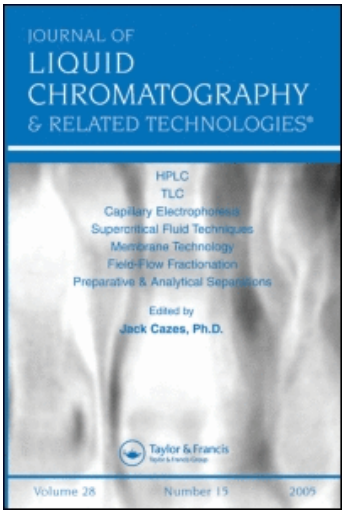

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Duk Hui Kang[a]; Ju Weon Lee[a]; Kyung Ho Row[a]
[a] Center of Advanced Bioseparation Technology and Department of Chemical Engineering, Inha University, Inchon, Korea

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EMPIRICAL CORRELATION OF RETENTION FACTOR OF MONONUCLEOTIDES TO BUFFER CONCENTRATION IN RP-HPLC

Duk Hui Kang, Ju Weon Lee, and Kyung Ho Row*

Center of Advanced Bioseparation Technology and
Department of Chemical Engineering, Inha University,
253 Yonghyun-Dong, Nam-Ku, Inchon 402-751, Korea

ABSTRACT

The retention factors of ionic samples with the types and concentrations of buffers were considered in RP-HPLC. The samples were the five mononucleotides and the buffers were acetic acid, sodium phosphate monobasic, potassium phosphate monobasic, and ammonium phosphate monobasic. The mobile phase was composed of water and 5 vol.-% methanol with the different concentrations of buffers. The three empirical correlations were suggested to predict retention factors of the ionized samples based on the assumption that the factor was proportional to the concentration of buffers. From the regression analysis of the correlation equations, the concentration of acetic acid is square root proportional to the retention factor, while the concentrations of other monobasic buffers are almost linearly proportional.

*Corresponding author.

INTRODUCTION

Nucleotides are regarded as the fundamental compounds in the field of biochemistry (1). They are implicated in nucleic acid metabolism and are also important as energy storage molecules and as cofactors of numerous biochemical reactions. In particular, inosine-5′-monophosphate dehydrogenase (IMPDH) catalyzes the nicotinamide adenim dinucleotide-dependent biosynthesis of xanthine-5′-monophosphate (2). The IMPDH-catalyzed reaction is the rate-limiting step in the biosynthesis of guanine nucleotides in mammals. In addition, adenosine-5′-triphosphate (ATP), one of the derivatives of nucleotides, is an important reactant in biochemical reactions. When ATP is hydrolyzed to adenosine-5′-diphosphate, this reaction discharges useful energy. Nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, coenzyme A, and vitamin B_{12} are important derivatives of nucleotides (3).

Several methods have been developed for the separation and purification of nucleotides. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a representative analytical apparatus for separation and purification of nucleotides (3–5). RP-HPLC is usually performed with C_{18} or C_8 as stationary phase (6). The optimization of RP-HPLC separation requires proper control of mobile phase conditions such as solvent composition and pH. Because an ionized sample with high polarity is not retained on the stationary phase, analyzing the sample is very difficult. Accordingly, the addition into the mobile phase of a counterion of a charge opposite to that of the molecule or control of pH has permitted ionized samples to be analyzed (1).

Studies of the retention behavior of solutes according to the pH of the mobile phase were published by many researchers (1,5,7–9). Because control of the pH in the mobile phase is extremely difficult, we used buffer solutions such as acetic acid, sodium phosphate monobasic, potassium phosphate monobasic, and ammonium phosphate monobasic instead of pH control or addition of pH in the mobile phase.

In this paper, we investigated the retention mechanisms of five mononucleotides, 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) with the concentrations of buffers of acetic acid, sodium phosphate monobasic, potassium phosphate monobasic, or ammonium phosphate monobasic in the binary mobile phase of water methanol. The retention factors were correlated into empirical equations with the various types of buffers and mobile phase compositions.

