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TEMPERATURE, METHANOL, AND STRUCTURE EFFECTS IN THE REVERSED-PHASE LIQUID CHROMATOGRAPHY OF GUANINE AND HYPOXANTHINE AND THEIR RIBONUCLEOSIDES AND RIBONUCLEOTIDES

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SUMMARY

The combined effects of changes in temperature and percentage of methanol in the mobile phase on the retention behavior of two sets of purine compounds were investigated. With the bases guanine and hypoxanthine, there was a linear relationship between the ln k' and the reciprocal of the absolute temperature at all concentrations of methanol. However, with the nucleosides, in the presence of methanol, the relationship was non-linear, and there appeared to be two components in the curve, indicating two retention mechanisms, one at the higher temperatures, and a different one at the lower temperatures. With the monophosphate nucleotides, there were two peaks when methanol was present; the temperature at which the second peak appeared was dependent on the methanol concentration and the structure of the base in the nucleotide. With the di- and triphosphate nucleotides, each nucleotide was represented by multiple peaks when methanol was present in the mobile phase.

The enthalpies of retention were calculated for all the purine analogues, and the trend in the enthalpies corresponded to the retention order of purine compounds, as predicted by Brown and Grushka [*Anal. Chem.*, 52 (1980) 1210].

INTRODUCTION

Purine and pyrimidine compounds are very important biochemically, and research in this field has been greatly accelerated by the development of the reversed-phase mode of high-performance liquid chromatography (RP-HPLC). With RP-HPLC, the concentrations of free nucleotides, nucleotides, and their bases in physiological fluids and cell tissues can readily be determined. Analyses such as these are necessary in studies of cell growth, metabolic processes, and disease states[1–9].

Results have been reported of systematic investigations of the separate effects of methanol concentration in the mobile phase and temperature on the RP-HPLC retention behavior of selected purine nucleosides[4,10,11]. It was found that the capacity factors of these nucleosides are decreased by increasing either the temperature or the

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methanol concentration of the eluent. However, there have been no systematic investigations of the combined effects of these two operating parameters on nucleosides and on other purine analogues. Moreover, although structure–retention relationships of groups of purine and pyrimidine compounds have been found[12], the effects of mobile phase conditions on these relationships have not been examined.

Therefore, we investigated the combined effects of changes in methanol concentration and temperature on the retention behavior of two sets of purine compounds; hypoxanthine and guanine, and their ribonucleosides and ribonucleotides*.

By using these model compounds we could study the effects of substitution on the purine ring and of the presence of a ribosyl group and a phosphoribosyl group; thus, we could determine the effects of selected mobile phase conditions on the structure–retention relationships of these purine compounds.

EXPERIMENTAL

Instrumentation

An isocratic liquid chromatographic system, equipped with a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), and a constant-temperature (*ca.* 0.1°C) column compartment (DuPont, Wilmington, DE, U.S.A.) housing a 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) was used. Retention times and peak areas were measured with an HP3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Detection was by UV absorbance at 254 and 280 nm.

Columns

Prepacked, stainless-steel columns, Zorbax ODS, μBondapak C_{18}, and Partisil 5-ODS-3 were obtained from DuPont, Waters Assoc., and Whatman (Clifton, NJ, U.S.A.), respectively. The columns were 250 × 4.6 mm I.D. The size of the particles in the Zorbax and μBondapak columns was 10 μm, and in the Partisil column, 5 μm. The void time (t_0) was determined on each column by the method of Neidhart *et al.*[13]

Chemicals

The nucleotides, nucleosides and their bases were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade potassium dihydrogen phosphate was obtained from Fischer Scientific (Fairlawn, NJ, U.S.A.). All chemicals were of the highest purity available. Methanol, distilled-in-glass (Burdick & Jackson, Muskegon, MI, U.S.A.) and double-distilled, deionized water were used for the preparation of mobile phases. A stock solution was made up for each compound. From the stock solutions, a working standard solution was prepared in such a way as to yield 2.5 mmol of each purine analogue. The stock and working solutions were stored at −20°C.

