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Chromatographic behaviour of aromatic acids in reversed-phase high-performance liquid chromatography

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ABSTRACT

Using reversed-phase high-performance liquid chromatography, the effect of the pH of the mobile phase on the retention of indole-3-acetic acid and some derivatives of benzoic acid was investigated. The capacity factors of the neutral form (log k'_n), and the anionic form (log k'_{ia}) and the pK^*_A values of solutes in given mobile phases were determined. The log k'_n values were correlated with 1-octanol–water partition coefficients (log P_{oct}). Both the log k'_n and log k'_{ia} values of the acids tested were correlated with their fungicidal activities examined against *Fusarium moniliforme* CCMF-180 and *Penicillium expansum* CCMF-567. Significant linear relationships between fungicidal activities and log k'_{ia} were found.

INTRODUCTION

Auxins are a group of plant hormones among which indole-3-acetic acid (IAA) is considered to be the major but perhaps not the sole active substance [1]. This compound has been identified in different plant materials [1,2].

Reversed-phase high-performance liquid chromatography (RP-HPLC) on silica gel with chemically bonded alkyl chains has proved to be a valuable method for separating polar ionogenic compounds [3–5]. However, substances such as benzoic and phenolic acids [6,7] present in plant can interfere with the determination of IAA. The chromatographic behaviour of ionizable compounds can be varied by changing either the pH or the modifier content of the mobile phase. The chromatographic separation of IAA, benzoic and phenolic acids is usually carried out on octadecylsilica (C_{18}) column eluted with mixtures of water and a water-miscible organic modifier [8–14].

The partition coefficient between 1-octanol and water, P_{oct}, is a good descriptor of the hydrophobicity of compounds and it can be used as an alternative method to control their chromatographic behaviour [15–17].

The aim of this work was to study the effect of the pH of the mobile phase in RP-HPLC on the chromatographic behaviour of IAA and some benzoic acids.

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THEORETICAL

The solute retention in HPLC is expressed by the capacity factor k'. Total protonation of carboxylic groups with $pK_A < 3.5$ in RP-HPLC requires an eluent with pH <2. However, this is not feasible owing to instability of the matrix of an alkyl-bonded stationary phase. For these compounds only the distribution coefficient can be measured. The distribution coefficient is defined as the ratio of the concentrations of a solute in the organic and aqueous phases at a given pH. The partition coefficient is defined as the ratio of the concentrations of the same form of the solute in the two phases. The capacity factor of a monoprotic acid can be expressed [3,4,18] as

$$\log k' = \log (k'_n + k'_{ia} K_A/[H^+]) - \log (1 + K_A/[H^+]) \tag{1}$$

where k'_n is the capacity factor of the neutral form, k'_{ia} the capacity factor of the anionic form of the acid, K_A the dissociation constant of the acid in the eluent and $[H^+]$ the concentration of solvated protons. Eqn. 1 represents the relationship between the capacity factor of a partially dissociated weak acid and the hydrogen ion concentration in the eluent. For pH $\ll$ pK_A, eqn. 1 can be simplified to eqn. 2, assuming that k'_{ia} is negligible compared with k'_n:

$$\log k' = \log k'_n - \log (1 + K_A/[H^+]) \tag{2}$$

When the capacity factor is measured at various pH values (at the same ratio of water and modifier), it is possible from delogarithmed and linearized eqns. 1 and 2 to determine the pK_A value of a solute in a given mobile phase:

$$k' = k'_n + (k'_{ia} - k')K_A/[H^+] \tag{3a}$$

$$k' = k'_n - k'K_A/[H^+] \tag{3b}$$

Hydrogen ion concentration plays an important role in eqns. 1 and 2. pH is generally reported as the pH of an aqueous buffer component before it is mixed with an organic modifier, or as the operational pH of the water–organic mixture as measured by a glass electrode. Generally, the latter approach is preferred when the pH of the mixture has to be finally adjusted. The thermodynamically valid pH value in mixed media, pH*[a], is expressed as

$$\text{pH}^* = \text{pH}^{\text{app}} - \delta \tag{4}$$

where pH^{app} is the apparent pH (measured by a pH meter) of the mixed medium and δ is a correction factor, which is available for the methanol–water system in the literature [19,20].

