sufficiently resolved under one set of chromatographic conditions using high-performance liquid chromatography with a reversed-phase column (Zorbax ODS) and the solvent 0.4 *M* $NH_4H_2PO_4$ (pH 3.5). The effect of pH and salt concentration in the solvent on the retention of these compounds in the column was thoroughly investigated. Proportionality of peak height to the content, and reproducibility and recovery of the four bases were satisfactory under appropriate conditions and as little as 1 μg of DNA could be analysed for its base composition by this method.

INTRODUCTION

A high-performance liquid chromatographic (HPLC) procedure was developed for analysis of nucleic acid bases, nucleosides and nucleotides [1]. In most cases, a cation-exchanger resin is employed for the separation of bases or nucleosides, and an anion exchanger is used for nucleotides. It is desirable to separate these nucleic acid constituents using a simple chromatographic system with a single column and a single solvent. Quantitative analysis of both nucleotides and nucleosides in a mixture of enzymatically degradated products of DNA is a necessary step for studying the mode of action of deoxyribonuclease. Simultaneous separation of components of these two sets of DNA constituents, if possible, would be especially useful for the determination of nucleoside composition, terminal nucleoside and chain length of DNA fragments produced by the action of deoxyribonuclease.

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Ho and Gilham [2] and Asteriadis et al. [3] reported a simultaneous separation of nucleotides and nucleosides with a polystyrene anion-exchange column using a solvent containing ethanol. But their procedure is rather tedious and time-consuming. Reversed-phase HPLC on a column of a quaternary ammonium derivative on polychlorotrifluoroethylene beads (RPC-5) was also used to separate mononucleotides and nucleosides in the sequence analysis of tRNAs [4,5], but the separation of nucleosides was not satisfactory even under the best conditions for nucleotides.

In order to simplify the routine work and to reduce the necessary time for analysis of these DNA constituents, we established an ideal chromatographic system with one reversed-phase column consisting of a porous silica containing a covalently bonded octadecyl hydrocarbon (Zorbax ODS) and one solvent.

MATERIALS AND METHODS

Standard samples

All nucleic bases, deoxyribonucleosides and deoxyribonucleoside 5'-monophosphate used in this study were purchased from Sigma (St. Louis, Mo., U.S.A.). These were all dissolved in 0.1 *N* HCl. The following millimolar extinction coefficients were used to check the true concentration of the standard solution of bases for the quantitative determination [6]: adenine, 13.2 at 262.5 nm; cytosine, 10.0 at 276 nm; guanine, 11.4 at 248 nm and thymine, 7.9 at 264.5 nm. Radioactive bases, [2-^{14}C]thymine, [8-^{14}C]adenine, [8-^{3}H]-guanine sulphate and [2-^{14}C]cytosine sulphate, were obtained from The Radiochemical Centre (Amersham, Great Britain) and purified once by chromatography as described in this article.

Chromatographic procedure

A Shimadzu—DuPont Model LC-1 high-performance liquid chromatograph equipped with a prepacked stainless-steel column of Zorbax ODS (25 cm × 2.1 mm I.D.) was used throughout this study. Routinely, the column was run at a pressure of 140 kg/cm^2 and a temperature of 40°. The flow-rate of solvent through the column was maintained at 0.6 ml/min under such conditions. The solvent of a certain concentration of ammonium phosphate was prepared by dissolving $NH_4H_2PO_4$ (analytical grade, Wako, Osaka, Japan) in water at the desired concentration and the pH was adjusted by the addition of concentrated phosphoric acid. The sample was applied to the column with the aid of a microsyringe (Hamilton, 801NR) and a septum injection valve interrupting the flow of the eluent momentarily. The effluent was monitored at 260 nm with a Shimadzu model SPD-1 spectrophotometric detector.

