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HYDROPHOBICITY AND CHROMATOGRAPHIC BEHAVIOUR OF AROMATIC ACIDS FOUND IN URINE*

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SUMMARY

In reversed-phase liquid chromatography, the capacity ratios of urinary compounds were related with their hydrophobicity calculated by Rekker's hydrophobic fragmental constants. The retention behaviour of these compounds differed from that of non-ionizable compounds whose retention time can be predicted in different mixtures of acetonitrile and water as eluent and on an octadecyl silica packing from their calculated hydrophobicity. However, the calculated values for hydrophobicity and/or those derived from the results obtained for non-ionizable compounds could be useful to analyze the metabolites of acidic compounds in urine. The retention behaviour of all the compounds on gradient elution is also discussed.

INTRODUCTION

One of the challenges in liquid chromatography (LC) is the development of qualitative analyses for organic metabolites. The metabolites are usually purified by LC or gas chromatography and identified by spectrophotometric methods.

For qualitative analysis by LC, different approaches (use of resonance energy[1], delocalization energy[2], number of carbon atoms in the alkyl chain, dipole moment[3,4], Van der Waals radius or electronegativity[3]) have been used to characterize the solutes. The thermodynamic equilibrium of the solutes between mobile and stationary phases[5,6] has also been investigated and applied to the separation of catecholamine derivatives[7].

Another approach was the use of hydrophobicity for compounds in their molecular form[8–10]. The hydrophobicity of solutes was calculated with Rekker's hydrophobic fragmental constants[11], which were derived from the partition coefficients by Hansch's method. The partition coefficients for several compounds were also directly measured in LC and related to observed capacity ratios[12–14].

In order to predict the retention time of solutes in LC, different packings and organic modifiers were previously tested to find a practical system. The system formed by acetonitrile–water and a chemically bonded octadecyl packing was found to be suitable for qualitative analysis. For aliphatic alcohols and polyaromatic hydrocarbons a linear relation was found between the calculated hydrophobicity and the

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TABLE I

HYDROPHOBICITY OF URINARY AND STANDARD COMPOUNDS

No.	Compound	log P_c*	k'			
			% Acetonitrile			
			90	80	70	60
1	Benzoic acid	1.79	0.512	0.581	0.702	0.890
2	2-Hydroxybenzoic acid	1.90	0.558	0.644	0.777	1.066
3	3-Hydroxybenzoic acid	1.25	0.402	0.414	0.445	0.575
4	4-Hydroxybenzoic acid	1.25	0.393	0.405	0.443	0.541
5	2,4-Dihydroxybenzoic acid	1.30	0.391	0.414	0.486	0.647
6	3,4-Dihydroxybenzoic acid	0.65	0.370	0.365	0.376	0.471
7	3,5-Dihydroxybenzoic acid	0.65	0.350	0.327	0.362	0.434
8	3,4,5-Trihydroxybenzoic acid	−0.011	0.333	0.356	0.333	0.373
9	3-Methoxybenzoic acid	1.86	—	—	—	—
10	4-Hydroxy-3-methoxybenzoic acid	1.26	0.419	0.431	0.454	0.558
11	Cyclohexanecarboxylic acid	1.93	0.529	0.595	0.725	0.887
12	Phenylacetic acid	1.75	0.417	0.538	0.673	0.864
13	2-Hydroxyphenylacetic acid	1.21	0.402	0.434	0.486	0.607
14	4-Hydroxyphenylacetic acid	1.21	0.385	0.396	0.443	0.529
15	2,5-Dihydroxyphenylacetic acid	0.61	—	—	—	—
16	3,4-Dihydroxyphenylacetic acid	0.61	0.368	0.342	0.382	0.434
17	3-Methoxyphenylacetic acid	1.82	0.477	0.520	0.665	0.884
18	4-Methoxyphenylacetic acid	1.82	0.466	0.532	0.639	0.858
19	4-Hydroxy-3-methoxyphenylacetic acid	1.22	—	—	—	—
20	Indoleacetic acid	1.75	—	—	—	—
21	Indolepropionic acid	1.99	0.469	0.523	0.673	0.904
22	5-Hydroxyindoleacetic acid	1.23	—	—	—	—
23	Tryptophan	1.13	1.181	0.751	0.694	0.541
24	5-Hydroxytryptophan	0.61	—	—	—	—
25	Cinnamic acid	2.26	0.541	0.624	0.800	1.037
26	4-Hydroxycinnamic acid	1.72	—	—	—	—
27	3,4-Dihydroxycinnamic acid	1.12	0.370	0.365	—	0.443
28	3-Methoxycinnamic acid	2.33	—	—	—	—
29	4-Hydroxy-3-methoxycinnamic acid	1.79	—	—	—	—
30	Hydrocinnamic acid	1.99	0.552	0.616	0.780	1.043
31	Mandelic acid	1.23	0.402	0.428	0.486	0.515
32	3-Methoxymandelic acid	1.30	0.411	0.425	0.486	0.529
33	4-Hydroxy-3-methoxymandelic acid	0.76	—	—	—	—
34	2-Naphthoic acid	2.72	0.673	0.881	1.424	1.559
35	3-Hydroxy-2-naphthoic acid	2.83	0.691	0.884	1.212	1.902
36	Hippuric acid	1.04	0.411	0.379	0.437	0.544
37	2-Hydroxyhippuric acid	1.15	—	—	—	—
38	Nicotinic acid	0.43	0.999	0.682	0.500	0.443
39	Uric acid	—	0.310	0.269	0.229	0.212
40	Caffeine	—	0.639	0.558	0.543	0.587
41	Theobromine	—	0.460	0.399	0.369	0.362
42	Xanthine	—	0.333	0.298	0.248	0.249
43	Phenacetin	1.62	0.613	0.653	0.774	1.020
44	Uracil	—	0.396	0.327	0.304	0.307
45	Benzylalcohol	0.93	0.569	0.616	0.694	0.846