THEORY

For RP-HPLC retention of ionic solute as a function of buffer concentration, it can be assumed that a given solute exists in both ionized and nonionized forms and its retention factor k is given by (10)

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

where k_0 and k_{-1} refer to k values for nonionized 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 with the cation in the buffer. Therefore, F_{-1} is expressed as

$$F_{-1} = \frac{[\mathrm{XMP^-}]}{[\mathrm{XMP^-CA^+}] + [\mathrm{XMP^-}]}, \tag{2}$$

where $[\mathrm{XMP^-}]$ is the concentration of ionized mononucleotide and $[\mathrm{CA^+}]$ is the concentration of cation in buffer.

When the equilibrium between ionized and un-ionized species in the mobile phase is attained, the proportional coefficient is defined by the equilibrium constant, K_S:

$$K_S = \frac{[\mathrm{XMP^-}][\mathrm{CA^+}]}{[\mathrm{XMP^-CA^+}]}. \tag{3}$$

F_{-1} is rearranged in terms of K_S to give a) in buffer of acetic acid,

$$F_{-1} = \frac{1}{1 + [\mathrm{H^+}]/K_S}; \tag{4-1}$$

b) for sodium phosphate monobasic,

$$F_{-1} = \frac{1}{1 + [\mathrm{Na^+}]/K_S}; \tag{4-2}$$

c) for potassium phosphate monobasic,

$$F_{-1} = \frac{1}{1+[K^+]/K_S}; \tag{4-3}$$

d) for ammonium phosphate monobasic,

$$F_{-1} = \frac{1}{1+[NH_4^+]/K_S}. \tag{4-4}$$

Putting Equations (4-1), (4-2), (4-3), and (4-4) into Equation (1) gives a) in buffer of acetic acid,

$$k = \frac{k_0[H^+]/K_S + k_{-1}}{1+[H^+]/K_S}; \tag{5-1}$$

b) for sodium phosphate monobasic,

$$k = \frac{k_0[Na^+]/K_S + k_{-1}}{1+[Na^+]/K_S}; \tag{5-2}$$

c) for potassium phosphate monobasic,

$$k = \frac{k_0[K^+]/K_S + k_{-1}}{1+[K^+]/K_S}; \tag{5-3}$$

d) for ammonium phosphate monobasic,

$$k = \frac{k_0[NH_4^+]/K_S + k_{-1}}{1+[NH_4^+]/K_S}. \tag{5-4}$$

When a small amount of solute is typically injected in an experimental run, it is assumed that only the cation in the buffer is involved to attach to the anionic solute. The concentration of cation is correlated by the following empirical equations: a) in buffer of acetic acid,

$$[H^+] = K_B C_B^{\ a}; \tag{6-1}$$

b) for sodium phosphate monobasic,

$$[Na^+] = K_B C_B^{\ a}; \tag{6-2}$$

c) for potassium phosphate monobasic,

$$[K^+] = K_B C_B^{\ a}; \tag{6-3}$$

d) for ammonium phosphate monobasic,

$$[NH_4^+] = K_B C_B^{\ a}, \tag{6-4}$$

where K_B and a are empirical constants.

Putting Equations (6-1), (6-2), (6-3), and (6-4) into Equations (5-1), (5-2), (5-3), and (5-4) gives

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

This equation is a semiempirical equation that indicates the variation of retention factor with the buffer concentrations in the mobile phase. The four parameters of Equation (7), k_0, k_1, K_B/K_S, and a were estimated by Levenberg-Marquardt optimization. The confidence and tolerance were set at 0.95 and 0.05, respectively. To confirm the validity of Equation (7), the following empirical equations were considered. The retention factor in Equation (8) is assumed to be linearly proportional to the concentration of buffer, C_B, in the numerator, and the denominator of the Equation (8), while that in Equation (9) is square root proportional.

$$k = \frac{b + c\ C_B}{1 + a\ C_B} \tag{8}$$

$$k = \frac{b + c\ \sqrt{C_B}}{1 + a\ \sqrt{C_B}} \tag{9}$$

The retention factors of mononucleotides with the types and concentrations of buffer were fitted with the three equations and compared.

