*Journal of Pharmaceutical & Biomedical Analysis*
Vol. 9, Nos 10–12, pp. 843–847, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

# Separation and isolation of the isomers of bacitracin by high-performance liquid chromatography and their relationship to microbial activity*

ROBERT G. BELL

*A.L. Laboratories, Inc., 400 State Street, Chicago Heights, IL 60411, USA*

**Abstract**: Bacitracin, a polypeptide antibiotic produced from strains of *Bacillus licheniformis*, is one of the most commonly used antibiotics in the world. Actually, the various products generally referred to as 'bacitracin' are mixtures of similar polypeptides which may differ by only one amino acid. The approved method of analysis for bacitracin is microbial. To correlate the microbiological method with an HPLC method, bacitracin was chromatographed using a YMC basic column with UV detection.

Adequate separation of the isomers were obtained to scale up this procedure to preparative HPLC using a 250 × 21 mm YMC basic column. The various fractions were separated, isolated and examined for microbial activity. 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.

**Keywords**: *Bacitracin; reverse-phase HPLC; preparative HPLC.*

## Introduction

Bacitracin, a polypeptide antibiotic produced by strains of *Bacillus licheniformis* and *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 keto-thiazole instead of an amino-thiazoline moiety [9, 10].

Traditionally, microbiological methods [6, 11, 12] have been used for qualitative and quantitative evaluation of bacitracin, although countercurrent distribution [13, 14] and column chromatography has also been used [15, 16]. Tsuji *et al.* [8] initially developed an 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 AOAC as an official procedure for bacitracin [18]. Recently Pavli [19–21] and Oka *et al.* [22] have described isocratic methods for the separation and quantitation of bacitracin components utilizing silica-based or polymer reverse-phase columns.

The above methods provide a great advantage over the microbial method of analysis for bacitracin in terms of time of analysis and specificity. The approved microbial method requires 16–24 h 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 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

* Presented at the "Third International Symposium on Pharmaceutical and Biomedical Analysis", April 1991, Boston, MA, USA.

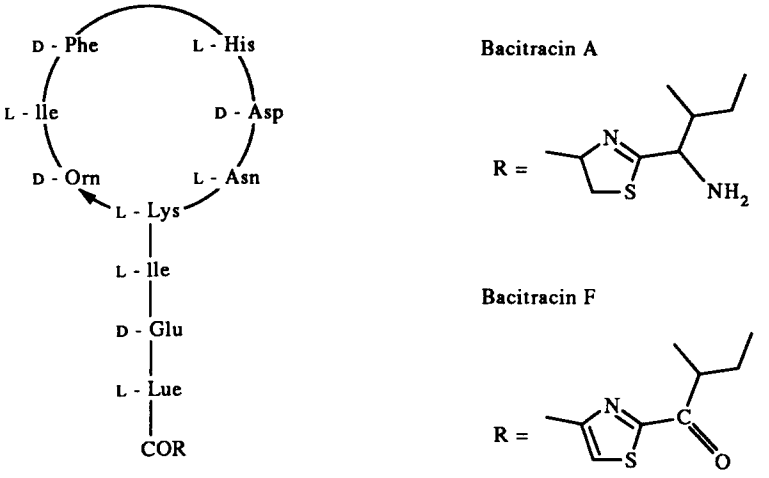


**Figure 1**
The structures of bacitracins A and F.

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 needed to be separated analytically, scaled up to semi-preparative mode for their isolation and subsequent microbial potency determination of the isolates. This information was entered into the integration scheme, and with mobile phase adjustment, a rapid analytical HPLC method was developed that successfully determined 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–2 ml, a SPD-6AV detector with analytical and preparative flow cells, and a SIL-601 integrator.

Stainless steel columns (250 × 4.6 mm, 250 × 21 mm) prepacked with YMC basic (YMC, Inc., Morris Plains, NJ, USA) were used for the analytical and preparative separations, respectively.

The mobile phase for the isocratic separation consisted of methanol–0.05 M potassium dihydrogen phosphate buffer, pH = 6.5 (59:41, v/v).

The mobile phase for gradient elution consisted of HPLC grade methanol (pump A) and 0.05 M potassium dihydrogen phosphate (pH = 6.5) (pump B), made with distilled, deionized water. A linear gradient was programmed (57% A initial, 63% A final) for 1 h.

*Procedure*

*Preparation of bacitracin.* Approximately 1 g of zinc bacitracin USP reference (U.S.P.C. Inc., Rockville, MD, USA) standard was dissolved in 100 ml of acidic methanol solution (20 mM HCl in 80% methanol). The solution was mixed, vortexed, centrifuged and filtered (0.45 μm).

