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Note

α-Cyclodextrin as selective agent for the separation of *o*-, *m*- and *p*-nitrobenzoic acids by reversed-phase high-performance liquid chromatography

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Conventional high-performance liquid chromatographic (HPLC) and thin-layer chromatographic systems show little selectivity towards *m*- and *p*-nitrobenzoic acid (NBA), which have similar dissociation constants. The selectivity factor, $\alpha_{m/p}$, usually does not exceed *ca.* 1.2. The recently reported resolution of *o*-, *m*- and *p*-NBA in clathrate-containing systems[1], although very selective ($\alpha_{m/p}$ = 14–16), has some major drawbacks, mainly that non-dissociated molecules of NBA but not anionic NBA^- species are absorbed by the lipophilic channel structure of the β-$Ni(NCS)_2$(4-methylpyridine)$_4$ clathrate sorbent. Acidic mobile phases have to be used, which results in a significant decrease in the clathrate column stability.

Our recent successful use[2] of β-cyclodextrin (β-CD) as a component for suitably modifying the separation properties of reversed-phase (RP) LC chromatographic systems and the known[3] differences between shifts of apparent dissociation constants of NBA isomers in the presence of α-cyclodextrin (α-CD) prompted us to try α-CD to enhance the efficient separation of isomers of NBA.

EXPERIMENTAL

Reagents

α-Cyclodextrin was supplied by Chinoin (Budapest, Hungary). All other materials were of analytical or reagent grade and were used without further purification.

Apparatus and procedure

Chromatographic measurements were performed using a Type 302 HPLC apparatus (Institute of Physical Chemistry, Polish Academy of Sciences, Warsaw, Poland) equipped with a 5-μl high-pressure injection valve and a spectrophotometric detector (254 nm) with a Z-shaped passage (volume 8 μl).

For HPLC use was made of stainless-steel columns (250 × 4 mm I.D. and 100 × 4 mm I.D.) slurry packed at 435 kg/cm^2 using the "balanced density" technique with 10 μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.).

pH measurements were performed using a Type N517 pH meter (Mera-Tronic, Poland).

The mobile phases were aqueous solutions of pH ≈ 3, 4 and 6, containing α-CD at concentrations of *ca.* $1 \cdot 10^{-3}$, $2 \cdot 10^{-3}$, $4 \cdot 10^{-3}$ and $8 \cdot 10^{-3}$ *M*. Buffer solutions

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were prepared by addition of 2.0 ml of concentrated orthophosphoric acid (85%) to 250 ml of water followed by titration to a given pH (checked with the pH meter) with 4 *M* sodium hydroxide solution.

Solutions of *o*-, *m*- and *p*-NBA dissolved in methanol ($4 \cdot 10^{-3}$–$5 \cdot 10^{-3}$ *M*) were injected onto the column. The flow-rate was 20 μl/sec.

All chromatographic experiments were performed at 20 ± 1°C.

RESULTS AND DISCUSSION

Table I gives the measured capacity factors (k') of *o*-, *m*- and *p*-NBA and the observed selectivity factors, $\alpha_{m/p}$ and $\alpha_{p/o}$, at different α-CD concentrations and different pH values of the mobile phase. It can be seen that in the absence of α-CD, $\alpha_{m/p}$ does not exceed 1.16 (at pH 4.18); the highest value of $\alpha_{m/p}$ (1.74) is observed at low pH (3.04) and high α-CD concentration (0.0078 *M*); at high pH of the mobile phase the observed selectivity is low in both the presence ($\alpha_{m/p} = 0.94$) and absence ($\alpha_{m/p} = 1.04$) of α-CD in the solution; and at intermediate pH, approximately within the limits $pK_a - 2 < pH < pK_a + 2$, high $\alpha_{m/p}$ values may be attained by an appropriate admixture of α-CD.

Fig. 1 shows a chromatogram of *o*-, *m*- and *p*-NBA obtained at a pH and α-CD concentration considered to be optimal.

When studying chromatographic equilibria in the system $(\text{ion exchanger})_{\text{solid}}$–$(\text{sorbate} + \text{CD})_{\text{dissolved}}$, Uekama *et al.*[4] derived an equation that relates observed

TABLE I

VALUES OF CAPACITY FACTORS (k') OF *o*-, *m*- AND *p*-NBA AND SELECTIVITY FACTORS, $\alpha_{m/p}$, $\alpha_{p/o}$, OBTAINED FROM CHROMATOGRAPHIC MEASUREMENTS AT VARIOUS pH VALUES AND CD CONCENTRATIONS

