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Determination of Acid Dissociation Constants by High-Performance Liquid Chromatography

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Values for the acid dissociation constant K_a of various acidic and basic compounds were determined from the pH-dependent capacity factor k' by reversed-phase high-performance liquid chromatography (HPLC) with octadecyl silane as the stationary phase. Both the neutral and ionic forms of acids and bases were retained on the stationary phase, and k' of the ionic form markedly influenced the calculation of K_a value. Values of K_a determined by HPLC agreed well with those determined by the conventional potentiometric method, indicating the availability of this method for the determination of K_a values. The "hydrophobicity" of the ionized forms of these compounds is discussed in terms of the partition coefficient of the neutral form between octanol and water.

Keywords——acid dissociation constant; reversed-phase high-performance liquid chromatography; capacity factor; partition coefficient; neutral form; ionic form

Recently, high-performance liquid chromatography (HPLC) has been recognized to be very useful for determination of some physical constants of organic compounds.[1] The partition coefficient between octanol and water (P_{oct}), which is one of the most important physical properties relevant to the biological effect of a bioactive compound, can be determined simply by HPLC, without the tedious and troublesome procedures inherent in the conventional shaking flask method.[2-6] Moreover, we recently reported that the partition coefficient of a compound with a $\log P_{oct}$ of more than 6 can be determined by this method,[7] whereas this is very difficult by the shaking flask method.[8]

The acid dissociation constant (K_a) is another important physical parameter for evaluation of biological activity in terms of quantitative structure–activity relationships. There have been some attempts to determine the pK_a by HPLC: Horvath *et al.*[9] and Unger *et al.*[10,11] showed that pK_a values can be determined by HPLC from the pH-dependent retention times of chemicals. Salto *et al.*[12] and Pashankov *et al.*[13] determined the pK_a values of various drugs, such as penicillins and antipyrines by HPLC. Despite such attempts, this method is still not established, mainly due to difficulties in exact determination of the retention times of the neutral form and the ionic form of a compound. Thus, we tried to establish an HPLC method for the determination of K_a, since it is expected to be useful, especially for compounds that are slightly soluble in water.

This paper deals with the determination of the pK_a values of various acidic and basic compounds from their pH-dependent retention times obtained by HPLC with octadecyl silane (ODS) as a stationary phase. It is demonstrated that the present HPLC method allows simple and rapid determination of pK_a values, using only small amounts of test compounds.

Experimental

The chemicals used in this study were obtained from commercial sources, and were used without further purification. These compounds were dissolved in methanol at about 1 mg/ml.

HPLC was carried out with a TRIROTAR-II (JASCO, Tokyo). The stationary phase was μ-Bondapak C_{18} (5 cm × 4.6 mm i.d.) (Waters Assoc., Milford, MS), and the mobile phase was a mixture of methanol (15% (v/v) final concentration, unless otherwise noted) and an aqueous solution of phosphoric acid. The aqueous phosphoric acid solution was adjusted to the desired pH value with mono- or disodium phosphate. When metanol was added to the buffer, it caused a slight upward shift of pH depending on its concentration. In the presence of 15% (v/v) methanol, the shift was about 0.15 at pH 1.9 and 0.2 at pH 4.0. The pH values in this study are given as those after addition of methanol. The final concentration of phosphate was kept at less than 0.01 M to avoid the effect of ionic strength on the acid dissociation of chemicals. Use of this concentration also avoided precipitation of phsophate when a higher methanol concentration than 15% (v/v) was used in the mobile phase. The pH of the mobile phase was not affected by injection of the sample. Chromatography was performed at 25 °C and the flow-rate of the mobile phase was 1 ml/min. Potassium iodide was used as an unretained reference compound. The capacity factor (k') of the sample compound was determined from the retention times of the sample (t_R) and the reference compound (t_0) by means of the following equation:

$$k' = (t_R - t_0)/t_0 \tag{1}$$

The accuracy of pK_a determined by the present method was very high: deviation of k' values in 5 runs was always less than 4%, resulting in a deviation of the pK_a value, at most, of less than 0.04.

Values for the capacity factors of the neutral form (k'_n) and ionic form (k'_i) of acid-dissociable compounds were determined along with K_a by the non-linear least-squares method (Marquardt method) with a Facom 230 computer, at Osaka City University.

