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Chromatographic characteristics of nucleic acid components on vinyl alcohol copolymer gel columns

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SUMMARY

The chromatographic characteristics of nucleic acid components on an Asahipak GS-320 vinyl alcohol copolymer gel column were investigated in comparison with those on an ODS column. On both columns the order of elution within each class (nucleotide, nucleoside or nucleic acid base) was generally pyrimidine compounds followed by purine compounds, the only exception being the late elution of thymine from the GS-320 and, particularly, from the ODS column.

Between the three classes of nucleic acid components, the two columns exhibited different elution orders. On the GS-320 column, the order was consistently nucleotide, nucleoside and base, *i.e.*, in order of increasing water solubility, with a consistent correlation between capacity factors (k') and Hansch's log P values, showing that hydrophobic adsorption between the gel and the base group is the predominant separation mechanism for both the nucleosides and the bases. In the separation of nucleotides on the GS-320 column, an ion-repulsion interaction appeared to be predominant, as the k' values were negative and the retention volumes were increased by methylation of the carboxylic groups in the gel but unaffected by addition of acetonitrile to the mobile phase. On the strongly hydrophobic ODS column, nucleoside elution occurred after nucleotide and base elution and more complex correlations were observed, suggesting hydrophobic interaction of the octadecyl group of the gel with the sugar group and also with the base group, particularly for the nucleosides. This inference was supported by an observed increase in the capacity factor of ribose in the presence of antichaotropic sodium chloride on the ODS but not on the GS-320 column.

The utility of the GS-320 column for the separation of nucleic acid components was also investigated. Nucleotides, nucleosides and bases were consistently separated, apparently by a hydrophobic adsorption mechanism, permitting the simple, efficient analysis of mixtures of these substances by isocratic elution.

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INTRODUCTION

The Asahipak GS-320 vinyl alcohol copolymer gel column, is a multi-purpose column for high-performance liquid chromatography (HPLC), which can be utilized for gel chromatography, reversed-phase partition or multi-mode chromatography, depending on the properties of the sample and the mobile phase[1–4]. Preliminary investigations of its suitability for the analysis of nucleic acid components showed their elution order to be nucleotides, nucleosides and nucleic acid bases, unlike that exhibited on the widely used ODS column, and led to the present investigation of the chromatographic characteristics of various nucleic acid components on a GS-320 column.

EXPERIMENTAL

Chromatography was performed with a Hitachi-638 chromatograph (Hitachi Seisakusho, Tokyo, Japan), equipped with a Uvidec spectrophotometer (JASCO, Tokyo, Japan), operated at 260 nm.

The following columns were used: Asahipak GS-320 (500 mm × 7.6 mm I.D.) and GS-320H (250 mm × 7.6 mm I.D.) HPLC columns (Asahi Chemical Industries, Tokyo, Japan), packed with vinyl alcohol copolymer gel, having a mean particle diameter of 9.0 μm and an exclusion limit of 40 000 Da; YMC PACK AQ-312 or AM-312 (both 150 mm × 6.0 mm I.D.; Yamamura Kagaku, Kyoto, Japan) HPLC columns packed with ODS gel; and methylated Asahipak GS-320 gel columns (250 mm × 7.6 mm I.D.).

The methylated gel was obtained by stirring Asahipak GS-320 gel and 14% boron trifluoride–methanol in a three-necked flask, equipped with stirrer and thermometer, for 16 h at room temperature[5]. Nucleotides, nucleosides and their bases were purchased from Wako (Osaka, Japan).

RESULTS AND DISCUSSION

In general terms, the chromatographic characteristics of the bases, nucleosides and nucleotides (structures and pK_a values are given in Table I) on the GS-320 column were found to be similar to those on the ODS column, but with significantly smaller retention volumes, particularly for nucleosides, and some differences in elution order. The overall results for both columns are shown in terms of the capacity factors at pH 6.0 in Table II, the relationship between mobile phase pH and retention volume in Figs. 1–3 and the elution orders in Table III.