EXPERIMENTAL

Materials

The five mononucleotides used in this work, 5′-CMP, 5′-UMP, 5′-GMP, 5′-IMP, and 5′-AMP, were supplied by Fluka (Buchs, Switzerland).

Water and methanol (J. T. Baker, Phillipsburg, NJ, USA), as the mobile phase, were used throughout the experiments and the volume fraction of methanol was constant at 5 vol.-%.

Acetic acid, sodium phosphate monobasic, potassium phosphate monobasic, and ammonium phosphate nonobasic were used as the buffer solutions. An HPLC column, 0.39 (inside diameter) × 30 cm, was packed in-house (Lichrospher 100 RP-18, 15 μm, MERCK, Darmstadt, Germany).

Apparatus

A Waters 600E solvent delivery system and 486 ultraviolet (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 m*M*. The concentration of the five mononucleotides dissolved in water was 0.150 mg/mL. The hold-up time was 0.46 min. This experiment was carried out in ambient temperature.

RESULTS AND DISCUSSION

The effect of the buffer concentration in the mobile phase containing 5 vol.-% methanol on the retention of mononucleotide was investigated. Numerous papers have been published on ion-suppressing (3) and ion-pair liquid chromatography (1,5,7,8). Nucleotides become ionic compounds in the aqueous solution so that they are not retained on the C_{18} surface.

The addition of a buffer solution of charge opposite to that of the nucleotide into the mobile phase has permitted ionized samples to be analyzed (1,3). In this work, acetic acid, sodium phosphate monobasic, potassium phosphate monobasic, and ammonium phosphate monobasic were used as buffer solutions.

To investigate the influence of buffer concentration on the retention factor, 12 concentrations in ranges of 0 to 16 m*M* for each buffer, were experimented with. In Figure 1, the retention factors of mononucleotides increased with the

