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NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF POSITIONAL ISOMERS OF SUBSTITUTED BENZOIC ACIDS WITH AMINE AND β-CYCLODEXTRIN BONDED-PHASE COLUMNS

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SUMMARY

The separation of positional isomers of several substituted benzoic acids was studied using two different columns, *i.e.* amine and β-cyclodextrin bonded silicas, at appropriate normal-phase conditions. Although some other columns do separate substituted benzoic acids, particularly the octadecylsilica column in a reversed-phase mode, the present separation under simple normal-phase, isocratic conditions is unique. In particular, the retention order of these acids for the amino bonded-phase column can be roughly predicted using the pK_a values of the analytes. On the other hand, due to the strong interaction between substituted benzoic acids and the β-cyclodextrin bonded phase, a small amount of acetic acid has to be added into the mobile phase to overcome band broadening and tailing problems. Possible retention mechanisms are also discussed.

INTRODUCTION

Aromatic acids are ubiquitous in the biological world. It was pointed out by Robinson that derivatives of hydroxylated cinnamic acid, such as caffeic, ferulic and *p*-coumaric acids, are included in most plants[1]. Various phenolic acids, including *p*-hydroxybenzoic acid, syringic and vanillic acids, have also been found in culture solutions from lignin decomposition by white rot fungi[2], in soil extracts[3], and in chemical degradation products of humic substances[4-7]. Thus, the analysis of aromatic acids has been an active field due to its practical concerns in clinical, biochemical, forensic and wood chemistry as well as in food inspections and environmental pollution control. During the past twenty years, a variety of studies by liquid chromatography on the separation of aromatic acids have been reported. Although separations by paper, thin-layer, and gas chromatographic techniques were attained, the time-consuming factor and the need of derivatization of analytes render them disadvantageous as compared to liquid chromatographic techniques.

Charpentier and Cowles developed a quick and reliable high-performance liquid chromatographic (HPLC) method for analysing the phenolic acid content in

hypocotyl portions of young, rapidly developing ping seedlings using a reversed-phase octadecylsilica (ODS) column and a mobile phase composed of acetonitrile, acetic acid and water[8]. HPLC methods for the analysis of phenoxy acid herbicides are also introduced and compared using different bonded-phase columns with either a normal- or a reversed-phase separation mode[9-12]. However, most reports on the separation of substituted benzoic acids have employed ODS columns under a reversed-phase mode[13-20].

Recently, a number of separations of a few selected benzoic acids using cyclodextrin (CD) bonded-phase columns were reported[21-26]. Cyclodextrin molecules were covalently bonded to silica packings via different amine or ethylenediamine linkages. Modifications of the CD molecules by esterification were also attempted in order to improve separation selectivity. However, problems such as poor hydrolytic stability, low cyclodextrin loading, low efficiency, tedions synthetic procedures, and poor reproducibility were encountered[27]. On the other hand, the separations of geometrical isomers of a few substituted benzoic acids were only tested in methanol-phosphate buffer mobile phases.

In this paper, we report our HPLC separation of several positional isomers of substituted benzoic acids using two different columns, *i.e.* amine and newly commercialized β-cyclodextrin (β-CD) bonded phases, under appropriate normal-phase isocratic conditions. It is noted that although gradient elution and other optimization techniques may always be used for the separation of any mixture, the simple isocratic method is preferred for easy application and reproducibility.

EXPERIMENTAL

Apparatus

Chromatographic data were obtained with two equipment systems: (i) A Beckman (Berkeley, CA, U.S.A.) Model 332 gradient liquid chromatography system was used for the β-CD column separation. This system was equipped with two Altex Model 110A pumps, a Model 210 sample injector valve and a Model 420 system controller. A Waters (Milford, MA, U.S.A.) Model 440 adsorbance detector (254 nm) and a Houston Instrument (Houston, TX, U.S.A.) Omniscribe Model D5000 recorder were also applied. (ii) A Micromeritics (Norcross, GA, U.S.A.) Model 7500 liquid chromatograph equipped with a Model 750 solvent delivery system, a Model 752 ternary solvent mixer, a Model 731 column compartment with a universal sample injector and variable temperature control from ambient to 150°C (accuracy ± 1°C), and a Model 786 variable-wavelength (200–600 nm) detector with a deuterium lamp was used. This system is used for the amine bonded-phase column separation. The recorder connected was a Linear Model 555 single-channel recorder.

