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PREDICTION OF RETENTION TIMES FOR AROMATIC ACIDS IN LIQUID CHROMATOGRAPHY*

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SUMMARY

The chromatographic behaviour of 30 aromatic acids was examined in a system of fine particle macroporous polystyrene-divinylbenzene copolymers and acetonitrile-water mixtures at different pH as eluents. The acids were benzoic, phenylacetic, cinnamic, mandelic, naphthoic and hippuric acids, and their hydroxy and/or methoxy derivatives.

At low pH, the logarithm of the capacity ratios of these acids was linearly related to the logarithm of their partition coefficients in the octanol-water system calculated after Rekker. By combining the above result and the dissociation constant of the acid, it was possible to predict the retention times of the acids at a given pH of the eluent.

INTRODUCTION

The aromatic acids and their metabolites are biologically important compounds. Therefore the understanding of their chromatographic behaviour and the development of a system which allows the prediction of their retention times in liquid chromatography could improve their analyses in clinical chemistry and the study of drug metabolism.

When the retention times for the molecular and ionic forms of an ionizable compound and its dissociation constants are known, the retention time in an eluent at a given pH can be calculated in aqueous phase liquid chromatography (LC)¹ and therefore fewer experiments are needed for the rapid optimization of the chromatographic separation conditions.

It is also possible to predict retention times of non-ionic compounds in reversed-phase LC²⁻⁴. The retention times of aliphatic alcohols, alkyl and/or halogenated benzenes and polyaromatic hydrocarbons were related to the logarithm of their partition coefficients in the octanol-water system ($\log P$) calculated by Rekker's method⁵. The same approach was extended⁶ to aromatic acid separations on an octadecyl-bonded silica gel packing with an eluent at pH 2.

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TABLE I

HYDROPHOBICITY ($\log P$) AND LOGARITHM OF CAPACITY RATIO ($\log k'$)

Experimental conditions: column, 25 cm \times 4.1 mm I.D., packed with Hitachi 3011; eluent, 0.05 *M* phosphoric acid in 20–50% acetonitrile in water; column temperature, 55°C.

Compound	Abbreviation	$\log P_1^*$	$\log P_2^*$	$\log k'$	Acetonitrile (%)			
					20	30	40	50
3-Hydroxy-2-naphthoic acid	3(OH)NA	2.83	3.05	—	1.59	1.12	0.84	0.84
2-Naphthoic acid	NA	2.72	2.84	—	1.49	1.04	0.74	0.74
3-Methoxycinnamic acid	3MeOCA	2.33	2.37	—	1.19	0.79	0.52	0.52
Cinnamic acid	CA	2.26	2.32	—	1.10	0.75	0.49	0.49
Indole-3-propionic acid	IPA	1.99	2.20	—	1.00	0.67	0.41	0.41
2-Hydroxybenzoic acid	2(OH)BA	1.90	2.18	1.28	0.94	0.65	0.43	0.43
3-Methoxybenzoic acid	3MeOBA	1.86	1.99	1.27	0.91	0.60	0.37	0.37
3-Methoxyphenylacetic acid	3MeOPhA	1.82	1.99	1.21	0.86	0.58	0.34	0.34
4-Methoxyphenylacetic acid	4MeOPhA	1.82	1.95	1.19	0.84	0.54	0.33	0.33
Phenylacetic acid	PhA	1.75	1.94	1.11	0.81	0.55	0.33	0.33
Benzoic acid	BA	1.79	1.94	1.11	0.80	0.57	0.34	0.34
Indole-3-acetic acid	IAA	1.75	1.92	1.17	0.80	0.52	0.28	0.28