*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 $min^{-1}$ 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

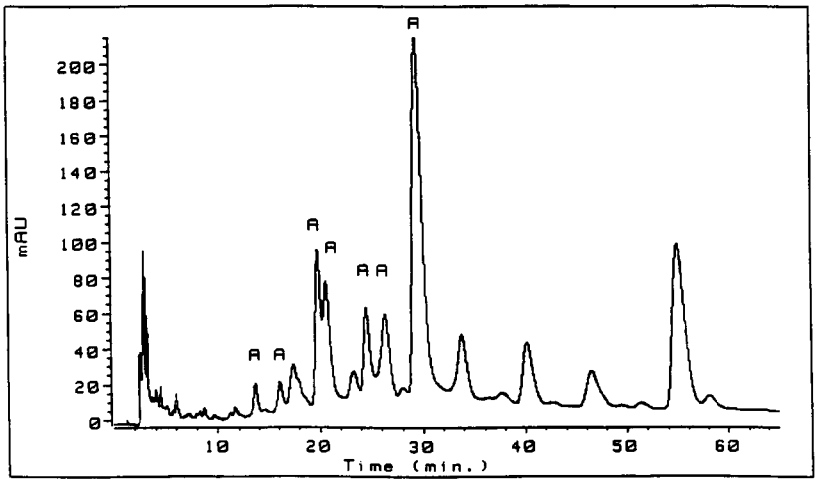


**Figure 2**
An HPLC chromatogram of the separation of the isomers of bacitracin. A YMC basic column (250 × 4.6 mm) was used with a linear gradient from 57 to 63% pump A (methanol) and 43–37% pump B (phosphate buffer:50 mM, pH = 6.5). Flow rate was 1 ml $min^{-1}$. An 'A' designates microbial activity.

incubation, the plates were analysed using a computerized zone reader.

## Results and Discussion

The chromatographic profile of bacitracin using gradient elution is shown in Fig. 2. Good separation exists for the isomers using these conditions. Heart cuts of each of these peaks on a preparative scale provide crude preparations of the bacitracin fractions. The methanol–phosphate mobile phase used for the chromatographic separations is ideally suited for microbiological plating without further modification. The gradient procedure is not ideally suited for routine analysis. Time of analysis and re-equilibration hamper this procedure. An isocratic mobile phase consisting of methanol–phosphate buffer (59:41, v/v) was developed. Adequate separation of the isomers exists for scale up to preparative HPLC. Scale up considerations for the flow rate and sample load were as follows:

$$\text{Flow rate} = \text{Flow rate anal} \times \frac{(D\ \text{prep})^2}{(D\ \text{anal})^2},$$

$$\text{Sample load} = \text{Load anal} \times \frac{(D\ \text{prep})^2\ L\ \text{prep}}{(D\ \text{anal})^2\ L\ \text{anal}},$$

where $D$ and $L$ are the diameter and length, respectively. The conversion from analytical to preparative chromatography in this situation involved approximately a 20-fold scale factor.

Figure 3 illlustrates 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 preparation columns, thus reducing the chances of lot-to-lot variability. The collected fractions were immediately plated to determine their microbiological potency. Accurate assessment of the identities of the individual peaks were not possible except for the A and F fraction. A corrected activity was calculated for the active peaks by

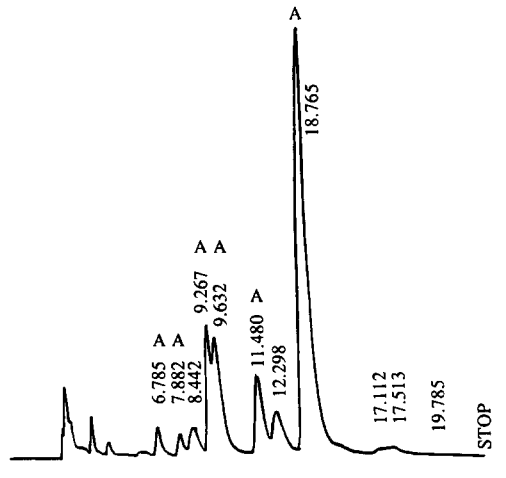


**Figure 3**
A preparative HPLC chromatogram of the separation of bacitracin isomers. A YMC basic column (250 × 21 mm) was used with a methanol–50 mM phosphate buffer (pH 6.5) (59:41, v/v). Flow rate was 20 ml $min^{-1}$. An 'A' designates microbial activity.

determining their individual potencies, chromatographic area and amount injected [Activity (units)/area/wt]. 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. Previous studies [4, 17, 21] only use bacitracins A, B, and $B_2$ to estimate the potency of bacitracin. These peaks usually account for ≈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 from 2 to 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 from 2 to 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 1 shows the comparisons of HPLC-determined potencies for 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 chromatographic method takes into account all of the active peaks, not just 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 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 determintion 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 do this. This procedure provides a more complete chromatographic profile than the other existing HPLC procedures, and by shortening the column length to 15 mm and increasing the organic modifier by 2%, will reduce the time of analysis to approximately 12 min. Time of analysis can be further shortened by the use of 50 mm columns packed with 3 μm particles.

**Table 1**
Comparison of bacitracin 50 g/lb potencies as determined microbiologically and by HPLC

| | Microbiologically | HPLC |
|---|---|---|
| $x$ | 50.6 g/lb | 50.0 |
| RSD | 1.23% | 0.94% |
| Range | 49.8–51.4 | 48.8–50.8 |
| $n$ | 509 | 509 |

*Acknowledgements* — The author wishes to thank A.L. Laboratories, Inc., Dr A. Hirsch, P. Arvia-Stonerock, K. Newman, L. Panozzo, M.K. Peterson and C. Reithel.

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*[Received for review 29 April 1991; revised manuscript received 3 June 1991]*