						log P_m**	log P_r***
50	*40*	*30*	*20*	*10*	*0*		
1.175	1.732	3.132	6.934	–	–	1.94	1.86, 1.87 (11)
1.522	2.280	4.424	10.05	–	–	2.18	2.26, 2.31 (11)
0.725	0.875	1.382	2.667	8.752	–	1.37	1.32, 1.36, 1.50 (11)
0.659	0.795	1.224	2.116	6.527	–	1.28	1.31, 1.36, 1.58 (11)
0.789	1.054	1.750	3.299	10.71	–	1.51	
0.494	0.564	0.763	1.193	3.440	18.45	0.99	
0.471	0.541	0.760	1.35	3.345	25.06	0.94	
0.393	0.443	0.535	0.699	1.634	9.03	0.72	
1.193	1.937	3.789	–	–	–	1.99	2.02 (11)
0.659	0.844	1.268	2.384	8.735	–	1.31	
1.279	1.787	3.045	7.050	–	–	1.96	
1.230	1.741	3.160	6.882	–	–	1.94	1.41, 1.42, 1.45, 1.96 (11)
0.797	1.011	1.556	3.065	9.127	–	1.47	
0.692	0.795	1.135	2.009	5.953	–	1.26	0.85 (11)[§]
–	–	0.673	0.916	1.949	10.86	0.86	
0.538	0.558	9.751	1.184	3.147	22.08	0.98	
1.222	1.827	3.466	8.550	–	–	1.99	1.46, 1.50 (11)
1.187	1.729	3.305	8.108	–	–	1.95	1.42, 1.46 (11) –
0.569	0.777	1.147	–	–	–	1.18	
–	1.637	3.287	8.342	–	–	1.92	1.39, 1.41 (11)
1.366	2.370	5.544	17.34	–	–	2.20	
–	0.702	1.121	1.822	6.363	–	1.17	
0.515	0.607	0.815	1.545	6.184	–	1.09	
–	0.365	0.483	0.656	1.885	23.23	0.62	
1.606	2.647	6.103	18.89	–	–	2.32	2.13, 2.25 (11)
0.720	1.043	1.891	–	–	–	1.46	
0.544	0.691	1.155	2.381	10.72	–	1.15	
1.608	2.976	7.223	–	–	–	2.37	
0.731	1.118	2.087	–	–	–	1.51	
1.562	2.405	5.174	13.89	–	–	2.25	1.84 (11)
0.665	0.789	1.230	2.061	4.724	–	1.26	
0.694	0.846	1.380	2.635	7.482	–	1.33	
–	–	0.670	0.832	1.692	7.03	0.84	
2.503	4.995	13.02	–	–	–	2.84	
3.077	5.463	18.45	–	–	–	3.05	
0.639	0.722	1.184	2.125	5.818	–	1.25	
–	–	1.738	3.830	13.23	–	1.55	
0.492	0.379	0.431	0.443	0.558	2.26	0.66	
0.180	0.209	0.321	0.359	0.561	4.83	0.18	
0.636	0.668	0.994	1.920	8.645	–	0.59	−0.07 (13)
0.376	0.368	0.558	0.771	2.381	–	0.69	
0.255	0.232	0.385	0.428	0.844	6.32	0.30	
1.351	1.986	3.942	9.828	–	–	1.47	
0.258	0.298	0.422	0.443	0.616	2.36	0.42	
1.161	1.764	2.332	3.821	–	–	1.19	

