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RETENTION BEHAVIOUR OF DISUBSTITUTED BENZENE DERIVATIVES ON SEVERAL β-CYCLODEXTRIN STATIONARY PHASES

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SUMMARY

β-Cyclodextrin has been immobilized on silica gel beads via three kinds of spacers. The retention behaviour of several disubstituted benzene derivatives on these phases and on their modified derivatives was investigated, as well as on stationary phases without β-cyclodextrin. The β-cyclodextrin stationary phase obtained from succinamidopropyl silica (whose unreacted carboxyl groups are not modified) is more efficient than the other β-cyclodextrin phases in the liquid chromatographic separation of the *ortho*, *meta* and *para* isomers. The unreacted carboxyl groups of the spacer arms do not interact significantly with solutes having various functional groups. The effects of ionic strength and column temperature on the retention were also studied briefly.

INTRODUCTION

In a previous paper[1] we reported the specific liquid chromatographic separations of various aromatic compounds on α- and/or β-cyclodextrin stationary phases. These phases were prepared by treating succinamidopropyl silica with ethylenediamine-monosubstituted α- or β-cyclodextrin. The carboxyl content of this succinamidopropyl silica was about 1000 μmol/g. The proportion of the carboxyl groups used for the immobilization of the α- or β-cyclodextrin derivative was only about 1/30 or 1/20, respectively. Therefore, the unreacted, terminal groups of the spacer arms will probably affect the selectivity of the cyclodextrin stationary phases. A study of this selectivity is expected to give information on the interactions between solutes and the cyclodextrin moiety, as well as the spacer-arm functional groups.

To date various functional groups have been introduced into cyclodextrins and attached to the surface of silica gel by chemical reactions. Among monosubstituted cyclodextrins, the amino derivatives from the primary monotosylates are readily prepared on a large scale and are well characterized.

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The objective of this work was to find an efficient β-cyclodextrin stationary phase by changing the spacer arms and to investigate their effect on retention. We used diethylenetriamine-monosubstituted β-cyclodextrin as the derivative and coupled it to carboxylated, epoxy-substituted or glutaraldehyde-activated silica. The retention behaviour on these stationary phases was studied for several disubstituted benzene derivatives. The corresponding stationary phases without β-cyclodextrin were also prepared for comparison. The α-cyclodextrin stationary phases were not investigated because of their poorer resolution compared with the β-cyclodextrin ones[1,2].

EXPERIMENTAL

Materials

β-Cyclodextrin was purchased from Hayashibara Biochemical Labs. (Okayama, Japan) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) from the Peptide Institute (Osaka, Japan). Silica gel (Wakogel LC-10, particle size 10 μm) and other chemicals were purchased from Wako (Osaka, Japan). The disubstituted benzenes used as solutes were purified by distillation or recrystallization. The mobile phases, deionized water and methanol, were distilled and filtered through a 0.45-μm membrane filter.

Preparation of β-cyclodextrin stationary phases

Silica gel was converted into the succinamidopropyl (Su-silica) or glutaraldehyde-activated (Gl-act-silica) derivative by a general procedure via the aminopropyl derivative[3], or into the epoxy-substituted one (Ep-sub-silica) by reaction with 3-glycidoxypropyltrimethoxysilane[4]. Diethylenetriamine-monosubstituted β-cyclodextrin (β-dtn), mono{6-deoxy-6-N-[(2-aminoethyl)-2-aminoethyl]amino}-β-cyclodextrin, was prepared from the primary monotosylate of β-cyclodextrin[5].

β-dtn was coupled to each silica derivative as follows (Fig. 1). In 80 ml of 0.15 *M* phosphate buffer (pH 5.5), 2 g of β-dtn were dissolved and the pH of the solution was adjusted to 5.5 by adding 1 *M* HCl. Su-silica (5 g) was suspended in this solution, and 1, 0.5 and 0.3 g of EDAC were added at intervals of 2 h with stirring. After stirring for 6 h, the bonded phase was filtered off, washed thoroughly with water and methanol and dried *in vacuo* at 80°C for 12 h. The unreacted silanol groups of this phase were silanized with hexamethyldisilazane (3 ml) in hexane (70 ml) for 8 h under refluxing. The resulting phase is denoted by β-dtn-Su-silica. β-dtn-Su-silica-Me was obtained by methylating the unreacted carboxyl groups of β-dtn-Su-silica with diazomethane. Freshly prepared ether solution was added dropwise until the solution developed a slightly yellow colour.