As pH* values are available, the pK^*_A values can be determined chromatographically using eqns. 3a and 3b.

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[a] The symbol marked with an asterisk refers to a water–organic mixture.

EXPERIMENTAL

Chemicals and equipment

Indole-3-acetic acid and benzoic acid derivatives were obtained from Lachema (Brno, Czechoslovakia), Fluka (Buchs, Switzerland) and Merck (Darmstadt, F.R.G.). They all were of analytical-reagent grade and used without further purification. The compounds used are listed in Tables I and IV. Solvents were redistilled before use.

Chromatography was performed on a Varian LC 8500 chromatograph with a UV-50 variable-wavelength detector (200–900 nm) using a CGC column (150 × 3.3 mm I.D.) (Tessek, Prague, Czechoslovakia) packed with Separon SCX-C_{18} (particle size 5 μm) silica gel with a C_{18} chemically bonded non-polar stationary phase and a Micro Pak CH-10 column (250 × 2.2 mm I.D., Merck, Darmstadt) packed with LiChrosorb RP-18 stationary phase (particle size 10 μm).

Conditions

Methanol–water (2:3) with the pH adjusted with sulphuric acid was used for studying the influence of mobile phase pH on the retention of tested acids. The separation of benzoic acids on the Micro Pak CH-10 column was carried out with methanol–water–acetic acid (30:65:5 and 40:55:5) as mobile phase at a flow-rate of 1.0 ml/min. Capacity facors were evaluated from the retention time of the solute, t_R, and the retention time of an unretarded component, $t_{R,0}$.

The pK_A values of acids [16,21], correction factor δ [19] and log P_{oct} values determined in the 1-octanol–water system [22–24] were taken from the literature.

RESULTS AND DISCUSSION

The values of K_A^* for methanol–water (2:3) as the mobile phase with different adjusted pH* values were calculated using eqn. 3b from the slope of the k' *vs.* $k'/[H^+]$ dependence. However, eqn. 3b is valid only for undissociated acids. It is assumed that this condition is satisfied for mobile phases with adjusted pH 2.59 and 3.15 (with respect to low k'_{ia} values determined in a mobile phase with pH 4.36 this assumption was fulfilled; see below). As the k'_{ia} values were not available for the mobile phase with pH 4.36, eqn. 3b was used also for the calculation of K_A^* and k'_n. These values were considered only as guesses and were used for the calculation of k'_{ia} values using eqn. 1 (first step). The k'_{ia} values obtained were used for the recalculation of the K_A^* and k'_n values using eqn. 3a (for the mobile phase of pH 4.36 only) (second step). The results are given in Table I. There were no significant differences between the first and second steps in the K_A^* values and in the k'_n values. A significant linear relationship was found between the pK_A values determined in water and pK_A^* (eqns. 5 and 6) (Table II). As the pK_A^* value of 4-hydroxybenzoic acid was outside the regression curve, it was excluded from the tested set.

The pK_A values of benzoic and 2-hydroxybenzoic acid were similar to those given by Van de Venne *et al.* [5]. The log k' and log k'_n values were correlated with the log P_{oct} values (Table III). A dominant influence of the values of the correlation coefficients was exhibited by 2-hydroxybenzoic acid, which had a lower pK_A value than the other acids. The chromatographic behaviour of this acid was evidently influenced (with regard to poor buffering of mobile phase) by the local changes of

TABLE I

THE EFFECT OF pH OF THE MOBILE PHASE ON THE SEPARATION OF AROMATIC ACIDS

System: Separon SGX-C_{18} column, methanol–water (2:3) mobile phase. k' = Capacity factor (uncorrected value); k'_n = capacity factor of the neutral form of an acid; k'_{ia} = capacity factor of the anionic form of an acid (for pH* = 4.36); K^*_A = dissociation constant determined chromatographically using methanol–water (2:3) mobile phase; K_A = dissociation constant determined in water [14].