Digestion of DNA

DNA from the sperm of the Pacific salmon (*Oncorhynchus keta*) was extracted by treatment with sodium dodecyl sulphate—phenol [7] and purified by successive treatment with ribonuclease A (Boehringer, Mannheim, G.F.R.) [8] and Pronase (Calbiochem, Los Angeles, Calif., U.S.A.) [9]. The DNA was hydrolysed with formic acid in a sealed tube (3 mm I.D.) according to the method of Bendich [10].

Radioactivity measurement

An aliquot (0.5—1.0 ml) of the column eluant with 0.2 ml of distilled water was mixed with 6 ml of PCS™ (Amersham—Searle, Arlington Heights, Ill., U.S.A.) in a counting vial. Radioactivity was measured in a Searle Analytical Model Mark II liquid scintillation counter and the quenching was corrected by the sample-channel ratio method.

RESULTS

Analytical conditions

Fig. 1 illustrates a typical separation of authentic samples of DNA bases, deoxyribonucleosides and deoxyribonucleoside 5'-monophosphate on the Zorbax ODS column with a single solvent system of 0.4 *M* ammonium phosphate (pH 3.5). Four components in three sets of DNA constituents were adequately resolved by this chromatographic system.

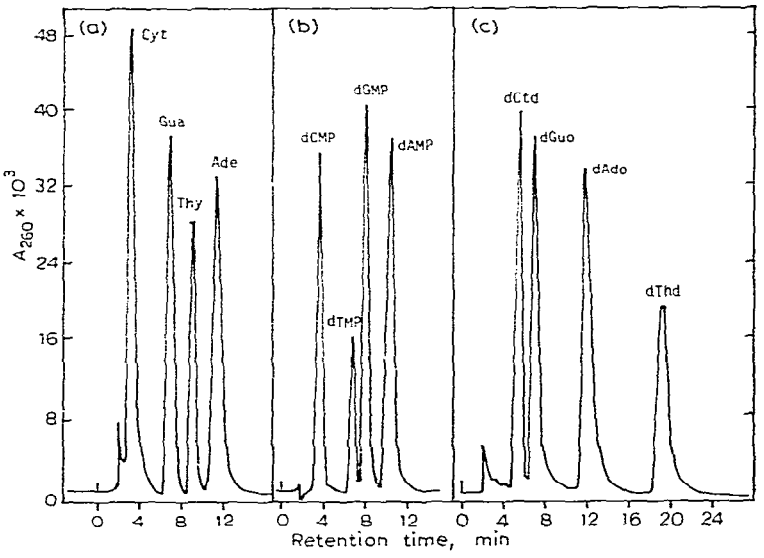


Fig. 1. Separation of: (a) DNA bases, (b) deoxyribonucleoside 5'-monophosphates, and (c) 2'-deoxyribonucleosides, on a Zorbax ODS column (25 cm × 2.1 mm I.D.) with 0.4 *M* ammonium phosphate (pH 3.5) as the solvent, under the conditions described in Methods. Of each compound, 1.0 nmole was applied to the column.

Fig. 2a shows the effect of the solvent pH (0.2 *M* ammonium phosphate) on the elution of DNA bases. The elution position of thymine and cytosine was not changed within the pH range of 2.5—4.0, whereas the retention of guanine and adenine was considerably affected by the pH. Adenine particularly had a higher retention time as the pH increased. Fig. 2b and c indicate the effect of the pH on the resolution of nucleosides and nucleotides. The elution of dAdo*

*Abbreviations and symbols: Ade=adenine; Thy=thymine; Gua=guanine; Cyt=cytosine; dAdo=2'-deoxyadenosine; dThd=thymidine; dGuo=2'-deoxyguanosine; dCyd=2'-deoxycytidine; dAMP=2'-deoxyadenosine 5'-monophosphate; dTMP=thymidine 5'-monophosphate; dGMP=2'-deoxyguanosine 5'-monophosphate; dCMP=2'-deoxy 5'-monophosphate.