(Continued on p. 530)

TABLE I *(continued)*

No.	Compound	log P_c*	k'			
			% Acetonitrile			
			90	80	70	60
46	Cinnaminal	1.49	0.621	0.699	0.864	1.129
47	Indole	2.06	0.644	0.783	1.100	1.654
48	Phenol	1.54	0.523	0.595	0.720	0.921
49	Naphthalene	3.21	0.985	1.424	2.185	3.850
50	Butylophenone	2.81	0.870	1.158	1.706	2.681
51	Propiophenone	2.28	0.754	0.971	1.363	1.960
52	Acetophenone	1.75	0.679	0.803	1.051	1.398
53	2-Hydroxyacetophenone	1.21	0.581	0.616	0.708	0.774
54	Isopentylbenzoate	4.15	1.305	2.087	3.564	6.897
55	Butylbenzoate	3.74	1.141	1.741	2.837	5.145
56	Isopropylbenzoate	3.09	0.962	1.380	2.087	3.432
57	Methylberzoate	2.15	0.771	0.962	1.317	1.926
58	4-Hydroxypropylbenzoate	2.72	0.630	0.740	1.010	1.525

* log P_c values were calculated by Rekker's hydrophobic fragmental constants.
** log P_m values are mean values of observed data in 20–60% acetonitrile in water with 0.04 *M* phosphoric acid.
*** log P_r values are mainly collected from ref. 11.
‡ This value is the one of 3-hydroxyphenylacetic acid.

logarithm of the capacity ratios[9]. It was not necessary to classify the solutes in different categories, including the ones with a surface of covalently bonded hydrogen atoms and the others with a surface of non-covalently bonded electrons[15].

In this article, a liquid chromatographic system was used for the separation of aromatic acids. Compounds found in urine (urinary compounds) were selected as solutes and their calculated hydrophobicity was related with their capacity ratios in reversed-phase LC separation with acetonitrile–water mixtures as eluents. The results obtained were compared with those of non-ionizable compounds.

EXPERIMENTAL AND RESULTS

The solutes listed in Table I were supplied from Sigma and Chem. Service. The instruments and solvents were used as previously described[9,10]. The capacity ratios obtained in the system of a 5 μm octadecyl silica packing (Chromosorb LC-7 from Johns Manville) and different acetonitrile–water mixtures with 0.04 *M* phosphoric acid are also listed in Table I.

