Note

Improvement of chemical analysis of antibiotics

XIV*. Identification of the components of bacitracin using normal- and reversed-phase thin-layer chromatography

HISAO OKA, YOSHITOMO IKAI, NORIHISA KAWAMURA and MASUO YAMADA

Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462 (Japan)

and

KEN-ICHI HARADA*, YUMIKO YAMAZAKI and MAKOTO SUZUKI

Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468 (Japan)

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Bacitracin (BC) is a basic and cyclic peptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis*[1]. Commercial BC consists of various components that have different antimicrobial activities, and their proportions are not always constant. The components have been classified into BC-A, -B, -C, -D, -E, -F and -G based on their UV spectra[2,3]. The major antimicrobial active components are BC-A and -B and major degradation product is BC-F, which is devoid of antimicrobial activity and also shows nephrotoxicity[1]. The structures of the components have not been clarified, except for BC-A and -F.

Although the analysis of BC has been often carried out by bioassays, we considered that a chemical method would be more suitable because it would be possible to analyse each component of BC, including deactivated ones such as BC-F. For chemical analysis high-performance liquid chromatographic (HPLC)[3-7], paper chromatographic (PC)[8] and thin-layer chromatographic (TLC)[8-16] methods have been reported. Most reversed-phase (RP) HPLC methods use solvent gradient systems with consequent difficulties in reproducibility and the ion-exchange HPLC method gave a poorer resolution of the components of BC than did RP-HPLC. PC is time consuming and gives poor resolution. In general, TLC is a simple and inexpensive technique, and a number of workers have tried to separate the components of BC using adsorbent layers of silica gel[8-12], cellulose[10,14], ion-exchange resin[15] and RP-type silica gel[16]. Although many components exists in BC, the previously reported TLC methods using silica gel, cellulose and ion-exchange showed only one or two spots on the TLC plates[8-15]. Aszalos and Aquilar[16] analysed only BC-A on an RP-type TLC plate but we obtained a poor resolution of the components of BC under their TLC conditions.

For the identification of the components of BC, we considered that the combined use of different modes such as normal-phase (NP) and RP- chromatography would be the most suitable approach, because BC contains various components as

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mentioned above. Therefore, we attempted to establish simple and reliable methods using NP- and RP-TLC for the components of BC.

EXPERIMENTAL

Materials

Chloroform, methanol, isopropanol, ethyl acetate, *n*-butanol, ethanol, acetic acid, trifluoroacetic acid, trichloroacetic acid, phosphoric acid, oxalic acid, sodium hydroxide, aqueous ammonia, triethylamine, zinc chloride, calcium chloride, potassium sulphate, sodium sulphate, magnesium chloride and ninhydrin were analytical-reagent grade chemicals. Bacitracin was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). TLC plates precoated with silica gel and C_8- and C_{18}-modified silica gel (E. Merck, HPTLC plates 15696, 13725 and 13724, respectively) were used.

Bacitracin solution

BC (100 mg) was weighed accurately into a 10-ml volumetric flask and diluted to volume with methanol.

Thin-layer chromatography

Solvent systems in the optimization step. Various solvent systems were prepared by mixing chloroform, methanol and aqueous acetic acid for NP-TLC and by mixing methanol, various salt solutions (potassium sulphate, sodium sulphate, calcium chloride, magnesium chloride, manganese chloride and zinc chloride) and triethylamine for RP-TLC.

Optimal solvent system in NP-TLC. Chloroform–methanol–0.75% aqueous acetic acid (30:20:4) was used.

Optimal solvent system in RP-TLC. Methanol–0.1 *M* aqueous potassium sulphate solution (pH 2.0) (7:3) containing 1.0% triethylamine was used. The solvent system was prepared by the following procedure; 1 g of triethylamine was dissolved in 30 ml of 0.1 *M* aqueous potassium sulphate solution and the pH value of the solution was adjusted to 2.0 with phosphoric acid and then 70 ml of methanol was added to the solution.

Development. After applying 1 μl of the BC solution to a TLC plate, chromatography was carried out by placing the plate in a 24 × 11 × 20 cm glass chamber at 25°C. The tank was saturated with the respective solvent system for 30 min before introducing the plate.

Detection of bacitracin. The TLC plate was sprayed evenly with 0.5% ninhydrin in *n*-butanol and then heated at 120°C to produce coloured spots.

RESULTS AND DISCUSSION

BC-A and -F are the major antimicrobial and nephtrotoxic components[1], respectively, and the structures of the other components have not yet been clarified. Therefore, the separation of BC-A and -F from the other components was investigated.

