ASIAN JOURNAL OF CHEMISTRY

Retention Behaviour of Weakly Ionizable Acidic Compounds in Ion-Suppression Reverse-Phase Liquid Chromatography with Ternary Mobile Phase

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Ternary elution system employing organic modifiers, water and simple acid solutions has been widely applied to suppress the ionization of weakly ionizable acidic analytes in reversed-phase liquid chromatography. This paper focuses on the retention behaviour of benzoic acid derivatives using acetic acid or perchloric acid as ion-suppression in such ternary mobile phase. The apparent *n*-octanol-water partition coefficient (K_{ow}'') was proposed to calibrate the *n*-octanol-water partition coefficient (K_{ow}) of weak acidic compounds. Consistent with binary mobile phase, log K_{ow}'' was found to have a better linear correlation with log k_w, the logarithm of the retention factor obtained by extrapolating to neat aqueous fraction of the mobile phase. This straightforward relationship offers a potential medium for direct measurement of K_{ow} data of weak acidic analytes and can be used to predict retention behaviour of these compounds in the ion-suppression reversed-phase liquid chromatography mode with ternary mobile phase.

Key Words: Apparent *n*-octanol-water partition coefficient (K_{ow}''), Ion-suppressor, Reversed-phase liquid chromatography, *n*-Octanol-water partition coefficient (K_{ow}), Retention behaviour, Ternary mobile phase, Weakly ionizable acidic compound.

INTRODUCTION

Most environmental and biomedical molecules are ionizable more or less. In order to improve chromatographic retention and peak shape of ionizable compounds in reversed-phase liquid chromatography (RPLC), acids, bases or buffers are added into aqueous mobile phase according to the nature of analytes to suppress dissolution of ionizable solutes[1-5]. Although buffers has been widely used in a variety of areas over years, it is more convenient to use simple acids or bases as mobile phase additives in ion-suppression (IS)-RPLC[6,7] since acids or bases are friendly to liquid chromatography tube and mass spectrometer (MS).

Employing ternary mobile phase consisting of organic modifiers, water and acid solutions as the eluents makes the practical operation easier and has also been widely used, especially in trials to get desired resolution of unknown samples[8,9]. In an organic modifier-water-acid solution ternary elution system, the range of adjustable pH is continually widened since the aqueous solution can be further diluted to get lower [H⁺] by automatically increasing the proportion of water in liquid chromatography instrumentation. Less preparation of different concentrations of aqueous acid solutions is needed while an appropriate higher concentration of acid solution can be prepared in a solvent reservoir, which leads to more simple, convenient and efficient experimental operation.

n-Octanol-water partition coefficient (K_{ow}) is a hydrophobicity parameter conventionally used in quantitative-structure activity relationship (QSAR) studies for various bioactive compounds[10,11]. Nowadays, the application of HPLC method for the determination of log K_{ow} grows fast[12] because of the simple linear relationship between log K_{ow} and the logarithm of hypothetical retention factor (log k_w) corresponding to neat aqueous fraction of mobile phase and from this relationship the log K_{ow} value of a compound can be obtained by the experimentally measured retention factor k easily.

In binary hydroorganic mobile phase, Snyder-Soczewinski equation relating k with volume fraction of organic modifier (Φ) for liquid chromatography is expressed in terms of[13]:

$$\log k = \log k_w - S\Phi \qquad (1)$$

where, S is the entropy function of an adsorbed solute and is considered as constant for a given solute-eluent combination. Considering water and acid together as aqueous phase, ternary mobile phase employed in this paper (organic modifier, water and acid solution) is analogous to binary hydroorganic mobile phase. Therefore, Snyder-Soczewinski equation is still applicable in this situation.

