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New Retention Mechanism of Mononucleotides with Buffer Concentrations in Ion-suppressing RP-HPLC

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Abstract HPLC separation of ionic samples tends to be more complicated and difficult to understand than that of non-ionic compounds. On the other hand, band spacing is much more easily manipulated for ionic than for neutral samples. Ion-suppressing RP-HPLC method was used with organic modifier and aqueous buffer solution. In this work, five mononucleotides of cytidine-5-monophosphate (5'-CMP) disodium salt, uridine-5-monophosphate disodium salt (5'-UMP), guanosine-5-monophosphate disodium salt (5'-GMP), inosine-5-monophosphate disodium salt (5'-IMP), and adenosine-5-monophosphate disodium salt (5'-AMP) were examined. Acetic acid and sodium phosphate were used as buffers, and methanol as an organic modifier. A new relationship between the retention factor and the buffer concentration at a fixed modifier content (5% of methanol) could be expressed by following: $k = (k_{-1} + k_0 (K_B/K_S) C_B^a)/(1 + (K_B/K_S) C_B^a)$, where C_B was the concentration of buffer. Using this relationship, the calculated values closely matched the experimental data. The derived relationship showed that as the buffer concentration increased, the retention factor approached a certain value, and this was buffer dependent.

Keywords: retention mechanism, mononucleotides, buffer concentration, RP-HPLC

INTRODUCTION

Nucleotides are composed of base, D-ribose, and phosphoric acid, and are the basic units of DNA. In particular, adenosine-5'-triphosphate (ATP) one of the nucleotide derivatives is a important elements of biochemical reactions. When ATP is hydrolyzed to adenosine-5'-diphosphate (ADP), this reaction discharges useful energy. ATP drives the reactions of the living body, and adenosine-3', 5'-monophosphate(cyclic AMP, cAMP), which contains phosphoric acids bonded to the 3rd and 5th carbon atoms of adenosine has an important metabolic function. NAD, NADP, FAD, coenzyme A, and vitamin B12 are important nucleotide derivatives [1].

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) is an analytical technique that allows the various analysis of liquid-phase mixtures, and its various applications have been widely published [2-4]. In RP-HPLC, water and methanol or acetonitrile, which are of lower polarity than water, were used as a mobile phase. The solute dissolved in the mobile phase was either polar or ionized, and C_{18} or C_8 packing was used as stationary phase. Because ionized solute with high polarity is not retained, its analysis is very difficult. Therefore, a buffer solution or salt is added to the aqueous mobile phase to modify the ionization of the solute. Non-ionized solute is retained by the stationary phase [5]. Studies corcerning retention behavior of solute in accord with the pH of mobile phase have been widely published [6-8]. But to measure pH of mobile phase and ensure the same pH of the mobile phase during every experiment is often very difficult. Moreover retention behavior of the solute according to the kind of buffer was not satisfactorily yet expressed. In order to overcome these difficulties, the concentration of buffer in the mobile phase was altered instead of the pH of the mobile phase, and the effect of buffer concentrations upon the retention of ionized solute was studied and the retention of solute predicted [9,10].

The present study was undertaken to investigate the retention mechanism of five nucleotides, cytidine-5'-monophosphate disodium salt (5'-CMP), uridine-5'-monophosphate disodium salt (5'-UMP), guanosine-5'-monophosphate disodium salt (5'-GMP), inosine-5'-monophosphate disodium salt (5'-IMP), adenosine-5'-monophosphate disodium salt (5'-AMP) with different concentrations as acetic acid or monobasic sodium phosphate buffers in the binary mobile phase of water-methanol.