Nucleic acid bases

The relationship between base retention volume and eluent pH on the GS-320 column is similar to that on the ODS column, as shown in Fig. 1. The pH–retention volume profiles, showing weak retention of the bases at ionizing pH levels, suggest analogous elution mechanisms for the two columns. On both columns, the order of elution was generally pyrimidine compounds, followed by purine compounds. The exception was thymine, which was eluted after hypoxanthine from the GS-320 column and after guanine from the ODS column. It appears that the elution of thymine,

TABLE I

ABBREVIATIONS AND pK_a VALUES OF NUCLEIC ACID COMPONENTS

Substance	*Abbreviation*	pK_a
Cytosine	C	4.5, 12.2
Uracil	U	9.5
Thymine	T	9.9
Adenine	A	4.15, 9.8
Guanine	G	3.2, 9.6, 12.4
Hypoxanthine	Hx	2.00, 8.90
Cytidine	CYD	4.15, 12.5
Uridine	URD	9.2, 12.5
Thymidine	THD	9.8
Adenosine	ADO	3.5, 12.5
Guanosine	GUO	1.6, 9.2, 12.4
Inosine	INO	1.2, 8.8, 12.3
Cytidine monophosphate	CMP	4.5, 6.3
Uridine monophosphate	UMP	6.4, 9.5
Thymidine monophosphate	TMP	10.0
Adenosine monophosphate	AMP	3.7, 6.1
Guanosine monophosphate	GMP	2.5, 6.1, 9.4
Inosine monophosphate	IMP	6.0, 8.9

TABLE II

CAPACITY FACTORS (k')

Determined at 30°C with a mobile phase of 10 m*M* sodium hydrogenphosphate (pH 6.0) on Asahipak GS-320 and YMC PACK AQ-312 columns at flow-rates of 1.0 and 0.6 ml/min, respectively.

Sample	*Log P*	k'	
		GS-320	*AQ-312*
AMP	–	−0.10	6.17
GMP	–	−0.14	2.05
IMP	–	−0.21	2.31
TMP	–	−0.19	5.17
UMP	–	−0.23	1.00
CMP	–	−0.23	0.62
Adenosine	−0.18	2.15	52.99
Guanosine	−0.92	1.08	17.22
Inosine	−0.92	0.49	15.04
Thymidine	–	0.78	26.89
Uridine	−0.92	0.35	5.30
Cytidine	−0.97	0.27	3.16
Adenine	0.33	2.86	10.79
Guanine	−0.35	1.53	4.03
Hypoxanthine	−0.27	0.80	3.92
Thymine	0.05	0.85	6.29
Uracil	−0.40	0.48	1.78
Cytosine	−0.68	0.35	1.02

TABLE III

ELUTION ORDER

Columns and conditions as in Table II.

Compound	*Elution order*		*Compound*	*Elution order*		*Compound*	*Elution order*	
	GS-320	*AQ-312*		*GS-320*	*AQ-312*		*GS-320*	*AQ-312*
Cytosine	1	1	Cytidine	1	1	CMP	1	1
Uracil	2	2	Uridine	2	2	UMP	1	2
Thymine	4	5	Thymidine	4	5	TMP	4	5
Hypoxanthine	3	3	Inosine	3	3	IMP	3	4
Guanine	5	4	Guanosine	5	4	GMP	5	3
Adenine	6	6	Adenosine	6	6	AMP	6	6

which is the only one of the six bases that contains a methyl group, is strongly retarded on the ODS column as a result of strong enhancement of the hydrophobic interaction with its long alkyl group by the methyl group, whereas on the GS-320 column, which does not have a long alkyl group, the enhancement and hence the retardation effect are far smaller.