Chromatography

The working buffer concentrate was prepared as a 0.020 *M* potassium dihy-

* The compounds investigated and their abbreviations are as follows: guanine (Gua), guanosine (Guo), guanosine-5′-monophosphate (GMP), guanosine-5′-diphosphate (GDP), guanosine-5′-triphosphate (GTP), hypoxanthine (Hyp), inosine (Ino), inosine-5′-monophosphate (IMP), inosine-5′-diphosphate (IDP) and inosine-5′-triphosphate (ITP).

drogen phosphate solution. The pH was adjusted to 5.5 with potassium hydroxide. If methanol was to be added to the buffer, the appropriate volume was added after 200 ml of water had been added to the buffer concentrate but before making the solution up to the final volume of 1 l with water. The amount of methanol in the eluent was 0, 2.5, 5.0, 7.5, 10.0, or 12.0%.

The buffer solutions were filtered through a Millipore (Milford, MA, U.S.A.) 0.45-μm filter, degassed by sonication, and purged with helium. All separations were obtained isocratically at a flow-rate of 1.0 ml/min and temperatures of 25, 35, 45, 55, and 65°C. The temperatures were constant within ± 0.1°C.

RESULTS AND DISCUSSION

Temperature effects in the presence and absence of methanol

The capacity factors (k') of all compounds, except the triphosphate nucleotides, decreased significantly with increasing temperature both in the absence and presence of 10% methanol (Table I). In the absence of methanol, the decrease was most pronounced for the nucleosides; for example, the k' value of guanosine was reduced from 12.93 at 25°C to 2.80 at 65°C, and the k' value of inosine from 10.94 at 25°C to 2.62 at 65°C. Less of a decrease in the k' values of the bases, mono- and diphosphate nucleotides was observed in going from 25 to 65°C, both in the presence and absence of methanol. Little or no effects of temperature were seen for the triphosphate nucleotides at 0 and 10% methanol. It was found that at 25°C with 10% methanol present, the k' values of all the nucleotides, except the triphosphates, were the same as the k' values at 65°C in the absence of methanol (Table I).

The order of retention (*i.e.*, nucleotides < bases < nucleosides), as found in

TABLE I

CAPACITY FACTORS (k')

The capacity factors were determined at temperatures of 25, 35, 45, 55, and 65°C with a mobile phase of 0.02 *M* potassium dihydrogen phosphate (pH 5.5), containing 0 and 10% methanol. The column was a Zorbax ODS (DuPont), and the flow-rate was 1.0 ml/min.

Compound	*k'*									
	0% Methanol					*10% Methanol*				
	25°C	*35°C*	*45°C*	*55°C*	*65°C*	*25°C*	*35°C*	*45°C*	*55°C*	*65°C*
Gua	4.53	3.55	2.69	2.12	1.71	1.82	1.60	1.35	1.17	1.04
Hyp	3.59	2.67	2.13	1.76	1.49	1.80	1.63	1.48	1.36	1.21
Guo	12.93	8.24	5.55	3.83	2.80	2.72	2.03	1.60	1.45	1.26
Inosine	10.94	7.15	4.94	3.51	2.62	2.35	1.82	1.60	1.45	1.26
GMP	2.23	1.67	1.28	1.03	0.85	0.94	0.86	0.81	0.54	0.52
IMP	1.94	1.62	1.33	1.13	1.02	1.06	0.98	0.90	0.67	0.65
GDP	1.74	1.72	1.31	1.04	0.80	0.91	0.84	0.80	0.65	0.52
IDP	1.02	0.95	0.84	0.75	0.66	0.65	0.62	0.62	0.61	0.59
GTP	–	–	1.10	1.05	0.88	0.45	0.46	0.48	0.49	0.49
ITP	0.57	0.56	0.54	0.54	–	0.49	0.49	0.45	0.45	0.45

the structure–retention studies of Brown and Grushka[12], was not affected either by elevated temperatures, 10% methanol, or the combination of the two factors.