The linear relations between log k' and the hydrophobicity log P_c (calculated after Rekker) for non-ionizable compounds (Nos. 49–57 in Table I) in 30–90% acetonitrile–water mixtures at pH 2 were obtained with correlation coefficients higher than 0.995. All the straight lines merged at a single point. This fact allows the prediction of the retention times of non-ionizable compounds in a system with an octadecyl bonded silica as packing and a known concentration of acetonitrile in the eluent[8]. The slopes of the lines obtained in 70, 80 and 90% acetonitrile–water mixtures with phosphoric acid are identical to those of eluents without acid. Thus, the

						*log P_m***	*log P_r****
50	*40*	*30*	*20*	*10*	*0*		
1.631	2.606	5.394	13.81	–	–	1.67	.95, 2.03 (11)
2.765	4.909	10.64	–	–	–	2.17	2.00, 2.06, 2.13, 2.14, 2.25 (11)
1.334	1.467	3.039	4.810	–	–	1.28	1.46, 1.48, 1.54 (11)
7.275	14.74	–	–	–	–	3.21	3.01, 3.18, 3.30, 3.37, 3.48, 3.59 (11)
4.877	9.828	–	–	–	–	2.77	
3.290	5.832	12.26	–	–	–	2.30	
2.125	3.299	5.924	–	–	–	1.83	1.58, 1.70, 1.73 (11)
1.034	1.372	2.024	–	–	–	1.07	
15.45	–	–	–	–	–	4.10	
10.68	–	–	–	–	–	3.68	
6.501	14.67	–	–	–	–	3.11	
3.031	5.391	10.98	–	–	–	2.24	2.12, 2.17 (11)
2.508	4.967	12.49	–	–	–	2.15	

retention behaviour of non-ionizable compounds was not influenced by addition of the acid in the eluents. From the least-square lines the hydrophobicities log P_0 of the different compounds were calculated using the experimental values of log k'. The mean value log P_m of the hydrophobicities log P_0 obtained for different eluents are also given in Table I.

The same approach was applied to study the retention behaviour of aromatic acids. The correlation coefficients between log k' and log P_c of aromatic acids were not as good as those of non-ionizable compounds and the values in 80, 70, 60, 50, 40, 30, 20 and 10% acetonitrile in water at pH 2 were 0.857 ($n = 26$), 0.939 ($n = 25$), 0.960 ($n = 26$), 0.942 ($n = 31$), 0.957 ($n = 34$), 0.964 ($n = 37$), 0.954 ($n = 30$) and 0.848 ($n = 20$) respectively (n is the number of compounds which were studied).

The hydrophobicities log P_0 for these compounds were calculated with the results obtained for the non-ionizable compounds and examples of the variation of log P_0 with the acetonitrile concentration are reported in Figs. 1 and 2.

For the analysis of compounds in urine, an elution gradient was required and the influence of the gradient length on the relative retention defined as $(V_r - V_0)/V_0$ is shown in Fig. 3. The relationships between the relative retention and log P_c or log P_m are shown in Figs. 4 and 5.

DISCUSSION

In Fig. 1, we can notice that the log P_0 values of non-ionizable compounds are not influenced by the acetonitrile concentration with the exception of phenolic compounds. An example of the behaviour of phenols is given (No. 58) in Fig. 1. In a water rich eluent, the log k' and consequently log P_0 increased for phenols and therefore the

