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MOBILE PHASE AND TEMPERATURE STUDIES IN THE REVERSED-PHASE LIQUID CHROMATOGRAPHY OF INOSINE AND GUANOSINE COMPOUNDS

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SUMMARY

The results are reported of a systematic investigation of the effects of the mobile phase methanol concentration and pH, temperature and flow-rate, separately or in combination, on the reversed-phase liquid chromatography of selected biologically important nucleosides. As expected in a reversed-phase separation, the retention times of each nucleoside decreased proportionally with increasing methanol concentration. The pH effects were dependent on the percentage of methanol present. A methanol selectivity factor is proposed for expressing numerically the effect of methanol concentration on retention.

Increasing temperature also decreased retention, but the extent of the effect was dependent on the structure of the nucleoside. Thus, chromatography of a sample at temperatures above ambient could cause peak mergers. A method using experimentally determined retention times of two nucleosides at two temperatures was proposed for determining exact t_0 (void time) values of a system. The t_0 values which were calculated by an equation and determined graphically were in good agreement.

INTRODUCTION

In earlier research on the liquid chromatographic (LC) profiling of nucleosides in urine, serum and/or plasma, noticeable differences were detected between the profiles of normals and individuals with various types of cancer (1—7]. Brown and co-workers [8—10] found an elevation of guanosine (G)**

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**The symbols used are those listed in H. Sober, R.A. Harte and E.K. Sober, Handbook of Biochemistry, The Chemical Rubber Co., Cleveland, OH, 1970.

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and inosine (I) levels, the presence of the N^2,N^2-dimethylguanosine (m_2^2G), and increased concentrations of N^2-methylguanosine (m^2G) and 1-methylinosine (m^1I) in the serum of patients with breast and lung cancer, as well as lymphocytic leukemia. Gehrke and co-workers [11—15] also reported elevated levels of m_2^2G, 1-methylguanosine (m^1G) and m^1I in the urine of patients with Burkitt's lymphoma, lung, colon, breast, and other types of cancer.

Furthermore, in a chemometric approach to classifying the high-performance liquid chromatographic (HPLC) profiles of acute and chronic leukemic patients and normal subjects by multivariant linear analysis [16] and pattern recognition techniques [17], it was demonstrated that inosine and guanosine compounds were very important in discriminating between normal groups and groups with leukemia.

Although reversed-phase liquid chromatographic (RP-LC) methods have been used routinely for the quantitative determination of inosine and guanosine nucleosides in physiological fluids [18—20], only a little work [21—25] has been done to study systematically the RP-LC retention behavior of these nucleosides.

Recently, we reported on a method of optimization using orthogonal experiments to optimize resolution of inosine and guanosine compounds [26]. It was found that the concentration of methanol and flow-rate in mobile phase were highly significant factors at constant temperature, and the conditions giving optimal isocratic resolution of the six nucleosides of interest were as follows: temperature, 22 ± 1°C; flow-rate, 2 ml/min; eluent, 0.02 *M* potassium dihydrogen phosphate containing 10% methanol, pH 4.5—6.5. In this paper we report on the results of a systematic study of the effects of methanol concentration, pH, temperature and flow-rate separately and in combination on the RP-LC of selected inosine and guanosine nucleosides. In addition, we present a method for determining t_0 (void time) values using analyses at two temperatures and a method for numerically expressing retention behavior as a function of methanol concentration.

EXPERIMENTAL

Instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) ALC 204 liquid chromatograph, equipped with Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model 440 dual-wavelength detector and a Model UGK injector, was used. An isocratic LC system, equipped with an M6000A pump (Waters Assoc.), a constant-temperature (± 0.1°C) column compartment (DuPont, Wilmington, DE, U.S.A.) housing a 7125 sample injector (50-μl loop, Rheodyne, Berkeley, CA, U.S.A.) and an M440 dual-wavelength detector (Waters Assoc.), was used in the temperature experiments. Retention times and peak areas were measured using an HP3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Detection was by UV absorbance at 254 and 280 nm.