Chromatographic Mechanism——When the acid AH is dissolved in water, H^+ dissociates according to eq. (2), where [ ] denotes the concentration. For simplicity, it is assumed in this study that $pH = -\log[H^+]$.

$$K_a = \frac{[A^-][H^+]}{[AH]} \tag{2}$$

The distribution of the acid between the mobile aqueous phase (m) and the stationary hydrophobic phase(s) can be depicted as shown in Fig. 1, where two molecular forms of the acid, the neutral from (AH) and ionic form (A^-), are present. The distribution constant (D) of these molecular species between these two phases is expressed by eqs. (3) and (4).

$$D_n = \frac{[AH]_s}{[AH]_m} \tag{3}$$

$$D_i = \frac{[A^-]_s}{[A^-]_m} \tag{4}$$

The capacity factor is expressed by eq. (5) (for an acidic compound) and by eq. (6) (for a basic compound), where q is the volume ratio of the two phases ($= V_s/V_m$).

$$k' = q\frac{[AH]_s + [A^-]_s}{[AH]_m + [A^-]_m} \tag{5}$$

$$k' = q\frac{[B]_s + [BH^+]_s}{[B]_m + [BH^+]_m} \tag{6}$$

From eqs. (2)—(6), k' with respect to acids and bases can be expressed by eqs. (7) and (8), respectively.

$$k' = \frac{k'_n + k'_i \dfrac{K_a}{[H^+]}}{1 + \dfrac{K_a}{[H^+]}} \tag{7}$$

$$k' = \frac{k'_n + k'_i \dfrac{[H^+]}{K_a}}{1 + \dfrac{[H^+]}{K_a}} \tag{8}$$

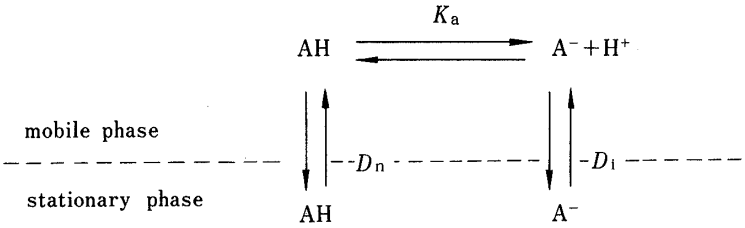

Fig. 1. Model of Chromatography of an Acidic Compound AH

where k'_n and k'_i are the capacity factors of the neutral and ionic forms of the molecule and are equal to $q \cdot D_n$ and $q \cdot D_i$, respectively.

With acidic compounds, eq. (7) indicates that k' decreases sigmoidally with increase in pH of the mobile phase, when $k'_n \gg k'_i$. With basic compounds, the pH-dependent change of k' is the reverse of that of acidic compounds, as shown in eq. (8). When the capacity factor can be measured over a wide range of pH, we can determine pK_a from the inflection point of the curve for k' plotted against pH. However, chemically bonded silica packings are usually stable only at pH 2—8, making it difficult to obtain the capacity factors to decide the inflection point exactly. Furthermore, even under conditions where the stationary phase is stable, the determination of k' in the acidic and/or alkaline mobile phase is not possible if the retention times of the compound under these conditions are too great or too small. Thus, in this study we determined K_a, k'_n and k'_i concomitantly by the non-linear least-squares method. The advantage of this computation is that K_a can be determined exactly from only a few data points in an appropriate pH range of the mobile phase.

Results and Discussion

Change of the Capacity Factor k' with pH

Figure 2 shows the dependence of the capacity factor k' of some acidic and basic compounds on the pH of the mobile phase. The k' value of acidic compounds, 4-nitrobenzoic acid and benzoic acid, begins to decrease abruptly at pH 2.5 and 3, respectively, as the pH of the mobile phase increases. Then the change becomes gradual and finally k' reaches a constant value. According to eq. (7), k' should be constant in the region of $\text{pH} \gg pK_a$, where k' represents k'_i. Thus the constant value of k' at alkaline pH in Fig. 2 represents k'_i. The change of k' of basic compounds, such as aniline and 4-methylaniline, is just the reverse of that of acidic compounds, as predicted from eq. (8). It is noteworthy that the k'_i of both acidic and basic compounds has a definite value, and is not zero, indicating that proportions of the ionic forms as well as the neutral forms of these compounds are distributed to the stationary phase, as is expected from Fig. 1.