K_{ow} employed in most studies on RPLC retention only refers to the hydrophobicity of neutral form of the compound. The hydrophobicity of weakly acidic compound should be expressed by the experimentally determined *n*-octanol-water

partition coefficient (K_{ow}')[14] that takes into account the contribution of both neutral and ionized forms. The K_{ow}' of a monobasic acidic solute is described as:

$$K_{ow}' = \frac{[HA]_0 + [A^-]_0}{[HA]_w + [A^-]_w} \qquad (2)$$

The subscripts *o* and w refer to the *n*-octanol (or oil) and aqueous phases, respectively. Using the expression of dissociation constant K_a, the Kow' can be trivially formulated as:

$$K_{ow}' = \frac{K_{ow} + K_{ow}^- \dfrac{K_a}{[H^+]}}{1 + \dfrac{K_a}{[H^+]}} \qquad (3)$$

where, K_{ow}^- is *n*-octanol-water partition coefficient of the A^- anionic form. Usually K_{ow}^- is thought to be very small compared to K_{ow} of its neutral form. The approximation of K_{ow}' was expressed by apparent *n*-octanol-water partition coefficient (K_{ow}'') as discussed in detail in our earlier work[15].

$$K_{ow}'' = \frac{[HA]_0}{[HA]_w + [A^-]_w} = \frac{K_{ow}}{1 + \dfrac{K_a}{[H^+]}} \qquad (4)$$

The linear relationship between $\log K_{ow}$ and $\log k_w$ known as Collander equation has been illustrated experimentally and theoretically[16]:

$$\log K_{ow} = m \log k_w + n \qquad (5)$$

where, m and *n* are constants. This correlation was expanded to acidic solutes in binary mobile phase by the authors[6,7]. In the case that mobile phase is not acidic enough to completely suppress the dissociation of acidic solute, k has the form as follows[17]:

$$k = k_0 x_0 + k_1 x_1 \qquad (6)$$

where k_0 and k_1 are retention factors of undissociated and dissociated acidic solute, respectively and x_0 and x_1 are molar fractions of the corresponding species.

In previous works[6,7], the retention behaviour of weakly ionizable acidic compounds utilizing acetic acid, perchloric acid or phosphoric acid as ion-suppressor in binary IS-RPLC have been systematically studied. The result showed that $\log K_{ow}''$ has a better linear correlation with $\log k_w$ than $\log K_{ow}$ has. Then this correlation was successfully applied to predict the retention behaviour of weakly ionizable acidic solutes, verify the K_{ow} values reported or software-computed and even forecast unknown K_{ow} values based on chromatographic measurement for weak acidic compounds regardless of category and amount of functional groups[6,7]. In this paper, an in-depth experimental study was made in order to investigate retention behaviour of benzoic acid derivatives in IS-RPLC with methanol-water-acid solution as ternary mobile phase using acetic and perchloric acid as ion-suppressors, respectively.

EXPERIMENTAL

A Varian 5060 HPLC system (Walnut Creek, CA, USA) consisting of a Rheodyne 7725i injector valve equipped with a 10 μL loop (Cotati, CA, USA), a Waters 486 tunable UV absorbance detector (Milford, MA, USA) and a JS-3050 chromatographic working station (Johnsson Sep. Sci. and Tech., Dalian, China) was employed. All retention data for the studied compounds were measured on a Kromasil C_{18}, 5 μm, 150 mm × 4.6 mm i.d. column (Hanbon Sci. & Tech., Huai'an, China) at 254 nm and obtained by averaging the results of at least two independent injections at 1.0 mL/min mobile phase flow rate. The column temperature was maintained at 30 ºC.

pH values of acid solutions were measured with a Seven Multi electrochemical analytical meter (Metter-Toledo, Schwerzenbach, Switzerland) calibrated using aqueous standard reference buffers of pH 2.00 and pH 4.01 at 25 ºC (Metter-Toledo).

Water for mobile phases was Robust pure water (Guangdong Robust Food and Beverage, Guangzhou, China). Mobile phases were prepared from methanol (HPLC, Hanbon), acetic acid (analytical-reagent, Sinopharm Group Chemical Reagent, Shanghai, China) or perchloric acid (70-72 %) (guaranteed-reagent, Tianjin Third Reagent Factory, Tianjin, China). 11-Benzoic acid derivatives investigated and the preparation of sample solutions were the same as in previous work[6].