Column

Prepacked, stainless-steel columns (250 × 4.6 mm), Zorbax ODS (octadecylsilica, particle size 10 μm) were obtained from DuPont. The t_0 value was

determined initially by the methods of Berendsen et al. [27] and Neidhart et al. [28].

Chemicals

The nucleosides I, G, m^1I, m^1G, m^2G, and m_2^2G were purchased from Sigma (St. Louis, MO, U.S.A.). The structures are shown in Fig. 1. HPLC-grade potassium dihydrogen phosphate was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other chemicals were of the highest purity available. Methanol, distilled in glass, from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), and double-distilled, deionized water were used for the preparation of mobile phase solutions.

Single compound stock solutions of nucleosides were prepared to yield concentrations of about 1.00 mol/ml (I, G, m^1I) or 0.25 mol/ml (m^1G, m^2G, m_2^2G) in double-distilled deionized water. The working standard solution was composed of 1.0 ml each of I, G and m^1I and 4.0 ml each of m^1G, m^2G and m_2^2G. The total mixture was diluted to a final volume of 100 ml. Of this solution 50 µl were used for the experiments. All stock and working solutions of nucleosides were stored at −20° C.

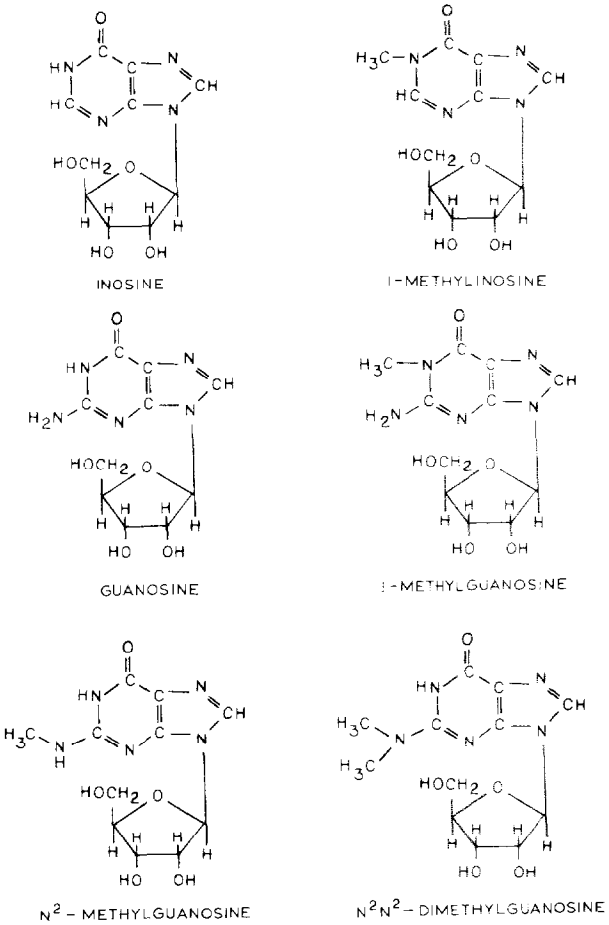

Fig. 1. Structures of the compounds investigated.

The working buffer concentrate was prepared as a 0.02 *M* potassium dihydrogen phosphate solution. The pH was adjusted with a few drops of either potassium hydroxide or orthophosphoric acid. If methanol was to be added to the buffer, the approriate 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 with double-distilled, deionized water. All buffers were filtered through a Millipore (Milford, MA, U.S.A.) 0.45-μm filter, degassed by sonication, and purged with helium.

Isocratic elution was carried out with the eluent of 0.02 *M* potassium dihydrogen phosphate containing various concentrations of methanol by volume. Where different chromatographic conditions were used, they are given in the text or in the legends of the figures.

RESULTS AND DISCUSSION

Effects of methanol concentration

Generally, the addition of an organic modifier, such as methanol, to a reversed-phase system decreases the retention of the solute. This effect has been attributed to a decrease of the surface concentration of the counter-molecule because of the competition by the co-solvent [29].