4-Hydroxy-3-methoxycinnamic acid	4(OH)3MeOCA	1.79	1.51	0.97	0.61	0.33	0.12
4-Hydroxycinnamic acid	4(OH)CA	1.72	1.46	0.81	0.50	0.23	0.049
2-Hydroxyhippuric acid	2(OH)HA	1.15	1.55	0.80	0.52	0.29	0.097
2-Hydroxyphenylacetic acid	2(OH)PhA	1.21	1.47	0.69	0.44	0.23	0.075
3-Hydroxybenzoic acid	3(OH)BA	1.25	1.37	0.60	0.37	0.17	-0.004
3-Methoxymandelic acid	3MeOMA	1.30	1.33	0.68	0.42	0.22	0.064
4-Hydroxy-3-methoxybenzoic acid	4(OH)3MeOBA	1.26	1.31	0.65	0.39	0.16	0.030
4-Hydroxybenzoic acid	4(OH)BA	1.25	1.28	0.52	0.31	0.11	-0.013
4-Hydroxyphenylacetic acid	4(OH)PhA	1.21	1.26	0.52	0.29	0.12	-0.018
Mandelic acid	MA	1.23	1.26	0.57	0.35	0.25	0.011
Hippuric acid	HA	1.04	1.25	0.56	0.32	0.19	0.000
4-Hydroxy-3-methoxyphenylacetic acid	4(OH)3MeOPhA	1.22	1.18	0.61	0.37	0.19	0.023
5-Hydroxyindole-3-acetic acid	5(OH)IAA	1.23	1.17	0.49	0.26	0.085	-0.050
3,4-Dihydroxycinnamic acid	3,4(OH) ₂ CA	1.12	1.15	0.52	0.28	0.076	-0.095
3,4-Dihydroxybenzoic acid	3,4(OH) ₂ BA	0.65	0.99	0.30	0.12	0.000	-0.16
3,4-Dihydroxyphenylacetic acid	3,4(OH) ₂ PhA	0.61	0.98	0.30	0.13	0.073	-0.14
3,5-Dihydroxybenzoic acid	3,5(OH) ₂ BA	0.65	0.94	0.22	0.084	-0.038	-0.16
2,4-Dihydroxyphenylacetic acid	2,4(OH) ₂ PhA	0.61	0.80	0.16	0.064	-0.058	-0.18
4-Hydroxy-3-methoxymandelic acid	4(OH)3MeOMA	0.76	0.84	0.19	0.065	-0.073	-0.17
3,4,5-Trihydroxybenzoic acid	3,4,5(OH) ₃ BA	-	0.72	0.051	-0.022	-0.092	-0.26
Uric acid	UA	-	0.18	-0.19	-0.25	-0.29	-

* $\log P_1$ are hydrophobicity values after Rekker^{5,6}; $\log P_2$ are calculated values obtained on an octadecyl-bonded silica gel⁶.

In this paper, the combination of the hydrophobicity and the acid dissociation constant K_a is used to predict the retention times of aromatic acids at a given pH and acetonitrile concentration. The retention times of *ca.* 30 aromatic acids were measured on macroporous polystyrene gels as packings with mixtures of acetonitrile and water as eluents. The ionization constants for the different acids were also measured by direct titration in different acetonitrile-water mixtures.

EXPERIMENTAL

A Waters 6000A chromatographic pump was used with a Rheodyne Model 7125 injector. A Hitachi Model 100-20 spectrophotometer with an Altex Model 155-01 8- μ l flow cell was used as a detector. Perkin-Elmer Model 56 or Shimadzu Model C-R1A were used as recorder. The packings used were 5- or 10- μ m macroporous polystyrene-divinylbenzene copolymers (Hitachi 3013 and 3011, respectively). The columns were slurry-packed in stainless-steel tubes (15 or 25 cm \times 4.1 mm I.D.). The column was thermostated, and the temperature was controlled by a Haake F5e water bath. Analytical grade chemicals supplied by Sigma and Chem. Service were used without further purification. Burdick & Jackson glass-distilled acetonitrile UV and distilled water treated through Milli-Q system (Millipore) were used as components of the eluent. The pH was controlled with a 0.05 M sodium phosphate buffer. A pH meter built at Université de Montréal was used with Fisher Scientific Model 13-639-6, 13-639-56 and 13-639-92 electrodes.

RESULTS AND DISCUSSION

The logarithms of the capacity ratios ($\log k'$) of the acids in their molecular form were measured in 20–50% acetonitrile in water mixtures with 0.05 M phosphoric acid at 55°C. Their values and the hydrophobicity expressed as $\log P$ are reported in Table I.

The $\log k'$ was linearly related to $\log P$ in this system, and the relation is given by eqn. 1:

$$\log k' = y \cdot \log P + m \quad (1)$$

where y and m are the slope and the intercept of the least-squares straight line, respectively.

The correlation coefficients obtained when $\log P$ values after Rekker⁵ ($\log P_1$ in the tables) were used in eqn. 1, were 0.947 ($n = 25$) in acetonitrile-water (20:80), 0.973 ($n = 31$) in acetonitrile-water (30:70), 0.959 ($n = 31$) in acetonitrile-water (40:60) and 0.965 ($n = 31$) in acetonitrile-water (50:50) mixtures ($n =$ the number of compounds used in the least-squares). The correlation coefficients obtained with $\log P$ values ($\log P_2$ in the tables) derived from measurements in reversed-phase LC on an octadecyl-bonded silica gel⁶ were 0.988 ($n = 27$) in acetonitrile-water (20:80), 0.996 ($n = 32$) in acetonitrile-water (30:70), 0.993 ($n = 32$) in acetonitrile-water (40:60) and 0.996 ($n = 32$) in acetonitrile-water (50:50) mixtures. All the lines merged at a single point and therefore the retention time in a given eluent (acetonitrile-water) for the molecular form of an acid can be predicted from its $\log P$ value.