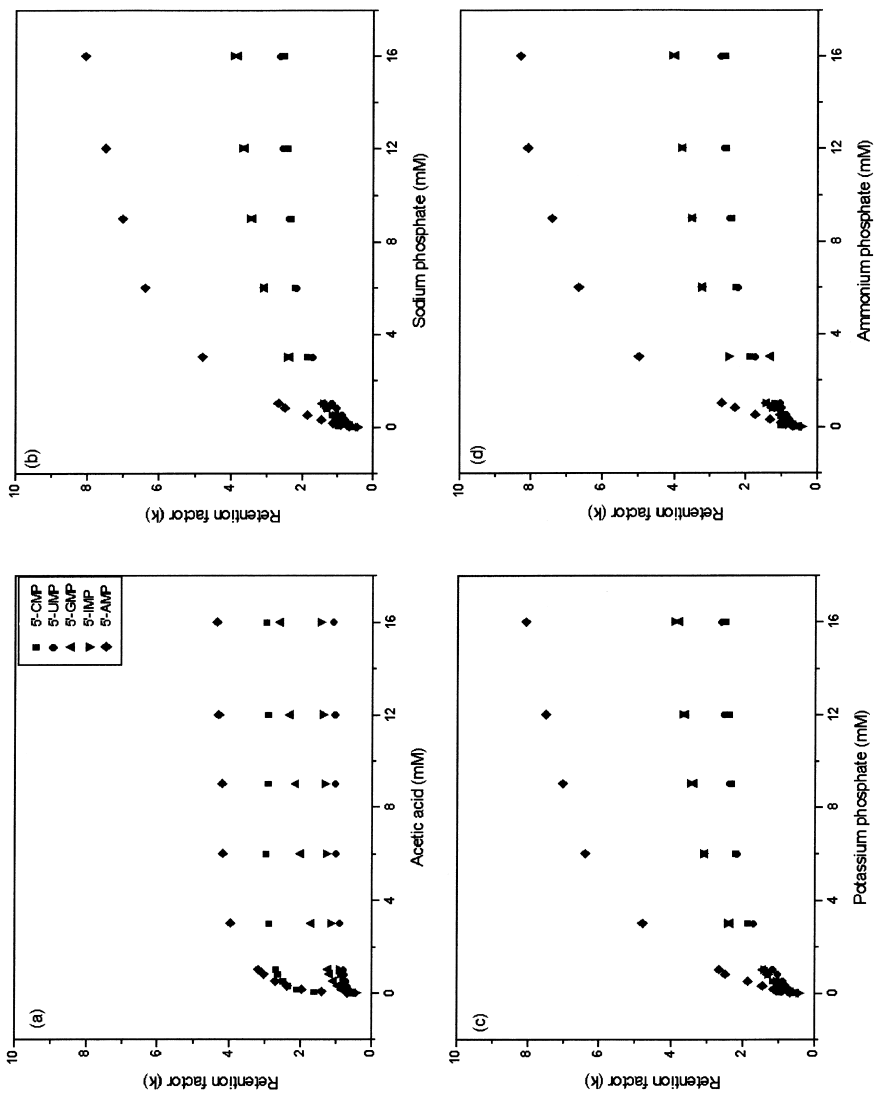

Figure 1. Effect of buffer concentration on retention factor of mononucleotides: a) acetic acid; b) sodium phosphate; c) potassium phosphate; d) ammonium phosphate.

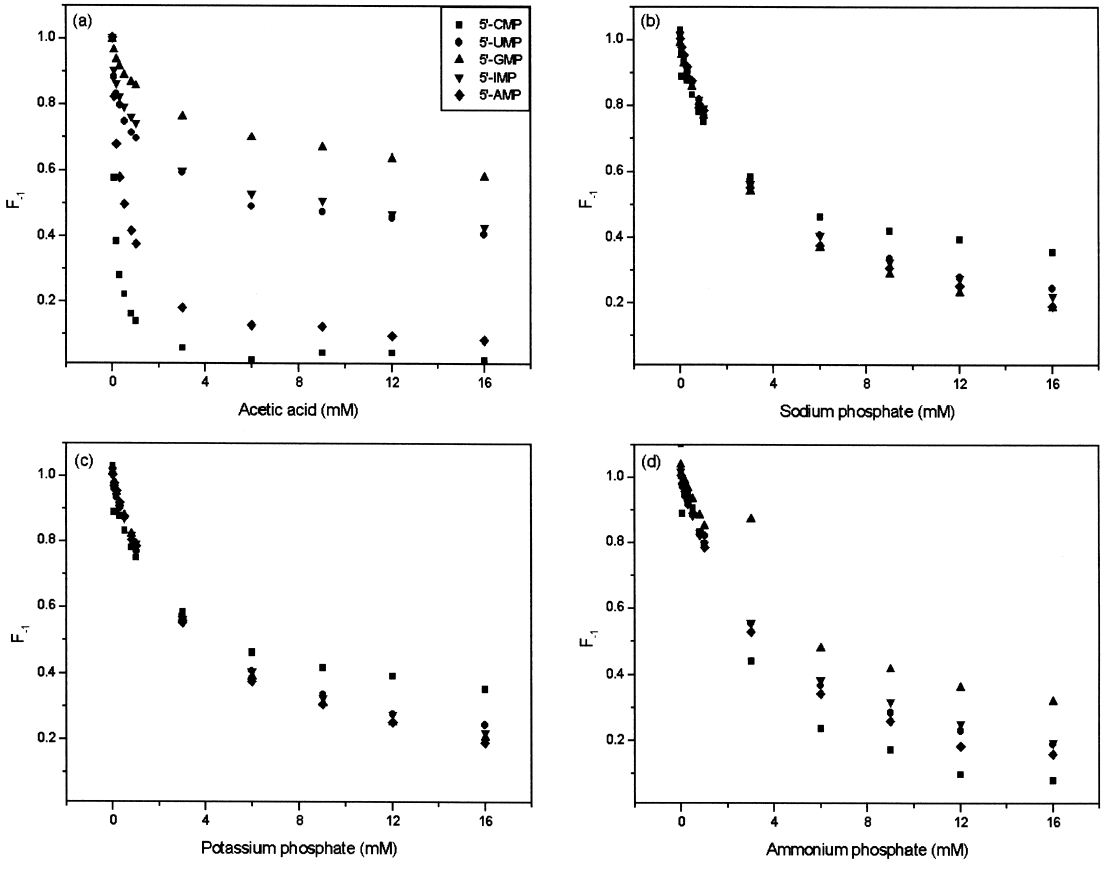

Figure 2. Shift of F_{-1} with buffer concentration: a) acetic acid; b) sodium phosphate; c) potassium phosphate; d) ammonium phosphate.