When k'_i is known, we can determine K_a and k'_n from the linear relation between k' and $(k'-k'_i)/[H^+]$ according to eq. (9), which is obtained from eq. (7). By analogy with the relation

$$k' = k'_n - K_a \frac{(k'-k'_i)}{[H^+]} \tag{9}$$

for acidic compounds, that for basic compounds is given by eq. (10). Results for 4-nitrobenzoic acid are shown in Fig. 3. The solid line, curve A, is the result of simulation with

$$k' = k'_n - \frac{(k'-k'_i)}{K_a \cdot [H^+]} \tag{10}$$

the values of $pK_a = 3.46$, $k'_n = 20.49$ and $k'_i = 0.81$, which were obtained by a non-linear least-squares calculation. If the k'_i of acidic compounds were zero, eq. (9) could be simplified to eq. (11). In this case k' should decrease linearly with increase in $k'/[H^+]$, and K_a and k'_n could be determined from the slope and the intercept, respectively, of the straight line, as proposed by Unger *et al.*[11]

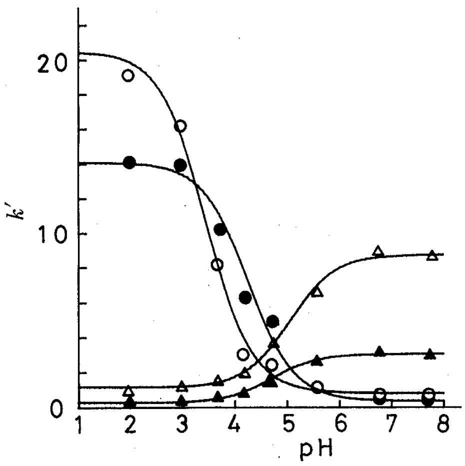

Fig. 2. Dependence of the Capacity Factor (k') of Acidic and Basic Compounds on the pH of the Mobile Phase

○, 4-nitrobenzoic acid; ●, benzoic acid; ▲, aniline; △, 4-methylaniline.

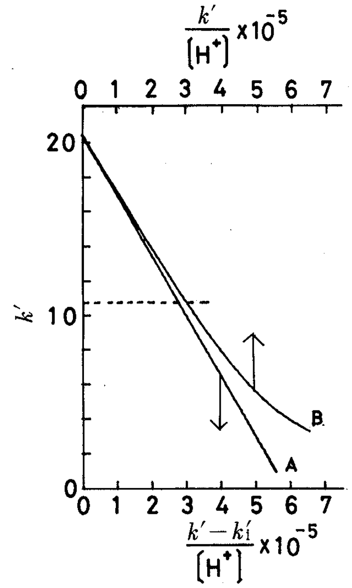

Fig. 3. Relation between k' and $k'/[H^+]$ and $(k'-k'_i)/[H^+]$ for 4-Nitrobenzoic Acid

curve A, simulated curve according to eq. (9); curve B, simulated curve according to eq. (11).

Table I. Values of Physical Parameters of Acids and Bases used in This Study

Compound	$\log P_{oct}$[a)]	pK_a (lit)[b)]	pK_a (HPLC)	k'_n	k'_i
Benzoic acid (BA)	1.87	4.20	4.25	14.10	0.36
4-CN–BA	1.56	3.55	3.64	13.29	0.55
3-NO_2–BA	1.83	3.49	3.56	18.51	0.90
4-NO_2–BA	1.89	3.42	3.46	20.49	0.81
3-F–BA	2.19	3.87	3.88	19.53	0.61
4-F–BA	2.07	4.14	4.20	18.13	0.61
3-Cl–BA	2.68	3.82	3.88	51.04	1.90
4-Cl–BA	2.65	3.98	4.02	53.36	2.11
3-Br–BA	2.87	3.81	3.87	76.08	2.73
3-I–BA	3.13	3.85	3.97	101.16	5.67
4-I–BA	3.02	3.93	4.11	108.17	5.40
Phenylacetic acid	1.41	4.31	4.30	11.08	0.67
2,4-Dinitrophenol	1.50	4.11	4.16	13.55	0.68
Aniline	0.90	4.62	4.72	3.09	0.30
4-CH_3–Aniline	1.39	5.12[c)]	5.08	8.81	1.19
N-CH_3–Aniline	1.82	4.85	4.84	10.59	0.98

a) From ref. 20. *b)* From ref. 19*a*. *c)* From ref. 19*b*.

$$k' = k'_n - K_a \frac{k'}{[H^+]} \tag{11}$$

However, this is not the case, as can be seen from the typical results in Fig. 3, curve B. The dotted line in Fig. 3 shows the k' value at pH $= pK_a$. It is clear that curve B does not decrease linearly with increase in $k'/[H^+]$ even at a pH near the pK_a, and that it deviates more from curve A in the pH region above the pK_a. This shows that k'_i cannot be considered as negligible in evaluating the pK_a value by means of these linear equations. We can determine K_a and k'_n by applying eq. (9) and (10), if k'_i is available. However, estimation of k'_i from a curve of k' *vs.*

pH, such as those in Fig. 2, is not always possible owing to the instability of the column packing and the small value of k'_i. Thus, we adopted the non-linear least-squares calculation in the determination of k'_n and k'_i as well as K_a.

