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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

XV*. ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS AND PREPARATIVE SEPARATION OF THE COMPONENTS OF BACITRACIN

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SUMMARY

Isocratic high-performance liquid chromatographic (HPLC) systems were established for analytical and preparative separation of the components of the antibiotic preparation bacitracin (BC). The best analytical results were obtained using a C_{18} modified silica gel column (Capcell Pak C_{18}) with a solvent system of 0.04 *M* disodium hydrogenphosphate buffer and methanol (4:6), pH 9–10. The calibration graphs showed good linear relationships between 50 and 1000 ng for BC-A and between 65 and 1000 ng for BC-F. With respect to the preparative HPLC, a Capcell Pak C_{18} column with methanol–0.05 *M* aqueous sodium sulphate solution (6:4) as a mobile phase gave satisfactory results. The isolation of BC-A and -F was readily achieved without decomposition of the components by using the present preparative HPLC followed by desalting on a prepacked C_{18} cartridge.

INTRODUCTION

Bacitracin (BC) is one of the most commonly used antibiotics in the world as an animal feed additive[1,2]. A large consumption of BC may increase the risk of residues of BC in food, so a simple, rapid and reliable method for the analysis of BC is required to monitor BC in food and feed. Although conventional microbiological assay procedures have good sensitivity, they are time consuming and cannot identify the individual components of BC. Therefore, a number of chemical analysis methods have been reported using high-performance liquid chromatography (HPLC)[3–5] and thin-layer chromatography (TLC)[6–15].

BC consists of more than twenty components with different antimicrobial activ-

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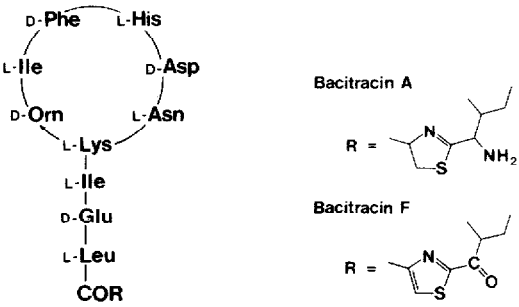

Fig. 1. Structures of bacitracin A and F.

ities. The main antimicrobial components are BCs A and B; the main degradation product is BC-F, which is devoid of antimicrobial activity and has nephrotoxicity[16]. The structures of the components have been determined only for BC-A and -F (Fig. 1). Therefore it is important to separate BC-A and -F from other components.

In the previous report[17], we established a combinational technique using normal-phase and reversed-phase (RP) high-performance thin-layer chromatography (HPTLC) for the identification of the components of BC. Although TLC can identify the various BC components, the advantages of HPLC are that it is useful in both the quantitation as well as the preparation of the individual components. All of the previously reported RP-HPLC methods used complicated gradient elution systems having long analysis times[3–5]. We could not obtain reproducible results under these chromatographic conditions. Additionally, ion-exchange HPLC gave low resolution of the BC components[6]. Therefore, we would like to establish a simple and reliable HPLC method for the analysis and preparative separation of the components of BC using isocratic elution.

According to preliminary studies, BC appears as tailing peaks on a conventional octadecylsilyl (C_{18}) silica gel column, probably due to interaction between the amino group of BC and residual silanol groups on the column. Use of a silanol-free stationary phase would prevent tailing of solute peaks. Capsule[18] and polymer[19] type RP-HPLC columns are made from silica gel coated with a silicone polymer and a porous polymer gel modified with hydrophobic materials such as C_{18}, respectively, so they are free from silanol groups. Accordingly, we decided to investigate the separation of the components of BC on capsule and polymer type RP-HPLC columns. In this paper, we report techniques for the analytical and preparative HPLC of the components using an isocratic system.

EXPERIMENTAL

Materials

Methanol, sodium hydroxide, phosphoric acid, disodium hydrogenphosphate, potassium phosphate, sodium sulphate, potassium sulphate, sodium chloride and potassium chloride were analytical grade reagents. Bacitracin was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.).

A Baker 10 C_{18} cartridge (7020-3) was obtained from J. T. Baker (Phillips-

burgh, NJ, U.S.A.). Capcell Pak C_8 (5 μm, 150 mm × 4.6 mm I.D., Lot No. 30006) and C_{18} (5 μm, 150 mm × 4.6 mm I.D., Lot No. 20138) columns were obtained from Shiseido (Tokyo, Japan). TSK gel Octadecyl-4PW (7 μm, 150 mm × 4.6 mm I.D., Lot No. OPWD0061) and Asahipak ODP-50 (5 μm, 150 mm × 6.0 mm I.D., Lot No. 8706439) columns were obtained from Tosoh (Tokyo, Japan) and Asahi Chemical Industry (Kawasaki, Japan), respectively.