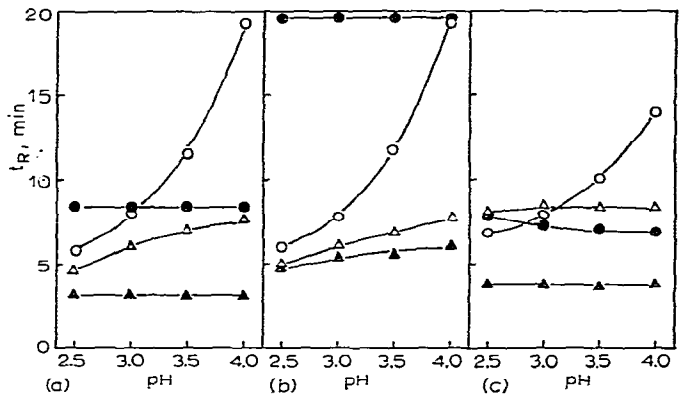


Fig. 2. Effect of pH of solvent (0.2 *M* $NH_4H_2PO_4$) on the separation of: (a) bases, (b) deoxyribonucleosides and (c) deoxyribonucleoside monophosphate. In (a): ○, Ade; ●, Thy; △, Gua; ▲, Cyt. In (b): ○, dAdo; ●, dThd; △, dGuo; ▲, dCyd. In (c): ○, dAMP; ●, dTMP; △, dGMP; ▲, dCMP.

and dAMP from the column was increasingly delayed as the pH increased. Other nucleosides and nucleotides were maintained at almost the same elution position within the pH range examined.

Fig. 3 depicts the effect of concentrations of ammonium phosphate in the solvent (pH 3.5). The resolution of four bases and four nucleosides was not satisfactory when the solvent of low salt concentration was used. As the concentration of ammonium phosphate was increased, Ade and dAdo were eluted more rapidly from the column, but dThd, dAMP, dCMP and dTMP were eluted more slowly. The retention of the other compounds was not affected by the change of the salt concentration in the solvent.

Increase of the pressure applied to the column caused a rapid separation of these materials without a significant change of resolution, within the range 130—150 kg/cm^2. Increasing the column temperature decreased the retention of all the bases on the column with a good separation.

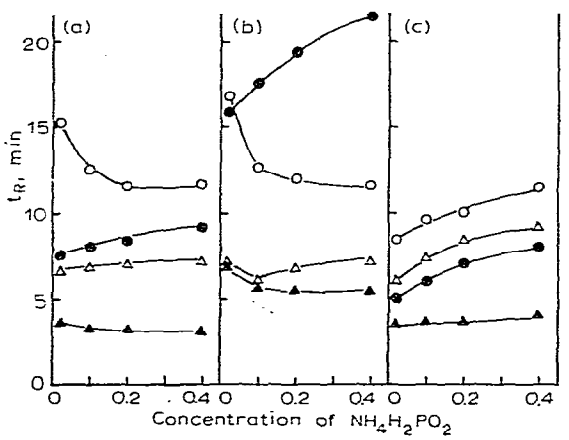


Fig. 3. Effect of $NH_4H_2PO_4$ concentration in solvent (pH 3.5) on the separation of: (a) bases, (b) deoxyribonucleosides and (c) deoxyribonucleoside monophosphate. The symbols are as in Fig. 2.

Reproducibility

The reproducibility of retention was examined by repeated analysis of the standard mixture of the four authentic bases of DNA under the same conditions. The peak positions of each base was fairly reproducible, as seen in Table I, except for Ade, the retention time of which was gradually reduced as the amount applied to the column was increased. Graphs of peak height vs. concentration were found to be linear for each base over the concentration range of interest, as shown in Fig. 4. Since measurement of peak width at half height was difficult, the peak height was chosen for quantitative calculation.