From Table I it can also be seen that in the separation of these nucleotides, nucleosides, and their bases, temperature in combination with methanol concentration can be used to optimize the RP-HPLC separation of all of these purine analogues. In addition, the combination of mobile phase conditions can be used to optimize separation for the determination of the concentration of only one compound in a set of compounds. For example, if the quantitative analysis of only IMP is required in the presence of the hypoxanthine and guanine analogues, the best conditions are phosphate buffer with no methanol at either 25 or 65°C, or phosphate buffer with 10% methanol at temperatures ranging from 25 to 45°C. On the other hand, to separate all the nucleosides and their bases in the presence of their nucleotides, phosphate buffer without methanol at any temperature from 25 to 65°C can be used; however, elevated temperatures will give more rapid separations, although the lower temperatures give better resolutions. Adequate resolution cannot be achieved for these compounds in the presence of 10% methanol at any temperature in the range 25–65°C.

Relationship between ln k' and reciprocal of temperature

In reversed-phase chromatography, the change in free energy, ΔG^0, associated with the reversible binding of solutes to the functional groups of the stationary phase is related to the natural logarithm of the capacity factor, which is readily obtained experimentally. We can express the relationship by

$$\ln k' = -\frac{\Delta G^0}{RT} + \ln \Phi \tag{1}$$

where R is the gas constant, T is the absolute temperature and Φ is the phase ratio which is constant for a given column[14–17].

TABLE II

RETENTION ENTHALPIES (ΔH_R)

The retention enthalpies were measured in RP-HPLC separations, obtained with a mobile phase of 0.02 *M* potassium dihydrogen phosphate (pH 5.5), containing 0, 2.5, 5.0, 7.5, and 10% methanol at temperatures in the range 25–45°C. The column was a μBondapak C_{18} (Waters Assoc.).

Compound	*ΔH_R (Kcal/mol)*				
	Methanol (%)				
	0	*2.5*	*5.0*	*7.5*	*10.0*
Gua	4.92	4.12	3.97	3.74	3.82
Guo	7.98	6.92	6.26	5.78	5.00
GMP	4.23	3.09*	2.99*	1.72*	1.40*
GDP	2.68	3.61*	2.92*	1.83*	1.22*
GTP	–	0.34*	0.36*	0.38*	0.40*

* With increasing methanol concentration, there were two or more peaks in the chromatograms of the ribonucleotides.

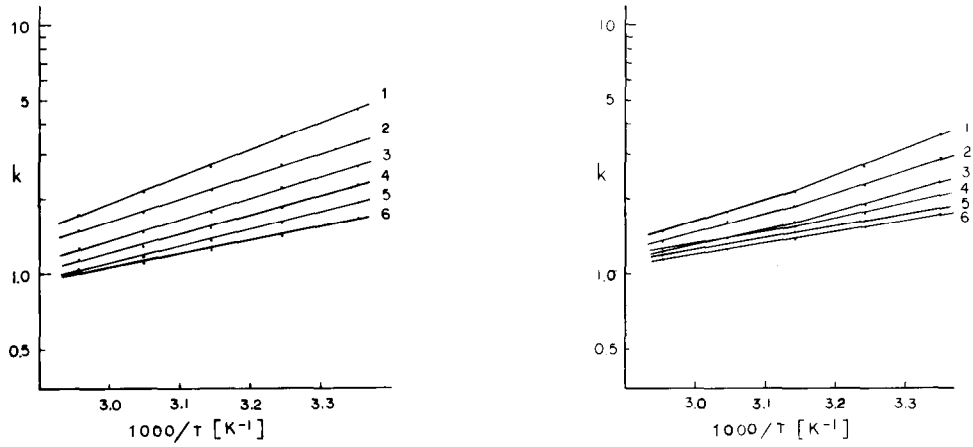

Fig. 1. Plots of the capacity factors of guanine on a logarithmic scale against the reciprocal temperature in reversed-phase chromatography. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10%; 6 = 12.5%.