THEORY

In the case of the RP-HPLC retention of an ionic solute as a function of buffer concentration it can be assumed that a given solute exists in both the ionized and

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non-ionized forms and that its retention factor k is given by [10]:

$$k = k_0(1-F_{-1}) + k_{-1}F_{-1} \quad (1)$$

where k_0 and k_{-1} refer to the k values of the non-ionized and ionic forms, respectively and F_{-1} is the fraction of ionized solute molecules. Mononucleotide is dissolved into water with buffer, and it is assumed that one of the two sodium ions in the mononucleotide is replaced by the buffer cation. Therefore F_{-1} is expressed as

$$F_{-1} = \frac{[XMP^-]}{[XMP^-CA^+] + [XMP^-]} \quad (2)$$

where $[XMP^-]$ is concentration of ionized mononucleotide and $[CA^+]$ is concentration of the cation in the buffer. When the equilibrium between ionized and non-ionized species in the mobile phase is attained, the proportional coefficient is defined by the equilibrium constant, K_S.

$$K_S = \frac{[XMP^-][CA^+]}{[XMP^-CA^+]} \quad (3)$$

When F_{-1} is rearranged in terms of K_S. In buffer of acetic acid,

$$F_{-1} = \frac{1}{1 + [H^+]/K_S} \quad (4\text{-}1)$$

and in a buffer of monobasic sodium phosphate,

$$F_{-1} = \frac{1}{1 + [Na^+]/K_S} \quad (4\text{-}2)$$

Substituting Eqs. (4-1), (4-2) into Eq. (1) gives :
In acetic acid buffer :

$$k = \frac{k_0[H^+]/K_S + k_{-1}}{1 + [H^+]/K_S} \quad (5\text{-}1)$$

In monobasic sodium phosphate buffer :

$$k = \frac{k_0[Na^+]/K_S + k_{-1}}{1 + [Na^+]/K_S} \quad (5\text{-}2)$$

As a small amount of solute is usually injected during an experimental run, it is assumed that only the cation in the buffer is involved to in the attachment of the anionic solute. The concentration of cation is determined by the following empirical equation. In acetic acid buffer,

$$[H^+] = K_B C_B{}^a \quad (6\text{-}1)$$

In monobasic sodium phosphate buffer,

$$[Na^+] = K_B C_B{}^a \quad (6\text{-}2)$$

where K_B and a are empirical constants.

Substituting the Eqs. (6-1), (6-2) into Eqs. (5-1), (5-2) gives :

$$k = \frac{k_0(K_B/K_S)C_B{}^a + k_{-1}}{1 + (K_B/K_S)C_B{}^a} \quad (7)$$

This equation is semi-empirical equation that demonstrates the relation between the retention factor with the buffer concentrations in the mobile phase. The four parameters of Eq. (7), k_0, k_{-1}, K_B/K_S, and a were estimated using the Levenberg-Marquardt optimization. The confidence and tolerance were set at 0.95 and 0.05, respectively.

EXPERIMENTAL

Five mononucleotides were used in this work, namely cytidine-5′-monophosphate disodium salt (5′-CMP), uridine-5′-monophosphate disodium salt (5′-UMP), guanosine-5′-monophosphate disodium salt (5′-GMP), inosine-5′-monophosphate disodium salt (5′-IMP), and adenosine-5′-monophosphate disodium salt (5′-AMP) as supplied by Fluka (Buchs, Switzerland). Water with the organic modifier of methanol (J. T. Baker, Phillipsburg, NJ, USA) were used as mobile phase throughout the experiments and the volume fraction of methanol was constant at 5 vol.%. Acetic acid and monobasic sodium phosphate were used as buffer. An HPLC column, 0.39 (I.D.)×30 cm, is in-house packed by Lichrospher 100 RP-18(15 μm, Merck, Darmstadt, Germany).

Waters 600E solvent delivery system and a 486 UV detector (Waters, Milford, MA, USA) were used as the HPLC system. The flow rate of the mobile phase and UV wavelength were fixed at 1.0 mL/min and 254 nm, respectively. The buffer concentrations of acetic acid and sodium phosphate monobasic (DUKSAN, Kyungkido, Korea) were changed from 0 to 16 mM. The concentration of the five mononucleotides dissolved in water was 0.150 mg/mL. The hold-up time was 0.46 min, and the experimental was conducted at ambient temperature.

RESULTS AND DISCUSSION

Equation (7) shows the relationship between the retention factor and buffer concentration in the mobile phase. Table 1 lists the parameters of Eq. (7) for the five mononucleotides and the two buffers, namely acetic acid and monobasic sodium phosphate. Regardless of the materials or buffers used, the regression coefficients were close to 1.0. Solutes with lower polarity are more retained because of hydrophobic packing, C_{18}. Therefore, the retention factors of non-ionized forms (k_0) are larger than those of ionic forms (k_{-1}).