High-performance liquid chromatography

A chromatograph equipped with a constant-flow pump (LC-5A; Shimadzu, Kyoto, Japan) was used with a variable wavelength UV detector (Shimadzu SPD-2AM) operated at 234 nm. The separations were performed on Capcell Pak C_{18} with methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4) and with methanol–0.05 *M* aqueous sodium sulphate solution (6:4) as the mobile phases for analytical HPLC and for preparative HPLC, respectively, at a flow-rate of 1.3 ml/min.

RESULTS AND DISCUSSION

Analytical HPLC conditions for the components of BC

The best result was obtained by elution with methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4) on a Capcell Pak C_{18} column. This is the first time that the components of BC have been completely separated into 22 peaks using an isocratic solvent system. A typical separation under the optimum conditions is illustrated in Fig. 2.

Various experimental results are discussed below using chromatographic behaviour of ten main components (peaks 11–18, 20 and 22 in Fig. 2).

Comparison of HPLC columns. We expected that capsule type and polymer type columns would give satisfactory results because their residual silanol groups are either covered with silicone polymer or essentially absent. The suitability of Capcell

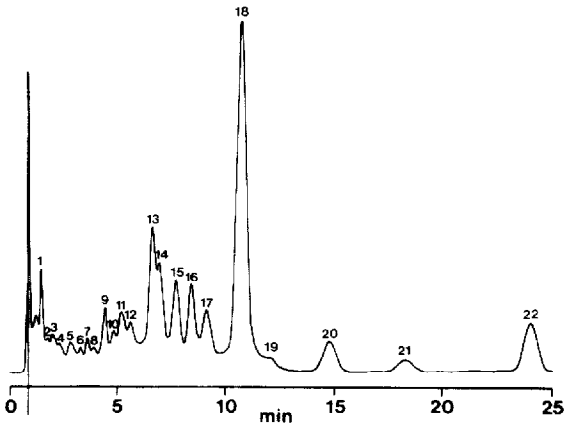

Fig. 2. Separation of the components of bacitracin using analytical HPLC conditions. Column: Capcell Pak C_{18} (5 μm, 150 mm × 4.6 mm I.D.). Mobile phase: methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4). Flow-rate: 1.3 ml/min. Detection: 234 nm. Peak identity: 18, BC-A; 22, BC-F.

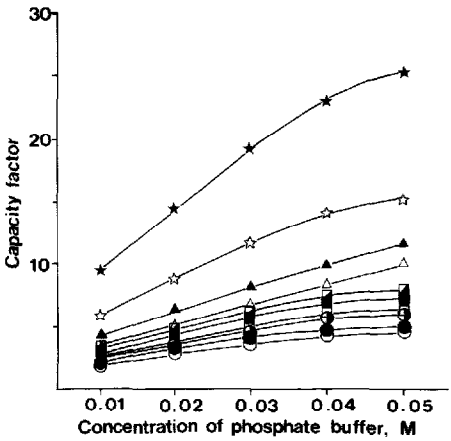

Fig. 3. Influence of disodium hydrogenphosphate concentration on the capacity factor. Column as in Fig. 2. Mobile phase: methanol–aqueous disodium hydrogenphosphate solution (pH 9.4) (6:4). Flow-rate and detection as in Fig. 2. Symbols: ○ = peak 11; ● = peak 12; ◐ = peak 13; □ = peak 14; ■ = peak 15; ◪ = peak 16; △ = peak 17; ▲ = peak 18 (BC-A); ☆ = peak 20; ★ = peak 22 (BC-F).

Pak C_8 and C_{18} as capsule type columns, and Asahipak ODP-50 and TSK gel Octadecyl-4PW as polymer type columns, for the analysis of the components was examined using methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4) as the mobile phase. Among these four columns, Capcell Pak C_{18} yielded 22 peaks with excellent resolution, whereas Capcell Pak C_8 and Asahipak ODP-50 columns produced 20 peaks, and TSK gel Octadecyl-4PW produced one broad peak. On the basis of these findings, we considered Capcell Pak C_{18} as the best choice in subsequent experiments.

Addition of disodium hydrogenphosphate. Because use of a mobile phase containing inorganic salt sometimes improves peak resolution in RP chromatography[20], we added disodium hydrogenphosphate to various mobile phases. Using methanol–*x M* aqueous disodium hydrogenphosphate solution (pH 9.4) (6:4) the influence of the concentration of the salt on the separation and resolution was investigated. Low resolution peaks of the components appeared on the chromatogram using disodium hydrogenphosphate-free mobile phase; however, the components were clearly resolved above 0.01 *M*. The capacity factors (k'), of the components show (Fig. 3) that the separation, especially among peaks 11–17, is improved with increasing salt concentration. Optimum separation is obtained above 0.04 *M*, but when 0.05 *M* is used the retention time of BC-F is too long. Accordingly, we chose 0.04 *M* aqueous disodium hydrogenphosphate solution in this study.