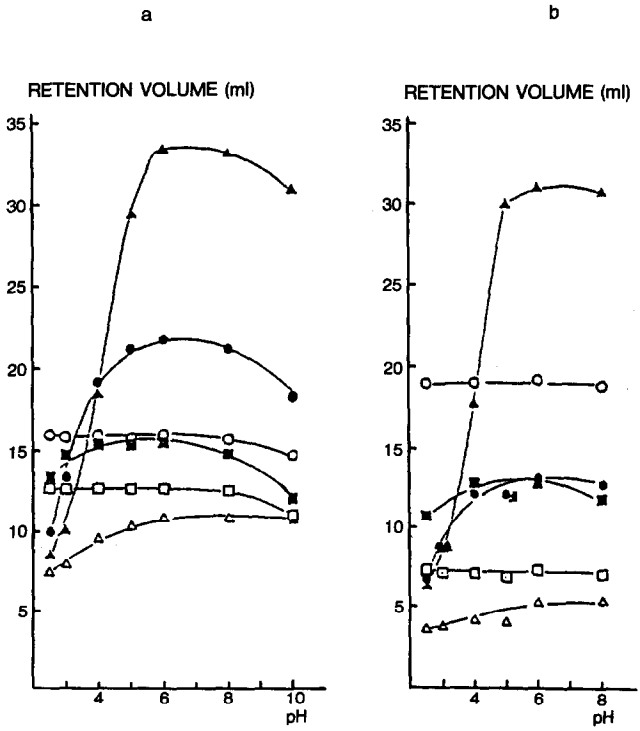


Fig. 1. Nucleic acid base retention volumes *versus* eluent pH. Columns: (a) Asahipak GS-320H; (b) YMC PACK AQ-312. Samples: 0.1 mg/ml solution, 10 μl for (a) and 6 μl for (b). ▲ = Adenine; ● = guanine; ■ = hypoxanthine; ○ = thymine; □ = uracil; △ = cytosine. Mobile phase: 10 m*M* sodium phosphate. Flow-rates: (a) 1.0; (b) 0.6 ml/min. Temperature, 30°C; detection at 260 nm (0.64 a.u.f.s.).

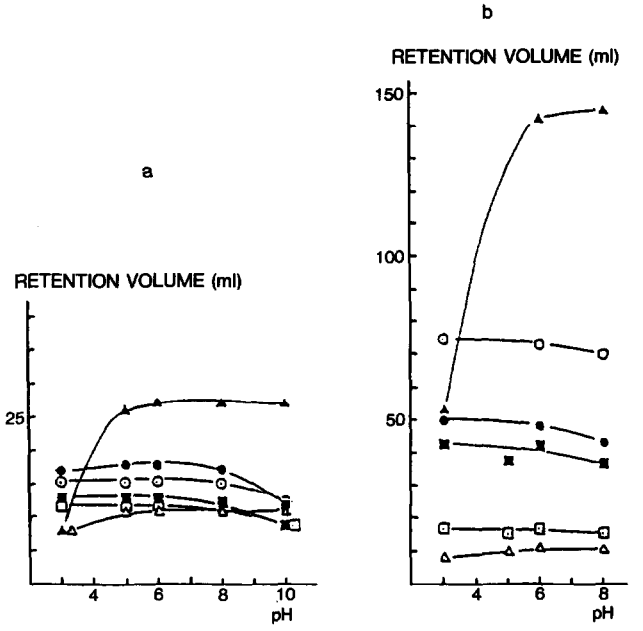


Fig. 2. Nucleoside retention volumes *versus* eluent pH. Samples: ▲ = adenosine; ● = guanosine; ■ = inosine; ○ = thymidine; □ = uridine; △ = cytidine. Detection at 260 nm (0.32 a.u.f.s.); other conditions and columns as in Fig. 1.

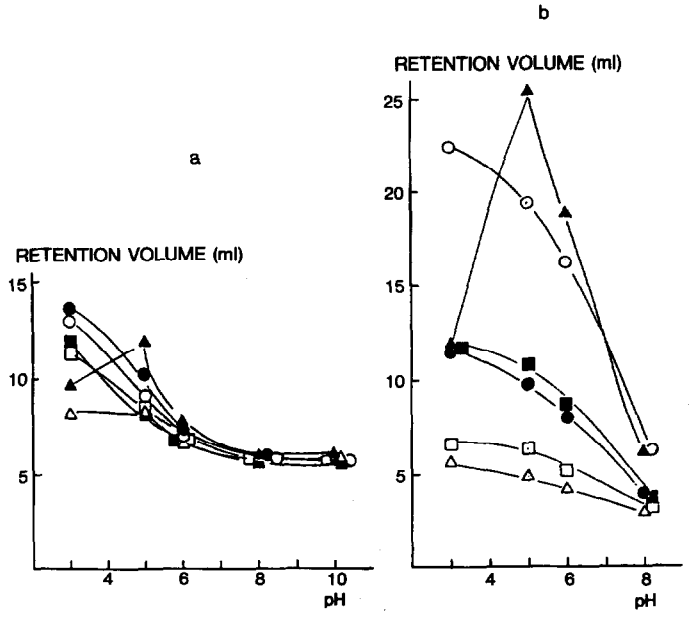


Fig. 3. Nucleotide retention volumes *versus* eluent pH. Samples: ▲ = AMP; ● = GMP; ■ = IMP; ○ = TMP; □ = UMP; △ = CMP. Columns and conditions as in Fig. 2.