Table I lists the values of K_a, k'_n and k'_i determined by the HPLC method by elution with phosphoric acid solution of various pH values containing 15% (v/v) methanol. Addition of methanol to the mobile phase makes it possible to perform HPLC over a wide pH range by increasing the solubility of the test compound in the mobile phase. Another advantage of addition of methanol is that it decreases the retention time to a certain degree, as reported previously,[7] thus enabling us to measure the exact retention times of compounds with large t_R values. As thown in the table, for all compounds k'_n is greater than k'_i, and k'_i takes a definite value that is not zero. Thus, k'_i must be considered when determining K_a. The physical meaning of k'_n and k'_i will be discussed later. It is also noteworthy in the table that values of pK_a determined by HPLC, pK_a (HPLC), agree well with reported values measured by the conventional method, pK_a (lit). The usefulness of the present method is also demonstrated in Fig. 4, by the linear relation with a slope of unity obtained between pK_a (HPLC) and pK_a (lit) for both acidic (open circles) and basic (closed circles) compounds.

In the mobile phase, methanol acts in two ways during HPLC: i) in the absence of methanol, the ODS-type stationary phase is not accessible to water molecules in the mobile phase, but the accessibility is improved by the presence of such organic solvents as methanol and ethanol as pointed out by McCormic and Karger,[14] and ii) it influences the acid dissociability of test compounds. As shown later in Fig. 6, the latter effect of methanol becomes apparent when the methanol concentration in the mobile phase exceeds 15% (v/v) for acidic compounds and 20% (v/v) for basic compounds. As shown in the next section, a concentration of 15% (v/v) methanol was appropriate for determination of the retention times of the compounds tested.

For chemially bonded column packings, such as ODS resins, the pH of the aqueous mobile phase should be below 8 to prevent the alkaline degradation of the silica matrix. Thus, high pK_a values cannot be determined by this method. It is of interest to know the highest pK_a value that can be determined under these conditions. The capacity factors, k', of benzoic acid and aniline were determined between pH 2 to 8, and are summarized in Tables II and III, respectively. Values of K_a, k'_n and k'_i were calculated with the data set of k' by successively deleting k' one by one from that at the highest pH. It is clear in Table II that the value of k'_n of benzoic acid is almost independent of the number of data points (n) used for calculation, but k'_i increases with decrease in n. This relation is reversed with the basic compound aniline: k'_i is constant, but k'_n decreases. However, in both cases, consistent pK_a values are obtained with k'

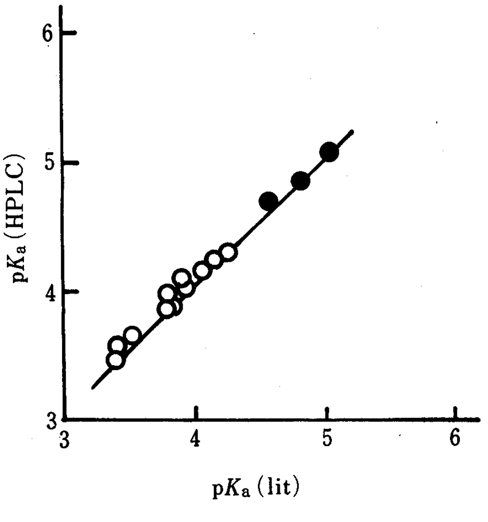

Fig. 4. Correlation between pK_a Values Determined by HPLC, pK_a (HPLC), and Those Obtained by the Conventional Potentiometric Method,[19] pK_a (lit)

●, basic compound; ○, acidic compound.