Influence of pH of aqueous disodium hydrogenphosphate solution. The influence of the pH of the aqueous disodium hydrogenphosphate solution on k' and the peak resolution of the components of BC were investigated using methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4). In general, conventional C_{18} silica gel is used under acidic conditions, because it is unstable under basic conditions. However, since the Capcell Pak C_{18} column can be used under basic conditions[18], the pH was varied between 2.0 and 10.0. The pH of the aqueous solutions was adjusted

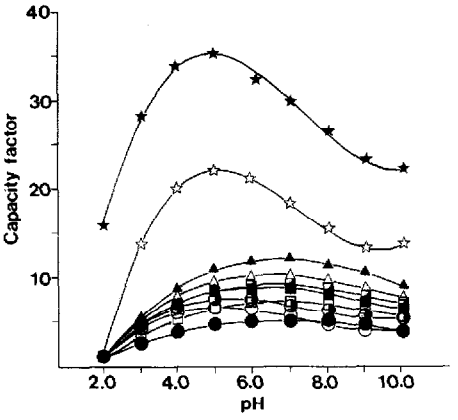

Fig. 4. Influence of pH of aqueous disodium hydrogenphosphate solution on the capacity factor. Column: as in Fig. 2. Mobile phase: methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4). Flow-rate and detection as in Fig. 2. Symbols as in Fig. 3.

with 0.04 *M* aqueous phosphoric acid and 0.04 *M* aqueous sodium hydroxide solutions. The k' of the components show that peak separation is improved with increasing pH of the mobile phase (Fig. 4). Satisfactory separation and resolution occur between pH values of 8.0 and 10.0. Peak 22 has too long a retention time below pH 9.0. Because the pH of 0.04 *M* aqueous disodium hydrogenphosphate solution is 9.4, we did not adjust the pH value in our subsequent work.

Proportion of methanol and aqueous disodium hydrogenphosphate solution. The ratio of the organic solvent and aqueous solution is one of the most important parameters in RP chromatography[21]. As the organic solvent we used methanol which produced better separations than acetonitrile. Various ratios of methanol and 0.04 *M* aqueous disodium hydrogenphosphate solution as mobile phases were examined to

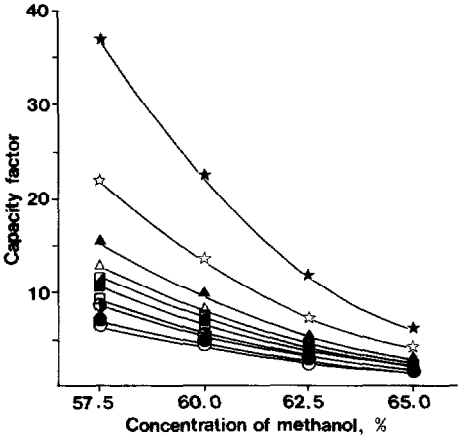

Fig. 5. Effect of the ratio of methanol and aqueous disodium hydrogenphosphate solution on the capacity factor. Column as in Fig. 2. Mobile phase: mixtures of methanol and 0.04 *M* aqueous disodium hydrogenphosphate solution. Flow-rate and detection as in Fig. 2. Symbols as in Fig. 3.

obtain the optimum proportion. Concentrations of methanol higher than 60% in the mobile phase result in unsatisfactory separation of BC components. The best separation was obtained below 60% (Fig. 5). Because a long analytical time was needed to elute all components using 57.5% methanol in the mobile phase, we recommend methanol–0.04 *M* aqueous disodium hydrogenphosphate solution (6:4) as the mobile phase.

Determination of BC-A and -F. It was reported that BC-A is unstable in solution[16], so we investigated the stability of components of BC during chromatography under the present HPLC conditions. After standing of BC in the mobile phase at 25°C for 30, 60, 90, 120, 150, 180 and 210 min, these samples were injected into the HPLC system and the peak heights of the components of BC were compared. No change in peak heights was observed within 90 min. Therefore, we used the present HPLC system for the quantitative analysis of BC-A and -F obtained as described in the next section. With purified samples, linear relationships were found between 50 and 1000 ng ($y = 0.055x + 2.76$) for BC-A and between 65 and 1000 ng ($y = 0.015x + 4.02$) BC-F.