Pressure-Lock series C-160 (Precision Sampling, LA, U.S.A.) 25-μl syringes were employed for sample injection.

Columns

(i) Amine bonded-phase column (Alltech Assoc., Deerfield, IL, U.S.A.), 10 μm particle size, 25 cm × 4.6 mm I.D. (ii) β-Cyclodextrin bonded phase (β-CD) column (Advanced Separation Technologies, Whippany, NJ, U.S.A.), 5 μm particle size, 25 cm × 4.6 mm I.D.

Reagents

Several positional isomers of substituted benzoic acids from Aldrich (Milwaukee, WI, U.S.A.) were used for the study. The substituents include amino (NH_2), carboxy (COOH), chloro (Cl), hydroxy (OH), methoxy (OCH_3), methyl (CH_3) and nitro (NO_2) groups. HPLC-grade solvents were obtained from Fisher (Fairlawn, NJ, U.S.A.).

Chromatographic procedures

Before the separation experiments, the columns were pre-equilibrated with the mobile phase for at least 3 h. After pre-equilibrium was achieved, a flow-rate of 1 ml/min was set for the chromatographic process.

In each separation, solutions of solute mixtures were prepared using the eluent. The concentration of each solute was *ca.* 1–2 mg/ml. A 3-μl aliquot of the solute mixture was injected into the system. A back-pressure of < 1000 p.s.i. was usually observed in the course of the normal-phase separation experiments, and for reversed-phase, 3000 p.s.i. All data points were collected by averaging more than three reproducible separations. Published methods were used to determine t_0 values for the amine column[28] as well as the β-CD column[29].

RESULTS AND DISCUSSION

Amine bonded-phase column

A chromatogram for some selected benzoic acids is shown in Fig. 1. The capacity factors of several isomeric substituted benzoic acids using an amine bonded-phase column with 2, 7.5 and 15% acetic acid in 2-propanol as mobile phases are
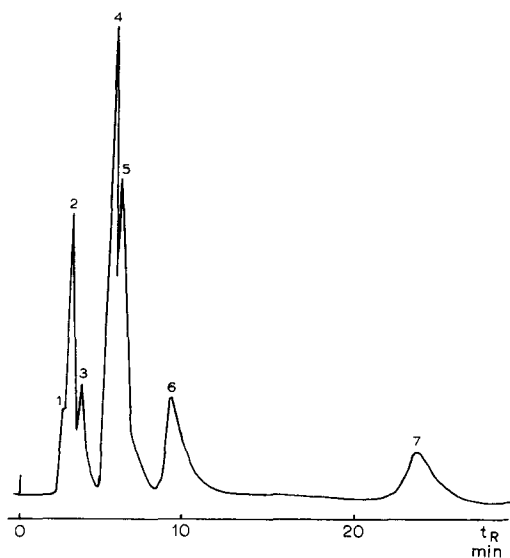

Fig. 1. Chromatogram of separation of seven substituted benzoic acids using an amine bonded-phase column, acetic acid–2-propanol (15:85), flow-rate = 1 ml/min. Peak identification: 1 = *p*-methylbenzoic acid; 2 = *o*-methoxybenzoic acid; 3 = *p*-aminobenzoic acid; 4 = *o*-chlorobenzoic acid; 5 = *p*-terephthalic acid; 6 = *o*-hydroxybenzoic acid; 7 = *o*-nitrobenzoic acid.