On the examination of the effects of percentage organic modifier in 0.02 *M* potassium dihydrogen phosphate eluent system at pH values of 4.5, 5.5 and 6.5, we also observed that the retention times of all the compounds decreased when there was 15% methanol in the mobile phase (Fig. 2B). The decrease in capacity factor (k') of m_2^2G was more pronounced than the k' values of the other compounds. However, when only 5% or 10% methanol was present in the mobile phase, the changes in the k' values were negligible (Fig. 2A).

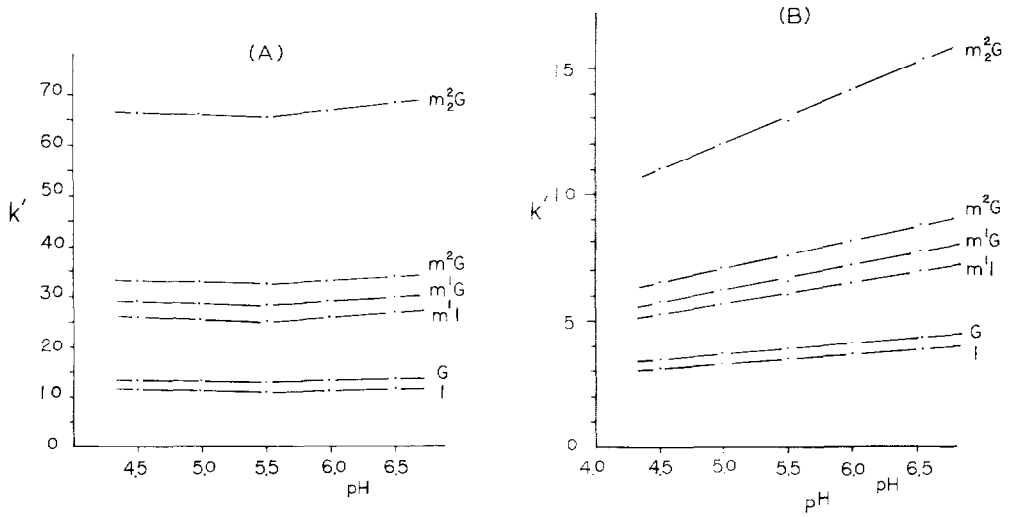

Fig. 2. Effect of pH of the mobile phase on capacity factor k' in the RP-LC isocratic separation of the inosine and guanosine compounds. Sample: 50 μl of standard solution. Column, Zorbax ODS (250 × 4.6 mm). Buffer: 0.02 *M* potassium dihydrogen phosphate with (A) 5% methanol, (B) 15% methanol. Flow-rate: 1 ml/min. Detector: 254 nm, 0.05 a.u.f.s. Temperature: 22 ± 1°C.