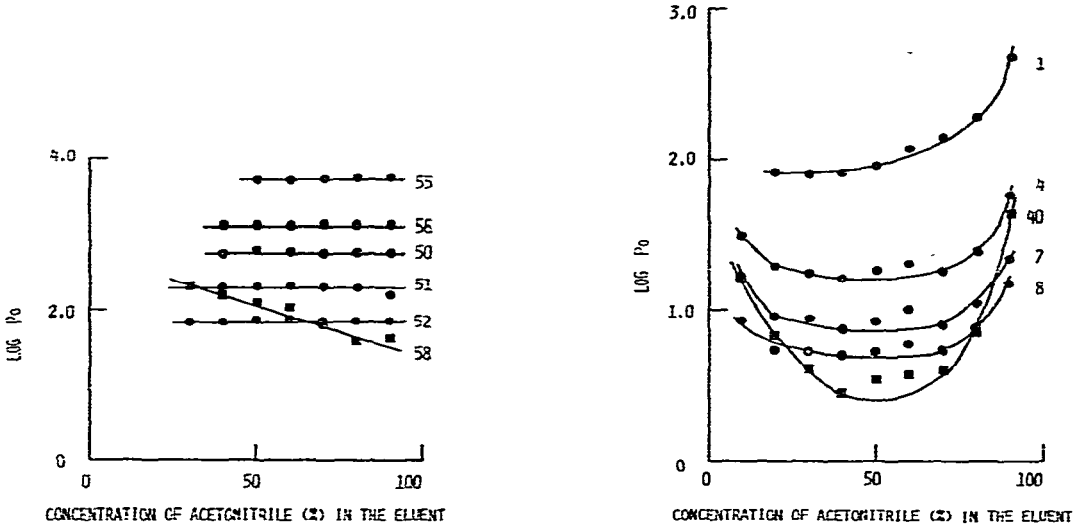

Fig. 1. Relation between log P_0 of non-ionizable compounds and concentration of acetonitrile in water. The column was an octadecyl silica packing (Chromosorb LC-7) and the eluents were acetonitrile–water mixtures with 0.04 *M* phosphoric acid. The numbers after the symbols refer to the compounds listed in Table I.

Fig. 2. Relation between log P_0 of ionizable compounds and concentration of acetonitrile in water. The experimental conditions are the same as in Fig. 1.

prediction of retention time for phenols will be discussed separately in a future paper.

For acidic compounds, the hydrophobicity increases in eluents with acetonitrile concentration higher than 70% (Nos. 1, 4, 7 and 8). Only the hydrophobicities log P_0 obtained for acetonitrile–water mixtures up to 60% acetonitrile were used for the calculation of log P_m in Table I.

The large capacity ratios and hence the high hydrophobicities for ionizable compounds in acetonitrile–water mixtures with a large proportion of acetonitrile could be explained by the poor solvation of these compounds in acetonitrile or by the direct adsorption on the surface of the packing.

The mean value log P_m obtained for the hydrophobicities in different eluent mixtures is usually similar to the values obtained with Rekker's hydrophobic fragmental constants (log P_c). The largest discrepancies were observed for 3,4,5-trihydroxybenzoic acid and nitrogen heterocyclic compounds.

The change of hydrophobicity for nitrogen containing heterocycles was important and irregular, especially for caffeine, theobromine, xanthine and tryptophan (Nos. 23, 40, 41 and 42). The behaviour of these nitrogen heterocycles is still unexplained in separation on bonded phase silica packings. One possible explanation could be the existence of unreacted silanol groups on the packing and therefore the interaction should not be only hydrophobic in nature[16].

From the above results it can be concluded that for eluents with acetonitrile concentrations between 20 and 60% the differences between log P_c and log P_m are small, the correlation between log P_c and log k' is good and it is therefore possible to predict the retention times from the values of log P_c. Better prediction for retention times can be made with the log P_m values.

The different chromatographic behaviour for polar and non-polar compounds made the discussion of the retention mechanism difficult. The change of the ratio of acetonitrile to water affects only slightly the dipole moment. This phenomenon differs from the one observed in mixtures of water and methanol or tetrahydrofuran, whereas the hydrogen bonding is more changed[24]. However, a preclassification of solutes according to their nature may allow the development of qualitative analysis in reversed-phase LC from the hydrophobicity log P or related values.