Figs. 1 and 2 show that the retention factor is dependent upon the buffer concentrations of acetic acid and monobasic sodium phosphate, respectively. The

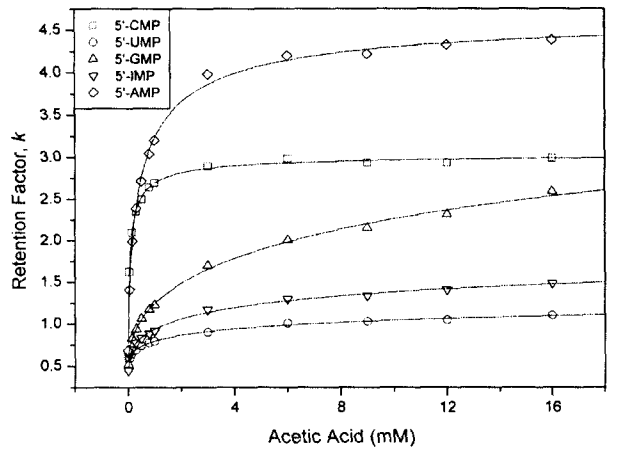

Fig. 1. Effect of concentration of acetic acid on retention factor. MeOH 5%, 10 μL injection volume, 0.15 mg/mL concentration.

Table 1. Parameters used in Eq. (7) and regression coefficients with buffers and materials

Buffer	Material	k_0	K_B/K_S	k_{-1}	a	Regression coefficient
Acetic acid	5'-CMP	3.039	6.046	0.595	0.708	0.99881
	5'-UMP	1.536	0.444	0.469	0.420	0.99748
	5'-GMP	5.480	0.173	0.513	0.497	0.99792
	5'-IMP	2.252	0.370	0.456	0.457	0.99688
	5'-AMP	4.715	1.709	0.700	0.704	0.99831
Sodium phosphate	5'-CMP	3.533	0.349	0.677	0.611	0.98935
	5'-UMP	3.311	0.291	0.522	0.867	0.99847
	5'-GMP	4.657	0.273	0.572	0.954	0.99961
	5'-IMP	4.930	0.279	0.516	0.910	0.99930
	5'-AMP	9.823	0.285	0.724	0.956	0.99989

points in the figures represent the experimental values and the solid lines were calculated value using Eq. (7) with the parameters in Table 1. With increasing buffer concentration (acetic acid and sodium phosphate monobasic), the retention factors gradually increase, and finally they converge. In the case of acetic acid (Fig. 1), the retention factors were increased up to concentrations of ca. 6 mM, but in the case of monobasic sodium phosphate (Fig. 2), the retention factors were increased up to a concentration of 16 mM. Especially in the case of acetic acid, the retention factor of 5'-CMP converged earlier than that of the other mononucleotides, and the k_0 of 5'-CMP is larger than that of 5'-UMP or 5'-IMP. However, in monobasic sodium phosphate, this tendency was not observed. This implies that the retention factor is manipulated by the types of buffer, and also that the retention mechanism is affected by its chemical species undergoing separation.

Figs. 3 and 4 show that the mole fractions of ionized solutes, F_{-1}, decrease with increased buffer concentration, acetic acid and monobasic sodium phosphate, respectively. The figures were calculated using Eq. (1) using the k_0 and k_{-1} values in Table 1, and the solid lines

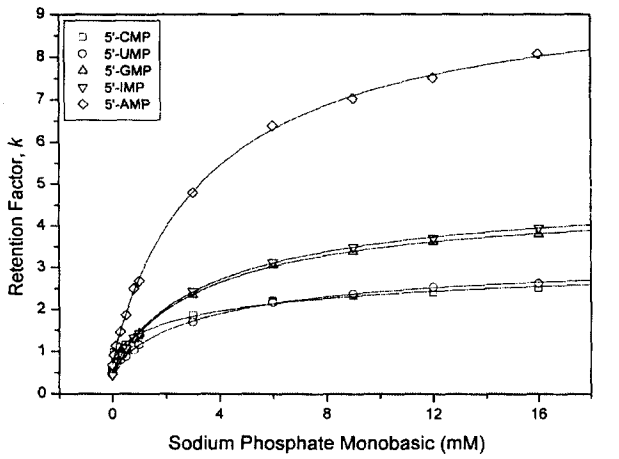

Fig. 2. Effect of concentration of sodium phosphate monobasic on retention factor. MeOH 5%, 10 μL injection volume, 0.15 mg/mL concentration.