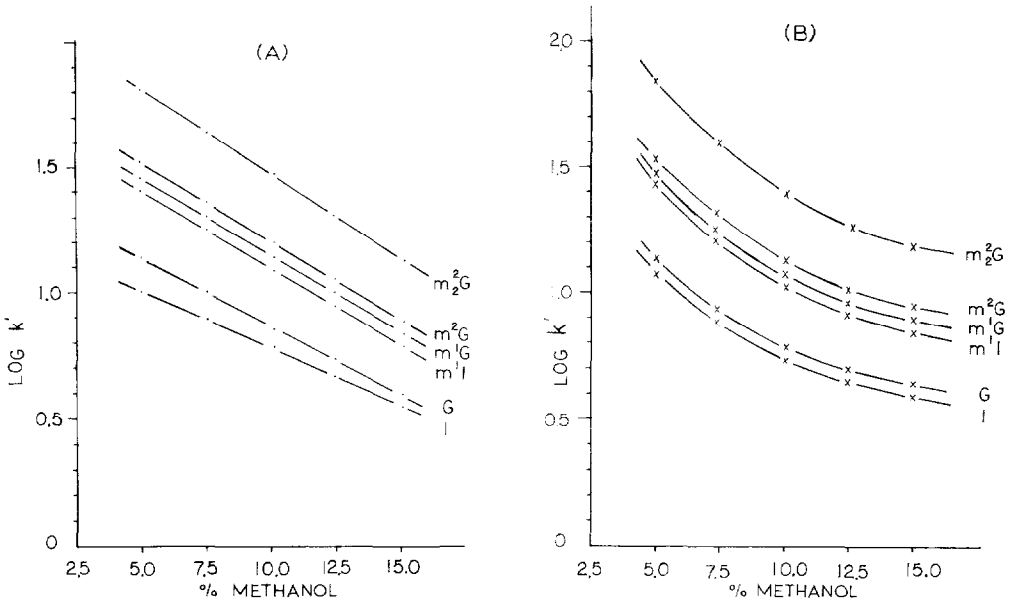

Fig. 3. Log k' of the inosine and guanosine compounds versus percentage methanol in 0.02 M potassium dihydrogen phosphate at (A) pH 5.5 and (B) pH 6.5. Sample: 50 μl of standard solution. Column: Zorbax ODS (250 × 4.6 mm). Flow-rate: 1 ml/min. Detector: 254 nm, 0.05 a.u.f.s. Temperature: 22 ± 1°C.

When the percentage of methanol was plotted versus the log k' at various pH values, the plots were linear at pH 5.5, a behavior that is expected if the solvophobic mechanism is operative (Fig. 3A). However, the plots were non-linear at pH 6.5, indicating that at that pH, retention is influenced by other processes in addition to or in place of solvophobic ones (Fig. 3B).

Methanol selectivity factor (MSF)

To compare the effects of methanol concentration under various conditions, a parameter called MSF was proposed. The MSF value expresses the retention of a nucleoside as a function of methanol concentration in the eluent. This factor is defined as the ratio of the adjusted retention time at a given methanol concentration to the adjusted retention time with no methanol in the mobile phase, i.e. MSF = $(t_R)_{i\%CH_3OH}/(t_R)_{0\%CH_3OH}$, where t_R is the adjusted retention time of a particular nucleoside.

For example, when 2.5% methanol was present in the eluent at 40°C, the MSF value for both I and G was 0.62, whereas at 10% methanol it was 0.32 for I and 0.30 for G. Thus, it can be seen from Table I that the MSF values increased with increasing temperature at each concentration of methanol and decreased with increasing methanol concentration at each temperature.

Therefore, the retention for any given nucleoside at various temperatures can be shown as a function of methanol, and this relationship can be expressed numerically (Table I). The larger number (i.e., nearly 1.00) indicated that the methanol caused little decrease in t_R.

TABLE I

METHANOL SELECTIVITY FACTOR (MSF) OF INOSINE AND GUANOSINE

Nuceloside	Temperature (°C)	Percentage methanol in buffer*			
		2.5	5.0	7.5	10.0
Inosine	25	0.57	0.39	0.29	0.24
	30	0.60	0.40	0.31	0.26
	35	0.60	0.44	0.38	0.32
	40	0.62	0.46	0.37	0.32
	45	0.64	0.48	0.39	0.37
Guanosine	25	0.56	0.38	0.29	0.23
	30	0.60	0.41	0.31	0.26
	35	0.61	0.43	0.33	0.28
	40	0.62	0.45	0.35	0.30
	45	0.64	0.47	0.38	0.33

*The buffer was a 0.02 *M* potassium dihydrogen phosphate solution, pH 5.5.

TABLE II

RETENTION TIMES OF GUANOSINE COMPOUNDS AT THREE TEMPERATURES

Temperature (°C)	Retention time (min)		
	G	m^2G	m_2^2G
35	5.29	9.37	16.16
40	4.74	8.10	13.91
45	3.76	5.83	9.74

Effect of temperature

In the early days of HPLC, elevated temperatures (approximately 70°C) were routinely used to separate nucleotides on pellicular packings [30, 31]. Although temperature programming is rarely used in HPLC, temperature can affect both the capacity factors and resolution in a given separation. Thus, for reproducibility of a given separation, constant temperature is required [30].