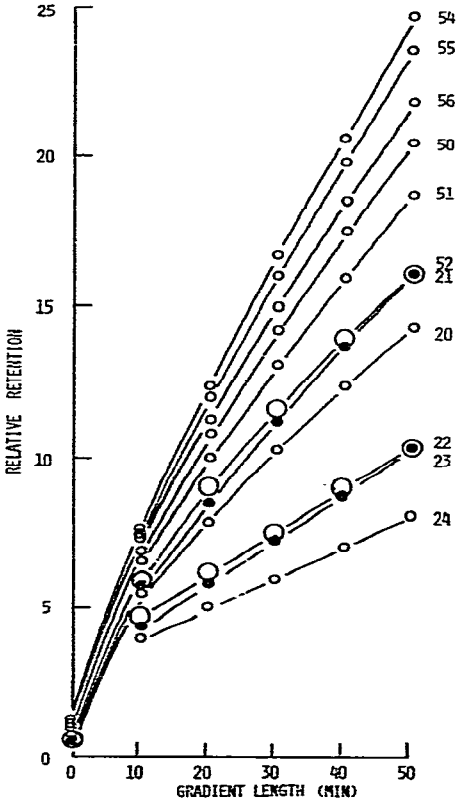

Fig. 3. Influence of the gradient length on relative retentions. The packing was Chromosorb LC-7, the flow-rate was 1ml/min. The linear gradient elution was from 0.04 M phosphoric acid to 90% acetonitrile–water with 0.04 M phosphoric acid. The detection was done at 254 nm. The numbers after symbols indicate the compounds listed in Table I.

Effect of hydroxylation on retention

The differences between the hydrophobicity of hydroxy-substituted compounds and the hydrophobicity of the unsubstituted parent compounds were calculated for both the observed and calculated values of log P. The values of the differences (Δ log P) are summarized in Table II.

The *ortho*-monosubstituted compounds for which an intramolecular hydrogen bond exists were more retained than their parent compounds (Δ log $P > 0$). The *meta*- and *para*-monohydroxy compounds were less retained than the parent com-

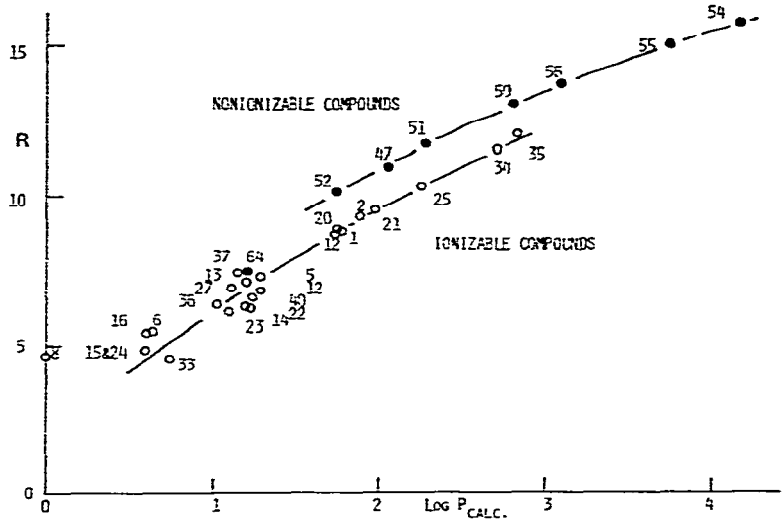

Fig. 4. Relation between log P_c and relative retention in a linear gradient elution. A 30-min gradient was used. For experimental conditions, see Fig. 3. R = relative retention.

pounds (Δ log $P < 0$). The di- and trisubstituted compounds were also less retained than their parent compounds.

For the di- and tri-substituted compounds in which intramolecular bonding can be expected (2-hydroxyphenylacetic acid, No. 13, and 2,4-dihydroxybenzoic acid, No. 5) the log P_m value and hence the retention was higher than for compounds with the same degree of substitution on the parent molecule (4-hydroxyphenylacetic acid, No. 14, and 3,4-dihydroxybenzoic acid, No. 6, respectively).