TABLE I

CAPACITY FACTORS (k) FOR SEVERAL SUBSTITUTED BENZOIC ACID ISOMERS USING AN AMINE BONDED-PHASE COLUMN AND AN ACETIC ACID-2-PROPANOL ELUTION SYSTEM AT 30.5°C

Substituent		Percentage acetic acid in 2-propanol			pK_a
		2	7.5	15	
NH$_2$	o-	0.92	0.37	0.19	6.97
	p-	1.77	0.92	0.33	4.92
OH	o-	—	5.03	1.95	2.97
	p-	0.73	0.25	0.18	4.48
OCH$_3$	o-	3.37	0.83	0.38	4.09
	p-	0.60	0.25	0.12	4.47
CH$_3$	o-	0.98	0.33	0.14	3.91
	p-	0.72	0.25	0.13	4.36
NO$_2$	o-	—	—	5.37	2.16
	p-	9.03	1.92	0.81	3.41
COOH	o-	—	—	—	2.97
	p-	—	2.82	1.02	3.51
Cl	o-	—	2.56	1.09	2.92
	o-	1.97	0.49	0.22	3.98

listed in Table I, respectively. It is observed that *para*-isomers usually elute faster than the corresponding *ortho*-isomers, except for aminobenzoic acids and dicarboxylic acids which show the opposite trend. If all acids are taken into account together, it is seen that the elution order also parallels their acidities with a few exceptions, *i.e.* aminobenzoic acids (Fig. 2). The more acidic the acid is, the longer it is retained in the column, which indicates that acid–base interaction is present during the elution process. When the percentage of the strong solvent, *i.e.* acetic acid, is increased in the eluent, the capacity factor decreases accordingly as expected. Also, the fact that o-Cl, o-OH and o-NO$_2$ benzoic acids are eluted as the last three compounds and the retention time for phthalic acid is too long to be measured within the mobile phase composition range, *i.e.* 1–15% acetic acid-2-propanol, seems to imply the presence of bifunctional interactions. (It is noted that a similar separation of some phenoxyacid herbicides using an amine bonded-phase column was also reported, but with a slightly different view point[10].)

β-Cyclodextrin bonded-phase column

The β-cyclodextrin (β-CD) bonded-phase column just commercialized in recent years is packed with a silica material covalently bonded with β-CD molecules[30-33]. The β-cyclodextrin is considered to be the host molecule chemically bonded to silica gel via a non-nitrogen-containing stable spacer 6–10 atoms in length. In the hollow
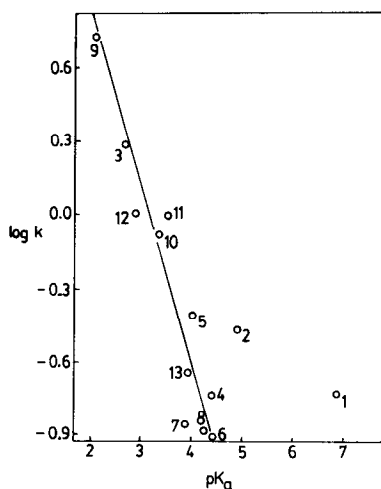
Fig. 2. Effect of acidity of benzoic acids on the capacity factors using an amine bonded-phase column, acetic acid–2-propanol (15:85), at 30.5°C. Peak identification: 1 = *o*-aminobenzoic acid; 2 = *p*-aminobenzoic acid; 3 = *o*-hydroxybenzoic acid; 4 = *p*-hydroxybenzoic acid; 5 = *o*-methoxybenzoic acid; 6 = *p*-methoxybenzoic acid; 7 = *o*-methylbenzoic acid; 8 = *p*-methylbenzoic acid; 9 = *o*-nitrobenzoic acid; 10 = *p*-nitrobenzoic acid; 11 = terephthalic acid; 12 = *o*-chlorobenzoic acid; 13 = *p*-chlorobenzoic acid.

truncated cone, there are seven primary hydroxyl groups on the side of torus with the smaller circumference and fourteen secondary hydroxyl groups, seven in a clockwise direction and seven counterclockwise, on that of the greater side. In brief, fourteen hydroxyl groups in two directions are around the entrance and seven hydroxyl groups are about the bottom of the cone. Inside the cavity there are no hydroxyl groups, which provides a hydrophobic environment. Because of the rigid cavity size of β-CD, only those guest molecules of proper size, such as naphthalene, etc., can form strong β-CD inclusion complexes. Due to its hydrolytic stability, it is often used with aqueous methanol eluent systems. This column has a unique selectivity toward a large number of enantiomers besides its applications in routine separations[34].