Today ambient temperatures are usually used in the RP-LC analyses of nucleosides [18—20]. Since it cannot be predicted how separations will be affected by elevated column temperatures, we investigated the effect of temperature from 25°C to 45°C on the retention times of the nucleosides of interest. Retention times of the guanosine compounds are shown in Table II. It was found that k' values of nucleosides decreased with increasing temperatures. However, since the amount of change in the k' value was different for each nucleoside, an increase in column temperature can cause a merger of some of the peaks. For example, at 35°C m^1I merges with m^1G, and at 45°C I also merges with G (Figs. 4 and 5). Thus, if the RP-LC analysis of a biological sample is run at 35°C and the m^1I—m^1G is identified as m^1I, the peak will appear to be larger than it actually is, and the concentration of m^1I will be erroneously reported as elevated. However, if the flow-rate is in-

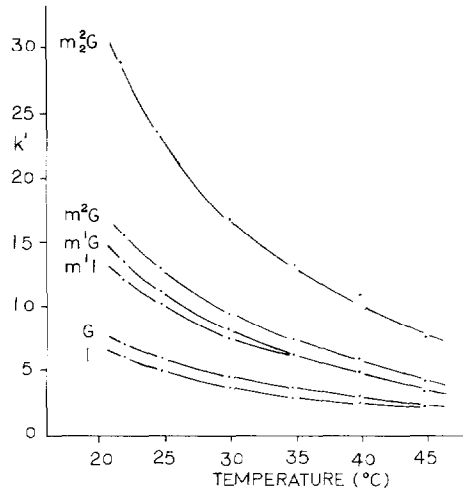

Fig. 4. The influence of the column temperature on the capacity factors (k') of inosine and guanosine compounds. Sample: 50 μl of a standard solution. Column: Zorbax ODS (250 × 4.6 mm). Buffer: 0.02 *M* potassium dihydrogen phosphate, pH 5.5 with 10% (v/v) methanol. Flow-rate: 1.0 ml/min. Detector: 254 nm, 0.05 a.u.f.s.

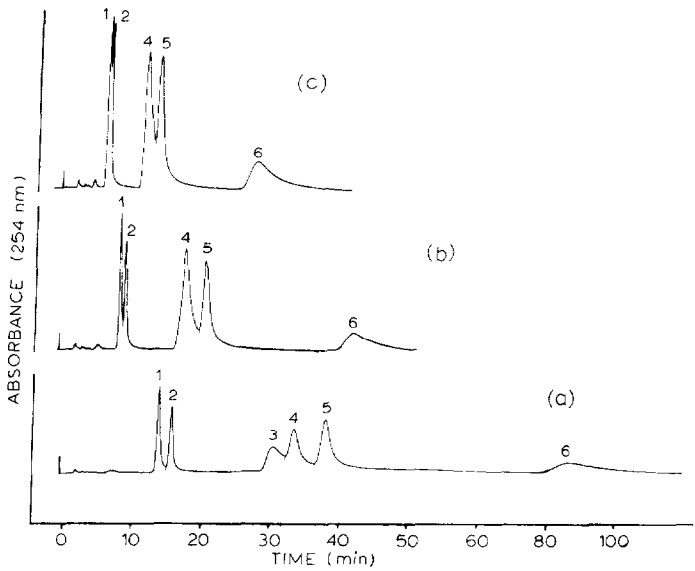

Fig. 5. The influence of column temperature on the RP-LC separation of inosine and guanosine compounds. Sample: 50 μl of a standard solution. Column: Zorbax ODS (250 × 4.6 mm). Buffer: 0.02 *M* potassium dihydrogen phosphate, pH 5.5 with 5% (v/v) methanol. Flow-rate: 1.0 ml/min. Detector: 254 nm, 0.05 a.u.f.s. Temperature: 22°C (a), 35°C (b) and 45°C (c). Peaks: 1 = I; 2 = G; 3 = m^{1}I; 4 = m^{1}G; 5 = m^{2}G; 6 = m$_2^2$G.

creased, the k' values decreases. With the flow-rates investigated, the k' values decreased but the peaks did not merge (Fig. 6).