A clear decrease in retention volume was observed for both columns when acetonitrile was present in the mobile phase, as shown in Fig. 4. For both columns, a positive slope was found in the plots showing the relationship between k' and Hansch's log P values[6], an index of the hydrophobicity of nucleic acid components.

All of the above results indicate that the chromatographic mechanism for nucleic acid bases on both columns is reversed-phase partition, based on hydrophobic interaction between base and gel.

Nucleosides

For nucleosides on both columns, the pH–retention volume profile (Fig. 2), the elution order (Table III) and the effect of acetonitrile concentration in the mobile phase (Fig. 5) were all similar to those obtained for the nucleic acid bases. The k' values for nucleosides on the GS-320 column were all far smaller than those found for the ODS column. The elution mechanism is discussed below.

Nucleotides

Negative k' values were obtained on the GS-320 column, in contrast to the positive k' values obtained on the ODS column (Table II). As shown in Fig. 6, the k' values on the GS-320 column were apparently unaffected by the presence of acetonitrile in the eluent, whereas those on the ODS column were decreased sharply by the

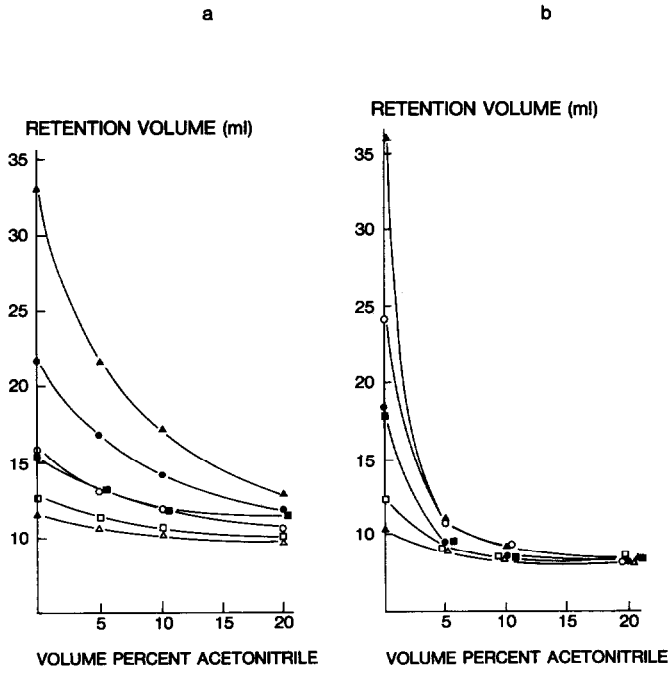


Fig. 4. Nucleic acid base retention volumes *versus* mobile phase acetonitrile content. Base designations as shown in Table I. Samples: 0.1 mg/ml solution, 20 μl for (a) and 6 μl for (b). ▲ = Adenine; ● = guanine; ■ = hypoxanthine; ○ = thymine; □ = uracil; △ = cytosine. Mobile phases: 10 mM sodium hydrogenphosphate (pH 6.0)–acetonitrile as indicated. Columns and other conditions as in Fig. 2.