The recovery rate of each base after chromatography was measured by the use of radioactive bases. Table II indicates that the recoveries of bases were

TABLE I

REPRODUCIBILITY OF RETENTION TIME (t_R)

Results represent the mean ± S.D. of five experiments. C.V.= Coefficient of variation.

Base	nMoles	t_R (min)	C.V. (%)
Cyt	0.2	3.20 ± 0.038	1.2
	2.0	3.24 ± 0.048	1.5
	10.0	3.20 ± 0.052	1.6
Gua	0.2	7.00 ± 0.060	0.9
	2.0	7.16 ± 0.080	1.1
	10.0	6.88 ± 0.080	1.2
Thy	0.2	9.04 ± 0.080	0.9
	2.0	9.28 ± 0.088	1.0
	10.0	9.12 ± 0.12	1.1
Ade	0.2	12.04 ± 0.056	0.5
	2.0	11.80 ± 0.092	0.8
	10.0	10.72 ± 0.19	1.8

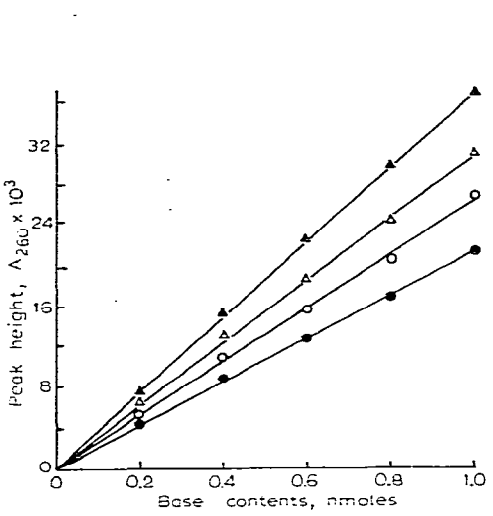
TABLE II

RECOVERY OF BASES

Results represent the mean disintegrations per minute ± S.D. of five experiments. C.V.=coefficient of variation.

Base	Amount applied (dpm)	Amount recovered (dpm)	Recovery (%)	C.V. (%)
[^{14}C]Cytosine	5567 ± 84	5286 ± 44	95.6 ± 0.81	0.85
[^{3}H]Guanine	16519 ± 480	14719 ± 1112	89.2 ± 6.7	7.5
[^{14}C]Thymine	9146 ± 127	7806 ± 164	85.4 ± 1.8	2.1
[^{14}C]Adenine	6807 ± 116	6432 ± 341	90.0 ± 4.5	5.4

between 85 and 95%. The radiochromatogram of each base in Fig. 5 shows that trace amounts of Thy, Gua, and Ade appeared near the position of Cyt and that Ade was eluted with slight tailing.



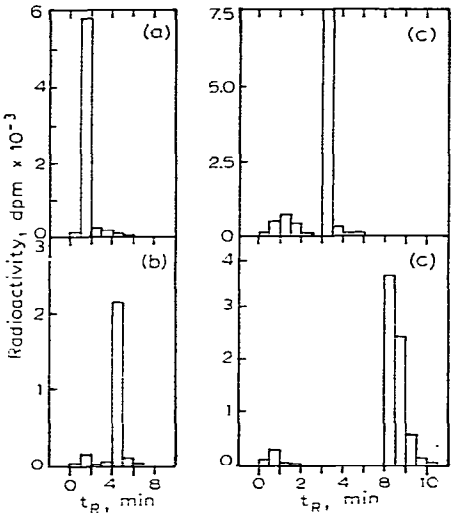


Fig. 4. The relationship between peak height and content of bases. The symbols are the same as in Fig. 2.

Fig. 5. Examination of elution profiles of bases by radiochromatography. (a) [^{14}C]Cytosine, (b) [^{3}H]guanine, (c) [^{14}C]thymine and (d) [^{14}C]adenine were eluted respectively by HPLC and fractions of the eluant at 1-min intervals were collected and the radioactivity measured in a liquid scintillation counter.