Gl-act-silica (5 g) was placed in 25 ml of phosphate buffer containing 2.5 g of β-dtn (whose pH was adjusted to 8.5 with HCl) and the mixture was stirred at 0°C for 4 h. After washing with cold water, the β-cyclodextrin phase was treated at 0°C for 2 h with 0.3 g of ethylamine in 10 ml of phosphate buffer at pH 8.1 and then reduced with 1% $NaBH_4$ at pH 4.5. The resulting β-cyclodextrin phase is denoted by β-dtn-Gl-silica-Et, and that obtained without the ethylamine treatment by β-dtn-Gl-silica. Both phases were also silanized with hexamethyldisilazane after the reduction.

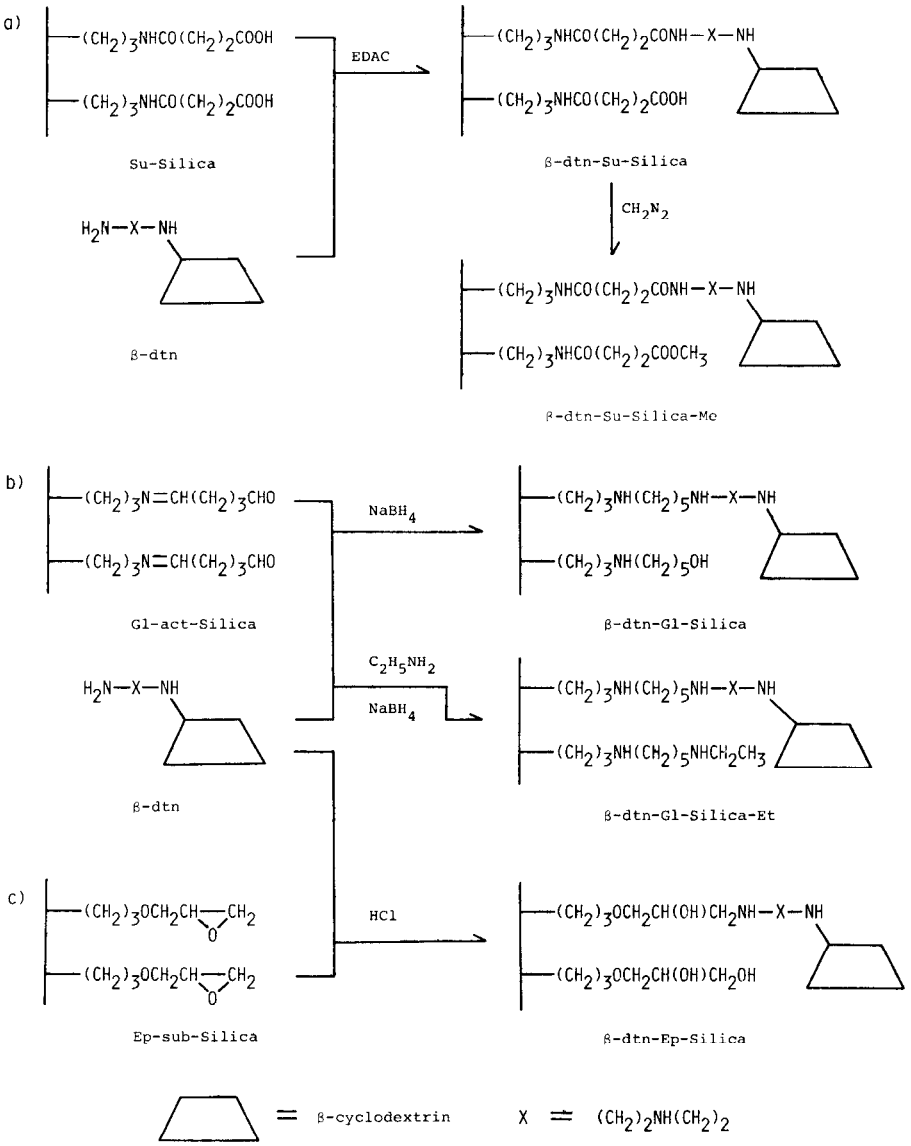

Fig. 1. Preparation of β-cyclodextrin stationary phases.

