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Preparative high-performance liquid chromatographic separation and isolation of bacitracin components and their relationship to microbiological activity

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ABSTRACT

Bacitracin, a polypeptide antibiotic, is one of the most commonly used antibiotics in the world. The approved method of analysis for bacitracin is microbial. To correlate the microbiological method with a high-performance liquid chromatographic (HPLC) method, bacitracin was chromatographed using HPLC with ultraviolet detection and a YMC basic column. Adequate separation of the isomers was obtained to scale up this procedure to preparative HPLC using a Prep HPLC system and a 250 × 21 mm YMC basic column. The various fractions were separated, isolated and examined for microbial activity. The individual fractions could be precipitated by adding zinc or methylene disalicylic acid and lowering the pH. The crude fractions were recycled to ensure chromatographic purity. The chromatograms can accurately predict (in minutes) the microbiologically determined potency which usually takes 16–24 h to develop. The chromatographic procedure also provides information on the amounts of isomers and degradation products present in the sample, whereas the microbiological assay only provides activities or potencies of the antibiotic. The reported HPLC method also possesses some advantages over some other published HPLC methods in terms of accuracy and time of analysis.

INTRODUCTION

Bacitracin, a polypeptide antibiotic produced by strains of *Bacillus licheniformis* and *B. subtilus*, is one of the most commonly used antibiotics in the world, especially as an animal feed additive [1,2]. Actually, the various products generally referred to as "bacitracin" are mixtures of similar polypeptides which may differ by only one amino acid. These similar polypeptides have been given the designations A, A_1, B, B_1, B_2, C, D, E, F_1, F_2, F_3, G and X [3–8]. Bacitracin A is of primary importance and is highly biologically active (Fig. 1). Bacitracin B differs from A by the replacement of isoleucine with valine, although the exact position of the replacement is not clear. Bacitracins C, D and E are active, but less so than A or B. Bacitracin F (Fig. 1) is the oxidative deaminated compound containing a ketothiazole instead of an aminothiazoline moiety [9,10].

Traditionally, microbiological methods [6,11,12] have been used for the qualitative and quantitative determination of bacitracin, although counter-current distribution [13,14] and column chromatography have also been used [15,16]. Tsuji *et al.* [8] initially developed a high-performance liquid chromatographic (HPLC) method using gradient elution for the separation of bacitracin. By comparing the microbial and HPLC values for bacitracin A and B, the HPLC method was further improved by Tsuji and Robertson [4]. Gallagher *et al.* [17] have developed an isocratic method which has also been accepted by the AOAC as an official procedure for bacitracin [18]. Recently Pavli and co-workers [19–21] and Oka *et al.* [22] have described isocratic methods for the separation and quantitation of bacitracin components utilizing silica-based or polymer reversed-phase columns.

The above methods provide a great advantage over the microbial method of analysis for bacitracin

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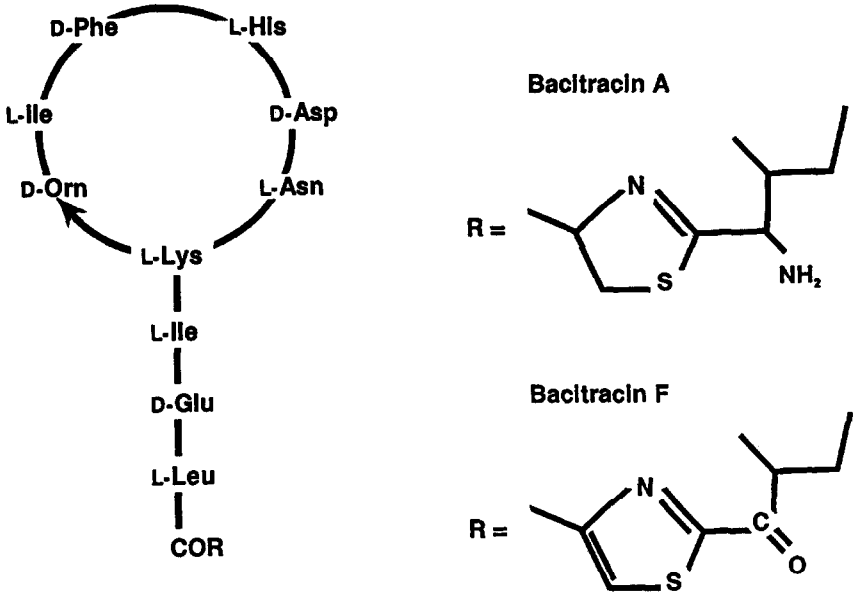

