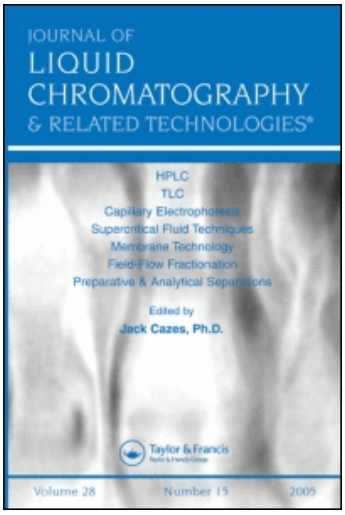

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The Effects on Separation of Cephalosporins by HPLC with β-Cyclodextrin Bonded Stationary Phase

Tai-Li Tsou[a]; Jing-Ran Wu[a]; Teen-Meei Wang[a]
[a] Institute of Preventive Medicine National Defense Medical Center, Taipei, Taiwan, R. O. C

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THE EFFECTS ON SEPARATION OF CEPHALOSPORINS BY HPLC WITH β-CYCLODEXTRIN BONDED STATIONARY PHASE

Tai-Li Tsou, Jing-Ran Wu
Teen-Meei Wang

Institute of Preventive Medicine
National Defense Medical Center
P. O. Box 90048-700
Taipei, Taiwan, R. O. C

ABSTRACT

A β-cyclodextrin bonded stationary phase is used for the liquid chromatographic separation of cephalosporins. A complete separation of nine cephalosporins with methanol and tetraethylammonium acetate (TEAA) buffer solution has been demonstrated. It is also found that a 100% of TEAA buffer solution is suitable for the separation of cephalosporins containing a hydrophilic α-amino moiety.

The effects of pH, TEAA concentration, organic modifier concentration of mobile phase, and the column temperature on the retention are examined and discussed.

INTRODUCTION

Cephalosporins are β-lactam antibiotics possessing antibacterial activities against both gram-positive and gram-negative bacteria. Determination of cephalosporins in pharmaceutical dosage forms and biological fluids has been performed by microbiology analysis,[1] UV absorption,[2] thin layer chromatography,[3,4] ion exchange chromatography.[5,6] and capillary electrophoresis chromatography.[7,8] It has also been of considerable interest in the utilization of reverse phase high performance liquid chromatography (HPLC) for rapid separation and accurate determination of antibiotics.[9-16] Other HPLC techniques, such as ion-pairing,[17-19] microbore column,[20] postcolumn,[21] multi-phase column,[22,23] copolymer column,[24] and column-switching[25] have been developed recently.

The β-cyclodextrin (β-CyD) is an oligosaccharide of seven glucose units cyclized together to form a toroidal structure with a hydrophilic outerior face and a hydrophobic inner cavity. Since Armstong[26] developed the first high efficiency bonded β-CyD phase on 5 μm silica gel as the packing material in HPLC, many pharmaceutics have reported the separation and determination of drugs simultaneously by employing bonded β-CyD column. In this study, we describe for the first time the successful separation of a mixture of cephalosporins by HPLC with β-CyD bonded stationary phase.

Characteristics such as the effects of inclusion complex formation between the solutes and β-CyD cavity, the influence of pH, TEAA concentration, organic modifier concentration of mobile phase, and the column temperature on retention are investigated. The observed behavior of the compounds during chromatographic processes is also discussed.

EXPERIMENTAL

Materials

All cephalorsporins were purchased from Sigma (Louis, MO, USA). The HPLC solvents such as acetonitrile, methanol, and glacial acetic acid were obtained from E. Merck (Frankfurter, Germany). Tetraethylammonium acetate (TEAA), Tetrabutylammonium acetate (TBAA) and hexanesulfonic acid (HSA) were obtained from Aldrich (Milwaukee, WI, USA) and Fluka (Buchs, Switzerland), respectively. Distillated water was deionized twice before use.

Instrumentation

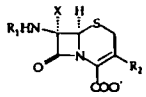
The liquid chromatographic system consists of a Waters Model 6000A pump connected to a U6K injector, a photodiode array detector model 990 set at 230 nm and a model 520 printer/plotter (Waters Associates, Milford, MA, USA). A model TCM temperature controller (Waters, MA, USA) was employed to adjust the temperature of the column from 30° to 55°C . The column employed was a 5 μm bonded β-CyD (250 mm x 4.6 mm i.d.) purchased from Advanced Separation Technologies Inc. (Whippany, NJ, USA). A model 112 pH meter (Photovolt, NY, USA) was used to measure the pH values of mobile phases.