α-CD concentration (mmole/l)	pH	k' of NBA			$\alpha_{m/p}$	$\alpha_{p/o}$
		o-	*m*-	*p*-		
0.0	2.95	4.99	33.94	32.38	1.05	6.49
0.0	4.18	1.63	8.60	7.39	1.16	4.53
0.0	5.92	1.28	3.89	3.50	1.11	2.73
0.9913	3.00	3.66	23.52	18.68	1.26	5.10
0.9892	3.87	1.69	9.61	8.29	1.16	4.90
0.9845	5.95	1.25	3.53	3.18	1.11	2.54
1.9509	3.05	3.58	19.16	15.27	1.25	4.26
1.9451	4.25	1.41	6.58	5.60	1.17	3.97
1.9350	6.05	1.27	3.42	3.30	1.04	2.60
3.9555	3.14	2.84	15.03	10.02	1.50	3.53
3.9449	4.57	1.26	4.25	3.79	1.12	3.01
3.9268	5.92	1.16	3.16	3.01	1.05	2.59
7.8745	3.04	3.11	12.01	6.89	1.74	2.21
7.8545	3.87	1.42	5.88	4.33	1.36	3.05
7.8208	6.00	1.09	2.60	2.77	0.94	2.54

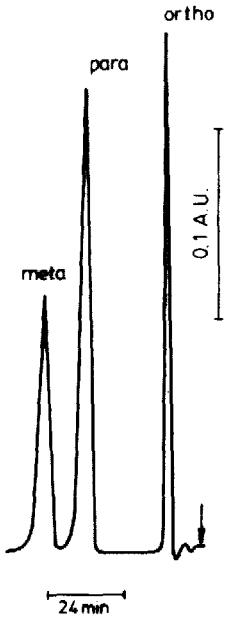

Fig. 1. Elution curve of a mixture of $5 \cdot 10^{-3}$ *M* *o*-, *m*- and *p*-NBA with an aqueous mobile phase of pH 2.93 and [α-CD] = 0.0099 *M*. Column: 250 × 4 mm I.D. LiChrosorb RP-18 (10 μm).

retention times (t_{obs}) of ionic species and the concentration of cyclodextrin in the mobile phase ([CD]):

$$t_{obs} = \frac{t'_0 + t_c K_c [CD]_m}{1 + K_c [CD]_m}$$

where t'_0 is the retention time of the sorbate, t_c that of the sorbate–CD complex and K_c is the stability constant of the 1:1 complex. If acid–base equilibria of the sorbate in the mobile phase:

$$HA \rightleftharpoons H^+ + A^-$$

and also adsorption of both neutral and anionic species on a reversed phase:

$$(HA\text{–}CD)_m \rightleftharpoons (HA\text{–}CD)_s$$

$$(A\text{–}CD)_m^- \rightleftharpoons (A\text{–}CD)_s^-$$

where the subscripts s and m denote the stationary and mobile phase, respectively, are taken into account then the following expression can be derived for the equilibrium distribution constant of HA:

$$C = \frac{[HA]_s + [A^-]_s + [HA\text{–}CD]_s + [(A\text{–}CD^-)]_s}{[HA]_m + [A^-]_m + [HA\text{–}CD]_m + [(A\text{–}CD)^-]_m}$$

and for the retention time:

$$t_{obs} = \frac{t_{HA} + t_A \ K_a/[H^+] + t_{HA-CD} K^\circ [CD] + t_{(A-CD)} K^-[CD] K_a/[H^+]}{1 + K_a/[H^+] + K^\circ [CD] + K^- [CD] K_a/[H^+]} \quad (1)$$

where t denotes retention time and the subscripts obs, HA, A^-, HA–CD and (A–CD)$^-$ refer to overall (measured) values and to the retention of neutral, anionic, neutral-complexed and anionic-complexed molecular species, respectively, and K is the acidity constant (K_a) or stability constant of CD (1:1) complexes of neutral