Fig. 1. Structures of bacitracins A and F.

in terms of time of analysis and specificity. The approved microbial method requires 16–24 to develop and is not able to identify and/or quantitate the degradative bacitracin F components. The above HPLC methods only use the bacitracin A and B fractions to quantitate the total microbial activity of bacitracin neglecting the minor components, which, depending on the source and starting materials of the fermentation process, may contribute significantly to microbial activity. The feed industry produces thousands of pounds of bacitracin daily. Determining the potency of bacitracin by HPLC allows constant monitoring of the fermentation process, as well as aiding in blending and quality control of bagging. This allows production to produce the antibiotic on a continual basis, assured that the previous lots were in specification and in good agreement with the pending microbiological assay. In order to provide a rapid, reliable and reproducible HPLC method that will successfully predict, within 2%, the microbiologically determined potency of bacitracin, the components of bacitracin need to be separated analytically, scaled up to the preparative mode for isolation and subsequent microbial potency determination of the isolates. These crude fractions are recycled to ensure chromatographic purity. This information is entered into the integration scheme and, with mobile phase adjustment, a rapid and reproducible analytical HPLC method can be developed that will successfully determine the microbial activity of bacitracin.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a modified Shimadzu chromatographic SCL-6B system controller, LC-6A analytical pumps, LC-8A preparatory pumps, SIL-6B injector capable of delivering accurately 1 μl to 2 ml, a SPD-6AV detector with analytical and preparative flow cells and a SIL-601 integrator.

Stainless-steel columns (250 × 4.6 mm I.D. and 250 × 21 mm I.D.) prepacked with YMC basic (C_8, 200 Å, 5 μm); YMC, Morris Plains, NJ, USA) were used for the analytical and preparative separations, respectively.

The mobile phase for the isocratic separation consisted of HPLC-grade methanol–50 m*M* potassium dihydrogenphosphate buffer (pH 6.5) (59:41), made with distilled, deionized water.

The mobile phase for gradient elution consisted of HPLC-grade methanol (pump A) and 50 m*M* potassium dihydrogenphosphate (pH 6.5) (pump

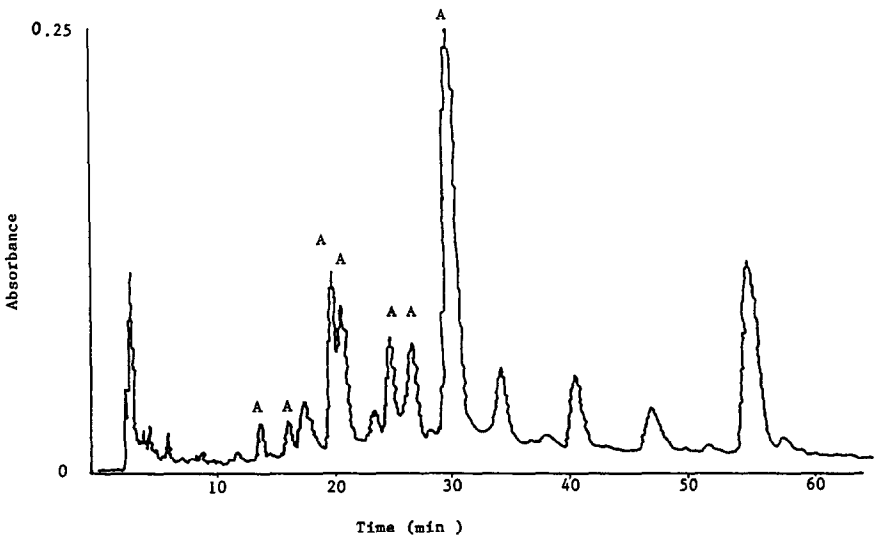

Fig. 2. HPLC profile of the separation of the isomers of bacitracin. A YMC basic column (250 × 4.6 mm I.D.) was used with a linear gradient from 57 to 63% methanol (pump A) and 43 to 37% 50 m*M* phosphate buffer (pH = 6.5) (pump B). Flow-rate, 1 ml/min. An "A" designates microbial activity.