Fig. 2. Plots of the capacity factors of hypoxanthine on a logarithmic scale against the reciprocal temperature in reversed-phase chromatography. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%; 6 = 12.5%.

It follows that

$$\ln k' = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \Phi \qquad (2)$$

Thus, the enthalpy of retention, ΔH_R, can be obtained from a plot of ln k' *vs.* the reciprocal of the absolute temperature (Table II).

The trend in the ΔH_R values supports the trend in capacity factors predicted by Brown and Grushka[12]; *i.e.*, nucleosides are retained longer than their bases, which are retained longer than their corresponding nucleotides.

Typical linear relationships between the ln k' of guanine and $1/T$ at various methanol concentrations are shown in Fig. 1. The same type of linear relationship was also exhibited by the base hypoxanthine (Fig. 2).

For the nucleosides, guanosine and inosine, the relationship between ln k' and $1/T$ was not linear when methanol was present in the mobile phase (Figs. 3 and 4). It appears that each curve includes two components, suggesting that in the presence of methanol, different retention mechanisms are operative in different temperature ranges.

With the monophosphate nucleotides, two peaks were present in the chromatogram whenever the eluent contained methanol. If ln k' of the peak with the longer retention time was plotted *vs.* $1/T$, the plots showed a typical linear relationship (Figs. 5 and 6). There were three or more peaks in the chromatograms of the di- and triphosphate nucleotides. However, by plotting only the peak with the longer

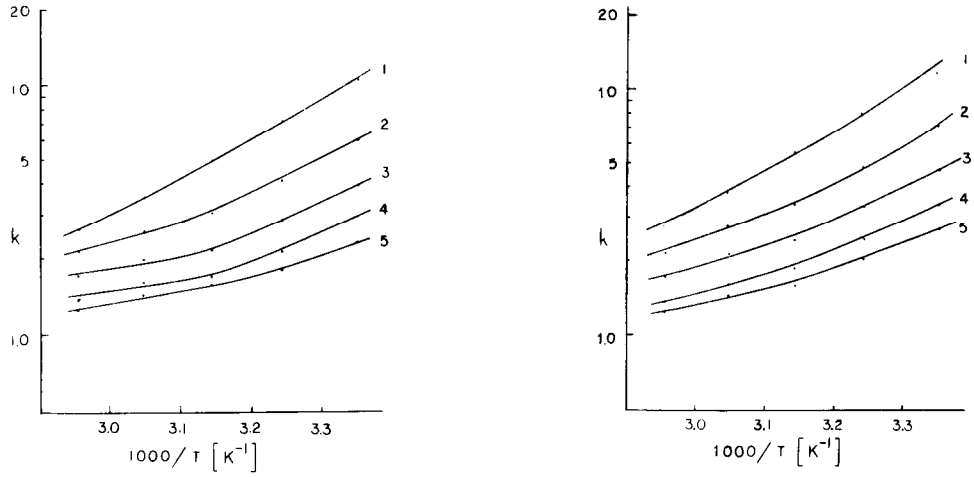

Fig. 3. Plots of the capacity factors of guanosine on a logarithmic scale against the reciprocal temperature in reversed-phase chromatography. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%.

Fig. 4. Plots of the capacity factors of inosine on a logarithmic scale against the reciprocal temperature in reversed-phase chromatography. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%; 6 = 12.5%.