Chromatographic condition

Buffers such as TEAA, TBAA, and HSA were prepared in water and glacial acetic acid was added until the desired pH was obtained. Mobile phase was prepared by mixing methanol with buffer solution and was degassed by bubbling helium into it for about 10 min. The mobile phase was filtered through a 0.45μ filter before use. Sample solutions were prepared by dissolving each drug in mobile phase to give a concentration of about 1 mg/mL. Typically, 10 μL of sample solution was injected. The flow rate was adjusted at 0.8 mL/min. The eluant was eluted and attenuated for full scale deflection at 2 units.

RESULTS AND DISCUSSION

The cephalosporins used in this work are listed in Figure 1. All compounds are β-lactam derivatives with C-7 amino substitutions or C-3 modified side chains. These compounds can be grouped in three types according to their structures. Type I, as compounds 1-5, containing a hydrophilic α-amino moiety gets stronger affinity in aqueous solution. Type II, as 7-ACA and 7ADCA, has the similar amino group at the C-7 position of β-lactam but with less polarity. Type III, as compounds 8-16, obtains bulky substituents at C-7 or C-3 side chain and gets different molecular sizes and polarity. Thus, separation of cephalosporins by HPLC with β-CyD bonded phase is a function of the formation of the inclusion complexes in the cavity of cyclodextrin that in turn would be primarily effected by many factors such as



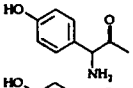
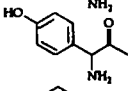
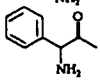
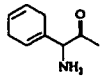
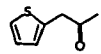
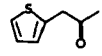
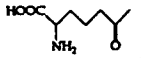
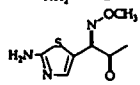
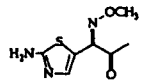
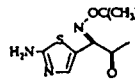
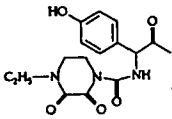
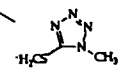
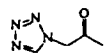
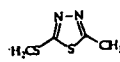
NO.	Name	R_1	R_2	X
1	Cefadroxil		$-CH_3$	H
2	Cefatrizine			H
3	Cefaclor		$-Cl$	H
4	Cephalexin		$-CH_3$	H
5	Cephradine		$-CH_3$	H
6	7-ACA	H	$-CH_2OCOCH_3$	H
7	7-ADCA	H	$-CH_3$	H
8	Cephaloridine			H
9	Cephalothin		$-CH_2OCOCH_3$	H
10	Cephalosporin C		$-CH_2OCOCH_3$	H
11	Cefotaxime		$-CH_2OCOCH_3$	H
12	Ceftizoxime		H	H
13	Ceftazidime			H
14	Cefoxitin		$-CH_2OCONH_2$	$-OCH_3$
15	Cefoperazone			H
16	Cefazolin			H

Figure 1. Structures of the analyzed cephalosporins.

Table 1

The Capacity Factors of Cephalosporins on β-Cyclodextrin Bonded Column with Cation and Anion (5mM) in MeOH/Buffer = 32/68, pH 3.6, Column Temperature 30°C.

		k'*		
No.	**Compound**	**TEAA**	**TBAA**	**HSA**
1	Cefadroxil	0.07	0.06	0.08
2	Cefatrizine	0.14	0.12	0.09
3	Cefaclor	0.14	0.13	0.12
4	Cephalexin	0.14	0.12	0.11
5	Cephradine	0.14	0.12	0.12
6	7-ACA	3.61	2.69	0.87
7	7-ADCA	2.49	3.01	0.65
8	Cefaloridine	0.81	0.59	0.47
9	Cephalothin	12.76	10.29	2.19
10	Cephalosporin C	7.23	6.57	1.52
11	Cefotaxime	8.04	7.26	1.87
12	Ceftizoxime	7.47	6.77	1.93
13	Ceftazidime	7.03	6.25	2.09
14	Cefoxitin	10.65	9.10	2.02
15	Cefoperazone	13.54	10.89	2.60
16	Cefazolin	16.65	12.96	2.46

*Capacity factor (k') = t_1 - t_0/t_0. t_0 is the retention time of unretained methanol, while t_1 is the average data in three times of retention time for each compound.

pH, ions, ionic concentration, organic modifier concentration of mobile phase, and the column temperature. This project plans to investigate these factors, so that a series of isocratic mobile phases can be developed.