TABLE II

DISSOCIATION CONSTANTS OF ACIDS (pK_a) IN WATER

The pK_a values were measured in different acetonitrile-water mixtures. They were converted into pK_a values in pure water by the experimentally derived relation:

$$pK_a(\text{water}) = pK_a(\text{acetonitrile-water}) - 0.022(\% \text{ acetonitrile}) \quad (7)$$

Compound	Chromatography		Titration	Literature
	Method 1*	Method 2**		
3(OH)NA	2.56	2.86	2.89	
NA	4.24	—	4.25	4.17***
3MeOCA	4.34	4.24	4.40	
CA	4.37	4.61	4.37	4.44***
IPA	4.79	4.73	4.81	
2(OH)BA	2.69	2.97	3.06	2.97***, 2.92 [‡] , 2.84 [‡] , 1.88 [‡]
3MeOBA	4.10	4.09	4.09	
3MeOPhA	4.37	4.45	4.27	
4MeOPhA	4.67	4.44	4.35	
PhA	4.43	4.58	4.29	4.25***, 4.14, 4.31, 4.10 [‡]
BA	4.18	4.20	4.19	4.19***, 3.78, 3.93 [‡]
IAA	4.68	4.60	4.65	4.75***
4(OH)3MeOCA	4.57	4.57	4.56	
4(OH)CA	4.56	4.56	4.57	
2(OH)HA	3.38	—	3.58	
2(OH)PhA	4.14	4.18	4.14	
3(OH)BA	4.12	4.15	4.16	4.06***
3MeOMA	3.28	3.36	3.47	
4(OH)3MeOBA	4.50	4.52	4.48	
4(OH)BA	4.56	4.73	4.53	4.48***
4(OH)PhA	4.43	—	4.40	4.30 [‡]
MA	3.40	3.41	3.42	3.85***, 3.37, 3.46, 3.49 [‡]
HA	3.42	—	3.58	3.80***
4(OH)3MeOPhA	4.34	4.30	4.37	4.29 [‡]
5(OH)IAA	4.61	4.65	4.57	
34(OH) ₂ CA	4.54	4.54	4.53	
34(OH) ₂ BA	4.48	4.59	4.49	4.48***
34(OH) ₂ PhA	4.37	4.38	4.34	4.20 [‡]
35(OH) ₂ BA	4.18	4.23	4.12	4.04***
25(OH) ₂ PhA	4.13	—	4.06	
4(OH)3MeOMA	3.58	3.71	3.55	3.25 [‡]
345(OH) ₃ BA	4.45	—	4.40	4.41***
UA	—	—	—	3.89***
24(OH) ₂ BA	—	—	3.32	3.20, 3.25 ^{‡‡}
34(OH) ₂ MA	—	—	3.50	

* Experimental conditions, see Table I.

** Obtained on Hitachi 3013 packing, with a 20% acetonitrile-water mixture with 0.05 M sodium phosphate at 55°C.

*** Ref. 6.

[‡] Ref. 8.

^{‡‡} Ref. 9.

TABLE III
COMPARISON OF PREDICTED AND OBSERVED CAPACITY RATIOS FOR ACIDS

Compound	$k_m(\text{obs.})^*$	$k_m(\text{calc.})^{**}$	$k_f(\text{obs.})^*$	k' at pH 4.0		k' at pH 5.09		
				Obs.*	Pred.†	Obs.*	Pred.†	
3(OH)NA	39.17	41.65	1.54	5.80	12.05	10.91	2.68	1.18
NA	30.73	29.47	1.02	24.49	26.36	26.25	12.49	11.88
3MeOCA	15.52	13.73	0.84	12.90	12.70	12.63	7.13	6.70
CA	12.67	12.60	0.78	10.90	11.56	11.53	6.35	5.93
IPA	11.10	10.38	0.70	9.92	10.06	10.04	7.64	7.40
2(OH)BA	8.76	10.02	0.68	2.20	3.90	3.45	1.07	0.41
3MeOBA	8.20	7.32	0.65	6.53	6.31	6.21	3.24	2.32
3MeOPhA	7.22	7.29	0.63	6.22	6.59	6.53	3.65	3.01
4MeOPhA	6.95	6.87	0.61	6.03	6.32	6.26	4.22	3.15
PhA	6.40	6.74	0.65	5.59	6.12	6.06	3.22	2.86
BA	6.33	6.77	0.58	5.38	6.01	5.93	2.92	2.50
IAA	6.33	6.51	0.64	5.69	6.24	6.21	3.98	4.10
4(OH)3MeOCA	4.09	3.32	0.56	3.67	3.16	3.13	2.52	1.92
4(OH)CA	3.18	3.07	0.52	2.83	2.93	2.90	1.76	1.79
2(OH)HA	3.28	3.52	0.64	1.98	2.47	2.23	0.99	0.44

