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SEPARATION OF DISUBSTITUTED BENZENE ISOMERS ON CHEMICALLY BONDED CYCLODEXTRIN STATIONARY PHASES

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SUMMARY

α- and β-cyclodextrins have been immobilized on polyacrylamide gel beads via two spacers. The resulting stationary phases containing α-cyclodextrin exhibit selectivity in the liquid chromatographic separation of disubstituted benzene isomers as models: the retention order is $o < m < p$ in all cases. Except for aminobenzoic acid, the *o*-, *m*- and *p*-isomers of each solute tested can be completely separated on the α-cyclodextrin stationary phases. The β-cyclodextrin stationary phases also interact more strongly with the *p*- than with the *o*- or *m*-isomers. In the case of dinitrobenzene, the *o*-isomer is retained most strongly.

INTRODUCTION

Cyclodextrins have been successfully utilized in chromatographic separations and purification methods (see the recent review by Hinze[1]).

Being interested in the specific interactions between organic compounds and cyclodextrins, we have prepared several α- and β-cyclodextrin polyurethane resins cross-linked with different diisocyanates[2–4]. These resins exhibited interesting, specific sorption behaviour which was considered to be based on the formation of inclusion compounds. We also prepared chemically bonded β-cyclodextrin gels[5] which are expected to be more efficient in chromatographic separation than the cyclodextrin polyurethane resins. A β-cyclodextrin stationary phase, obtained by coupling ethylenediamine monosubstituted β-cyclodextrin to the succinylhydrazide derivative of polyacrylamide beads, showed efficient, liquid chromatographic separations of *o*-, *m*- and *p*-isomers of the disubstituted benzenes.

In the present work, α- and β-cyclodextrins were immobilized on polyacrylamide beads by the above succinylhydrazide procedure or by a general acyl azide method, and the retention behaviours on these stationary phases were studied for several disubstituted benzene derivatives.

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EXPERIMENTAL

Materials

The polyacrylamide beads, Bio-Gel P-2 (200–400 mesh), were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) from Peptide Institute (Osaka, Japan) and α- and β-cyclodextrins from Hayashibara Biochemical Labs. (Okayama, Japan). All other chemicals (Wako, Osaka, Japan) were of analytical reagent grade. Disubstituted benzenes used as solutes were purified by distillation or recrystallization.

Immobilization of cyclodextrins on succinylhydrazide Bio-Gel

Ethylenediamine monosubstituted α-cyclodextrin (α-en), mono[6-deoxy-6-N-(2-aminoethyl)amino]-α-cyclodextrin, was prepared via the primary monotosylate[6] of α-cyclodextrin in much the same way as mono[6-deoxy-6-N-(2-aminoethyl)amino]-β-cyclodextrin[7] (β-en).

According to a modified method for preparing boronate affinity gel[8], α-en or β-en was coupled to succinylhydrazide Bio-Gel[9] (SuHz-Bio-Gel) as follows (see Fig. 1). Five grams (dry weight) of SuHz-Bio-Gel (1.59 mmol/g) were allowed to swell overnight in 150 ml of 0.1 *M* NaCl. The calculated amount of α-en or β-en dissolved in 100 ml of 0.1 *M* NaCl was added to the suspension. The pH of the suspension throughout the coupling reaction was adjusted to 5.5–6.0 by addition of 0.5 *N* HCl. One gram of solid EDAC was added with mechanical stirring, followed by three further amounts of 0.27 g at intervals of 3 h. The reaction mixture was stirred overnight at room temperature to give a total reaction time of 24 h, then filtered and thoroughly washed successively with 0.1 *M* NaCl and water.

Immobilization of cyclodextrins by the acyl azide procedure

Diethylenetriamine monosubstituted-α-cyclodextrin (α-dtn) and -β-cyclodextrin[10] (β-dtn) (*i.e.*, mono[6-deoxy-6-N-[(2-aminoethyl)-2-aminoethyl]amino]-α-cyclodextrin and -β-cyclodextrin, respectively) were prepared via the corresponding primary monotosylates.