B), made with distilled, deionized water. A linear gradient was programmed (57% A initial, 63% A final) for 1 h.

Preparation of bacitracin standards and samples

Approximately 1 g of zinc bacitracin USP reference standard (U.S.P.C., Rockville, MD, USA) was dissolved in 100 ml of acidic methanol solution (20 m*M* hydrochloric acid in 80% methanol). The solution was mixed, vortexed, centrifuged and filtered (0.45 μm). Samples of bacitracin were prepared in the same manner.

Chromatographic conditions

The column temperature was 25°C. Injection volumes were 100 μl and 2 ml for the analytical and preparative procedures, respectively. The flow-rates were 1 ml/min for the analytical procedure and 20 ml/min for the preparative procedure. The detector used both analytical and preparative flow cells that were monitored at 215 nm.

Microbiological conditions

Bacitracin potency was determined microbiologically following the USP XXII <81> procedure [12]. The cylinder plate assay was used utilizing *Micrococcus luteus* as the test organism. After incubation, the plates were analyzed using a computerized zone reader which consisted of a video imager that, in conjunction with a computer, will construct a linear curve for five standards and calculate the concentration of the unknown by imaging the zone of inhibition and comparing it to the standard curve.

RESULTS AND DISCUSSION

The chromatographic profile of bacitracin using gradient elution is shown in Fig. 2. Good separation exists for the isomer using these conditions. Heart cuts of each of these peaks on a preparative scale provide crude preparations of the bacitracin fractions. These crude fractions were recycled to improve their chromatographic purity. The methanol–phosphate mobile phase used for the chromatographic separations is ideally suited for microbiological plating without further modification. The crude fractions can be precipitated by adding zinc chloride or methylene disalicyclic acid and adjusting the pH accordingly. The gradient procedure is not ideally suited for routine analysis. Time of analysis and reequilibration hamper this procedure. An isocratic mobile phase consisting of methanol–phosphate buffer (59:41) was developed. Adequate separation of the isomers exists for scale up to pre-