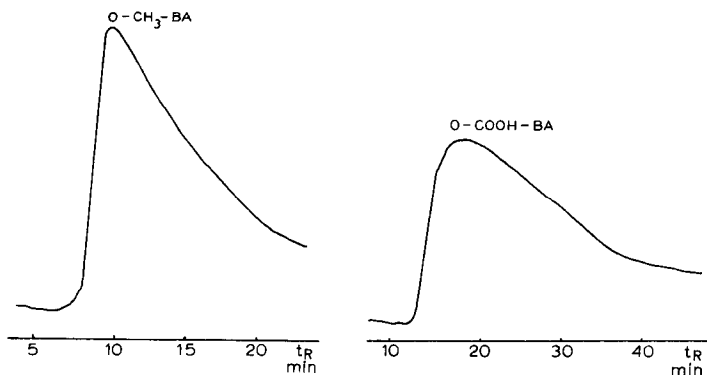Fig. 3 shows the chromatographic peak profile of *o*-methylbenzoic acid and

Fig. 3. Chromatograms of *o*-methylbenzoic acid and phthalic acid using a β-CD column and 100% methanol.

TABLE II

CAPACITY FACTORS (k) OF SEVERAL SUBSTITUTED BENZOIC ACID ISOMERS USING A β-CD COLUMN

The relative errors are within 2%; BA = benzoic acid.

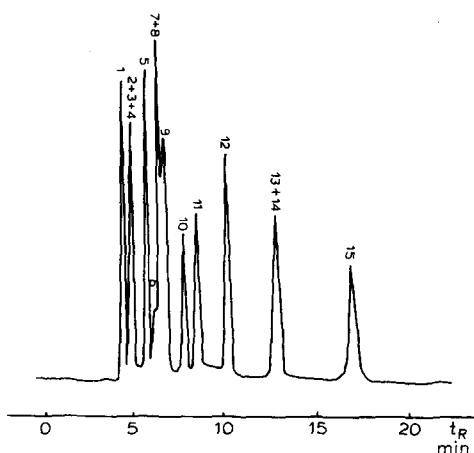| Samples	100% methanol + 0.1% acetic acid	5% water in methanol + 0.1% acetic acid	7.5% water in methanol + 0.1% acetic acid	20% water in methanol + 0.1% acetic acid	40% water in methanol + 0.1% acetic acid
p-NH$_2$-BA	0.33	0.34	0.34	0.40	0.79
m-NH$_2$-BA	0.98	0.98	0.98	1.00	1.23
o-NH$_2$-BA	0.5	0.5	0.5	0.52	0.81
p-CH$_3$-BA	0.93	0.95	0.95	1.07	2.13
m-CH$_3$-BA	0.98	0.98	0.98	1.12	1.90
o-CH$_3$-BA	1.34	1.34	1.34	1.36	2.09
p-OCH$_3$-BA	0.77	0.79	0.79	0.89	1.62
m-OCH$_3$-BA	1.66	1.66	1.70	1.78	1.95
o-OCH$_3$-BA	2.04	1.99	2.0	2.29	2.75
p-Cl-BA	2.95	2.95	2.95	3.38	5.75
m-Cl-BA	4.36	4.57	4.57	4.62	7.08
o-Cl-BA	2.95	3.01	3.02	3.54	6.02
p-OH-BA	0.51	0.51	0.51	0.59	1.17
m-OH-BA	1.12	1.12	1.12	1.17	1.78
o-OH-BA	0.44	0.44	0.44	0.57	1.15