Normal-phase TLC

For NP-TLC, eight spots of the components of BC were observed on a silica gel HPTLC plate using chloroform–methanol–0.75% aqueous acetic acid (30:20:4) as the mobile phase. A typical separation obtained using the optimal conditions is illustrated in Fig. 1A. Various examinations were carried out to obtain these optimal conditions on the silica gel TLC plate, but we describe mainly the optimization of the concentration of acetic acid in the solvent system below.

In order to optimize the concentration of acetic acid in the aqueous solution, using chloroform–methanol–aqueous acetic acid (30:20:4) the influence of the acid concentration on the R_F values and the shape of the spots was examined. When 0.75% aqueous acetic acid was used, the most suitable R_F values and a good shape of the spots were obtained. Therefore, we chose chloroform–methanol–0.75% aqueous acetic acid (30:20:4) as the optimal solvent system with a silica gel TLC plate to separate the components.

RP-TLC

Using RP-TLC, as shown in Fig. 1B the components of BC were separated into ten spots on a C_8 TLC plate using methanol–0.1 M aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system.

Comparison of C_8 and C_{18} plates. The suitability of C_8 and C_{18} TLC plates for the separation of the components of BC was tested using methanol–0.1 M aqueous potassium sulphate solution (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system. When a C_{18} TLC plate was used, two spots with $R_F \approx 0.6$ showed tailing and overlapping. With a C_8 TLC plate, the components that overlapped on the C_{18} TLC plate were separated into two spots, so that we could observe ten spots of the components. Accordingly, we chose the C_8 TLC plate for subsequent work.

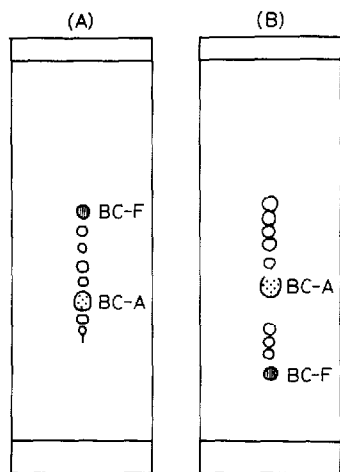*Addition of triethylamine.* In general, BC gives extremely tailing spots on RP-

Fig. 1. Separation of the components of bacitracin. (A) Silica gel TLC plate; solvent system, chloroform–methanol–0.75% acetic acid (30:20:4); (B) C_8 TLC plate; solvent system, methanol–0.1 M potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine.
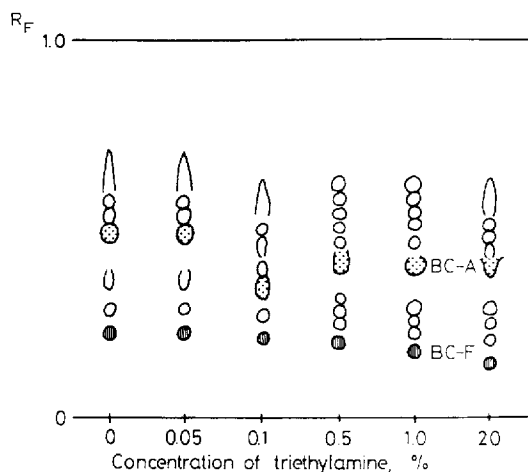
Fig. 2. Effect of triethylamine concentration in RP-TLC. C_8 TLC plate; solvent system. methanol 0.1 M potassium sulphate (pH 2.0) (7:3) containing 0 2% of triethylamine.

TLC plates. We tried using a solvent system containing triethylamine[17] to avoid this tailing. Using methanol–0.1 M aqueous potassium sulphate (pH 2.0) (7:3) containing triethylamine as the solvent system. the influence of the triethylamine concentration on the separation and the shape of spots was examined. As shown in Fig. 2, only seven spots with tailing appear on the chromatogram using a triethylamine-free solvent system, but the separation and the shape of the spots are improved with increasing concentration of triethylamine and satisfactory results were obtained between 0.5 and 1.5%. However, because the spots showed tailing when triethylamine concentrations above 1.5% were used. we chose to use 1.0% triethylamine in the solvent system in subsequent work.
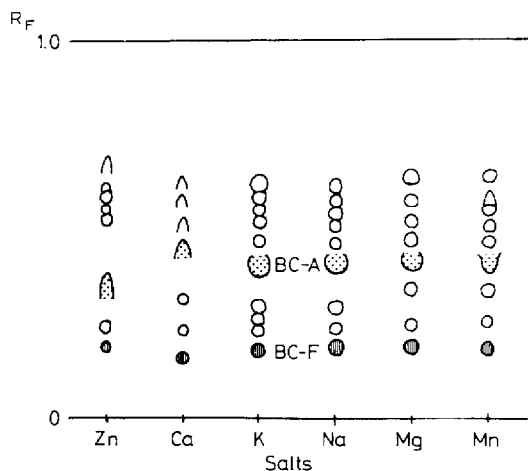

Fig. 3. Comparison of salts in the solvent system on the resolution of the spots. C_8 TLC plate; solvent system, methanol–0.1 M salt (pH 2.0) (7:3) containing 1.0% of triethylamine.