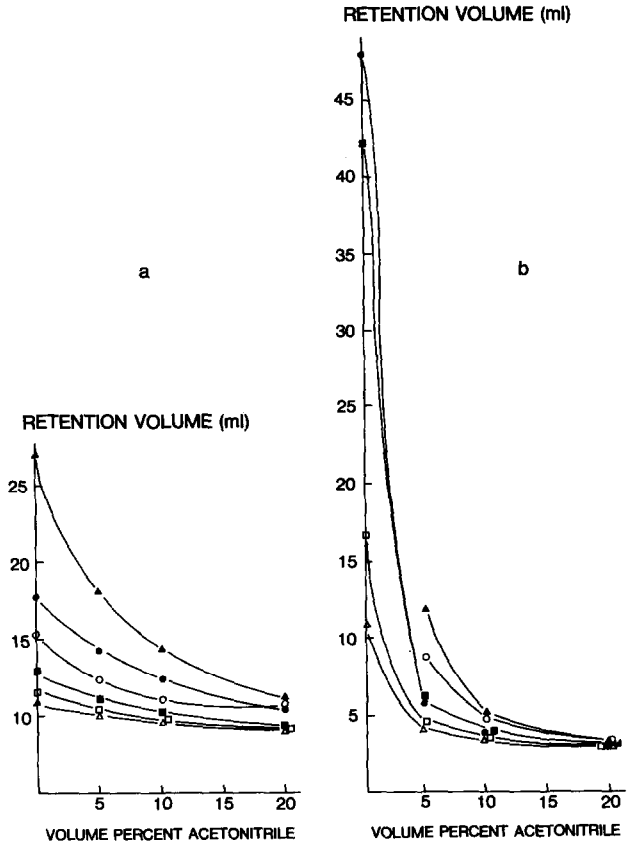


Fig. 5. Nucleoside retention volumes *versus* mobile phase acetonitrile content. Samples: ▲ = adenosine; ● = guanosine; ■ = inosine; ○ = thymidine; □ = uridine; △ = cytidine. Columns and conditions as in Fig. 4.

presence of as little as 5% of acetonitrile. Methylation of the GS-320 gel, which inherently contains a small amount of carboxyl groups, was found to result in larger retention volumes for nucleotides. For bases and nucleosides, on the other hand, no difference in retention volume was observed between the GS-320 and the methylated GS-320 gel columns. Overall, these results indicate that the elution mechanism for nucleotides was mainly ion repulsion on the GS-320 column and, as expected, reversed-phase partition on the ODS column.

Selectivity between a base and its derivatives

Between the three classes of nucleic acids (nucleotide, nucleoside and nucleic acid base), the two column exhibited significantly different elution orders. On the GS-320 column, the order was consistently nucleotide, nucleoside and base, whereas on the ODS column, it was nucleotide, base and nucleoside. The reasons for this difference may be of particular interest, and were therefore investigated further.

In plots of the relationship between the capacity factors (k') and Hansch's log P

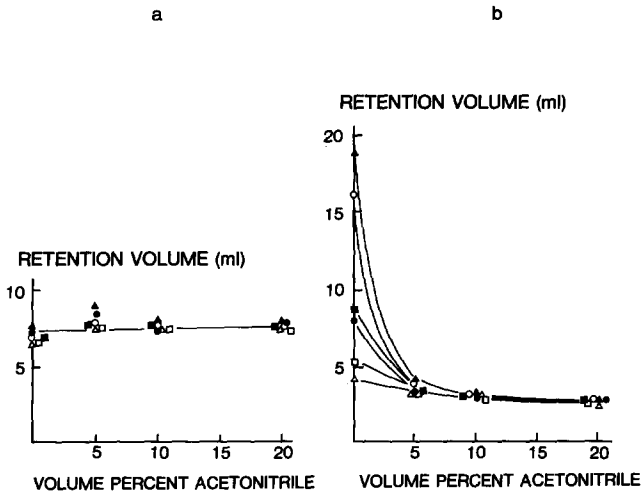


Fig. 6. Nucleotide retention volumes *versus* mobile phase acetonitrile content. Samples: ▲ = AMP; ● = GMP; ■ = IMP; ○ = TMP; □ = UMP; △ = CMP. Columns and conditions as in Fig. 4.

values of nucleosides and bases, one correlation curve was found to apply to both nucleosides and bases on the GS-320 column, but two separate correlation curves were found for the ODS column as a result of the high capacity factors of nucleosides on this column.

Linear van 't Hoff plots were obtained for both columns, as shown in Fig. 7, indicating that the nucleoside and base elutions involved reversed-phase partition, based on hydrophobic interaction, but that this interaction is remarkably strong for nucleosides on the ODS column.