As shown in Fig. 2, α-dtn or β-dtn was coupled to hydrazide Bio-Gel (Hz-Bio-Gel) by a method similar to that of Inman and Dintzis[9]. In 0.1 *M* NaCl, 5.05 g (dry

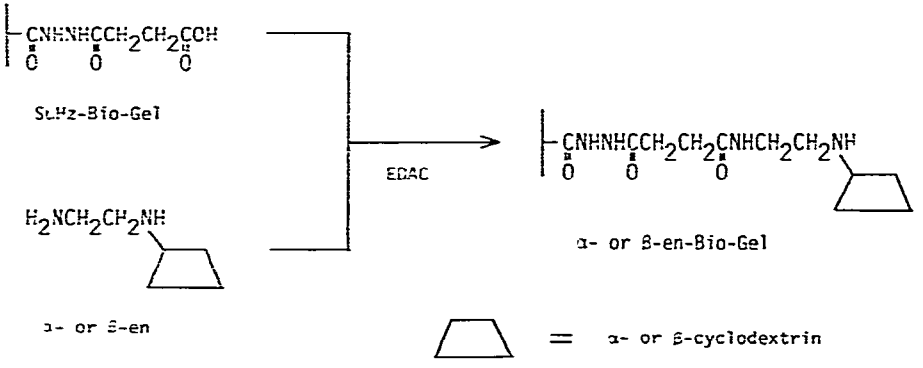

Fig. 1. Immobilization of cyclodextrins on SuHz-Bio-Gel.

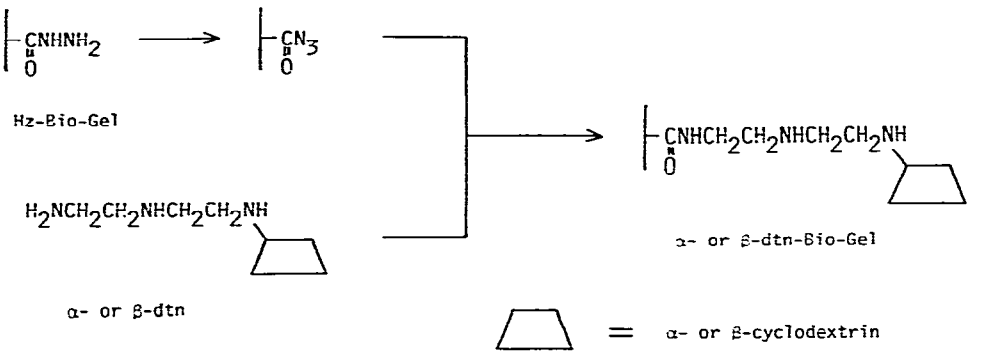

Fig. 2. Immobilization of cyclodextrins on Hz-Bio-Gel by the acyl azide procedure.

weight) of Hz-Bio-Gel (1.54 mmol/g) were allowed to swell overnight. The swollen gel was resuspended to a volume of 200 ml with 0.5 *N* HCl after washing with the same HCl solution. The suspension was then cooled to 0°C in an ice-bath and stirred for 6 min after addition of 20 ml of 1 *M* $NaNO_2$. The acyl azide gel obtained was repeatedly washed with water at 0°C until the pH of the suspension increased to near 3.8, and resuspended in cold water. Then α-dtn or β-dtn in small quantities of water was added to the suspension, and the total volume was made up to 200 ml with cold water. The mixture was stirred in the ice-bath for 1.5 h. Unreacted azide was reconverted into the hydrazide by adding 8 ml of hydrazine hydrate and stirring for 1.5 h. The derivative was washed well with 0.1 *M* NaCl and then with water. The hydrazide produced was converted into the acetylhydrazide form with sodium acetate solution containing acetic anhydride.

Instead of α-dtn or β-dtn, 0.048 g of diethylenetriamine were treated with 5.05 g of Hz-Bio-Gel as described above. Thus the stationary phase containing no cyclodextrin units, dtn-Bio-Gel, was prepared.

Apparatus

Gel particles were packed by a slurry packing technique in a glass column (0.6 cm I.D.) to form a gel bed 30 cm long. An Atto SJ-1541 UV monitor equipped with a 254- or 280-nm UV detector with 60-μl flow cell and Adzuma Model MF-1 syringe-driving equipment with a 100-ml syringe were used. A 2-ml syringe was used to inject 0.6 ml of sample solution (0.1–1 m*M*) into the chromatographic system. Elution was carried out with water for α- and β-dtn-Bio-Gels or with phosphate buffer (pH 6.8) containing 0.05% sodium azide for α- and β-en-Bio-Gels at a flow-rate of 9 ml/h and at a column temperature of 25°C.