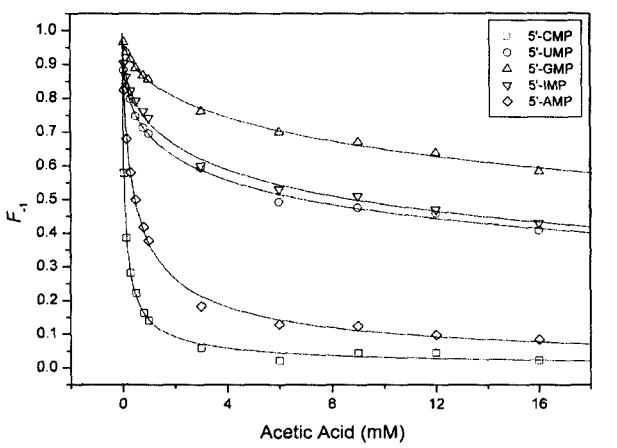

Fig. 3. Effect of concentration of acetic acid on F_{-1}. MeOH 5%, 10 μL injection volume, 0.15 mg/mL concentration.

were calculated values from Eq. (4). As the buffer concentration increases, F_{-1} gradually approaches zero. Higher values of mononucleotide F_{-1} mean that ionic forms are more abundant in the mobile phase, and the solute elutes earlier. However, the elution order is not consistent with the value of F_{-1}, which is attributed to the fact that the adsorption of mononucleotides on C_{18} packings and the ionic status of the solutes both contribute to the retention mechanism.

In the case of monobasic sodium phosphate, the cation in the aqueous mobile phase is the sodium ion, which is the same as the dissociated ion in mononucleotides, but acetic acid supplies only the hydrogen ion, which also combines with the. Therefore, the k_0 values in the two buffers are different as shown in Table 1. Fig. 5 shows the ratios of nonionic to ionic solutes at different buffer concentrations. The left Y axis denotes $[XMP^- H^+]/[XMP^-]$, while the right denotes $[XMP^- Na^+]/[XMP^-]$. The ratio is almost linearly dependent

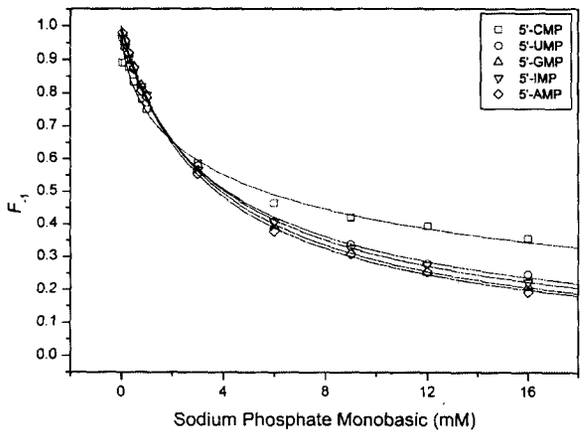

Fig. 4. Effect of concentration of sodium phosphate monobasic on F_{-1}. MeOH 5%, 10 μL injection volume, 0.15 mg/mL concentration.

upon buffer concentrations of monobasic sodium phosphate (SP), but nonlinear at low concentrations of acetic acid (AA). At the concentrations, anionic mononucleotides are combined with both sodium and hydrogen ions, but they are combined mainly with the hydrogen ion at higher acetic acid concentrations. This causes the nonlinearity in terms of the overall range of the concentration of acetic acid.

CONCLUSION

Earlier researchers predicted solute retention factors in terms of pH of the mobile phase. In the case of RP-HPLC, pH of the mobile phase may be changed by the addition of buffer. The right selection of buffer to separate a complex mixture is very critical. Research on the retention behavior with different types of buffer is difficult to find. In this work, a new retention model for buffer concentration was developed, for two types of buffer, namely acetic acid and monobasic sodium phosphate. In another respect, it is interesting to calculate the shift in a retention factor upon changing the ionic strengths of the sample and buffer. We intend to investigate this aspect further.