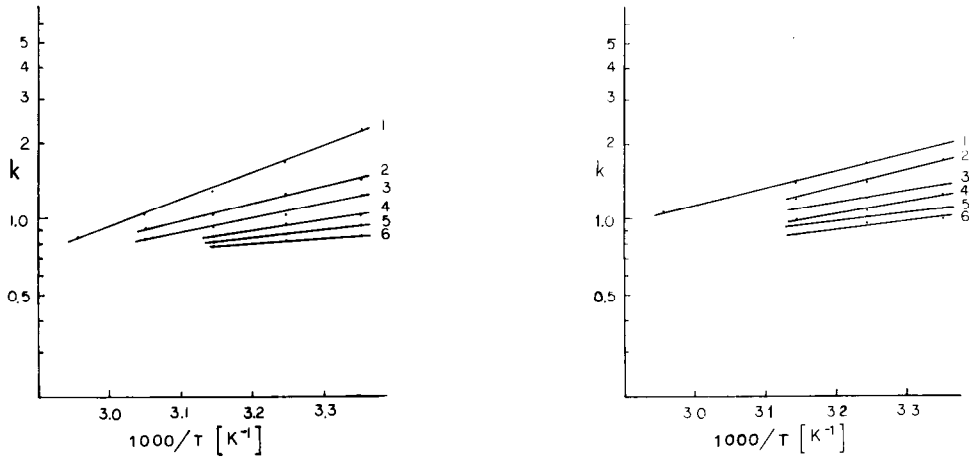

Fig. 5. Plots of the capacity factors of GMP on a logarithmic scale against the reciprocal temperature in reversed-phase chromatography. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%; 6 = 12.5%.

Fig. 6. Plots of the capacity factors of IMP on a logarithmic scale against the reciprocal temperature in reversed-phase chromatography. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%; 6 = 12.5%.

TABLE III

CAPACITY FACTORS (k') OF HYPOXANTHINE

Capacity factors of hypoxanthine, determined at temperatures of 25, 35, 45, 55, and 65°C with a mobile phase of 0.02 *M* potassium dihydrogen phosphate (pH 5.5), containing 0, 2.5, 5.0, 7.5, 10.0, and 12.5% methanol. The column used for this determination was a Partisil 5-ODS-3 (Whatman). The flow-rate was 1.0 ml/min.

Methanol (%)	*Temperature (°C)*				
	25	*35*	*45*	*55*	*65*
0	6.57	5.05	4.16	3.55	3.11
			5.52	4.66	4.10
2.5	5.28	4.32	3.70	3.30	2.86
			4.37	3.88	3.65
5.0	4.46	3.73	3.26	2.91	2.64
7.5	4.01	3.49	3.19	2.92	2.70
10.0	3.62	3.34	3.10	2.88	2.64
12.5	3.56	3.16	2.88	2.67	2.53

retention times, linear relationships were also obtained between ln k' and $1/T$; thus, ΔH_R could be calculated from the graphs (Table II).

Appearance of multiple peaks

In some peaks of the chromatograms, doublet or multiplet peaks were observed. In order to check the validity of these peaks, *i.e.* that they were not artifacts caused by the column or contamination of the solution, fresh solutions of each compound were prepared. Each solution was chromatographed alone on the column in use, on a new column from the same manufacturer and on columns from different manufacturers. It was found that two peaks were observed in the chromatograms of hypoxanthine in the upper temperature range (45, 55 and 65°C), either in the absence of methanol or when only 2.5% methanol was present in the mobile phase (Table III). At all other temperatures and methanol concentrations, only one peak was seen. Double peaks were not seen in the chromatograms of guanine, guanosine or inosine. However, double or multiple peaks were present in many of the chromatograms of the ribonucleotides. In the chromatograms of the monophosphate nucleotides, the appearance of the second peak depended upon the temperature, methanol concentration, and substitution on the purine ring (Figs. 7 and 8). For example, with IMP, the second peak did not appear in the absence of methanol at any temperature [Fig. 8(1)]. However, at 25°C, a small shoulder, representing the second peak, appeared where the eluent contained 10% methanol, and a sizeable peak was present with 12.5% methanol [Fig. 8(5) and 8(6)]. In general, the second peak appeared to form at lower temperatures with higher methanol concentrations (Figs. 7 and 8). With GMP the general trends were the same; *i.e.*, the second peak formed at lower temperatures with higher methanol concentration. However, the time of appearance and size of the second peak was dependent on the purine base structure. For example, with GMP, two peaks were approximately of equal size at 45°C in the presence of 10% methanol [Fig. 7(5)], but with IMP two peaks of equal size were not seen at the

temperatures used in this study. In the absence of methanol, only one peak was seen in the GMP chromatograms [Fig. 7(1)], and a small amount of the second peak was present at 25°C only when there was 12.5% methanol in the mobile phase [Fig. 7(6)].