TABLE III

ANALYSIS OF BASE COMPOSITION OF SALMON SPERM DNA

Data represented by the mean ± S.D. of five or more determinations. Numerals in parentheses indicate the coefficient of variation (%).

Amount of DNA examined (μg)	Base composition (%)			
	Cyt	Gua	Thy	Ade
1	20.4 ± 0.65 (3.1)	21.4 ± 0.82 (1.3)	29.9 ± 1.68 (5.6)	28.3 ± 1.86 (6.5)
5	20.6 ± 0.34 (1.6)	20.9 ± 0.12 (0.5)	29.7 ± 0.00 (0.0)	28.9 ± 0.46 (1.5)
20	21.4 ± 0.07 (0.3)	21.0 ± 0.04 (0.1)	30.0 ± 0.04 (0.1)	27.5 ± 0.10 (0.3)

TABLE IV

COMPARISON OF ANALYTICAL RESULTS OF BASE COMPOSITION OF SALMON SPERM DNA

Results are expressed as percentage base composition.

Chromatography	Cyt	Gua	Thy	Ade
Zorbax ODS column	21.4	21.0	30.0	27.5
Cation-exchange column*	21.0	20.8	30.3	28.0
Paper partition**	20.0	21.5	30.4	27.6
Paper partition***	20.4	20.8	29.1	29.7

*From Ref. 12

**From Ref. 13

***From Ref. 14

Base composition of salmon sperm DNA

The base composition of salmon sperm DNA was analysed by this method after formic acid digestion in a small-bore glass tube. The result obtained from different amounts of the DNA showed that as little as 1 μg of DNA could be analysed for its base composition using this method (Table III).

DISCUSSION

Four components in each group of three sets of DNA constituents were readily separated in a simple chromatographic system consisting of one reversed-phase column and a single solvent using a high-performance liquid chromatograph. The Zorbax ODS column proved to be suitable for these purposes. We attempted to separate eight components of nucleotides and nucleosides simultaneously by this system. But the present results revealed that the separation of dGuo from dTMP and dAdo from dAMP were not satisfactory. An improved resolution would be expected by a system with a longer column or employing gradient elution.

The chromatographic behavior of Ade, dAdo and dAMP was somewhat different from that of the others. The retention time of these three compounds was greatly increased as the pH of the solvent increased. Of these, dAMP was found to have a somewhat shorter retention time than the other two. The retention of only Ade and dAdo was reduced as the ammonium phosphate concentration increased, the retention of dAMP and other compounds not being affected or being slightly increased. Another peculiarity of Ade in chromatographic elution was that its retention decreased as the amount applied to the column increased. We could not explain the reason for these phenomena. Singhal and Cohn [11] observed a similar behaviour of adenosine on cation-exclusion chromatography on an anion-exchange column (Aminex A-25); that is, adenosine appears nearer the front as the pH of solvent (0.01 *M* ammonium

formate) is lowered. They concluded that this is due to its positive charge and its purine nature.

The elution profiles of bases traced by radioactivity indicated that small amounts of Gua, Thy and Ade were inclined to spill near the position of Cyt and that Ade eluted with tailing. These would be reasons for the somewhat lower recoveries of these compounds. Moreover, that this method would inevitably result in a slightly higher content of Cyt in DNA than the other methods may not be excluded.

According to this method at least 1 μg of DNA can be analysed for its base composition as shown in Table III. Our previously published results for the base composition of salmon sperm DNA are presented together with the data obtained by the present study in Table IV. The data in the second row of the table were obtained from DNA of the same origin as used in the present study, but the DNA used by Chargaff et al. [14] was from sperm of Atlantic salmon (*Salmo salar*), a different species from our source, and that of Darlington and Randall [13] was of commercial origin, the source of which is unknown. Although the methods and the respective origins of DNA are different, the results are in good accord with each other.

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