Ep-sub-silica (2.2 g) was stirred in 5 ml of phosphate buffer pH 8.8 containing 5 g of β-dtn at 40°C for 3 days. After washing with water, the β-cyclodextrin phase was placed in 50 ml of 0.01 *M* HCl and heated at 75°C for 30 min to hydrolyze unreacted epoxy groups. The washing, drying and silanization treatments were performed as described above. The phase obtained is denoted by β-dtn-Ep-silica.

Preparation of stationary phases without β-cyclodextrin

Gl-act-silica was reduced directly or after reaction with ethylamine and then

silanized as described above. The resulting phases are denoted by Gl-silica and Gl-silica-Et, respectively.

Su-silica was methylated with diazomethane (Su-silica-Me), or maltose was immobilized instead of β-cyclodextrin on Su-silica (Mal-Su-silica).

Chromatography

The chromatographic system was as described previously[1]. Each stationary phase was packed by a balanced density slurry method into a stainless-steel column (15 × 0.4 cm I.D.). The eluent flow-rate (water, methanol–water or 1/15 *M* phosphate buffer) was 1 ml/min. The wavelength used for detection was 254 nm. The concentration of the sample solutes was 0.2 m*M*, and a volume of 20 μl was injected, except for benzoic acids (10 μl).

RESULTS AND DISCUSSION

The amounts of β-cyclodextrin immobilized in β-dtn-Su-silica, β-dtn-Gl-silica and β-dtn-Ep-silica were evaluated spectrophotometrically by determining the D-glucose formed after hydrolysis with H_2SO_4[1]. Table I gives the β-cyclodextrin contents and the results of elemental analyses. β-dtn-Gl-silica has the lowest β-cyclodextrin content, although it was prepared under the optimum conditions. On the other hand, more β-cyclodextrin can be immobilized on Su-silica under milder conditions than those employed for preparing the other two stationary phases.

TABLE I

ANALYTICAL DATA FOR β-CYCLODEXTRIN STATIONARY PHASES

Phase	*Amount of β-cyclodextrin immobilized (μmol/g)*	*Elemental analysis (%)*		
		C	*H*	*N*
β-dtn-Su-silica	29.1	9.28	1.80	1.41
β-dtn-Su-silica-Me	29.1	9.42	1.78	1.40
β-dtn-Gl-silica	18.1	11.12	1.89	1.17
β-dtn-Gl-silica-Et	18.1	11.82	1.79	1.32
β-dtn-Ep-silica	26.2	8.17	1.56	–

Comparison of column efficiency and retention times

In this work, β-cyclodextrin was immobilized on silica gel via three spacers. The HETP values for *p*-cresol on β-dtn-Su-silica, *p*-iodoaniline on β-dtn-Gl-silica and *m*-cresol on β-dtn-Ep-silica, which give similar retention times as shown in Table II, are 0.13, 0.19 and 0.37 mm, respectively. Fig. 2 shows typical liquid chromatograms of a mixture of *o*-, *m*- and *p*-cresol on these three stationary phases. The resolution was estimated from *o*- and *m*-cresol: 1.97 for β-dtn-Su-silica; 1.17 for β-dtn-Gl-silica and 1.22 for β-dtn-Ep-silica. The complete separation of the three isomers can be accomplished only on β-dtn-Su-silica.

In general, a decrease in retention was found for the six disubstituted benzene derivatives (Table II) on the three β-cyclodextrin stationary phases with increasing methanol–water ratio in the eluent from 0:100 to 40:60. Aminobenzoic and nitro-

TABLE II

RETENTION TIMES (min) OF DISUBSTITUTED BENZENE DERIVATIVES ON β-CYCLODEXTRIN STATIONARY PHASES

Eluent: methanol–water (20:80).