concentrations of all the buffers used in this work. The elution order of mononucleotides was changed in acetic acid, compared with the other buffers, sodium phosphate monobasic, potassium phosphate monobasic, and ammonium phosphate monobasic. Notably, the elution of 5′-CMP was fourth in acetic acid while, in monobasic buffers, it eluted as the first peak. There might be a difference in binding force between ionized 5′-CMP and buffer solution, so the retention times of 5′-CMP were different with acetic acid and monobasic buffers.

5′-CMP combines with acetic acid more strongly than with sodium phosphate, potassium phosphate, and ammonium phosphate. Also, the elution order of each of the mononucleotides seems to be the same with sodium phosphate monobasic and potassium phosphate monobasic buffers. On the other hand, the elution orders of mononucleotides were different between ammonium phosphate monobasic and the other phosphates buffers. This implies that the retention factors of mononucleotides were affected by not only the types of buffers but also their chemical structures.

Figure 2 shows the mole fractions of ionized samples, F_{-1}, of Equation (1) with buffer concentrations. F_{-1} was calculated using the values, k_0 and k_{-1} in Table

Table 1. Empirical Constants with Buffers by Equation (7)

Buffer	Materials	k_0	K_B/K_S	k_{-1}	a	R^2
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	3.310	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	9.8238	0.285	0.724	0.956	0.99959
Potassium phosphate	5′-CMP	3.526	0.350	0.677	0.612	0.98935
	5′-UMP	3.310	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	9.8238	0.285	0.724	0.956	0.99959
Ammonium phosphate	5′-CMP	2.743	0.274	0.793	1.358	0.98620
	5′-UMP	3.199	0.261	0.555	1.024	0.99744
	5′-GMP	5.500	0.107	0.710	1.124	0.96069
	5′-IMP	4.961	0.268	0.531	0.971	0.99888
	5′-AMP	9.755	0.270	0.737	1.089	0.99966

1. All of the mole fractions of ionized samples decrease as the concentrations of buffer solutions increase. When the buffers were not added in the mobile phase, in pure water, all the mononucleotides were completely dissolved and F_{-1} was 1. That is, mononucleotides became completely ionic compounds in the mobile phase. However, they approached zero as the concentrations of buffers increased.

The value of zero means that anionic solutes combine with the cations supplied by the buffer, so that the solute presents a nonionic compound in the mobile phase. Consequently, mononucleotides are retained on the hydrophobic C_{18} surface, and they may be separated with the retention times.

This trend is coincident with the results in Figure 1 and those of other researchers (1,5,11). k_0 and k_{-1} were estimated from Equation (7) by regression analysis. If the buffers were not added in the mobile phase, $C_B = 0$ and $k = k_{-1}$ [refer to Eq. (7)]. For example, the retention factor of 5′-IMP without buffer added was 0.449 in Figure 1 and that of ionic form of 5′-IMP was 0.456 in Table 1 using acetic acid as the buffer. If the concentrations of buffers are sufficiently high (say above 12 m*M*), k is close to k_0 as depicted in Equation (7). This means that prediction of the retention factor by Equation (7) is reasonable.