In addition, the effects of both temperature and methanol were investigated on the separation of I and G (Table III). It was found that the temperature effects were moderated by the presence of methanol in the eluent. For

TABLE III

CAPACITY FACTORS (k') AND RELATIVE RETENTIONS (α) OF INOSINE (I) AND GUANOSINE (G)

Sample: 50 μl of a standard solution. Column: Zorbax ODS (250 × 4.5 mm). Buffer: 0.02 *M* potassium dihydrogen phosphate, pH 5.5. Flow-rate: 1.0 ml/min. Detector: 254 nm, 0.05 a.u.f.s.

Methanol concentration (%)	45°C			40°C			35°C			30°C			25°C		
	k'		α	k'		α	k'		α	k'		α	k'		α
	I	G		I	G		I	G		I	G		I	G	
0	8.77	9.79	1.12	10.35	11.71	1.13	12.41	14.21	1.15	15.19	17.64	1.16	18.66	21.93	1.18
2.5	5.66	6.27	1.10	6.47	7.24	1.12	7.54	8.60	1.14	9.18	10.61	1.15	10.54	12.26	1.16
5.0	4.24	4.64	1.09	4.75	5.27	1.11	5.40	6.09	1.13	6.09	7.06	1.15	7.20	8.38	1.16
7.5	3.45	3.72	1.09	3.79	4.15	1.10	4.20	4.68	1.11	4.72	5.33	1.13	5.48	6.33	1.16
10.0	3.27	3.27	1.00	3.34	3.60	1.08	3.65	4.00	1.10	3.99	4.44	1.11	4.51	5.12	1.14

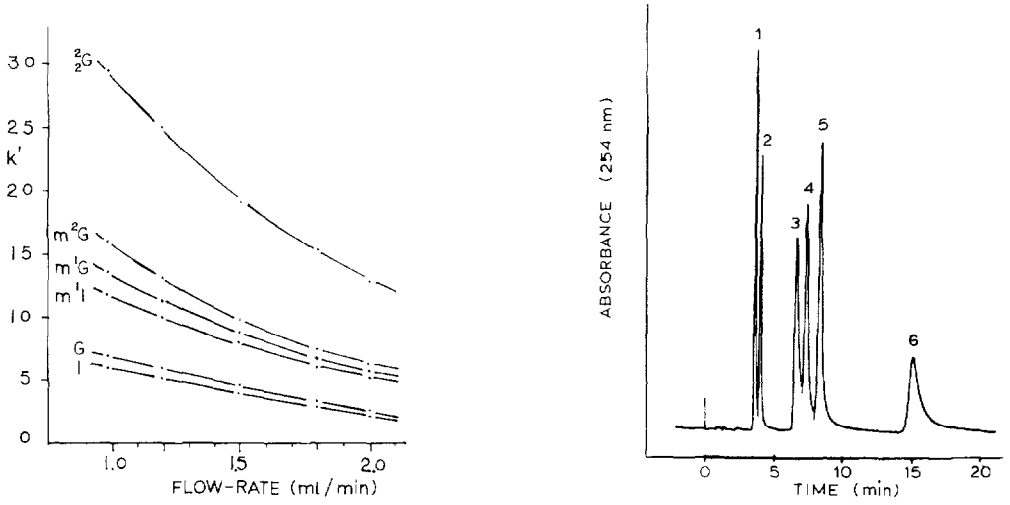

Fig. 6. The influence of flow-rate on the RP-LC separation of inosine and guanosine compounds. Sample: 50 μl of standard solution. Column: Zorbax ODS (250 × 4.6 mm). Buffer: 0.02 *M* potassium dihydrogen phosphate, pH 5.5 with 10% (v/v) methanol. Detector: 254 nm, 0.05 a.u.f.s.