Solute	*β-dtn-Su-silica*			*β-dtn-Gl-silica*			*β-dtn-Ep-silica*		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	5.11	6.50	9.99	3.66	4.14	4.96	8.13	10.50	16.75
Iodoaniline	8.30	14.41	29.20	5.10	6.95	9.49	4.98	15.62	50.77
Toluidine	3.40	3.70	4.90	1.89	1.73	1.60	4.38	4.50	5.85
Nitroaniline	6.58	5.41	21.62	4.83	3.70	9.79	12.79	10.88	53.06
Nitrophenol	19.40	10.21	59.45	23.52	9.10	–*	94.34	27.50	–*
Dinitrobenzene	7.30	3.00	3.27	4.55	2.29	2.16	17.58	4.03	4.33

* Not eluted.

benzoic acids could not be eluted in any case. However, they can easily be eluted by using phosphate buffer as the eluent instead of methanol–water. Table II gives the retention times of the solutes on these β-cyclodextrin stationary phases in methanol–water (20:80). Nitrophenol is retained strongly, and the *p*-isomer cannot be eluted with methanol–water (20:80) on either β-dtn-Gl-silica or β-dtn-Ep-silica. Considering the separation of the three isomers and their retention times, of the three phases studied, β-dtn-Su-silica gave the best results. β-dtn-Ep-silica was unsuitable for practical usage because it yielded broad peaks, poor selectivity and strong retention, and was not studied further.

Comparison of unreacted terminal groups

The proportion of the functional, terminal groups on Su-silica or Gl-act-silica used for the immobilization of β-dtn is only about 1/40 or 1/60, respectively. It is

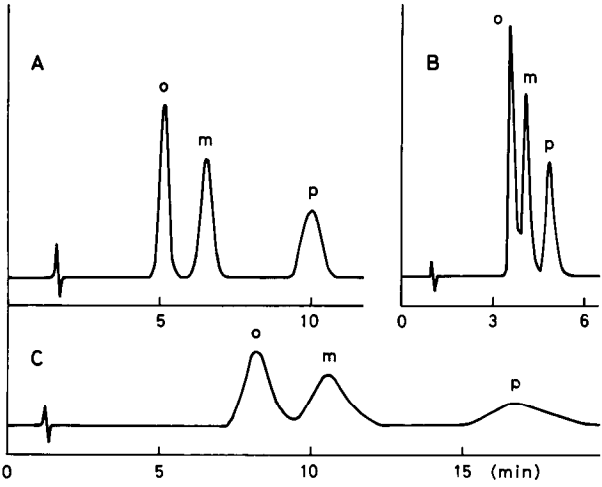

Fig. 2. Liquid chromatograms of cresol isomers on β-dtn-Su-silica (A), β-dtn-Gl-silica (B) and β-dtn-Ep-silica (C). *o*, *m* and *p* denote *ortho*, *meta* and *para* isomers, respectively.

assumed that the unreacted, terminal groups will have some effect on the liquid chromatographic properties of the β-cyclodextrin stationary phases. In order to deduce the specificity of the β-cyclodextrin stationary phases as effectively as possible, we investigated the retention behaviour of the solutes on several stationary phases with or without β-cyclodextrin.

As mentioned above, *p*-nitrophenol cannot be eluted from β-dtn-Gl-silica with methanol–water (20:80); *o*-nitrophenol exhibits a much longer retention time of 23.52 min, compared with the other solutes (Table II). These results suggest the participation of the spacer arms in retention. When β-dtn-Gl-silica was treated with ethylamine before the reduction with $NaBH_4$, *p*-nitrophenol could still not be eluted from the resulting stationary phase, β-dtn-Gl-silica-Et (Table III). It is apparent that both Gl-silica and Gl-silica-Et, which contain no cyclodextrin units, also retain *o*- and *p*-nitrophenol quite strongly. On the other hand, the other solutes in Table II were not retained so strongly on Gl-silica and Gl-silica-Et (not shown). Conversion of the –OH groups of the spacer arms on β-dtn-Gl-silica into the $-NHC_2H_5$ groups brought about little change in retention and no appreciable improvement in the separation of the isomers. The amount of β-cyclodextrin immobilized on β-dtn-Gl-silica was strongly dependent upon the pH of the reaction medium. We could not immobilize more than 18.1 μmol/g of β-dtn, although several attempts were made under the optimum conditions.