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SYMBOLS

a : empirical constant
C_B : concentration of buffer in mobile phase [mM]

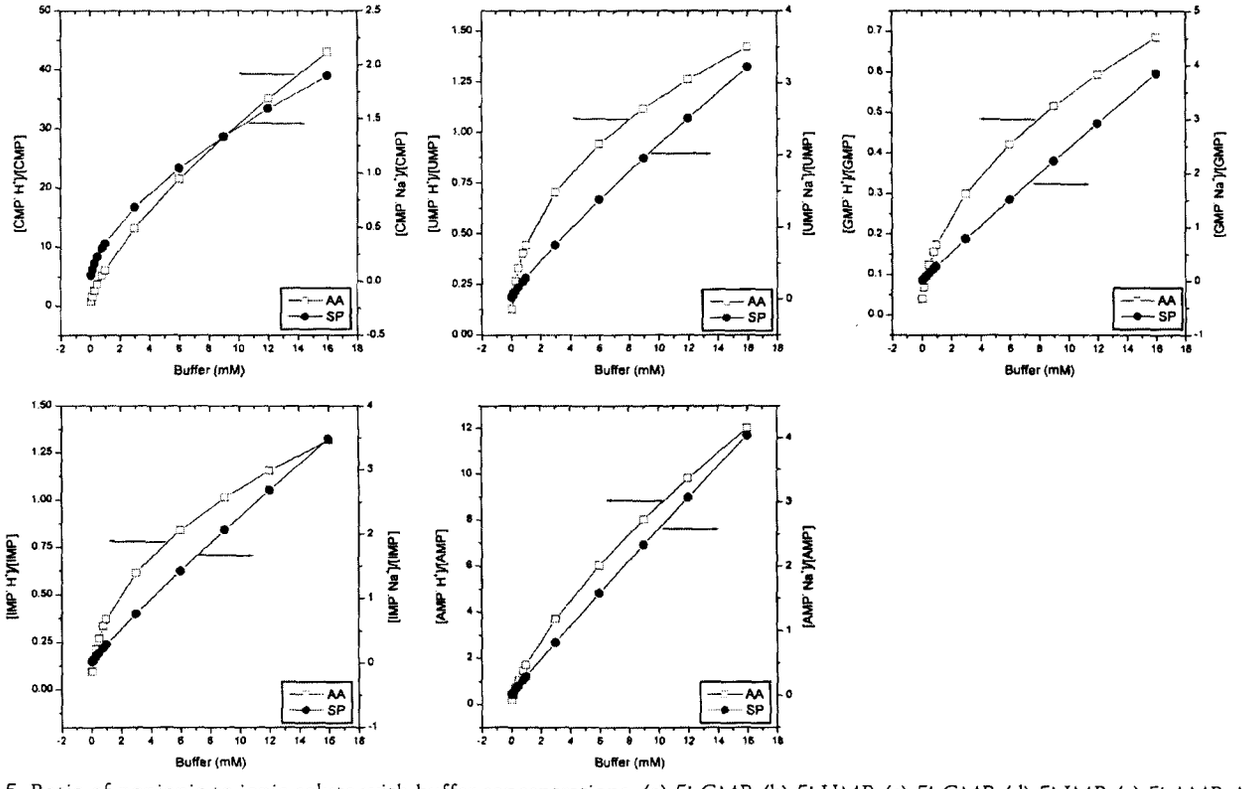

Fig. 5. Ratio of nonionic to ionic solute with buffer concentrations. (a) 5'-CMP, (b) 5'-UMP, (c) 5'-GMP, (d) 5'-IMP, (e) 5'-AMP, AA : acetic acid, SP : sodium phosphate monobasic.

F_{-1} : mole fraction of ionized solute
k : retention factor
k_0 : retention factor of non-ionized solute
k_{-1} : retention factor of ionized solute
K_B : empirical constant
K_S : equilibrium constant of solute

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