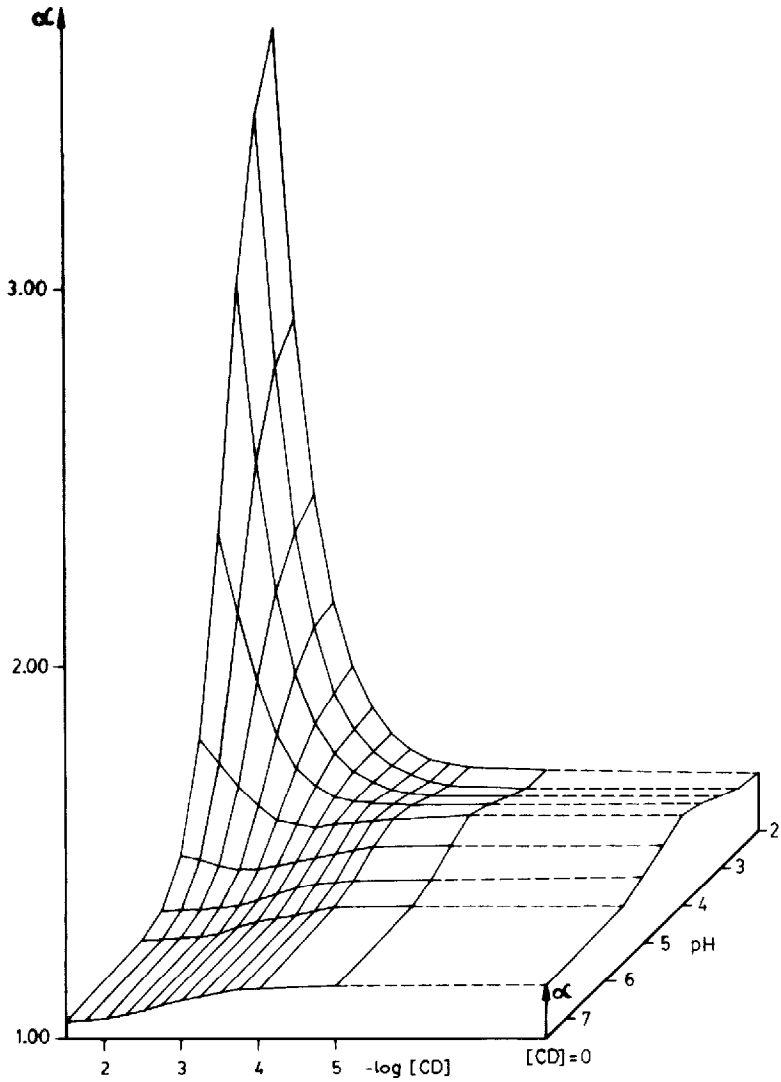

Fig. 2. Selectivity factor, $\alpha_{m/p}$, as a function of pH and log[α-CD] calculated from eqn. 1 and the data in Table II.

$$\left(K^{\circ} = \frac{[\text{HA–CD}]_m}{[\text{HA}_m[\text{CD}]_m}\right)$$

and anionic

$$\left(K^{-} = \frac{[(\text{A–CD})^{-}]_m}{[\text{A}]_m[\text{CD}]_m}\right)$$

species.

For numerical procedures it is useful to expand the non-linear function $t_{obs} = t([H^+][CD])$ into a Taylor series and neglect the non-linear components. In this way we calculated the parameters K°, K^{-} and individual capacity factors k'_{HA}, k'_{A^-}, $k'_{HA-\alpha-CD}$ and $k'_{(A-\alpha-CD)^-}$ corresponding to adsorption on a reversed phase of individual species by using a set of twelve values for t_{obs} measured at different pH values and [α-CD] concentrations for each isomer. Some results are given in Table II.

TABLE II

CALCULATED STABILITY CONSTANTS (K°, K^{-}) AND CAPACITY FACTORS [k'_{HA}, k'_{A^-}, $k'_{HA-\alpha-CD}$, $k'_{(A-\alpha-CD)^-}$] FOR α-CD COMPLEXES OF *m*- AND *p*-NBA

Compound	k'_{HA}	k'_{A^-}	$k'_{HA-\alpha-CD}$	$k'_{(A-\alpha-CD)^-}$	*Literature* pK_a *values*[5]	K°	K^{-}
m-NBA	42.85 ± 0.10	3.63 ± 0.02	5.62 ± 0.34	2.98 ± 0.05	3.47	408 ± 21 (lit.: 155[6]; 152[7])	486 ± 16 (lit.: 50[7])
p-NBA	37.30 ± 0.28	3.19 ± 0.04	0.28 ± 0.27	2.86 ± 0.04	3.41	473 ± 16 (lit.: 155[6]; 490[7])	359 ± 16 (lit.: 75[7])

These results seem inteesting but their reliability can hardly be evaluated in view of large discrepancies in literature data on the association constants of α-CD inclusion complexes of NBA and even on dissociation constants. Nevertheless, some qualitative conclusions may perhaps be given as reasonable. In particular, the decrease in adsorption on an RP-18 stationary phase of α-CD inclusion complexes of NBA molecules, if related to the adsorption of NBA isomers themselves (k'_{HA-CD} compared to k'_{HA}), is very significant: k'_{HA-CD} found for the *o*-NBA is 35% of k'_{HA}, for *m*-NBA only 13% of k'_{HA} and for the *p*-isomer k'_{HA-CD} is zero within the limits of error.

The sequence of the calculated capacity factors for individual species is as follows: $k'_{HA} > k'_{A^-}$; $k'_{HA} > k'_{HA-\alpha-CD}$; and $k'_{A^-} > k'_{(A-\alpha-CD)^-}$.

A deeper physico-chemical interpretation of these results requires further studies, which are in progress.

Eqn. 1 with the derived values of the parameters listed above can be used to illustrate the relationship between $\alpha_{m/p}$ and the concentration of α-CD and the pH of the mobile phase. The result is shown in Fig. 2.

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