TABLE II. Dependence of pK_a (HPLC) Values of Benzoic Acid on the Number of Data Points (n) used for Calculation[a)]

$n^{b)}$	pH	k'	pK_a (HPLC)	k'_n	k'_i
1	2.00	14.33	—[c)]	—	—
2	3.00	14.24	—	—	—
3	3.71	10.33	—	—	—
4	4.19	7.39	3.95	14.83	2.96
5	4.40	5.98	3.94	14.89	3.10
6	4.73	5.11	3.89	14.93	3.53
7	5.13	1.93	4.22	14.32	0.71
8	5.59	1.22	4.22	14.29	0.66
9	6.79	0.42	4.26	14.12	0.43
10	7.79	0.27	4.25	14.10	0.36

a) The value of pK_a (lit) of benzoic acid is 4.20. Values of pK_a (HPLC), k'_n and k'_i were determined by the non-linear least-squares method from the k' values by deleting them one by one from that at the highest pH.

b) Number of data points used for calculation of pK_a, k'_n and k'_i.

c) Values cannot be determined due to the small number of data points (n).

TABLE III. Dependence of pK_a (HPLC) Values of Aniline on the Number of Data Points (n) used for Calculation[a)]

$n^{b)}$	pH	k'	pK_a (HPLC)	k'_n	k'_i
1	2.00	0.26	—[c)]	—	—
2	3.00	0.40	—	—	—
3	3.71	0.63	—	—	—
4	4.19	0.92	3.86	1.21	0.26
5	4.40	1.21	4.21	1.73	0.27
6	4.73	1.71	4.58	2.67	0.29
7	5.13	2.31	4.68	2.99	0.30
8	5.59	2.59	4.64	2.88	0.30
9	6.79	3.23	4.72	3.09	0.30
10	7.79	3.10	4.72	3.09	0.30

a) The value of pK_a (lit) of aniline is 4.62. Values of pK_a, k'_n and k'_i were determined by the non-linear least-squares method from the k' values by deleting them one by one from that at the highest pH.

b) Number of data points used for calculation of pK_a, k'_n and k'_i.

c) Values cannot be determined due to the small number of data points (n).

values obtained from pH 2 to one pH unit higher than the pK_a of the test compound. Thus, it is concluded that pK_a values up to about 7 can be determined with the ODS column, because the highest pH at which the column is usable is about 8.

Effect of Methanol on K_a

As reported previously,[7)] the $\log k'_n$ of various compounds decreases linearly with increase in the concentration of methanol added to the aqueous mobile phase up to about 30% (v/v). Thus, methanol may have an effect on the value of K_a, even under conditions where it does not affect the acid dissociability itself. Figure 5 shows the effect of methanol on the pH-dependent $\log k'$ of 4-nitrobenzoic acid.

As shown in the figure, methanol causes a decrease in $\log k'$ at all pH values. The effect of methanol is the same over a wide pH range, since the value of k'_n/k'_i is almost the same at various methanol concentrations: with 0, 10, 15, 20 and 30% (v/v) methanol, k'_n/k'_i takes

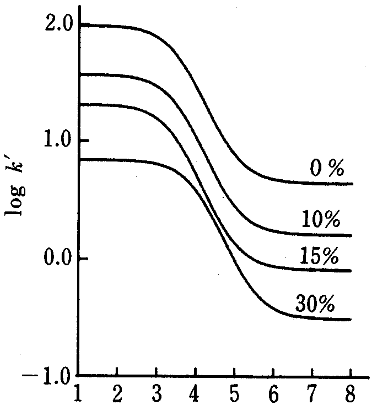

Fig. 5. Change in the k' of 4-Nitrobenzoic Acid with pH of the Mobile Phase at Various Methanol Concentrations

The percentages of methanol in the mobile phase are shown.

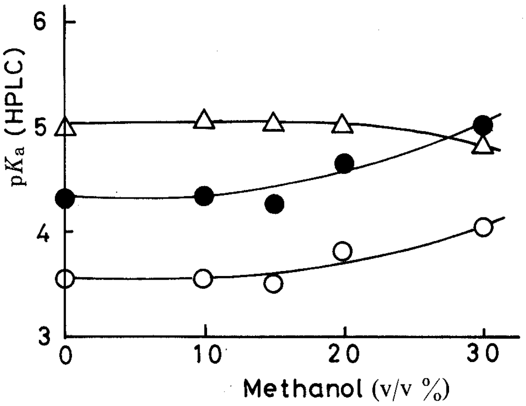

Fig. 6. Effect of Methanol in the Mobile Phase on pK_a of Compounds as Determined by HPLC, pK_a (HPLC)

●, benzoic acid; ○, 4-nitrobenzoic acid; △, aniline.

values of 22.2, 22.9, 25.3, 26.6 and 22.3, respectively. However, the inflection point shifts to higher pH at 30% (v/v) methanol. These results indicate that the capacity factor of the ionized form of the acid, k_i', is also affected by methanol and the shift of the inflection point is due to the effect of methanol on the acid dissociation.