Preparative HPLC conditions for the components of BC

In order to isolate the components, preparative HPLC was attempted using the above analytical HPLC conditions. However, it was impossible to concentrate the chromatographic fractions, because BC-A was decomposed by disodium hydrogenphosphate during evaporation. Therefore, we tried to develop preparative HPLC conditions using a suitable mobile phase which can preserve all components of BC during evaporation as judged by the changes in peak height. As mentioned above, if disodium hydrogenphosphate is eliminated from the mobile phase, the chromatography produced low resolution peaks, probably due to dissociation of amino groups in BC. Addition of neutral and inert inorganic salt to the solvent system is sometimes effective for control of the dissociation of analyte[22], so mobile phases containing several neutral and inert salts (potassium and sodium chloride or sulphate) instead of disodium hydrogenphosphate were tried on a Capcell Pak C_{18} column using methanol–0.05 *M* aqueous salt solution (6:4). With sodium and potassium chloride there were no clearly resolved peaks. We could not make a mixture of methanol and 0.05 *M* potassium sulphate (6:4) because of precipitation of the latter. However, addition of sodium sulphate to the mobile phase resulted in good separation. As shown in Fig. 6, although it shows lower resolution in comparison with that with the mobile phase containing disodium hydrogenphosphate, we consider that a mobile phase containing sodium sulphate is the most suitable for the preparative HPLC of the components, because the components were not decomposed in the mobile phase during evaporation.

Finally, we isolated the major peak (peak 18) and one of minor peaks (peak 22) using the present preparative HPLC conditions followed by chromatography on a prepacked C_{18} cartridge. Peaks 18 and 22 were fractionated, and after concentration below 30°C, the desalting was carried out using a prepacked C_{18} cartridge. The solution was applied to a Baker 10 C_{18} cartridge activated with methanol and water, and the cartridge was washed with 10 ml of water. The components were eluted with 10 ml of methanol and were evaporated under vacuum below 30°C. Purified peaks 18 and 22 were analysed using the analytical HPLC described above, which showed that

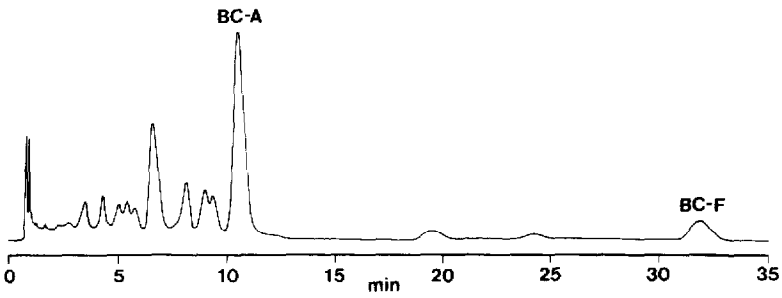

Fig. 6. Separation of the components of bacitracin using preparative HPLC conditions. Column as in Fig. 2. Mobile phase: methanol–0.05 *M* aqueous sodium sulphate solution (6:4). Flow-rate and detection as in Fig. 2.

both components were isolated without any decomposition. Peaks 18 and 22 were finally identified as BC-A and -F, respectively[16], by secondary ion mass spectrometry which produced a strong protonated molecule, $(M+H)^+$ (*m/z* 1422 for BC-A and *m/z* 1419 for BC-F) indicating that minor decomposition products are not coeluted with intact BC-A or -F in this HPLC system. Therefore, we recommend a combination of a Capcell Pak C_{18} column and methanol–0.05 *M* aqueous sodium sulphate solution (6:4) for the preparative HPLC of the components of BC.

CONCLUSIONS

The analytical and preparative HPLC methods for the identification and isolation of BC components were first established using an isocratic mobile phase on a capsule type C_{18} modified silica gel column (Capcell Pak C_{18}). These HPLC methods have the following characteristics. The best separation was obtained by the combination of a Capcell Pak C_{18} column and a methanol–0.04 *M* aqueous disodium phosphate solution (6:4) as a mobile phase. By this system the components of BC were successfully separated within 25 min and the calibration graphs showed good linear relationships between 50 and 1000 ng for BC-A and between 65 and 1000 ng for BC-F. With respect to the preparative HPLC, optimum conditions were found on a Capcell Pak C_{18} column using methanol–0.05 *M* aqueous sodium sulphate solution (6:4) as the mobile phase. The isolation of BC-A and -F was readily achieved without any decomposition of the components by the preparative HPLC conditions. Therefore, we recommend these HPLC methods for analytical and preparative separation of BC. At present we are attempting to characterize the structures of the components of BC and to develop a sample preparation system using a prepacked cartridge for detection of BC in feed and biological samples. These results will be reported elsewhere in the near future.

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