Compound	No.	Log k'			Log k'_n		Log k'_{ia}		pK^*_A		pK_A
		I[a]	II[a]	III[a]	a[b]	b[b]	a[b]	b[b]	a[b]	b[b]	
IAA	I	0.215	0.268	0.276	0.276	0.276	−2.112	−1.824	4.98	5.04	4.65
$HOOCC_6H_4R$: R =											
H	II	0.310	0.386	0.417	0.412	0.412	−1.521	−1.222	4.73	4.72	4.19
2-OH	III	0.093	0.042	0.523	0.528	0.529	−1.912	−1.620	3.92	3.91	2.97
3-OH	IV	−0.319	−0.227	−0.214	−0.213	−0.213	−2.585	−2.720	4.72	4.72	4.06
4-OH	V	−0.444	−0.418	−0.411	−0.412	−0.412	−2.070	−1.765	5.27	5.26	4.48
2-OCH_3	VI	0.033	0.090	0.143	0.125	0.125	−1.645	−1.326	4.78	4.77	4.04
3-OCH_3	VII	0.401	0.488	0.508	0.506	0.506	−1.849	−1.525	4.72	4.72	4.27
4-OCH_3	VIII	0.447	0.542	0.558	0.558	0.558	−2.011	−1.698	4.70	4.70	4.36

[a] I, pH* = 4.36; II, pH* = 3.15; III, pH* = 2.59.

[b] a = 1st step of calculation; b = 2nd step of calculation (the procedure is given in the text).

mobile phase pH due to the mentioned acid injection. This influence was much stronger at higher mobile phase pH. The mobile phase without buffer was used in order to attempt to avoid the effect of the association of the acid anions with buffer cations on chromatographic retention [5].

Methanol–water–acetic acid mobile phases of various composition were used to determine the log k' values of the set of benzoic acid derivatives (Table IV). Significant linear relationships between log P_{oct} and log k' were calculated (eqns. 15 and 16, Table III). The influence of the"ortho effect" on chromatographic behaviour was not important. The relationships were calculated only for uncorrected log k' values because the pK^*_A values for given mobile phases were not available.

TABLE II

VALUES OF REGRESSION COEFFICIENTS FOR EQNS. 5 AND 6

n = Number of compounds in the set; r = correlation coefficient; s = standard deviation; t_a, t_b = Student's characteristics for the coefficients a and b of the regression equation $Y = a + bX$ (the coefficients were tested on hypothesis $a = 0$, $b = 0$).

Compound Nos.	Equation	n	r	s	t_a	t_b
I–IV, VI–VIII	pK_A = $-2.979 + 1.517$pK^{*a}_A (5)	7	0.962	0.145	3.634[c]	8.627[d]
I–IV, VI–VIII	pK_A = $-2.717 + 1.459$pK^{*b}_A (6)	7	0.966	0.137	3.662[c]	9.180[d]

[a] 1st step of calculation.

[b] 2nd step of calculation.

[c] Statistically significant difference ($P < 0.05$).

[d] Statistically significant difference ($P < 0.01$).

TABLE III

REGRESSION EQUATIONS FOR THE LOG P_{oct}–LOG k' CORRELATIONS

Systems: A = CGC glass column packed with Separon SGX-C_{18}, methanol–water (2:3) mobile phase; B = Micro Pak CH-10 column packed with LiChrosorb RP-18, methanol–water–acetic acid (30:65:5) mobile phase. n, r, s and t_b as in Table II.

Compound Nos.	System	pH*	Equation: log P_{oct} =		n	r	s	t_b
II–VIII	A	4.36	$1.695 + 0.813\log k'$	(7)	7	0.836	0.186	3.733[b]
II, IV–VIII	A		$1.628 + 0.801\log k'$	(8)	6	0.988	0.048	14.262[c]
II–VIII	A	3.15	$1.599 + 0.844\log k'$	(9)	7	0.948	0.107	7.323[c]
II, IV–VIII	A		$1.575 + 0.765\log k'$	(10)	6	0.986	0.052	13.069[c]
II–VIII	A	2.59	$1.575 + 0.828\log k'$	(11)	7	0.958	0.097	8.192[c]
II, IV–VIII	A		$1.559 + 0.753\log k'$	(12)	6	0.981	0.048	11.460[c]
II–VIII	A	–	$1.557 + 0.830\log k'^{a}_{n}$	(13)	7	0.962	0.093	8.594[c]
II, IV–VIII	A	–	$1.562 + 0.756\log k'^{a}_{n}$	(14)	6	0.983	0.056	12.051[c]
II–VIII, X–XIII	B	2.51	$1.204 + 1.176\log k'$	(15)	13	0.966	0.099	13.001[c]
II, IV, V, VII, VIII	B		$1.217 + 1.248\log k'$	(16)	10	0.974	0.096	12.916[c]