Effect of Cation and Anion on Retention

Table 1 shows the retention behaviors of sixteen cephalorsporins in the mobile phase (MeOH/buffer = 32/68, pH 3.6) with adding different ion reagents. Addition of either cation or anion decreased the retention of Type II and III compounds. This is due to the added ions competing with solutes for

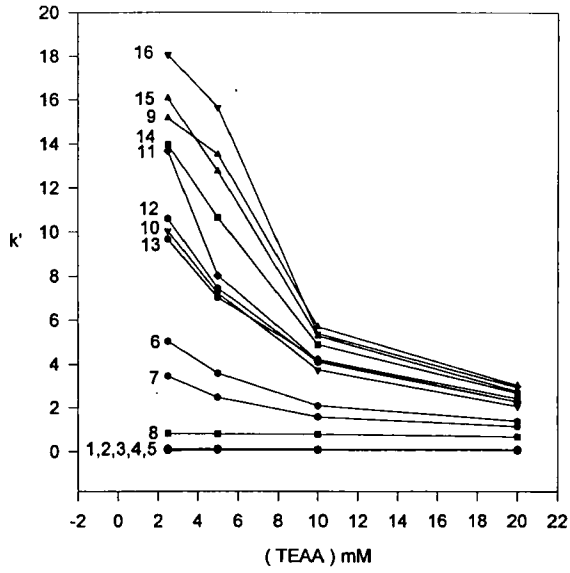


Figure 2. Effect of TEAA concentration on the capacity factors of cephalosporins. Chromatographic condition: Column, 250 x 4.6mm i.d. Cyclobond I; mobile phase, MeOH/TEAA buffer = 32/68, pH 3.6, column temperature 30°C; flow rate, 0.8 mL/min. Labels: 1, Cefadroxil; 2, Cefatrizine; 3, Cefaclor; 4, Cephalexin; 5, Cephradine; 6, 7-ACA; 7, 7-ADCA; 8, Cefaloridine; 9, Cephalothin; 10, Cephalosporin C; 11, Cefotaxime; 12, Ceftizoxime; 13, Ceftazidime; 14, Cefoxitin; 15, Cefoperazone; 16, Cefazolin.

the β-CyD cavity and thus renders the strength of inclusion complexation between the solutes and the β-CyD cavity weaker. By comparison, HSA has less separative selectivity than the other ion reagents, although it has more influence on retention. It also shows that the Type I compounds containing a hydrophilic α-amino moiety exhibit less retention and thus separation.

Effect of TEAA Concentration on Retention

The retention behaviors of cephalosporins over a wide range of TEAA concentration from 2.5 to 20 mM were studied. Figure 2 shows the capacity factors (k') of cephalosporins in mobile phase (MeOH/buffer = 32/68, pH 3.6) with different concentration of TEAA. A relatively fast decreasing in the k' values of Type II and III compounds is observed with increasing TEAA

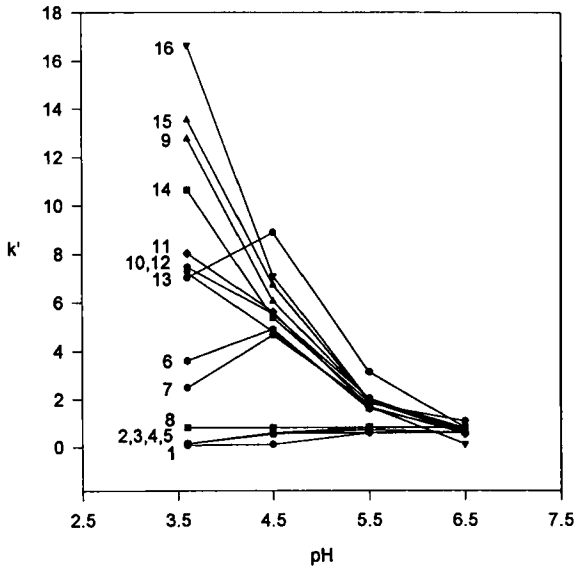


Figure 3. Effect of pH on the capacity factors of cephalosporins. Chromatographic condition: Mobile phase, MeOH/buffer = 32/68, TEAA = 5 mM, column temperature 30°C. Others are the same as in Figure 2.

concentration. The results indicate that higher concentration of TEAA in the mobile phase saturates the β-CyD cavities and causes the solutes to have less competition to form the inclusion complexation with β-CyD cavities resulting in faster decreasing on the retention of solutes. It is noted that in this case a suitable retention and selectivity is reached between 3 and 6 mM of TEAA in the mobile phase.