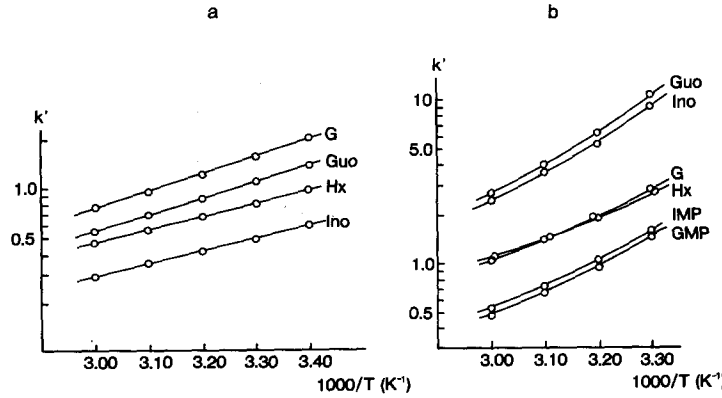


Fig. 7. Capacity factors as a function of absolute temperature. Columns: (a) Asahipak GS-320H; (b) YMC PACK AQ-312. Samples: (a) 20 μl of 0.1 mg/ml solution of guanine, guanosine, hypoxanthine or inosine; (b) 6 μl of 0.1 mg/ml solution of guanosine, inosine, guanine, hypoxanthine, IMP or GMP. Mobile phase, 10 m*M* sodium hydrogenphosphate (pH 6.0); flow-rate, (a) 1.0 ml/min, (b) 0.6 ml/min; temperature, 30°C; detector, 260 nm (0.32 a.u.f.s.).

We further found that the order of elution nucleotide, base and nucleoside observed here for the ODS column was also exhibited on the Asahipak ODP-50 column, which is packed with a gel containing C_{18} groups on a vinyl alcohol copolymer (results not shown). This suggests that the strong retention of nucleosides on the ODS column results from the strong hydrophobic character of its C_{18} group and not from its silanol group.

In a previous report, we described the retention behaviour of non-ionic surfactants, represented by the series C_9H_{19}–$C_6H_4(p)$–$(OCH_2CH_2)_nOH$, on an Asahipak GS-310 vinyl alcohol copolymer gel column, in comparison with that on an ODS column[7]. The GS-310 column, which is a hydrophobic polymer gel column without C_{18} groups, apparently interacted hydrophobically only with the alkylaryl groups at temperatures of $\leq$ 30°C in a mobile phase of aqueous acetonitrile at low to medium concentration, whereas the strongly hydrophobic ODS column apparently interacted hydrophobically with both the alkylaryl and the polyoxyethylene groups under the same conditions.

This suggests that an analogous interaction, involving the ribose groups of the nucleosides, might occur on the ODS gel. To explore this possibility for both the ODS and GS-320 gels, we investigated the effect of mobile phase NaCl and NaSCN contents on the retention of ribose alone by both columns, and on their retention of guanosine and inosine. As shown in Table IV, the capacity factor of ribose alone with a mobile phase containing no NaCl or NaSCN was nearly zero on the GS-320 column, indicating little or no hydrophobic interaction, and 0.34 on the ODS column. The addition of NaCl, an antichaotropic agent generally known to enhance hydrophobic interaction, effected no change in the ribose capacity factor on the GS-320 column, but substantially increased that on the ODS column. The addition of NaSCN, a chaotropic agent known to inhibit hydrophobic interaction, had no significant effect on ribose elution from either the GS-320 or the ODS column. These results are in accord with the assumption of hydrophobic interaction of nucleosides with both column gels. Although the results for ribose are not definitive, particularly because of the absence of a decrease in k' in the presence of NaSCN, they suggest that

TABLE IV

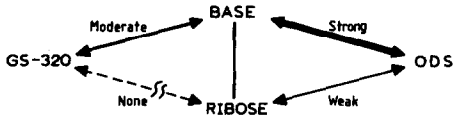
CAPACITY FACTORS (k')

Determined at 30°C with a mobile phase of pH 6.0 and indicated content on Asahipak GS-320H and, in this experiment only, YMC PACK AM-312 columns, at flow-rates of 1.0 and 0.6 ml/min, respectively.

Column	*Mobile phase*	k'		
		Ribose	*Guanosine*	*Inosine*
GS-320H	10 m*M* sodium hydrogenphosphate	0.02	1.10	0.49
	2 *M* sodium chloride	0.02	1.42	0.61
	2 *M* sodium thiocyanate	−0.03	0.32	0.05
AM-312	10 m*M* sodium hydrogenphosphate	0.34	10.85	9.33
	2 *M* sodium chloride	0.41	18.71	14.86
	2 *M* sodium thiocyanate	0.33	4.23	3.22

a weak hydrophobic interaction also occurs between the ribose group of nucleosides and the ODS gel, but not the GS-320 gel.