With the diphosphate nucleotides, two or three peaks were present at higher temperatures and methanol concentration. Multiple peaks, which were not well resolved, appeared in the chromatograms of the triphosphate nucleotides under the same conditions.

There is no obvious explanation for the appearance of more than one peak for a single compound in an RP-HPLC chromatogram. Whether two isomeric forms are present in each commercial sample of a nucleotide and we are able to separate them only under selected conditions of methanol concentration and temperature, or whether a reaction is taking place is not known. It has been found previously that a "poly-peak phenonomen", *i.e.* the presence of two or more peaks for one compound, has been observed when temperature, pressure, or flow-rate are elevated[18–20]. We find that there appears to be a synergistic effect of the methanol concentration with temperature. For example, elevation of the temperature may affect selectively the solubility of one solute in methanol. Possibly the changes affect certain functional groups in a compound differently; *e.g.*, the phosphate groups are affected by these dual changes to a far greater extent than is the purine ring or the riboside.

In addition, the following factors may also be affected by the increases in temperature and methanol concentration: the viscosity of the solvent system, the relative mass transport of a solute, the relative solvation effects, as well as "stacking" or "destacking" of the purine compounds[21–27]. Furthermore, the combination of

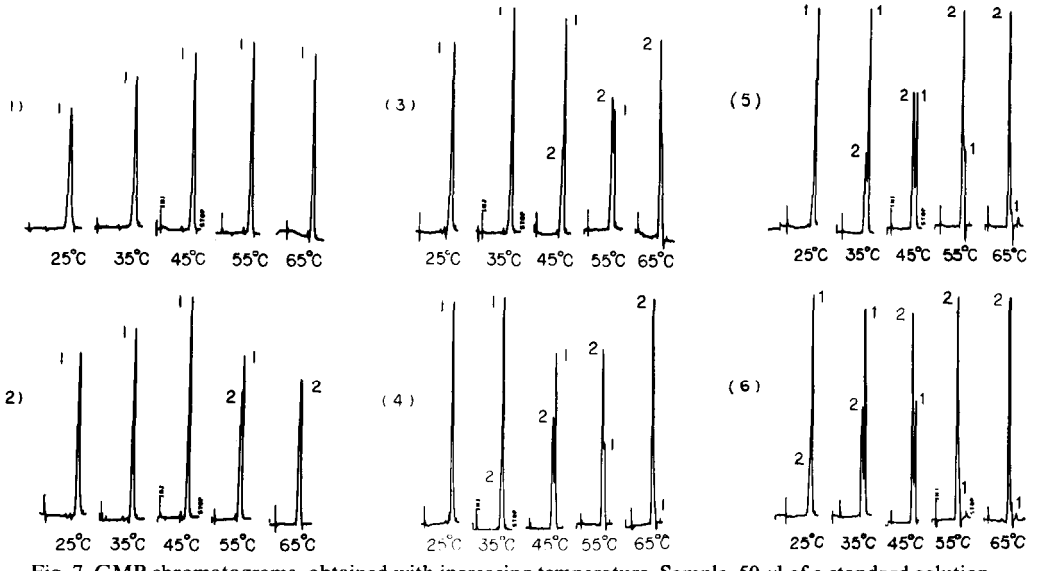

Fig. 7. GMP chromatograms, obtained with increasing temperature. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The scale on the asbscissa is 1 cm = 1 min. The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%; 6 = 12.5%. Peak 1 is the peak that appears first in the chromatogram. Peak 2 is the peak with shorter retention time that appears with increasing temperature.