RESULTS AND DISCUSSION

In order to obtain efficient cyclodextrin phases, the effect of the feed composition, (mmol cyclodextrin derivative)/(g polyacrylamide derivative), was investigated on the separation of *o*-, *m*- and *p*-isomers of dinitrobenzene. These three isomers cannot be separated on stationary phases containing no cyclodextrin units.

TABLE I

PREPARATION OF CYCLODEXTRIN STATIONARY PHASES AND RESOLUTION OF DINITROBENZENE ISOMERS

Phase	*Feed composition, cyclodextrin derivative/polyacrylamide derivative (mmol/g)**		*Retention time (min)*			*Resolution***
			o-	*m-*	*p-*	
α-en-Bio-Gel	α-en SuHz-Bio-Gel	*0.39*	103	128	177	1.5
β-en-Bio-Gel	β-en SuHz-Bio-Gel	0.17	275	114	134	1.1
		0.25	325	129	158	1.6
		0.34	395	141	177	1.6
		0.85	819	210	267	1.6
α-dtn-Bio-Gel	α-dtn Hz-Bio-Gel	0.22	90	105	125	0.8
		0.34	97	115	138	1.2
		0.51	112	138	179	1.6
β-dtn-Bio-Gel	β-dtn Hz-Bio-Gel	0.15	200	102	119	1.1
		0.22	433	140	178	1.7
		0.45	983	303	390	2.5

* Dry weight.

** Estimated from *o-* and *m*-isomers on the α-cyclodextrin stationary phases and from *m-* and *p*-isomers on the β-cyclodextrin stationary phases.

SuHz-Bio-Gel and dtn-Bio-Gel. Table I shows the retention times of the dinitrobenzene isomers and the peak resolutions for two isomers whose peaks are adjacent: the *o-* and *m*-isomers on the α-cyclodextrin stationary phases and the *m-* and *p*-isomers on the β-cyclodextrin stationary phases. The retention times of the three isomers increase with increasing content of cyclodextrin derivative in the feed. In the cases of α- and β-dtn-Bio-Gels, the peak resolutions also increase as the ratios of α-dtn/Hz-Bio-Gel and β-dtn/Hz-Bio-Gel increase. On the other hand, β-en-Bio-Gel gives a constant resolution of 1.6 in the range of β-en/SuHz-Bio-Gel = 0.25–0.85. Considering the retention time and peak resolution, each cyclodextrin stationary phase obtained from the feed composition italicized in Table I was investigated in further experiments.

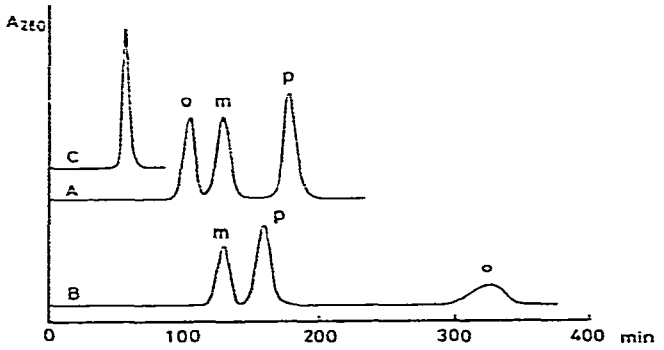

Fig. 3. Liquid chromatograms of dinitrobenzene isomers. Stationary phases: A = α-en-Bio-Gel; B = β-en-Bio-Gel; C = SuHz-Bio-Gel. Peaks: o, m and p denote *o-*, *m-* and *p*-isomers, respectively.

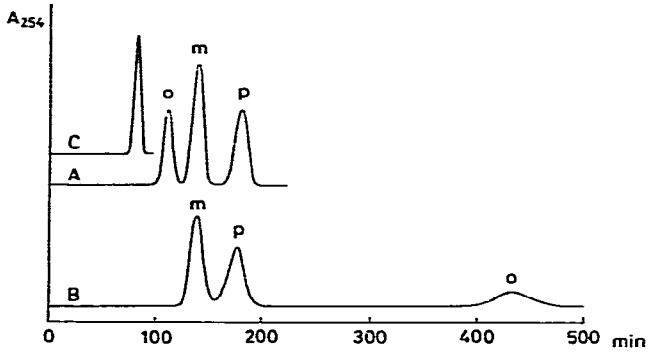

Fig. 4. Liquid chromatograms of dinitrobenzene isomers. Stationary phases: A = α-dtn-Bio-Gel; B = β-dtn-Bio-Gel; C = dtn-Bio-Gel.