As already stated, β-dtn-Su-silica is more efficient than β-dtn-Gl-silica. Therefore, we prepared several stationary phases derived from Su-silica, in order to study the effect of the unreacted–COOH groups. On β-dtn-Su-silica (about 1200 μmol/g) these groups were methylated with diazomethane. Fig. 3 shows the retention of eight disubstituted benzene derivatives both on β-dtn-Su-silica and β-dtn-Su-silica-Me. It is apparent that, except for toluidine, the solutes are retained more strongly on the latter stationary phase than on the former. The nitrophenol isomers, especially, interact very strongly with β-dtn-Su-silica-Me, and the *p*-isomer is not eluted with methanol–water (20:80). However, by using the phosphate buffer at pH 5.5, *p*-nitrophenol can be eluted as well as aminobenzoic and nitrobenzoic acids. This buffer was used as the eluent for the separation of the isomers of these three solutes.

Su-silica containing no β-cyclodextrin units was also methylated (Su-silica-Me). Table IV gives the retention times of typical four of the eight solutes on these

TABLE III

RETENTION TIMES OF NITROPHENOL ISOMERS ON STATIONARY PHASES OBTAINED FROM Gl-act-SILICA

Eluent: methanol–water (20:80).

Stationary phase	*Retention time (min)*		
	o-	*m-*	*p-*
β-dtn-Gl-silica	23.52	9.10	–*
β-dtn-Gl-silica-Et	73.45	15.15	–*
Gl-silica	–*	19.83	–*
Gl-silica-Et	92.40	16.38	–*

* Not eluted.

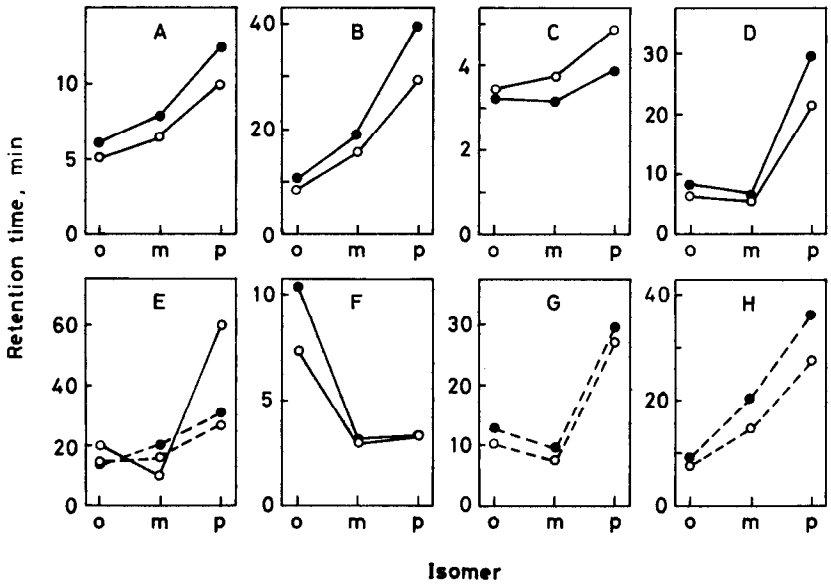

Fig. 3. Retention of disubstituted benzene isomers on β-dtn-Su-silica (○) and β-dtn-Su-silica-Me (●). Eluents: ———, methanol–water (20:80); ---------, phosphate buffer at pH 5.5. Solutes: A = cresol; B = iodoaniline; C = toluidine; D = nitroaniline;E = nitrophenol; F = dinitrobenzene; G = aminobenzoic acid; H = nitrobenzoic acid.

two stationary phases in water and/or the phosphate buffer at pH 5.5. The toluidine isomers are retained more strongly on Su-silica than on Su-silica-Me. Thus, in both β-dtn-Su-silica and Su-silica, methylation of the –COOH groups results in decreased retention of the toluidine isomers. This is reasonably interpreted as due to the stronger interaction of the $-NH_2$ group in toluidine with –COOH than with $-COOCH_3$ groups. On the other hand, the methylation results in an increase in retention of the other solutes. When eluted with water, the *o*- and *p*-isomers of nitrophenol are retained much more strongly on Su-silica-Me than on Su-silica. On β-

TABLE IV

RETENTION TIMES (min) OF DISUBSTITUTED BENZENE DERIVATIVES ON Su-SILICA AND Su-SILICA-Me

Eluent: water. Values in parentheses are the retention times in phosphate buffer at pH 5.5.