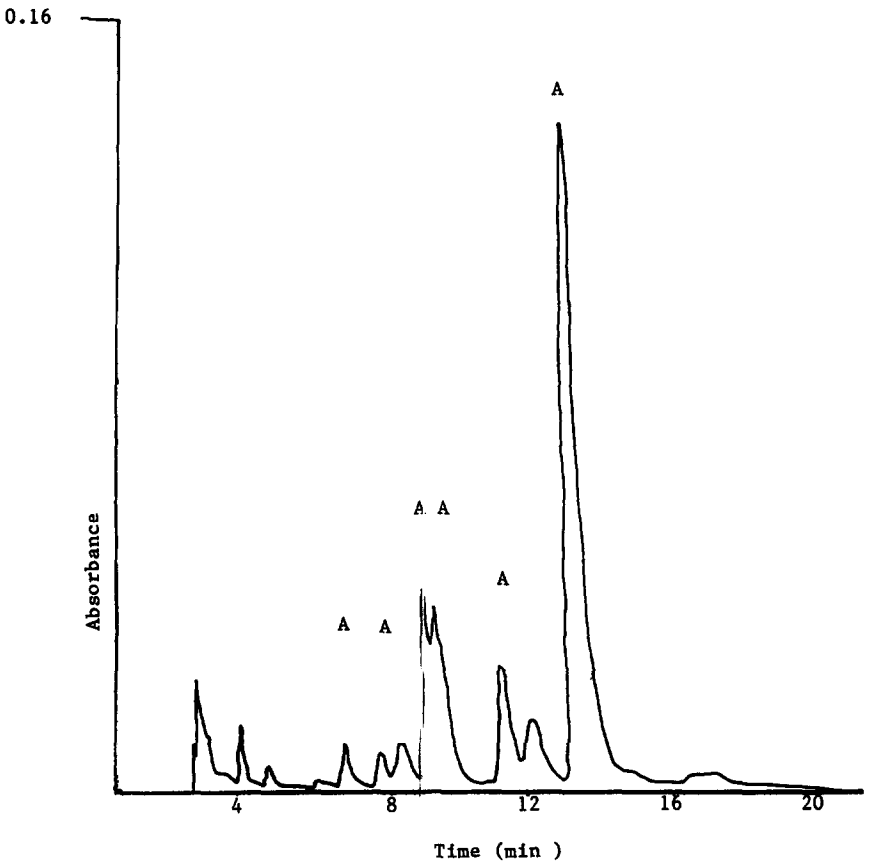

Fig. 3. Prep HPLC profile of the separation of bacitracin isomers. A YMC column (250 × 21 mm I.D.) was used with a methanol–50 m*M* phosphate buffer (pH 6.5) (59:41). Flow-rate 20 ml/min. An "A" designates microbial activity.

parative HPLC that allows all of the isomers to elute within 22 min. Scale up considerations for the flow-rate and sample load were as follows:

$$\text{flow-rate} = \text{flow-rate}_{\text{anal}} \cdot \frac{(D_{\text{prep}})^2}{(D_{\text{anal}})^2}$$

$$\text{sample load} = \text{load}_{\text{anal}} \cdot \frac{(D_{\text{prep}})^2 \, L_{\text{prep}}}{(D_{\text{anal}})^2 \, L_{\text{anal}}}$$

where D and L are the diameter and length, respectively. The conversion from analytical to preparative chromatography in this situaton involved approximately a twenty-fold scale factor. The linear range for this assay is 10–2000 I.U. (68.9 I.U./mg).

Fig. 3 illustrates the preparative chromatogram of bacitracin. There is excellent scale up between the analytical and the preparative procedure. This is due in part to the identical lots of packing material used for both the analytical and preparative columns, thus reducing the chances of lot-to-lot variability. The collected fractions were immediately plated to determine their microbiological potency. (An "A" indicates microbial activity as shown in Figs. 2 and 3.) Accurate assessment of the identities of the individual peak were not possible except for the A and F fractions. A corrected activity was calculated for the active peaks by determining their individual potencies, chromatographic area and amount injected [activity (U)/area/weight]. A response factor was then calculated relative to bacitracin A. The corrected responses were approximately equal, which allows the peak areas of the active peaks to approximate bacitracin potency.

TABLE I

COMPARISON OF 50 g/lb BACITRACIN POTENCIES AS DETERMINED MICROBIOLOGICALLY AND BY HPLC

Method	Concentration (g/lb)		R.S.D. (%)	*n*
	Range	Mean		
Microbial	49.8–51.4	50.6	1.23	509
HPLC	48.8–50.8	50.0	0.94	509

Previous studies [4,17,21] only use bacitracins A, B and B_2 to estimate the potency of bacitracin. These peaks usually account for approximately 80–95% of the total area of active components. Comparison of their HPLC potencies range from 1 to 15% of the microbiologically determined potencies. Pavli and Sokolic [21] reported a range of 2–16% average difference between the HPLC and the microbial-determined bacitracin potency. The HPLC-determined potency was consistently lower than the microbial-determined potency. Tsuji *et al.* [8] reported a range of 2–7% average difference for the HPLC- and microbial-determined potencies. The HPLC-determined potencies tended to be higher than the microbial-determined potencies. Both studies involved a small number of samples and indicated that the HPLC-determined potency compared statistically to the microbial-determined potency. Table I shows the comparisons of HPLC-determined potencies for 509 different lots of bacitracin (50 g/lb) *versus* the microbiologically determined potencies. There is excellent agreement between the means of the two methods for a large number of samples. This agreement can be attributed to several factors. This chromtographic method takes into account all of the active peaks, not only bacitracin A_1, B_1 and B_2. The microbiological assay employs automated media preparation of the plating media and analyte, and a computerized zone reader utilizing linear regression analysis of the zones of inhibition, thus reducing some of the variability [manual reading involves technician reading errors, scale reading errors (± 1 mm) etc.] associated with microbial assays.

It is important to note that different producers of bacitracin will have unique profiles with differing microbial activities. For this system, to accurately assess the microbial potencies, the manufactured product as well as the USP bacitracin potencies of the individual active peaks must be examined.