Fig. 4. Chromatogram of separation of fifteen substituted benzoic acids using a β-CD column and 0.1% acetic acid in methanol. Peak identification: 1 = p-aminobenzoic acid; 2 = o-hydroxybenzoic acid; 3 = o-aminobenzoic acid; 4 = p-hydroxybenzoic acid; 5 = p-methoxybenzoic acid; 6 = p-methylbenzoic acid; 7 = m-aminobenzoic acid; 8 = m-methylbenzoic acid; 9 = m-hydroxybenzoic acid; 10 = o-methylbenzoic acid; 11 = m-methoxybenzoic acid; 12 = o-methoxybenzoic acid; 13 = p-chlorobenzoic acid; 14 = o-chlorobenzoic acid; 15 = m-chlorobenzoic acid.

phthalic acid using a β-CD column and 100% methanol as the mobile phase. The long retention time and broad peak shape indicate that the interactions between substituted benzoic acids and the stationary phase are very strong and the sorption–desorption kinetics is very slow[35].

To improve the situation, 0.1% (v/v) acetic acid was introduced into the mobile phase. It should be noted that the separation of similar substituted benzoic acids cannot be achieved using a methanol–water mixture as the mobile phase in a reversed-phase mode[25]. Fig. 4 shows the chromatogram of the separation of eleven
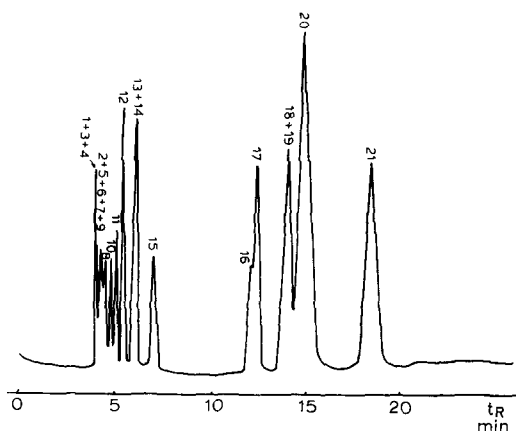

Fig. 5. Chromatogram of separation of twenty-one substituted benzoic acids using a β-CD column and 0.5% acetic acid in methanol. Peak identifications are the same as Fig. 4 except: 16 = m-carboxybenzoic acid; 17 = phthalic acid; 18 = m-nitrobenzoic acid; 19 = p-nitrobenzoic acid; 20 = o-nitrobenzoic acid; 21 = terephthalic acid.

and of fifteen substituted benzoic acids within 18 min. It is observed that separation is drastically improved with sharp peaks. If 0.5% (v/v) acetic acid was added to methanol as the mobile phase, additional separation of strong interacting nitro- and carboxy-substituted benzoic acids resulted in some sacrifice of the resolution of the earlier eluted compounds (Fig. 5). The capacity factor of each compound decreases as the content of acetic acid in methanol increases, which indicates a normal phase behavior. On the other hand, no simple correlation of the retention time with any of the physical properties of these benzoic acids can be found. It is not certain whether the inclusion process is significant.

Further addition of water into the mobile phase causes the capacity factor to increase with increasing water content, especially when 40% water is added (Table II). This indicates a reversed-phase separation mode. Under these conditions, the inclusion process becomes more significant as the water content increases because the cavity of the β-CD is more and more hydrophobic as compared to the mobile phase. Nevertheless, the resolution is not significantly improved.

CONCLUSION

In this paper, we have demonstrated that by using rather strong interacting columns such as the amine and β-CD bonded phases one is able to separate a mixture of strong interacting analytes such as substituted benzoic acids. It is clear that the two columns operate with different retention behaviors on the basis of the elution order of the substituted benzoic acids. Nevertheless, each can be used at least for separating the strong interacting analytes from the weak. In particular, the β-CD bonded-phase column shows great promise in separating many structural analogues.

ACKNOWLEDGEMENTS

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