Eleven benzoic acid derivatives were eluted by the mobile phase composed of methanol, water and acid solution with different pH (2.29, 2.74, 2.84 and 3.84 adjusted by acetic acid and 2.44, 2.77, 2.90 and 3.50 adjusted by perchloric acid). pH values of aqueous acid solutions were evaluated before mixing with methanol, *i.e*, $_w^w pH$.

At each pH, acid solution proportion was fixed at 20 % and these compounds were analyzed isocratically at a minimum of four methanol-water ratios. k Value was calculated according to the equation $k = (t_R - t_0)/t_0$, where t_0 was determined by using $NaNO_3$ as the holdup time marker. For each solute, the logarithm of k was plotted against Φ and $\log k_w$ was subsequently obtained by extrapolation *via* Eq. 1. Statistical analysis was accomplished by SPSS V17.0.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

pH of mobile phase: Among the three commonly used descriptions for the pH of HPLC mobile phase ($_w^w pH$, $_w^s pH$ and $_s^s pH$), $_w^w pH$, pH of aqueous fraction before mixing with organic modifiers, is the most simple and useful one. Although no information reveals about the real pH of the hydroorganic mobile phase after dilution with the organic modifier, it has several remarkable advantages that it is much more practical and convenient to obtain in automated HPLC systems and no corresponding hydroorganic pH reference solution is needed. Moreover, if the contribution of organic modifier to pH is considered, pK_a in hydroorganic solution should be used correspondingly. However, experimental pK_a data of analytes in various hydroorganic solutions are usually more difficult to get than the most widely used aqueous pK_a data. When both pH and pK_a are factors in the separation process, it is much more meaningful to use $_w^w pH$ and aqueous pK_a simultaneously. Besides, in present paper, ternary mobile phase were employed and the proportion of acid solution were fixed at 20 %. When extrapolated to neat aqueous phase, the mole concentration of the ion-suppressor in mobile phase was precisely diluted five times compared to original acid solution. Therefore, $_w^w pH$ values

of acid solutions were evaluated before mixing with methanol and water and the corresponding extrapolated pH of neat aqueous phase (Table-1) was calculated and used as mobile phase pH.