The time of analysis can be shortened by adjusting the organic composition and the pH. Increasing the organic modifier or reducing the pH will result in shorter run times without compromising the accuracy of the assay. This affords the producer accurate determination of the potency of bacitracin in minutes, instead of 16–24 h that are needed for incubation and development of the microbial assay. It also provides information on the degradation products of bacitracin that can be caused by production. The microbiological assay cannot. This procedure provides a more complete chromatographic profile than the other existing HPLC procedures, and shortening the column length to 150 mm and increasing the organic modifier by 2% will reduce the time of analysis to approximately 12 min this making this isocratic procedure quicker than the existing chromatographic procedures published to date. Time of analysis has been further shortened by the use of 50-mm columns packed with 3-μm particles, while still maintaining the resolution of the isomers.

CONCLUSIONS

This chromatographic procedure possesses several advantages over the existing HPLC procedures for bacitracin. All of the microbiologically active peaks are considered for potency determination, not only the A and B peaks. The time of analysis is considerably shorter than those of the other HPLC procedures. This HPLC-determined potency of bacitracin has better agreement with the microbiologically determined potency than the other HPLC procedures for bacitracin. This HPLC procedure and the other existing HPLC procedures provide information on the amounts of the individual components of bacitracin as well as information on the degradation and non-active components present in the sample. The HPLC procedures can accurately estimate bacitracin potency in minutes *versus* the 16–24 h needed for the microbiologically determined potency.

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REFERENCES

1 D. J. Hanson, *Chem. Eng. News*, 63 (1985) 7.
2 T. Yagasaki, *J. Food Hyg. Soc. Jpn.*, 27 (1986) 451.
3 G. G. F. Newton and E. P. Abraham, *Biochem. J.*, 53 (1953) 597–664.
4 K. Tsuji and J. H. Robertson, *J. Chromatogr.*, 112 (1975) 663–672.
5 O. Froyshov, in E. J. Vandamme (Editor), *Biotechnology of Industrial Antibiotics*, Marchel Dekker, New York, 1983, pp. 665–694.
6 G. A. Brewer, in K. Florey (Editor), *Analytical Profiles of Drug Substances*, Vol. 9, Academic Press, New York, London, 1980; pp. 1–69.
7 L. C. Craig and W. Konigsberg, *J. Org. Chem.*, 22 (1957) 1345–1353.
8 K. Tsuji, J. H. Robertson and J. A. Bach, *J Chromatogr.*, 99 (1974) 597–608.
9 E. Manekata, T. Shiba and T. Kaneko, *Bull Chem. Soc. Jpn.*, 46 (1973) 3835.
10 G. G. F. Newton, E. P. Abraham, H. W. Florey and N. Smith, *Br. J. Pharmacol.*, 6 (1951) 417.
11 *The British Pharmacopoeia 1980*, Vols. I and II, Department of Health and Social Security, London, 1980; pp. 42–43.
12 *The United States Pharmacopeia XXII*, National Formulary United States Pharmacopeial Convention, Rockville, MD 1989, pp. 1488–1493.
13 L. C. Craig, J. R. Weisiger, W. Hausmann and E. J. Harfenist, *J. Biol. Chem.*, 199 (1952) 259.
14 L. C. Craig, W. F. Phillips and M. Burachik, *Biochemistry*, 8 (1969) 2348.
15 W. Konigsberg and L. C. Craig, *J. Am. Chem. Soc.*, 81 (1959) 3452.
16 D. R. Storm and J. L. Strominger, *J. Biol. Chem.*, 248 (1973) 3940.
17 J. B. Gallagher P. W. Love and L. L. Knots, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 1178–1185.
18 W. Horwitz (Editor), *Official Methods of Analysis-AOAC*, 2004, The Association of Official Analytical Chemists, Washington, DC, 1982, pp. 491–493.
19 V. Pavli and J. Mohorič and R. Dobrovoljc, *8th Yugoslav Pharmaceutists Symposium, Bled, 1980*, Abstract, pp. 84–85.
20 V. Pavli and A. Krbavcic, *Sci. Pharm.*, 549 (1986) 293–294.
21 V. Pavli and M. Sokolic, *J. Liq. Chromatogr.*, 13 (1990) 303–318.
22 H. Oka, Y. Ikai N. Kawamura, M. Yamada, K. Harada, Y. Yamazaki and M. Suzuki, *J. Chromatogr.*, 462 (1989) 315–322.