Solute	*Su-silica*			*Su-silica-Me*		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	4.04	4.41	4.22	4.22	4.70	4.60
Toluidine	3.55	4.35	4.22	2.47	2.23	1.90
Nitrophenol	5.05	4.59	4.33	45.73	9.88	61.45
	(5.20)	(4.58)	(4.28)	(5.25)	(4.30)	(4.16)
Nitrobenzoic acid	3.46	2.80	2.90	58.90	87.62	63.73
	(2.31)	(3.03)	(2.43)	(6.79)	(9.65)	(7.40)

dtn-Su-silica-Me, the *p*-isomer is not eluted with methanol–water (20:80), as described above, and the *o*-isomer has a retention time of 68.90 min. This quite strong retention of *o*- and *p*-nitrophenol on β-dtn-Su-silica-Me can be ascribed mainly to the strong interaction of these isomers with the spacer arms.

A similar increase in retention upon methylation of the –COOH groups of Su-silica is also true for the nitrobenzoic acid isomers. They, as well as the aminobenzoic acid isomers, interact quite strongly with the β-cyclodextrin stationary phases and cannot be eluted by using methanol–water (20:80). The three isomers of nitrobenzoic acid exist mainly as their anionic forms in water because of their small pK_a values. It is assumed that the –COOH groups on Su-silica are also ionized. Consequently, owing to the repulsion between the negatively charged sites, the isomers of nitrobenzoic acid are eluted quickly from Su-silica in water. The methylation of the –COOH groups on Su-silica removes this repulsion and results in a strongly attractive interaction between the solutes and Su-silica-Me. However, this attractive interaction can be greatly reduced by use of the phosphate buffer (addition of ions), suggesting that the negative charge on each isomer participates in the retention. Evidence in supports of this may be obtained from the similar behaviour of the nitrophenol isomers.

3-(2-aminoethylamino)propyl silica (Dia-silica) was used by Fujimura *et al.*[2] to immobilize β-cyclodextrin monotosylate. On Dia-silica, which possesses $-NH_2$ groups, solutes with a $-NH_2$ group are eluted rapidly, but the two benzoic acids cannot be eluted with water as expected. This quite strong retention of aminobenzoic and nitrobenzoic acids on Dia-silica is in striking contrast to the easy elution of the aniline derivatives on Su-silica: they are eluted in the range 3.55 (*o*-toluidine)–7.24 min (*m*-iodoaniline). It is conducted that the succinamidopropyl spacer arm is the best of those studied because of its weak interaction with the various functional groups.

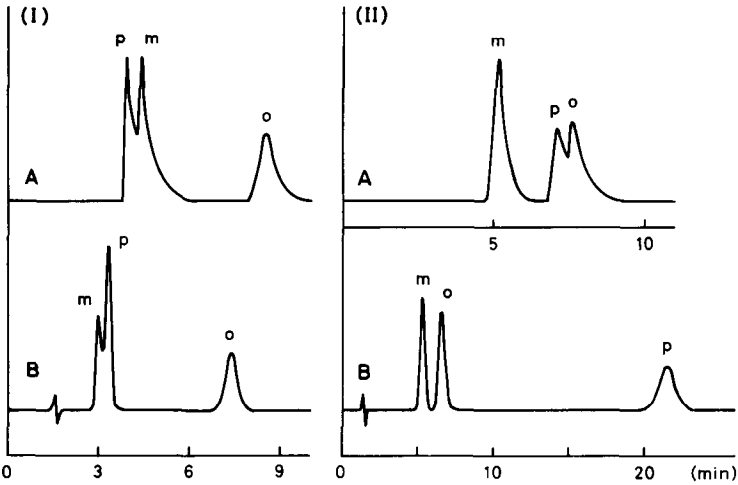

Fig. 4. Liquid chromatograms of dinitrobenzene isomers (I) and nitroaniline isomers (II) on Mal-Su-silica in water (A) and on β-dtn-Su-silica in methanol–water (20:80) (B).