Addition of salt. Effect of the addition of zinc chloride, calcium chloride, potassium sulphate, sodium sulphate, magnesium chloride and manganese chloride on the separation and the shape of the spots was examined using methanol–0.1 *M* aqueous salt solution (pH 2.0) (7:3) containing 1.0% of triethylamine. As shown in Fig. 3, only when potassium sulphate was used we observe ten spots of the components on the plate. Next, the optimization of the concentration of aqueous potassium sulphate was investigated using methanol–aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% triethylamine. The best separation was obtained when a 0.1 *M* aqueous solution is used and we therefore chose 0.1 *M* aqueous potassium sulphate for subsequent work.

pH of aqueous solution. The influence of the pH of the aqueous solution on the separation and the shape of spots was investigated using methanol–0.1 *M* aqueous potassium sulphate (7:3) containing 1.0% triethylamine. The pH values were varied with 85% phosphoric acid as described under Experimental. The best separation of the components was given at pH 2.0 (Fig. 4).

Proportions of methanol and aqueous potassium sulphate solution. We investigated the influence of the proportions of methanol and aqueous potassium solution on the separation of the components using various mixtures of methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) containing 1.0% of triethylamine as solvent systems. As shown in Fig. 5, the best separation was achieved when methanol and the aqueous solution were in the ratio 7:3. We therefore recommend methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system for separating the components of BC on a C_8 HPTLC plate.

CONCLUSION

A technique for the identification of components of BC using NP- and RP-TLC plates has been established with the following characteristics. For NP-TLC, a solvent
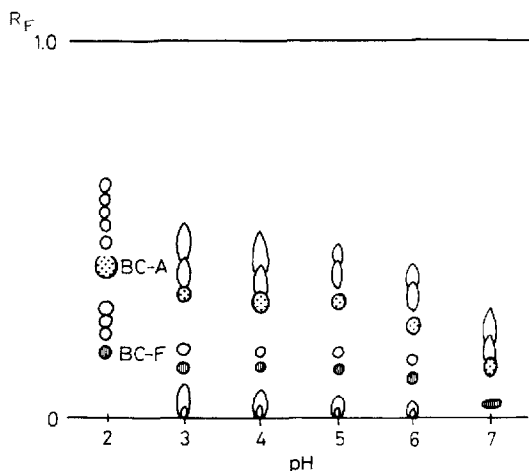

Fig. 4. Influence of pH of aqueous potassium sulphate solution. C_8 TLC plate; solvent system, methanol–0.1 *M* potassium sulphate (7:3) containing 1.0% of triethylamine.
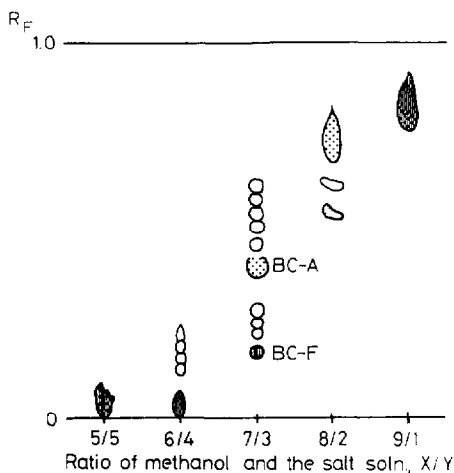
Fig. 5. Influence of ratio of methanol and 0.1 M aqueous potassium sulphate. C_8 TLC plate; solvent system, methanol–0.1 M potassium sulphate (pH 2.0) (X:Y) containing 1.0% of triethylamine.

system containing acetic acid made possible a reliable separation of the components of BC. The separation of the spots was dependent on the concentration of acetic acid and good separation was obtained at 0.75%. A combination of a silica gel TLC plate and chloroform–methanol–aqueous acetic acid (30:20:4) as the solvent system gave a satisfactory separation of the components. With respect to RP-TLC, a solvent system containing triethylamine and potassium sulphate gave a reliable separation of components. The separation and the shape of the spots were improved with increasing potassium sulphate and triethylamine concentrations and good results were obtained above 0.1 M and 1.0%, respectively. A satisfactory separation was achieved on a C_8 TLC plate using methanol–0.1 M aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system. Both TLC methods gave better separation of the components of BC than previously reported TLC methods. Because with this combination of NP- and RP-TLC techniques the identification of the components was able to be carried out simply, especially for BC-A and -F, we intend to apply it to the study of the structural characterization of the components of BC in the near future.

ACKNOWLEDGEMENT

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