Fig. 7. The RP-LC isocratic separation of the inosine and guanosine compounds. Sample: 50 μl of a standard solution. Column: Zorbax ODS (250 × 4.6 mm). Buffer, 0.02 *M* potassium dihydrogen phosphate with 10% methanol, pH 5.5. Flow-rate: 2.0 ml/min. Temperature: 22 ± 1°C. Detector: 254 nm, 0.05 a.u.f.s. Peaks: 1 = I; 2 = G; 3 = m^{1}I; 4 = m^{1}G; 5 = m^{2}G; 6 = m$^2_2$G.

example, at 45°C the k' value of I was 8.77 when there was no methanol in the eluent. With 5% methanol k' was 4.24 and with 10% methanol k' was 3.27. The I and G peaks had a relative retention (α) value of 1.00 (i.e., the peaks merged) at 45°C in 10% methanol, but the peaks did not merge in 2.5% methanol until the temperature was 65°C. An optimized separation of all six nucleosides is shown in Fig. 7. For this separation the temperature was kept constant at 22°C, the flow-rate was 2.0 ml/min, and the eluent was a 0.020 *M* potassium dihydrogen phosphate solution containing 10% methanol (pH can be from 4.5 to 6.5).

Determination of zero retention time

The zero retention time (t_0) or the retention time of a compound which is not retained on the column is necessary for the calculation of k', mass distribution coefficients (K), α, resolution (R_S) and efficiency (HETP). A number of different experimental methods have been used for the determination of t_0 values [27, 28]. However, these methods depend on the porous structure of the stationary phase; thus, the exact t_0 values cannot be determined. Since the t_0 values determined by the reported methods are usually too high, the k' values are low. Therefore, there is a need for a simple universal method by which t_0 values can be determined more exactly.

Since the t_0 value is defined as the retention time of non-retained compounds in the mobile phase, t_0 values should be temperature-independent. Eqn. 1 is a common equation for mass distribution coefficients.

$$k'_A = a \cdot g_A(T) \text{ and } k'_B = b \cdot g_B(T) \quad (1)$$

Here a and b are temperature-independent factors, and A and B are components A and B, and $g_A(T)$ and $g_B(T)$ are the sorption enthalpies of components A and B. Since $g_A(T) = g_B(T)$ for chemically related substances, then it follows that:

$$t_0 = \frac{t_{R_A}(T_1) \cdot t_{R_B}(T_2) - t_{R_A}(T_2) \cdot t_{R_B}(T_1)}{t_{R_A}(T_1) + t_{R_B}(T_2) - t_{R_A}(T_2) - t_{R_B}(T_1)} \quad (2)$$

where $t_{R_A}(T)$ and $t_{R_B}(T)$ are the retention times for A and B at temperature T.

To determine experimentally the t_0 values in our system, two sets of related compounds, for example G and m^2G or G and m_2^2G were used. Using a flow-rate of 1.0 ml/min with an eluent of 0.02 M potassium dihydrogen phosphate, pH 5.5 containing 10% methanol on a 4.6 × 250 mm Zorbax ODS column at 35°C, 40°C or 45°C, the mean values of t_0 were calculated from 2. The experimental $t_R(T)$ values for the three nucleosides are shown in Table II, and were calculated using these experimental data.

To determine t_0 graphically using these nucleosides, only two temperature points are necessary (Figs. 8 and 9). The t_0 values obtained graphically (Table IV) were in excellent agreement with the t_0 values calculated with eqn. 2 and had a precision of less than ± 3%.

Therefore, for the practical determination of t_0, the following procedure is recommended: (1) Measure t_R for nucleosides A and B (t_{R_A} and t_{R_B}) at two