Next, we determined the pK_a values of several compounds at various methanol concentrations in the mobile phase. Figure 6 depicts the dependence of pK_a (HPLC) on the methanol concentration up to 30% (v/v). With acids, pK_a (HPLC) is almost the same up to about 15% (v/v) and then increases as the concentration of methanol increases. Such a tendency is in agreement with that observed by the conventional potentiometric method.[15] In contrast to the case with acids, the effect of methanol on the dissociation of bases is small and pK_a (HPLC) values are almost constant up to 20% (v/v) and decrease with increase in the methanol concentration. For the determination of pK_a (HPLC), the methanol concentration should be as low as possible, consistent with sufficient solubility of the test compound in the mobile phase and a practical range of retention times.

Relation between k_n' and k_i'

In this study, we observed that ionic molecular species of acids and bases have affinity for the stationary phase and have a definite value of the capacity factor k_i', as observed by Mirrlees *et al.*[2] It has been shown that the capacity factor of the neutral form ($\log k_n'$) of a wide variety of compounds increases linearly with $\log P_{oct}$.[2−7] From this relation, the partition coefficient of organic compounds can be determined exactly and easily.[7] However, little attention has been paid to the hydrophobicity of ionic molecular species,[16−18] though the biological activity of some ionizable compounds is suggested to be closely related to the hydrophobicity of their ionic forms.[18] Thus examination of the relation between k_n' and k_i' as a function of the partition coefficient is of interest.

Figure 7 shows plots of $\log k_n'$ and $\log k_i'$ for a wide variety of compounds (listed in Table I) against the partition coefficients of the neutral forms of these compounds between octanol and water ($\log P_{oct}$). The $\log P_{oct}$ values are taken from the literature.[20] As can be seen, $\log k_n'$ and $\log k_i'$ both increase linearly with increase in $\log P_{oct}$. The difference between $\log k_n'$ and $\log k_i'$ is about 1.4, for acids, indicating that the affinity of the ionized form for the stationary phase is about 1/25 of that of the neutral form. For bases, values of $\log k_i'$ are larger than

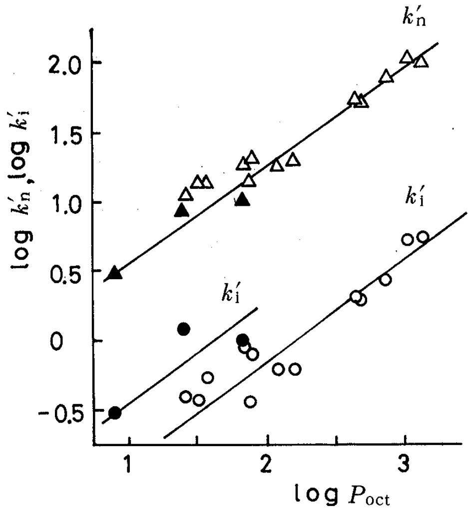

Fig. 7. Linear Relations between log k′ of Acidic and Basic Compounds and the Partition Coefficient, log P_{oct}

k'_n: k' for the neutral molecular form (△, acids; ▲, bases).
k'_i: k' for the ionic form (○, acids; ●, bases).
P_{oct}: Partition coefficient of the neutral form between octanol and water (cited from ref. 20).

those of the acids having the same log P_{oct}, and the differences between log k'_n and log k'_i is about 1. This discrepancy between acid and base may be due to the effect of the silanol moieties on the surface of the stationary phase; they interact with the protonated amine in the acidic mobile phase.[21] The "hydrophobicity" of the ionized form of acids is about 25 times less than that of the neutral form, when the "hydrophobicity" is evaluated by HPLC. However, this value seems too great in view of the fact that the partition coefficients of 2,4-dinitrophenol and indomethacin anions between octanol and water are about 1000 times less than those of the neutral form.[16,17] This discrepancy is presumably due to the difference in the partition mechanism in two immiscible liquid phases from that of partition between a solid stationary phase and an aqueous mobile phase. Recently, Deming and Kong[22] reported that k'_i is linearly correlated with k'_n for some aromatic carboxylic acids and k'_n/k'_i is about 16 for benzoic acids, 10 for cinnamic acids and 7 for phenylacetic acids and hydrocinnamic acids. Further study on the "hydrophobicity" of ionized molecular species would be useful for understanding the mechanisms of action of ionic bioactive compounds.

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