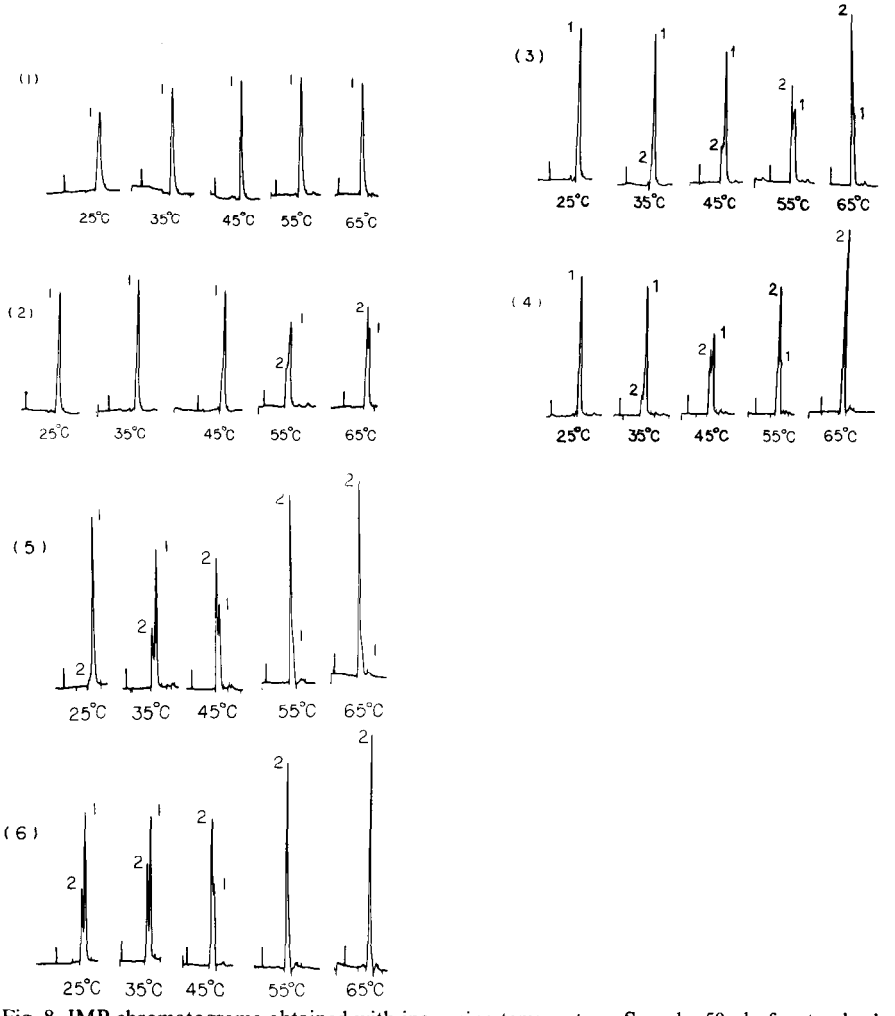

Fig. 8. IMP chromatograms obtained with increasing temperature. Sample, 50 μl of a standard solution. Column, μBondapak C_{18}. Buffer, 0.020 *M* potassium dihydrogen phosphate (pH 5.5). Flow-rate, 1.0 ml/min. Detector, 254 nm (0.05 a.u.f.s.). The scale on the abscissa is 1 cm = 1 min. The amount of methanol in the eluent: 1 = 0%; 2 = 2.5%; 3 = 5.0%; 4 = 7.5%; 5 = 10.0%; 6 = 12.5%. Peak 1 is the peak that appears first in the chromatogram. Peak 2 is the peak with the shorter retention time that appears with increasing temperature.

elevated temperature and increased methanol concentration may affect the stationary phase and may increase the selectivity. Further studies are in progress to determine the structures of the compounds represented by the two peaks and the reasons for the double peak formation.

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