The effects of organic solvent and antichaotropic salt additions to the mobile phase, the correlations between Hansch's log P and log k' and the linearity of the Van 't Hoff plots are in accord with the following scheme of hydrophobic interactions:



Hydrophobic interactions occur on the weakly hydrophobic GS-320 gel, but are relatively weak and are limited to a nucleic acid base, as it occurs alone or as part of its derivative. On the strongly hydrophobic ODS gel, on the other hand, hydrophobic interactions are strong with all three classes of nucleic acid components, and appear to occur with both the ribose group as well as the base group, particularly in nucleosides.

This gel–ribose interaction would account for the particularly high k' values of nucleosides on the ODS column, and hence for the difference in the order of elution of nucleic acid components between the ODS and GS-320 columns.

Utility of weakly hydrophobic GS-320

Isocratic analyses were performed on the GS-320 column, based on the above indications of its weakly hydrophobic effect and in particular its apparent freedom from pronounced nucleoside retention. Efficient separation of nucleic acid components was observed, as shown in the chromatograms in Fig. 8.

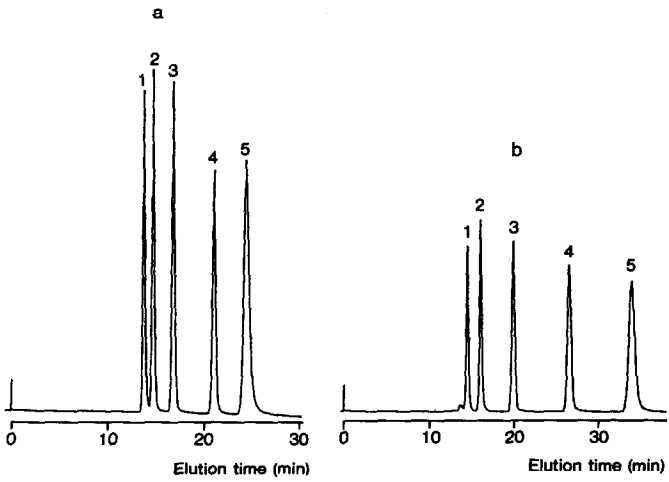


Fig. 8. Analysis of nucleic acid component mixtures. Column, Asahipak GS-320 (500 × 7.6 mm I.D.). Samples: (a) 15 μl of (1) ATP (23 μg/ml), (2) ADP (26 μg/ml), (3) AMP (22 μg/ml), (4) adenosine (22 μg/ml) and (5) adenine (8 μg/ml); (b) 15 μl of (1) GTP (23 μg/ml), (2) GDP (23 μg/ml), (3) GMP (20 μg/ml), (4) guanosine (21 μg/ml) and (5) guanine (12 μg/ml). Mobile phase, 200 mM phosphoric acid (pH 3.0); flow-rate, 1.0 ml/min; temperature, 30°C; detector, 260 nm (0.08 a.u.f.s.).

CONCLUSION

The results show that the elution of nucleic acid components on the GS-320 is effected primarily by hydrophobic interaction, similarly to that on the ODS column but substantially weaker and also differing in the following important respects.

(1) Within each class (nucleotide, nucleoside or base) a general tendency for elution of pyrimidines other than thymine before purines; elution of thymine after hypoxanthine from the GS-320 column, but after guanine from the ODS column, apparently because of interaction between the methyl group of thymine and the long alkyl group of the ODS gel.

(2) Between each base and its derivatives, elution in the order nucleotide, nucleoside and base from the GS-320 column but nucleotide, base and nucleoside from the ODS column; the difference in order is presumably attributable to restriction of interaction to the base group of nucleosides on the GS-320 column, and interaction with both the ribose as well as the base group of nucleosides on the ODS column.

(3) Elution of nucleotides before ethylene glycol, which is the non-retained solute, from the GS-320 column but after ethylene glycol from the ODS column, increased k' values for nucleotides on methylated GS-320 gel and the absence of any apparent effect by mobile phase acetonitrile on the k' values for nucleotides on the GS-320; all suggesting interaction dominated by ion repulsion between the phosphoric group of the nucleotide and the small number of carboxylic groups present in the GS-320 gel, in contrast to a predominant hydrophobic interaction between the octadecyl group of the ODS gel and the base group in the nucleotide.

The weakly hydrophobic polymeric structure of the GS-320 gel, and the resulting differences between its elution characteristics and those of the ODS column, permit highly practical, efficient analyses of complex molecules, based on the hydrophobicity of their components, as shown here for nucleic acid components by the chromatograms of component mixtures.

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