In the actual experimental procedure, with the change of mobile phase composition, acids used as ion-suppressor and analytes underwent complex dissociation due to the different pK_a values in aqueous and hydroorganic solution. To get a clearer view of ion-suppression in hydroorganic solutions, trends of true pH of mobile phase with different composition in binary and ternary eluents were illustrated in Fig. 1. In ternary elution system, for strongly ionizable perchloric acid, pH of mobile phase was consistent since perchloric acid, approximately, dissociated completely in either aqueous or hydroorganic solution. For weakly ionizable acetic acid, pK_a value increased while organic modifier was added in solution. Therefore, the actual pH of mobile phase varied as the content of organic modifier changes when acetic acid was used. However, in binary elution system, total amount of acid in mobile phase decreased as the content of organic modifier increased. True pH of mobile phase declined along with the increase of methanol content when perchloric acid was used. When acetic acid was involved, total amount of acetic acid decreased while pK_a of acetic acid increased, which lead to the fastest change of mobile phase pH among all four situations. Overall, in ternary elution system, pH varied slower and the changes of variables were much clearer in extrapolation.
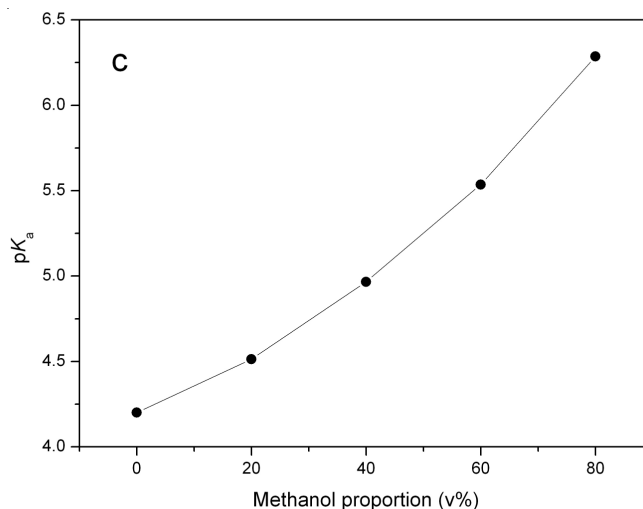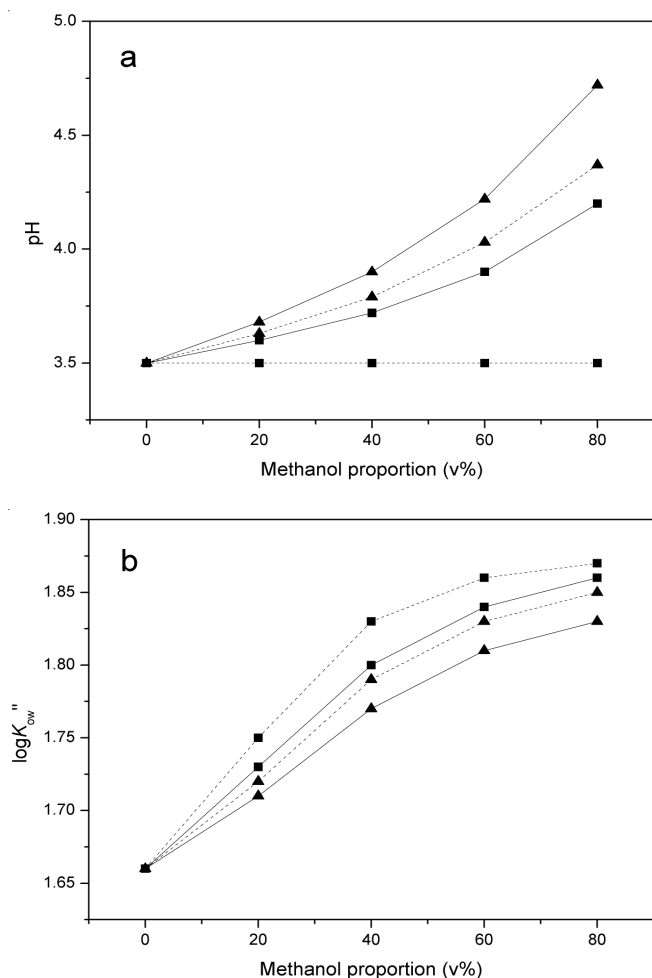

Fig. 1.　Relationship between true mobile phase pH and methanol content (a), relationship between log K_{ow}'' of benzoic acid and methanol content (b) and relationship between pK_a of benzoic acid and methanol content (c) in different mobile phases with extrapolated pH 3.50: methanol-perchloric acid solution (—■—), methanol - acetic acid solution (—▲—), methanol-water-perchloric acid solution (-■-) and methanol-water-acetic acid solution (--▲--). True mobile phase pH of perchloric acid hydroorganic solution was calculated by the dilution factor and that of acetic acid hydroorganic solution was calculated using both dilution factor and pK_a data of acetic acid in methanol-water solutions from[18]. log K_{ow}'' of benzoic acid was calculated using pK_a data of in methanol-water solutions from[18] and log K_{ow} value as the same[6]

Relationship between log K_{ow}'' and mobile phase composition: For benzoic acid derivatives studied, the relationships between pK_a and content of organic modifier are shown in Fig. 1 using benzoic acid as an example[18]. As methanol content increased, the difference between true mobile phase pH and pK_a of the solute was enlarged and the effect of ion-suppression was accelerated. K_{ow}'' value of benzoic acid under different mobile phase composition was calibrated from K_{ow} value according to actual pH of the mobile phase and corresponding pK_a. With the increase of organic modifier content, pK_a increased faster than pH. The greater the difference between pK_a and pH, the higher log K_{ow}'' value would be obtained. Therefore, magnitude orders of log K_{ow}'' values were the opposite of pH in four different mobile phases. When methanol content was raised up to 60 %, log K_{ow}'' of benzoic acid was higher than 1.80, which was close to the log K_{ow} value of neutral species (1.87). That is, although mobile phase pH did not meet the demand that should be three units lower than pK_a of the solute, dissociation of benzoic acid was almost entirely suppressed by the mobile phase with methanol content higher than 60 % since pK_a of solute changed in hydroorganic solution.