Effect of pH on Retention

The retention behaviors of cephalosporins with the same mobile phase (MeOH/5 mM TEAA = 32/68) at different pH values are indicated in Figure 3. The retention of Type I compounds has no significant changes at various pH values, while the Type III compounds except ceftazidime show the lower capacity factors with increasing pH. This can be explained by considering the effect of pH on the bonding strength and function of the solutes to the hydroxyl group of β-CyD. It had been reported that the OH^- ion exhibited a higher hydrogen bonding ability to the hydroxy groups of ROH molecules.[27]

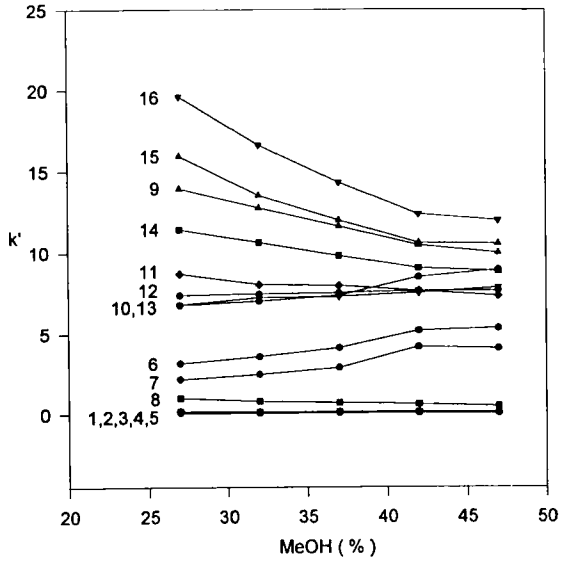


Figure 4. Effect of methanol concentration on the capacity factors of cephalosporins. Chromatographic condition: Mobile phase, TEAA = 5 mM, pH 3.6, column temperature 30°C. Others are the same as in Figure 2.

Therefore, the increase of OH^- ion concentrations by increasing pH of the mobile phase. The ions will compete with the carboxylate group of cephalosporin to interact with the hydroxy group of β-CyD. As a result, a decreased retention time accompaned with the increase of pH is observed. However, a variant retention model for 7-ACA (compound 6), 7-ADCA (compound 7), and ceftazidime (compound 13) is observed with increasing pH. This indicates that other factors that exercise influences on this β-CyD bonded stationary phase may be involved. The longest retention time appears near its isoelectric point, pH 4.5.

Effect of Organic Concentration on Retention

The effect of methanol concentration on the cephalosporin retention was also studied by changing the methanol-water ratio in the mobile phase. Figure 4 shows the typical plots of capacity factors *versus* the methanol contents from 27% to 47%. The TEAA concentration is 5 mM, and the pH of the mobile phase is 3.6 in all experiments. It is found that an increase in methanol content

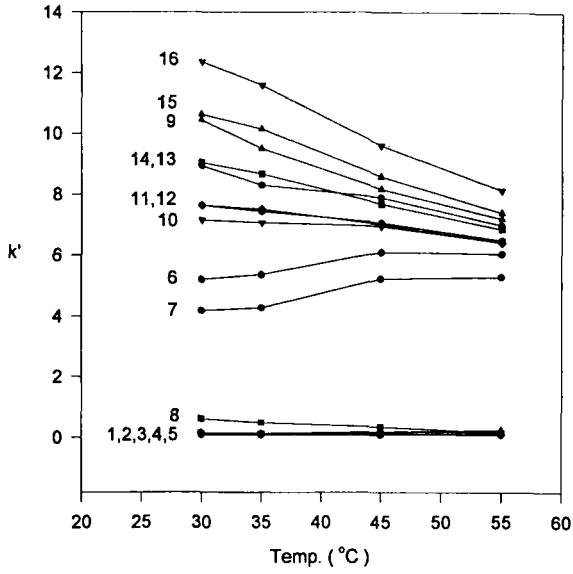


Figure 5. Effect of column temperature on the capacity factors of cephalosporins. Chromatographic condition: Mobil phase, MeOH/buffer = 42/58, pH 3.6, TEAA = 5mM. Others are the same as in Figure 2.

results in a decreased retention on Type III compounds. It is well explained from the cyclodextrin-binding studies that an increase in organic content in the mobile phase will weaken the strength of inclusion complexation between guest molecules and β-CyD.[28,29]

In contrast, for the Type II compounds and ceftazidime a medium increase on retention is obtained with an increasing of methanol content. This may be due to the stronger ion-pairing formation between solute and TEAA in the less aqueous content solution.