2(OH)PhA	2.77	3.08	0.68	2.38	2.75	2.66	1.38	1.50	1.05
3(OH)BA	2.35	2.64	0.50	2.01	2.36	2.29	1.12	1.25	0.93
3MeOMA	2.63	2.47	0.60	1.52	1.67	1.42	0.73	0.79	0.24
4(OH)3MeOBA	2.45	2.39	0.42	2.23	2.26	2.23	1.50	1.47	1.27
4(OH)BA	2.04	2.25	0.43	1.86	2.14	2.11	1.36	1.52	1.26
4(OH)PhA	1.95	2.18	0.51	1.75	2.04	2.00	1.22	1.32	1.06
MA	2.25	2.20	0.60	1.48	1.47	1.20	0.71	0.74	0.20
HA	2.09	2.16	0.54	1.43	1.57	1.37	0.74	0.74	0.27
4(OH)3MeOPhA	2.35	1.91	0.52	2.13	1.80	1.75	1.37	1.17	0.90
5(OH)AA	1.81	1.90	0.52	1.73	1.82	1.79	1.28	1.32	1.11
34(OH) ₂ CA	1.90	1.84	0.35	1.69	1.75	1.73	1.23	1.19	1.03
34(OH) ₂ BA	1.33	1.40	0.44	1.23	1.34	1.31	0.91	0.96	0.75
34(OH) ₂ PhA	1.34	1.38	0.43	1.17	1.29	1.25	0.85	0.86	0.62
35(OH) ₂ BA	1.21	1.29	0.45	1.07	1.17	1.11	0.72	0.73	0.43
25(OH) ₂ PhA	1.16	1.14	0.48	1.18	1.03	0.96	0.69	0.68	0.34
4(OH)3MeOMA	1.16	1.09	0.43	0.83	0.84	0.67	0.55	0.51	0.13
345(OH) ₃ BA	0.95	0.90	0.41	0.83	0.86	0.83	0.67	0.65	0.44
UA	0.56	—	0.34	0.52	—	—	0.49	—	—

* Experimental conditions are given in Table I.

** Calculated by eqn. 1.

*** Calculated by eqn. 2 with observed k'_i .

† Calculated by eqn. 3.

3MeOMA	1.41	0.17	0.64	0.70	0.60	0.27	0.21	0.04
4(OH)3MeOBA	1.36	0.17	1.27	1.22	1.20	0.50	0.47	0.34
4(OH)BA	1.27	0.17	1.00	1.15	1.14	0.49	0.46	0.34
4(OH)PhA	1.22	0.16	1.00	1.08	1.06	0.43	0.39	0.26
MA	1.24	0.16	-	0.59	0.49	0.19	0.19	0.03
HA	1.21	0.16	0.76	0.67	0.59	0.28	0.20	0.05
4(OH)3MeOPhA	1.06	0.16	1.19	0.93	0.91	0.44	0.34	0.22
5(OH)AA	1.05	0.15	0.97	0.96	0.95	0.51	0.41	0.30
34(OH)2CA	1.02	0.15	0.84	0.93	0.91	0.45	0.38	0.27
34(OH)2BA	0.75	0.13	0.58	0.68	0.67	0.31	0.29	0.19
34(OH)2PhA	0.74	0.13	0.60	0.65	0.63	0.30	0.25	0.14
35(OH)2BA	0.69	0.12	0.55	0.56	0.53	0.18	0.20	0.09
25(OH)2PhA	0.60	0.12	0.46	0.47	0.44	0.20	0.17	0.07
4(OH)3MeOMA	0.57	0.11	0.28	0.33	0.27	0.17	0.13	0.02
345(OH)3BA	0.46	0.10	0.36	0.42	0.40	0.20	0.18	0.10
UA	-	-	0.09	-	-	0.09	-	-

* k'_m was calculated by eqn. 1.