Typical liquid chromatograms of a mixture of *o*-, *m*- and *p*-dinitrobenzene isomers on these α- and β-en-Bio-Gels are shown in Fig. 3 together with that on SuHz-Bio-Gel. Fig. 4 shows the separations of the same isomers on α- and β-dtn-Bio-Gels. It is apparent that a complete separation of the three isomers can be obtained on each cyclodextrin stationary phase.

Table II gives the retention times of seven kinds of disubstituted benzene derivatives as model solutes on α- and β-en-Bio-Gels, and Table III those on α- and β-dtn-Bio-Gels. The stationary phases derived from SuHz-Bio-Gel, α- and β-en-Bio-Gels, have terminal carboxyl groups. Therefore, in order to keep the degree of dissociation of the terminal carboxyl groups constant, aqueous phosphate buffer (pH 6.8) was used as eluent. On the other hand, deionized water was used in the case of α- or β-dtn-Bio-Gel, because the unreacted azide on its surface was converted into the neutral acetylhydrazide form.

The *o*-, *m*- and *p*-isomers of each solute tested can be completely separated on both α-en-Bio-Gel and α-dtn-Bio-Gel with the exception of the *o*- and *m*-isomers of aminobenzoic acid. The resolution for the last two peaks on α-en-Bio-Gel is 0.6. The isomers of each solute are retained in the order $o < m < p$ on both the α-cyclodextrin

TABLE II

RETENTION TIMES OF DISUBSTITUTED BENZENE DERIVATIVES ON ETHYLENEDIAMINE-SUBSTITUTED CYCLODEXTRIN STATIONARY PHASES

Solute	*Retention time (min)*								
	α-en-Bio-Gel			*β-en-Bio-Gel*			*SuHz-Bio-Gel*		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	103	144	177	242	308	482	54	54	54
Ethylphenol	260	353	485	343	449	594	51	51	51
Toluidine	95	137	197	153	164	244	50	50	53
Nitroaniline	161	265	934	315	224	864	76	67	80
Nitrophenol	89	401	707	372	292	615	70	52	44
Dinitrobenzene	103	128	177	325	129	158	58	58	58
Aminobenzoic acid	39	67	285	65	54	164	20	20	20

TABLE III

RETENTION TIMES OF DISUBSTITUTED BENZENE DERIVATIVES ON DIETHYLENETRIAMINE-SUBSTITUTED CYCLODEXTRIN STATIONARY PHASES

Solute	*Retention time (min)*								
	α-dtn-Bio-Gel			*β-dtn-Bio-Gel*			*dtn-Bio-Gel*		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	103	133	152	224	301	495	76	76	76
Ethylphenol	228	369	663	352	480	666	76	76	76
Toluidine	132	218	473	158	183	324	139	216	427
Nitroaniline	161	283	1531	370	270	1305	112	94	121
Nitrophenol	116	342	504	92	252	152	74	104	90
Dinitrobenzene	112	138	179	433	140	178	82	82	82
Aminobenzoic acid	87	87	339	38	33	73	28	28	28

stationary phases. This order may be reasonably interpreted as follows. The *p*-isomers can enter deeply and fit well into the α-cyclodextrin cavity, with one of the substituents entering first (perhaps the more hydrophobic one). The *o*- or *m*-isomers cannot enter so deeply because of the steric effect of the substituents, the order of which is $o > m$. The *p*-isomers thus undergo the strongest hydrophobic interaction with the α-cyclodextrin cavity. In particular, *p*-nitroaniline exhibits the largest retention time on each of the cyclodextrin stationary phases.