Effect of Temperature on Retention

The effect of column temperature on the retention behaviors of cephalosporins is also examined. As shown in Figure 5, the retention and resolution of Type III compounds is decreased as the column temperature increased. This result is due to the fact that the higher temperature weakens

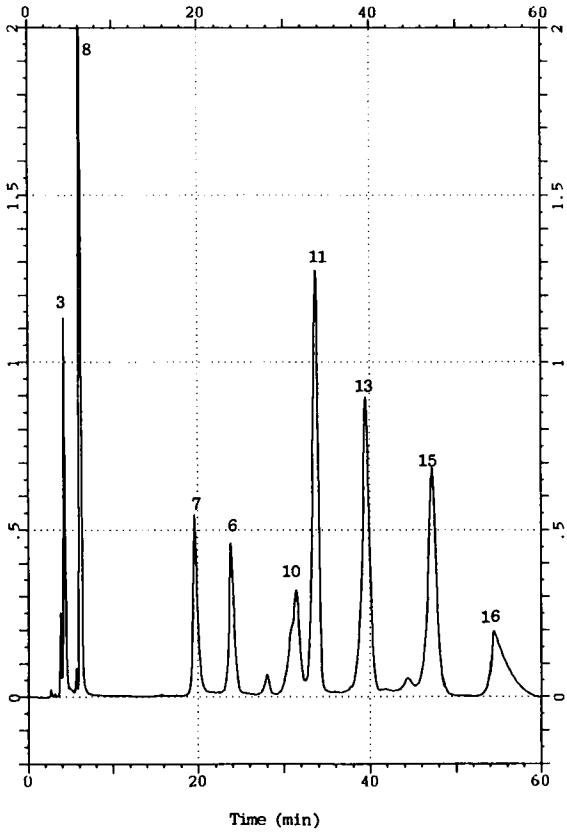


Figure 6. Chromatogram of the nine cephalosporins separated by using the optimized mobile phase of MeOH/buffer = 42/58, 5mM TEAA, at pH 3.6, 30°C, flow rate = 0.8 mL/min. Labels: 3, Cefaclor; 6, 7-ACA; 7, 7=ADCA; 8, Cefaloridine; 10, Cephalosporin C; 11, Cefotaxime; 13, Ceftazidime; 15, Cefoperazone; 16, Cefazolin.

the attraction strength between solutes and the β-CyD cavities. However, there is still a medium increasing retention for the Type II compounds at higher temperature.

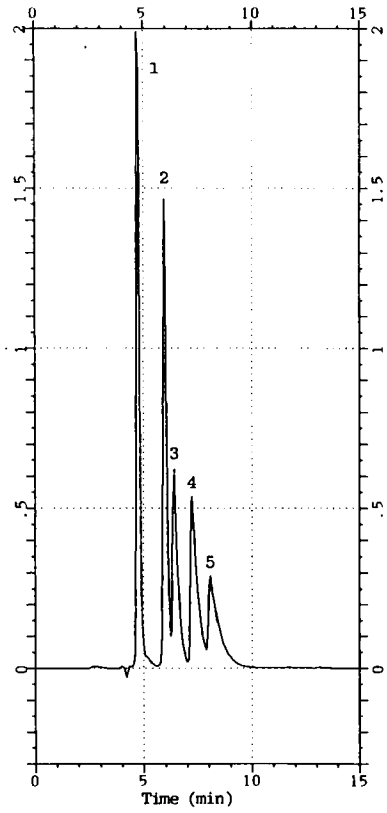


Figure 7. Chromatograms of the five cephalosporins containing a hydrophilic α-amino moiety separated by using 100% 5mM TEAA buffer solution at pH 3.6, 30°C, flow rate = 0.8 mL/min. Labels: 1, Cefaxroxil; 2, Cefatrizine; 3, Cefaclor; 4, cephalexin; 5, Cephradine.

Chromatogram of Cephalosporins

Figure 6 shows the optimal separation of a mixture of nine cephalosporins in the mobile phase under the condition of MeOH/buffer = 42/58, 5 mM TEAA, and pH 3.6. In chromatography, the adjustment on retention of Type II compounds will be predominated by changing the methanol content or TEAA concentration rather than the other factors. For the Type III compounds, the conditions of pH 3.6, 3-5 mM of TEAA, and MeOH content ranged in 37-42%

are recommended. If the hydrophilic Type I compounds need to be separated, the less methanol content in the mobile phase is suitable for the purpose. Figure 7 demonstrates the typical chromatogram of these compounds.

In conclusion, the isocratic separation of a mixture of cephalosporins on cyclodextrin bonded column has been demonstrated. The effects of methanol and TEAA concentration in the mobile phase, pll, and column temperature on retention suggest that the inclusion complexes formation between The type III compounds and the cavities of β-CyD is more predominate than the Type I and II. It shows that the bulky substituents on C-7 or C-3 side chain of cephalosporins are the important factors for the separation. Although the separation of five hydrophilic Type I cephalosporins is intrigued, the separation performance can be improved by using 100% TEAA buffer solution as mobile phase.

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