** k'_1 was calculated by eqn. 1.

*** The column was 15 cm long, 4.1 mm I.D. and packed with Hitachi 3013. The eluent was 30% acetonitrile-water with 0.05 M sodium phosphite. The column temperature was 55°C.

† Calculated by eqn. 2.

‡ Calculated by eqn. 3.

The better correlation between the $\log k'$ and $\log P_2$ values suggests that the $\log P$ values measured on an ODS silica gel can also be used with a different packing.

The dissociation constants K_a of the different acids were measured by chromatography in 20–50% acetonitrile–water mixtures with 0.05 *M* sodium phosphate (Table II). The values obtained by titration in acetonitrile–water mixtures with and without 0.1 *M* sodium sulfate are also given in Table II.

The dissociation constants obtained by LC were similar to those obtained by direct titration. If the dissociation constant is known, it should be possible to predict the retention times of an acid in an eluent at different pH values and/or concentrations of acetonitrile. To verify this, two eluents with different pH values were prepared in an 30% acetonitrile–water mixture with 0.05 *M* sodium phosphate. The capacity ratios k' of the acids at a given pH were calculated from eqns. 2 and 3:

$$k' = (k'_m + k'_i \cdot K_a/[H^+]) / (1 + K_a/[H^+]) \quad (2)$$

$$k' = k'_m / (1 + K_a/[H^-]) \quad (3)$$

Eqn. 2, where k'_m is the capacity ratio of the acid in its molecular form, k'_i the capacity ratio of the acid in its ionic form and K_a the dissociation constant of the acid in the eluent, can be simplified to eqn. 3 by assuming that k'_i is negligible compared with k'_m (ref. 7).

The capacity ratio of the molecular form of an acid (k'_m calc.) in acetonitrile–water (30:70) was calculated from eqn. 1 with the $\log P_2$ value in Table I. The γ value for a given eluent was obtained from the experimental results in Table I as previously described⁴.

The capacity ratio k'_i was measured in acetonitrile–water (30:70) mixtures with 0.05 *M* dibasic sodium phosphate, and the dissociation constants of the acids were obtained by direct titration.

The measured and calculated capacity ratios are listed in Table III. The average difference between the calculated and observed capacity ratios was *ca.* 10%. The largest differences were found for 3(OH)NA and 2(OH)BA. The main reason for such a difference was probably the poor reproducibility of the dissociation constant obtained by titration.

The capacity ratios predicted from eqn. 3 were usually found satisfactory, and this approach could be used as a first approximation for the calculation of the capacity ratios.

The agreement between the observed and the calculated capacity ratios was usually within $\pm 10\%$. The compounds for which the largest deviation was found are those for which the largest error was found in K_a .

To extend the study to another system, a new column was packed with a Hitachi 3013 polystyrene gel. The eluents were 10–60% acetonitrile in water mixtures with 0.05 *M* sodium phosphate. Six standard* compounds were selected to characterize the system following the procedure described previously⁴.

For each eluent (0.05 *M* phosphoric acid with acetonitrile) the capacity ratios k'_m of the standard compounds were measured and related to their hydrophobicity

* The standard compounds were: 4-hydroxy-3-methoxy mandelic acid, 4-hydroxy-3-methoxybenzoic acid, 2-hydroxynippuric acid, indole-3-acetic acid, 3-methoxybenzoic acid and cinnamic acid.

(log P). From a least-squares calculation the slope y and the intercept m in eqn. 1 were obtained for each eluent. With these values the capacity ratios k'_{m} for all acids used previously were calculated by eqn. 1.

The same procedure was used to calculate the k'_i values of the acids. The standard compounds used were the same as previously and in addition 3,4,5-trihydroxybenzoic and 2-naphtoic acids were also used. The eluent was 0.05 M dibasic sodium phosphate in acetonitrile–water (30:70). The dissociation constants obtained by titration were used (Table II). The capacity ratios were measured and calculated by eqns. 2 and 3 in eluents with 30% acetonitrile at pH 4.26 and 5.62. All data are collected in Table IV. Here again, the agreement between the observed and predicted k' were within $\pm 10\%$. For 3-hydroxy-2-naphtoic acid an error higher than 10% was attributed mainly to the error in K_a .

CONCLUSION

The hydrophobicity calculated after Rekker can be used as a first approach to predict retention times for both non-ionic⁴ and ionic compounds on different packings (ODS, polystyrene gels) used in reversed-phase LC. The relationship between hydrophobicity (log P) and capacity ratios (log k') is linear for ionic and non-ionic compounds but different slopes were obtained. The combination of hydrophobicity and dissociation constant with solvent concentration effect also allows us to predict the retention times for acids.

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