Table 2. Empirical Constants with Buffers by Equation (8)

Buffer	Materials	*a*	*b*	*c*	R^2
Acetic acid	5′-CMP	11.622	0.644	34.089	0.98960
	5′-UMP	0.976	0.537	1.067	0.97124
	5′-GMP	0.359	0.671	0.982	0.98470
	5′-IMP	0.706	0.559	1.060	0.97895
	5′-AMP	2.282	0.898	10.064	0.99076
Sodium phosphate	5′-CMP	0.446	0.799	1.215	0.98496
	5′-UMP	0.300	0.559	0.916	0.99788
	5′-GMP	0.274	0.589	1.243	0.99954
	5′-IMP	0.284	0.554	1.321	0.99903
	5′-AMP	0.287	0.761	2.743	0.99952
Potassium phosphate	5′-CMP	0.446	0.799	1.215	0.98496
	5′-UMP	0.300	0.559	0.916	0.99788
	5′-GMP	0.274	0.589	1.244	0.99954
	5′-IMP	0.284	0.554	1.321	0.99903
	5′-AMP	0.287	0.761	2.743	0.99952
Ammonium phosphate	5′-CMP	0.293	0.731	0.894	0.98347
	5′-UMP	0.261	0.549	0.844	0.99742
	5′-GMP	0.112	0.668	0.676	0.96056
	5′-IMP	0.269	0.543	1.311	0.99886
	5′-AMP	0.270	0.664	2.766	0.99940

Table 3. Empirical Constants with Buffers by Equation (9)

Buffer	Materials	*a*	*b*	*c*	R^2
Acetic acid	5′-CMP	3.629	0.568	11.626	0.99267
	5′-UMP	0.585	0.479	0.790	0.99662
	5′-GMP	0.175	0.514	0.949	0.99792
	5′-IMP	0.429	0.465	0.882	0.99668
	5′-AMP	1.166	0.578	6.223	0.99252
Sodium phosphate	5′-CMP	0.264	0.630	1.163	0.98872
	5′-UMP	0.138	0.385	0.962	0.99199
	5′-GMP	0.114	0.335	1.357	0.99008
	5′-IMP	0.121	0.282	1.449	0.99147
	5′-AMP	0.117	0.200	3.026	0.98941
Potassium phosphate	5′-CMP	0.264	0.630	1.163	0.98872
	5′-UMP	0.138	0.385	0.962	0.99199
	5′-GMP	0.114	0.335	1.357	0.99008
	5′-IMP	0.121	0.282	1.449	0.99147
	5′-AMP	0.117	0.200	3.026	0.98941
Ammonium phosphate	5′-CMP	0.138	0.573	0.916	0.96782
	5′-UMP	0.110	0.373	0.922	0.98726
	5′-GMP	−0.003	0.408	0.936	0.95364
	5′-IMP	0.108	0.267	1.452	0.98949
	5′-AMP	0.106	0.077	3.121	0.98489

For nonionic compounds, it is well known that in RP-HPLC, a linear relationship between the logarithm of retention factor and the percentage of organic modifier in the mobile phase exists over a limited concentration range (11). In this work, for ionic compounds at 5 vol.-% of methanol, in addition to Equation (7), we further tried two empirical equations [(8) and (9)] to investigate the relationship of the retention factors of mononucleotides with buffer concentration. Their empirical parameters are shown in Tables 2 and 3, respectively.

The comparisons between the values calculated by Equations (7), (8), and (9) and experimental data are shown in Figure 3. The empirical constants from Equation (7) are presented in Table 1.

The exponents of buffer concentrations differ with Equations (7), (8), and (9). In Equation (7), the value of *a* was obtained by nonlinear regression, while that of *a* was fixed as 1 and 0.5 in Equations (8) and (9), respectively. Apparently, Equation (7) shows the best linear relationship between empirical data and calculated values in Figure 3, irrespective of buffers used. The regression coefficients, R^2, of Equation (7) were more than 0.996 for the four parameters in the equations, compared to three parameters in Equations (8) and (9).