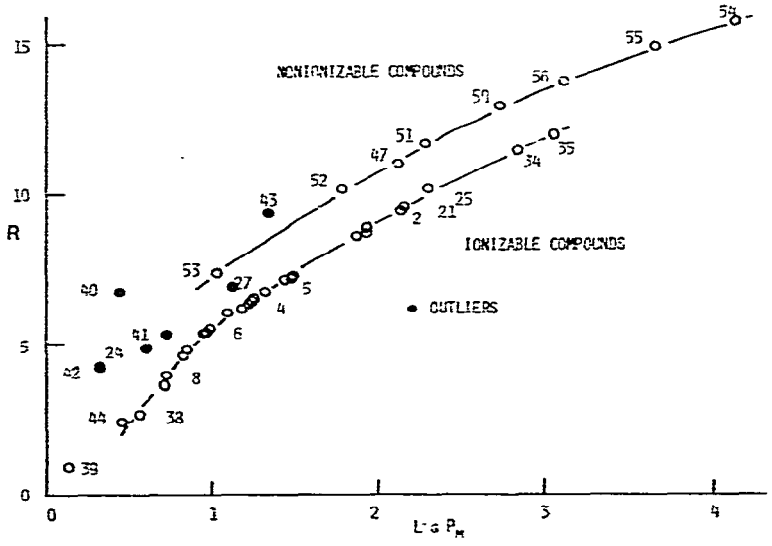

Fig. 5. Relation between log P_m and relative retention on a linear gradient. For experimental conditions, see Fig. 4. R = relative retention.

It is therefore important to introduce correcting factors based on the number and position of substituents for a more accurate calculation of hydrophobicity and hence a better prediction of retention times.

Effect of gradient

For practical analyses of urine samples, a gradient has to be used for fast separation, and it is therefore important to examine the change of the retention time (or volume) with the gradient length on one hand, and on the other hand, the change of the retention time (or volume) with the log P_0 for a given gradient. The relative retention was calculated from $(V_r - V_0)/V_0$. A linear gradient with a starting eluent of 0 or 10% acetonitrile in water mixture with 0.04 *M* phosphoric acid and a final eluent of 90% acetonitrile in water with 0.04 *M* phosphoric acid was used. The variation of the relative retention with the length of the gradient is shown in Fig. 3.

TABLE II

Δ log *P* FOR HYDROXYLATION*

Type of compounds	*Number of the compound*	$\Delta \log P_{calc}$	$\Delta \log P_{obs}$
Monosubstituted with intramolecular bonding	2,35,37	0.11	0.23 ± 0.03
Monosubstituted	3,4,10,14,19,22,26,53	−0.54	−0.70 ± 0.11
Disubstituted	6,7,15,16,27	−1.14	−1.03 ± 0.12
Trisubstituted	8	−1.80	−1.20

* Δ log *P* is the difference between log *P* of the hydroxy-substituted compound and log *P* of the parent compound.

The relative retention of different types of compounds were affected by the gradient length and no linear relation was observed. The examples are separation of No. 21 from No. 52 and No. 23 from No. 22. These compounds are indicated as fused circles and large circles in Fig. 3. Even if the column efficiency was good, some separation became difficult in longer gradient length due to the change of selectivity. This phenomenon was expected from the results in Figs. 1 and 2. Such selectivity change was also seen in the separation of caffeine and phenacetin. The elution order was reversed in different mixtures of methanol and water[23] and therefore, in discussion of selectivity, the compounds have to be carefully selected.

In Figs. 4 and 5, the relations between the relative retention and log P_c (or log P_m) of the standard compounds are shown. The chromatography was done with a 30-min linear gradient.

The behaviour of the two classes of compounds, *i.e.* ionizable and non-ionizable, is more apparent in Fig. 5. Most compounds in each class were located on the same curve. As mentioned before, even by using log P_m instead of log P_c, some compounds, especially nitrogen heterocyclic compounds showed a different chromatographic behaviour in this system.

CONCLUSION

The qualitative analysis in reversed-phase liquid chromatography is different from that with an organic solvent as eluent[17–22]. The selection of the organic modifier is limited, hence the main solubility parameter could be the hydrophobicity. The use of Rekker's hydrophobic fragmental constants was shown to be a simple and useful approach to qualitative analysis.

The retention times for aromatic acids were related with their log P values even with gradient elution. Regular changes were found for log P on hydroxylation of aromatic acids. Further studies are still required to understand the different steric effects and to introduce correction factors for the calculation. Nevertheless the log P can be used as a first approach to qualitative analysis. The log k' values differ from column to column, but the log P values are relatively constant.

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