Relationship between log K_{ow}'' and log k_w for benzoic acid derivatives: It was concluded that in IS-RPLC with organic modifier-aqueous acid solution binary mobile phase, the relationship between log K_{ow}'' and log k_w for weak acids displayed a better linearity than that between log K_{ow} and log k_w regardless of the kind of functional groups and of the chromatographic conditions[6,7]. The same circumstances were observed in methanol-water-acid solution ternary mobile phase. K_{ow}'' value of each benzoic acid derivatives was calibrated from

TABLE-1
RELATIONSHIPS BETWEEN log K_{ow}'' AND log k_w AND RELATIONSHIPS BETWEEN
log K_{ow} AND log k_w, RESPECTIVELY, FOR 11-BENZOIC ACID DERIVATES

Ion-suppressors	pH of acid solutions	Extra-polated pH	log K_{ow} = m log k_w + n					log K_{ow}'' = m'log k_w + n'				
			m±SD[a]	n±SD[b]	r	SD[c]	F	m'±SD[a]	n'±SD[b]	r	SD[c]	F
Acetic acid	2.29	2.64	1.09±0.06	-0.20±0.13	0.986	0.06	307.60	1.15±0.06	-0.38±0.13	0.988	0.06	357.52
	2.74	3.09	1.06±0.08	-0.20±0.19	0.972	0.09	153.98	1.24±0.07	-0.70±0.16	0.985	0.08	290.81
	2.84	3.19	0.95±0.08	0.04±0.18	0.969	0.09	138.03	1.16±0.03	-0.53±0.07	0.996	0.04	1278.37
	3.84	4.22	0.67±0.11	0.77±0.23	0.891	0.17	34.72	1.23±0.08	-0.91±0.16	0.983	0.11	258.86
Perchloric acid	2.44	3.14	0.96±0.06	-0.02±0.14	0.980	0.07	222.96	1.13±0.05	-0.51±0.11	0.991	0.06	508.68
	2.77	3.47	0.93±0.08	0.07±0.18	0.967	0.10	127.79	1.24±0.07	-0.79±0.15	0.987	0.08	330.98
	2.90	3.60	0.86±0.09	0.26±0.20	0.951	0.12	84.66	1.21±0.06	-0.74±0.13	0.989	0.08	392.31
	3.50	4.20	0.67±0.11	0.78±0.23	0.890	0.17	34.34	1.21±0.08	-0.86±0.17	0.980	0.12	221.10

a) Standard deviations of constants m and m'; b) Standard deviations of constants n and n'; c) Standard deviations of equations

corresponding K_{ow} value according to its aqueous pK_a and extrapolated pH at which the analyte was eluted. Thus equations that relate log K_{ow}'' with log k_w with various mobile phases were respectively derived. Linear regressions and statistical data shown in Table-1 proved that log K_{ow}'' had notable superiority to log K_{ow} when correlating with log k_w. Moreover, this superiority increased along with the increase of pH. The excellent linearity of log K_{ow}''-log k_w would enable more precise straightforward prediction of the retention behaviour of weak acids to optimize separations with satisfactory accuracy. Also, this correlation provided a powerful way for the determination and correction of K_{ow} values by simple measurements of retention data of weak acidic solutes.

Conclusion

Retention behaviours of a group of benzoic acid derivatives in ion-suppression reversed phase liquid chromatography with organic modifier-water-acid solution ternary as mobile phase were systematically studied using perchloric acid or acetic acid instead of buffers as ion-suppressors. The changes in actual pH of mobile phase and log K_{ow}'' of solute along with mobile phase composition were discussed. It was demonstrated that the linear relationship between log K_{ow}'' and log k_w was better than that of log K_{ow} - log k_w within the wide pH range adjusted by ion-suppressors, which was consistent with the results obtained in IS-RPLC with binary organic modifier - acid solution as the eluent. This correction implies a simple and accurate method to improve the prediction of retention behaviours and to determine or correct K_{ow} values of weak acidic compounds.

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