The *o*- and *m*-isomers of aminobenzoic acid cannot be separated on the stationary phases of β-en-Bio-Gel and β-dtn-Bio-Gel. The peak resolution for *o*- and *m*-toluidine is 0.7 on β-en-Bio-Gel. Except for the above two cases, the three isomers can be completely separated on the β-cyclodextrin stationary phases. The retention order of *o*-, *m*- and *p*-isomers for each solute on β-en-Bio-Gel is the same as that on β-dtn-Bio-Gel, except for nitrophenol. The behaviour of nitrophenol is unexpected and so far not explainable. Considering the consistency of the retention order on dtn-Bio-Gel with that on β-dtn-Bio-Gel, the spacer (diethylenetriamine) may strongly affect the elution order in this case. The strong interaction of the spacer with the solute can be seen for toluidine in Table III: the retention times of *o*-, *m*- and *p*-toluidine are 83, 96 and 122 min on Hz-Bio-Gel, respectively; 139, 216 and 427 min on dtn-Bio-Gel. The three isomers can be completely separated on the stationary phase containing no cyclodextrin units only in the case of toluidine (see Fig. 5).

Except for dinitrobenzene, the *p*-isomers are also eluted last on the β-cyclodextrin stationary phases. (The retention order of nitrophenol on β-dtn-Bio-Gel is, of course, exceptional as mentioned above.) In the cases of nitroaniline, nitrophenol, dinitrobenzene or aminobenzoic acid, the elution order of the three isomers on the α-cyclodextrin stationary phase is inconsistent with that on the corresponding β-cyclodextrin phase. It is reasonable to consider that this inconsistency is due to the difference in the cavity diameters of α- and β-cyclodextrins. The cavity diameter of β-cyclodextrin is larger than that of α-cyclodextrin. Therefore, the *o*- and *m*-isomers can also enter deeper into the β-cyclodextrin cavity than into the α-cyclodextrin cavity. The orientation of the guest molecule in the β-cyclodextrin cavity is not confined so severely as that in the α-cyclodextrin cavity. In the case of dinitrobenzene,

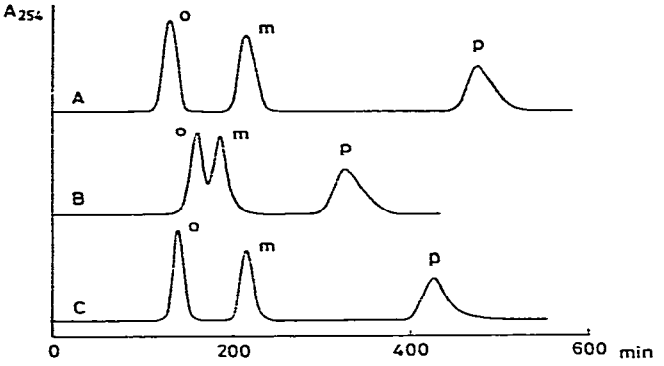

Fig. 5. Liquid chromatograms of toluidine isomers. Stationary phases: A = α-dtn-Bio-Gel; B = β-dtn-Bio-Gel; C = dtn-Bio-Gel.

the *o*-isomer is retained most strongly on both β-en-Bio-Gel and β-dtn-Bio-Gel. Obviously, the retention behaviour of *o*-dinitrobenzene is not caused by interaction with the spacers, but by that with the β-cyclodextrin units: the *o*-, *m*- and *p*-isomers are not separated at all on SuHz-Bio-Gel or dtn-Bio-Gel. We speculate that *o*-dinitrobenzene enters into the β-cyclodextrin cavity with the benzene ring first and that the nitro groups can structurally interact strongly with the hydroxyl groups on the side of the torus. Thus, an important rôle for hydrogen-bonding interaction is suggested in addition to the hydrophobic one. The same may be possible for nitroaniline, aminobenzoic acid, etc. The details are now under investigation.

β-en-Bio-Gel italicized in Table I contained 0.18 mmol of β-cyclodextrin per gram of starting SuHz-Bio-Gel, and α-en-Bio-Gel contained 0.25 mmol α-cyclodextrin. These capacities were calculated from the amounts taken up (see ref. 5). Unfortunately, the precise cyclodextrin capacities for the gels prepared by the acyl azide procedure could not be obtained either by the above mentioned absorption method or the method based on differences in the gel weights before and after the immobilization reaction. This seems to be due to the instability of the acyl azide intermediate. However, the acyl azide procedure is simpler, cheaper and yields stationary phases which perform as well as those obtained via SuHz-Bio-Gel.

In conclusion, stationary phases containing α- and β-cyclodextrins provide efficient, liquid chromatographic separations of the isomers of disubstituted benzenes, as expected. Attempts are now being made to separate the isomers on stationary phases containing cyclodextrins immobilized on silica gels in order to shorten the analysis times.

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