[a] 2nd step of calculation.
[b] Statistically significant difference ($P < 0.05$).
[c] Statistically significant difference ($P < 0.01$).

TABLE IV

BENZOIC ACID DERIVATIVES: RP-HPLC CAPACITY FACTORS (LOG k'), 1-OCTANOL–WATER PARTITION COEFFICIENTS (LOG P_{oct}) AND pK_A VALUES

Systems: B = Micro Pak CH-10 column packed with LiChrosorb RP-18, methanol–water–acetic acid (30:65:5) mobile phase; C = Micro Pak CH-10 column packed with LiChrosorb RP-18, methanol–water–acetic acid (40:55:5) mobile phase.

Substituent	Compound No.	Log k'		Log P_{oct}[c]	pK_A
		B[a]	C[b]		
H	II	0.627	0.269	1.87	4.19
2-OH	III	0.701	0.362	2.18[d]	2.97
3-OH	IV	0.112	−0.262	1.36[e]	4.06
4-OH	V	−0.026	−0.368	1.31	4.48
2-OCH_3	VI	0.417	0.000	1.59	4.04
3-OCH_3	VII	0.761	0.321	2.02	4.33
4-OCH_3	VIII	0.695	0.291	1.96	4.53
2-CH_3	IX	0.898	0.400	–	3.91
3-CH_3	X	0.963	0.460	2.37	4.27
4-CH_3	XI	0.940	0.366	2.27	4.36
2-Cl	XII	0.657	0.173	1.98	2.92
3-Cl	XIII	1.103	0.610	2.68	3.82
4-Cl	XIV	–	0.616	2.65	3.98
2-NO_2	XV	0.898	−0.281	–	3.91
3-NO_2	XVI	0.604	0.238	1.83	3.49
4-NO_2	XVII	0.660	0.291	1.89	3.43

[a] pH* = 2.51.
[b] pH* = 2.55.
[c] From ref. 22.
[d] From ref. 23.
[e] From ref. 24.

TABLE V

RELATIONSHIP BETWEEN THE FUNGICIDAL ACTIVITY OF AROMATIC ACIDS AND THEIR CAPACITY FACTORS IN ANIONIC FORM

$\log 1/C_{ia} = a + b \log k'_{ia}$. C_{ia} = Fungicidal activity of the anionic form of acid; other symbols as in Table II.

Compound Nos.	a	b	Fungi[a]	n	r	s	t_b	Eqn. No.[c]
I–IV, VI–VIII	2.829	0.224	F	7	0.940	0.040	6.769[b]	17
	2.836	0.201	P	7	0.923	0.041	5.859[b]	18

[a] F = *Fusarium moniliforme* CCMF-180; P = *Penicillium expansum* CCMF-567.
[b] Statistically significant difference ($P < 0.01$).
[c] In eqns. 17 and 18 the k'_{ia} values obtained in the 2nd step of their calculation were used.

For the mutual correlations of physico-chemical parameters, the limit of the allowable precision of a correlation was taken to be a value of the correlation coefficient [25] of $r \geq 0.9$.

Both log k'_n and log k'_{ia} values of the tested acids were correlated with their fungicidal activities [26] examined against *Fusarium moniliforme* CCMF-180 and *Penicillium expansum* CCMF-567. Non-significant linear relationship between fungicidal activities and log k'_n values were found ($r = 0.468$ and 0.446, respectively). In contrast, significant relationships between fungicidal activities and log k'_{ia} were found (Table V). This result agrees with the well known experience that salts of benzoic acid derivatives are fungicidally active [27]. It is interesting that IAA, a plant growth regulator, was also fungicidally active.

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