Effect of β-cyclodextrin units on retention

We prepared a stationary phase containing maltose units instead of β-cyclodextrin. This stationary phase, Mal-Su-silica, was then used as a reference in order to estimate the inclusion process of the β-cyclodextrin units. The isomers of cresol or iodoaniline cannot be separated on Mal-Su-silica. As shown in Fig. 4, the isomers of dinitrobenzene or nitroaniline are partially separated on Mal-Su-silica, but the elution order is different from that on β-dtn-Su-silica. The *p*-isomers except for dinitrobenzene are retained most strongly on β-dtn-Su-silica (Fig. 3). These facts suggest a positive contribution of the inclusion process of β-cyclodextrin to the retention. On Mal-Su-silica, the nitrobenzoic acid isomers are not eluted with water, but are with the phosphate buffer. The benzoic acid isomers interact quite strongly with the glucose units regardless of the existence of the inclusion.

Effects of ionic strength and column temperature

The effect of the ionic strength on the retention was investigated by changing the concentration of the phosphate buffer from 1/15 to 1/150 *M*. The retention of aminobenzoic and nitrobenzoic acids on β-dtn-Su-silica increases with decreasing phosphate concentration: for instance, the retention time of *p*-aminobenzoic or *p*-nitrobenzoic acid increases about 2.3 or 3.2 times, respectively. The retention of the nitrophenol isomers are affected similarly, although the increase is smaller (about 1.3 times for *p*-nitrophenol). In the case of cresol, nitroaniline or dinitrobenzene, the retention is scarcely affected by the change in ionic strength. The effect of the ionic strength on the retention is large for negatively charged solutes.

Fig. 5 shows the effect of the column temperature on the retention of the isomers of cresol, toluidine and nitroaniline. The retention decreases with increasing temperature. For the *p*-isomer, which interacts strongly with the β-cyclodextrin units, the decrease is larger compared with that for the *o*- or *m*-isomer. This is consistent with the observation that the stability of various cyclodextrin inclusion complexes in solution decreases significantly as the temperature increases[6]. Fig. 6 shows the separation of the cresol isomers on β-dtn-Su-silica in methanol–water (20:80) at various temperatures. The increase in the column temperature results in a shortening of the analysis time and a sharpening of the peaks.

In conclusion, β-cyclodextrin stationary phases have been prepared by coup-

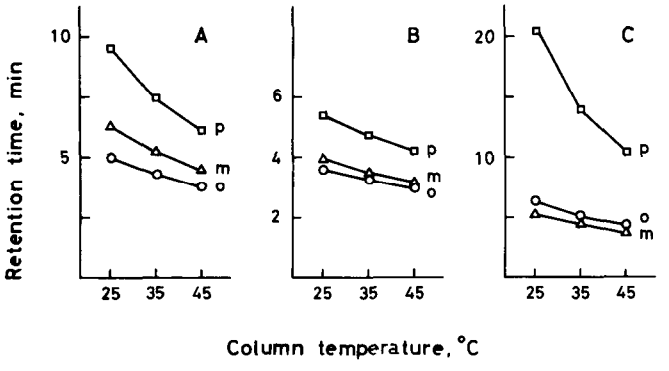

Fig. 5. Effect of column temperature on the retention times of cresol (A), toluidine (B) and nitroaniline (C) on β-dtn-Su-silica in methanol–water (20:80).

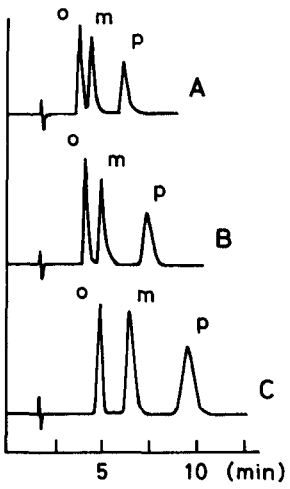

Fig. 6. Liquid chromatograms of cresol isomers on β-dtn-Su-silica at 45 (A), 35 (B) and 25°C (C).

ling β-dtn to three kinds of silica derivatives. It is found that β-dtn-Su-silica is superior to the others, and that the unreacted carboxyl groups do not interact strongly with the various functional groups of the solutes.

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