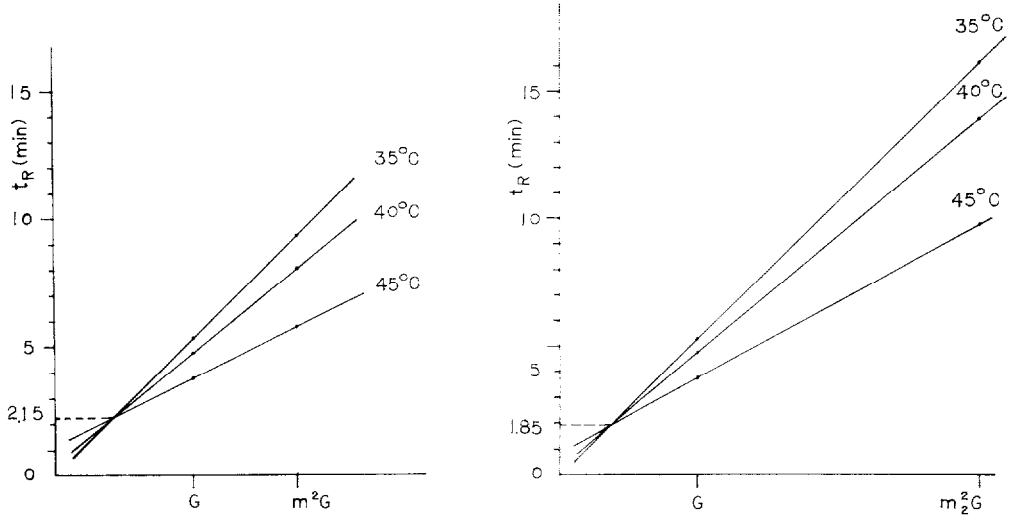

Fig. 8. Graphic evaluation of t_0 via temperature-dependent t_R values of guanosine and N^2-methylguanosine. Sample: 50 µl of a standard solution. Column: Zorbax ODS (250 × 4.5 mm). Buffer: 0.02 M potassium dihydrogen phosphate, pH 5.5 with 10% methanol. Detector: 254 nm, 0.05 a.u.f.s.

Fig. 9. Graphic evaluation of t_0 via temperature-dependent t_R values of guanosine and N^2,N^2-dimethylguanosine. Sample: 50 µl of a standard solution. Column: Zorbax ODS (250 × 4.5 mm). Buffer, 0.02 M potassium dihydrogen phosphate, pH 5.5 with 10% methanol. Detection: 254 nm, 0.05 a.u.f.s.

TABLE IV

ZERO RETENTION TIMES AS DETERMINED BY CALCULATION AND BY THE GRAPHICAL METHOD

Related substances	t_0 (min)		
	Calculated*	Mean ± S.D.	Graphical method**
m^2G	1.85	2.07 ± 0.19	2.15
	2.17		
	2.19		
m_2^2G	1.89	1.86 ± 0.08	1.85
	1.77		
	1.92		
Mean		1.97	2.00

*Calculated from eqn. 2 and t_R in Table II.
**Determined from the graphs shown in Figs. 8 and 9.

different temperatures (T_1 and T_2). In order to minimize the error of t_0, the intervals between T_1 and T_2 should be as large as possible. (2) Plot t_{R_A} and t_{R_B} for both temperatures at arbitrary positions x_1 and x_2 on the abscissa. (3) The y value of the point of intersection of the two straight lines for T_1 and T_2 is equal to the t_0 value of this system.

CONCLUSIONS

The decrease in retention time with increasing methanol concentration is the behavior expected in reversed-phase separations when the solvophobic mechanism is operative. When 5% or 10% methanol was present in the eluent, changes in pH in the range of 4.5—6.5 had no effect on retention. When 15% methanol was present, the k' values were higher at a pH of 6.5 than at 4.5 and m_2^2G showed a greater increase in retention than the other five nucleosides. Thus, greater solvophobicity is suggested at the higher methanol concentration and pH than under other conditions.

The effects of temperature are complex as we know from the Van Deemter equation. The most obvious reason for the reduction of k' values with the elevation of temperature is that increasing temperature decreases viscosity, which in turn affects flow-rate. Therefore, an advantage of an elevated temperature is that flow-rate can be increased without increasing pressure. However, temperature will also affect the mass transfer terms, both in the mobile and stationary phase, as well as solute—solvent and solute—stationary phase interactions. Although elevated temperatures decreased the retention times of all the nucleosides, the effects were not uniform and some of the peaks began to merge at 35°C. From the graph of k' versus temperature, indications exist that at temperatures higher than 45°C, all the peaks would eventually merge, eliminating resolution. However, if flow-rate is increased, lower k' values can be obtained and the effects are relatively the same for each nucleoside. Therefore, if faster separations are necessary, it is preferable to increase flow-rate rather than temperature. It is also evident from these data that it is important to work

at temperatures near room temperature and to control carefully the separation temperature both for reproducibility of retention times and prevention of peak mergers.

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