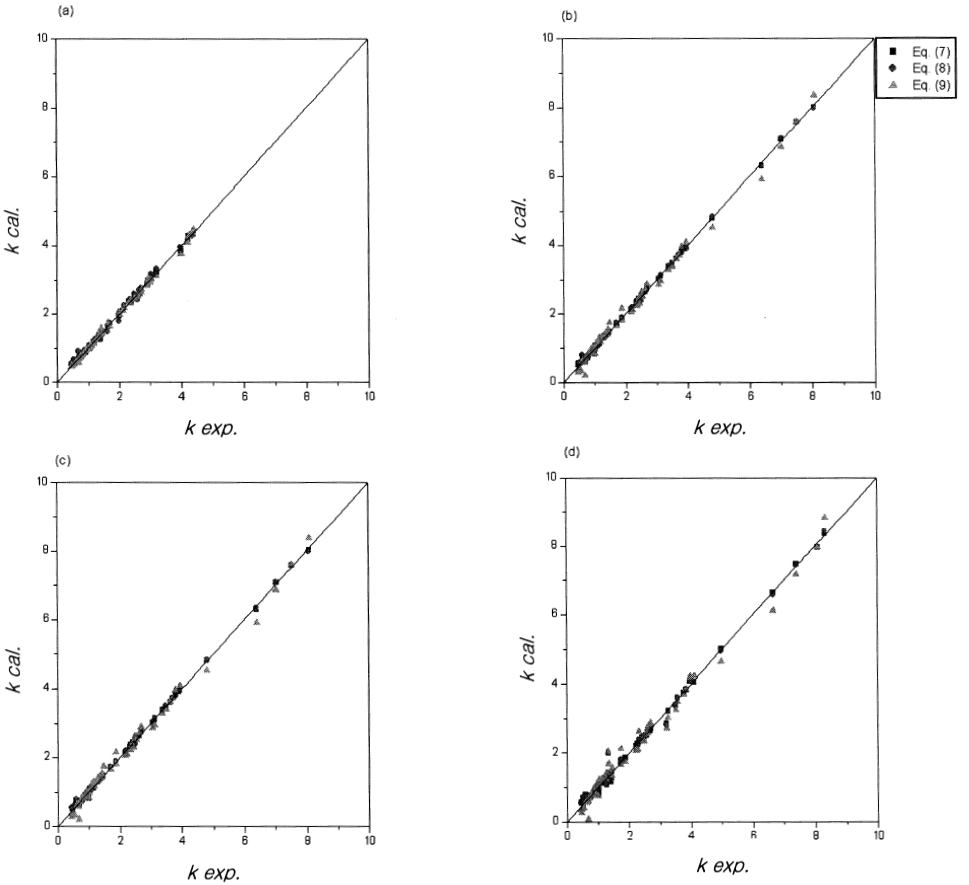

Figure 3. Comparison of the experimental data and calculated value of *k* a) acetic acid; b) sodium phosphate; c) potassium phosphate; d) ammonium phosphate.

The regression coefficients calculated by Equation (8), 0.971–0.989, are relatively low, especially with acetic acid as buffer, but they are better with other phosphate buffers. On the other hand, the regression coefficients of Equation (9) with acetic acid, 0.992–0.998, are higher than those with the other phosphates buffers, 0.954–0.992.

From these experimental results, we might want to insist that the concentration of acetic acid is square root proportional to the retention factor, while those of other monobasic buffers are almost linearly proportional.

CONCLUSIONS

The great advantage of RP-HPLC lies in the separation of the various samples, nonionic or ionic. It is necessary to add appropriate buffer into the mobile phase to analyze the ionic samples. The right selection of buffer requires a knowledge of its type and concentration. In this work, the concentration of buffers was adjusted rather than the pH of the mobile phase. The retention mechanism of ionic samples is hard to judge, but the approach of the empirical correlation might be helpful. The three empirical equations contain three or four empirical constants, which should be determined by regression analysis. The regression coefficient, the agreement of calculated values to experimental data, is relatively close to 1.0 with good reliability.

NOMENCLATURE

a	empirical constants of Equations (7), (8), and (9)
b, c	empirical constants of Equations (8) and (9)
C_B	concentration of buffer in mobile phase (mM)
F_{-1}	mole fraction of ionized solute
k	retention factor
k_0	retention factor of nonionized solute
k_{-1}	retention factor of ionized solute
K_B	empirical constant
K_S	equilibrium constant of solute
R^2	regression coefficient

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