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Optimization of HPLC method for stability testing of bacitracin

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Abstract

A stability indicating HPLC assay for bacitracin has been developed and validated. The assay is based on a gradient elution, reversed phase column and UV diode array detection. On the basis of our previous analytical work several additional systematic HPLC tests for optimization of analytical method were performed. In order to achieve the highest selectivity of HPLC method, tests were conducted with extremely complex samples – zinc bacitracin feed grade as food additive for animals. The influence of pH of mobile phase and type of columns on chromatographic separation of active (A, B_1 and B_2) and inactive (F) polypeptide components of bacitracin were investigated in detail. It was found also that the peak B_1 comprises three and the peak F two subunits – probably isomers. The obtained analytical procedure proved to be very selective and effective for the simultaneous determination of active polypeptide A, B_1 and B_2, impurities, known and unknown degradation products and ballast material.

Keywords: Bacitracin; Feed grade; Reversed-phase HPLC; Step gradient elution

1. Introduction

The polypeptide antibiotic bacitracin (Fig. 1) produced by the strains of *Bacillus licheniformis* and *Bacillus subtilis* exhibits a potent antimicrobial activity primarily against Gram-positive bacteria [1,2]. It is one of the most important antibiotics used in human medicine, and one most commonly used in animal feed additives [1,3]. Bacitracin (Bc) is actually a mixture of several similar polypeptide components differing by some amino acids and is often available in the form of more stable salt with zinc (ZnBc) [4]. The components designated as A, B_1 and B_2 are known to be the most microbiologically active [5]. The main degradation product of bacitracin is an inactive component F (Fig. 1) which is known to be nephrotoxic. Bacitracin F is the oxidative deaminated derivative of bacitracin containing a keto-thiazole instead of an amino-thiazoline moiety [6].

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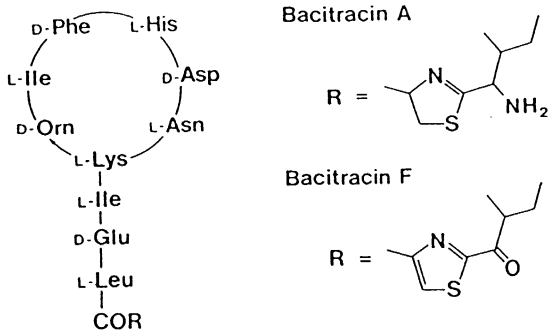

Fig. 1. The structures of bacitracin A and F.

Only the structures of bacitracin A and F have been determined to date [6]. The sequence of amino acids of bacitracin A and F is known (Fig. 1).

Along with traditional microbiological methods for qualitative and quantitative evaluation of bacitracin [2,7], several HPLC procedures were published using isocratic as well as gradient elution on silica or polymer based reverse phases [5,8–11]. Official analytical method for bacitracin determination, however, is a microbiological assay [2,7]. A more selective and rapid analytical method with degradation products separation would be preferred for product quality evaluation as well for stability studies. The aim of the study was to develop a stability-indicating HPLC method for the assay of bacitracin. This was achieved by a suitable modification of our method, which had been previously published [10]. Several changes were introduced based on screening several different stationary phases, optimization of the mobile phase pH and the addition of a step gradient approach. Good performance characteristics of the method were ascertained by method validation and subsequent accelerated stability study.

2. Materials and methods

2.1. Apparatus

HPLC system Hewlett Packard model 1050 controlled with Chemstation software consisted of a binary gradient pump, a diode array detector, an autosampler and a column heater.

2.2. Chemicals and reagents

KH_2PO_4 p.a. (J.T. Baker, NY,); water: deionized, distilled; acetonitrile, HPLC grade (Rathburn Wakerburn, Scotland); methanol, HPLC grade (Merck, Darmstadt, Germany); H_3PO_4, HPLC grade (Merck); KOH, p.a. (Merck); bacitracin (Fluka, Buchs, Switzerland); ZnBc reference standard, Lot No: 230548, declared activity 67.9 IU/mg (Krka d.d., Novo mesto, Slovenia) – dried before use in vacuum at 60°C for 3 h (USP 24); ZnBc feed grade 10% (Krka d.d.); selected HPLC columns: Nucleosil 5 μm C-18, 100 Å, 150 × 4.6 mm ID and Nucleosil 5 μm C-18, 100 Å, 250 × 4.6 mm ID (Macherey Nagel, Düren, Germany); Kromasil 5 μm C-8, 100 Å, 150 × 4.6 mm ID (Ekka Nobel, Surte, Sweden); Eurospher 5 μm C-18, 100 Å, 150 × 4.6 mm ID (Knauer, Berlin, Germany). All above-mentioned columns were packed by BIA d.o.o. Ljubljana, Slovenia.

2.3. Chromatographic conditions

Optimal mobile phase composition:

- *Mobile phase A*. Methanol–acetonitrile (1:1 v/v) – KH_2PO_4 (0.05 M, pH = 6.0): (49:51 v/v).
- *Mobile phase B*. Methanol–acetonitrile (1:1 v/v) – KH_2PO_4 (0.05 M, pH = 6.0): (60:40 v/v).

For the study of the impact of pH on chromatographic separation of bacitracin, water solution of 0.05 M KH_2PO_4 was adjusted to pH 2; 4; 6 and 8 with H_3PO_4 (10% v/v) and KOH (20%), respectively.

Flow rate: 1.4 ml/min; detection: UV, 254 nm (only occasionally at 230 nm); injecting: 10 μl; temperature: 30°C.

Gradient program. After injecting of the standard or sample solution in the equilibrated chromatographic system using 100% mobile phase A, the isocratic elution with mobile phase A was kept for 7.5 min. Then the content of mobile phase B was linearly increased to 100% in the time interval from 7.5 to 12 min. After isocratic elution of mobile phase B between 12.0 and 18.0 min the system was equilibrated again with 100% mobile phase A between 18.1 to 22.0 min.

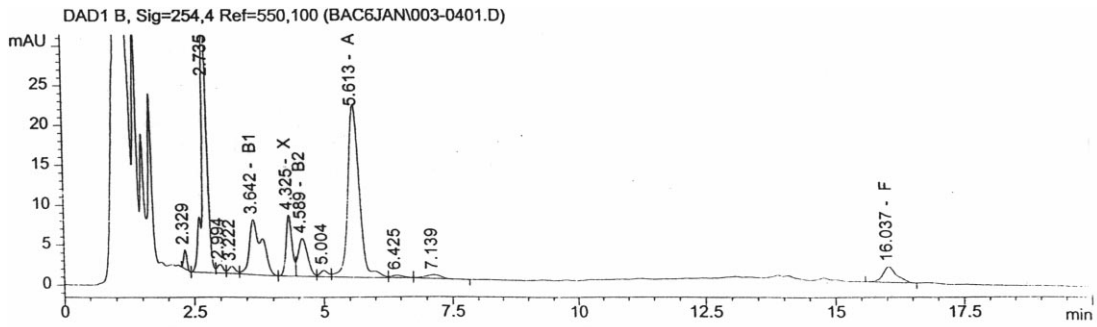

Fig. 2. The HPLC step gradient separation of ZnBc feed grade on Kromasil 5 μm C-8, 100 Å (150 × 4.6 mm ID) without pH adjustment of the mobile phase ($\lambda = 254$ nm).

2.4. Solvent used for preparation of sample and standard solutions

Ninty-nine volume parts of water solution of 0.05 M KH_2PO_4, methanol and acetonitrile (2:1:1 v/v/v) + 1 vol. part of H_3PO_4 (85%).

2.4.1. Preparation of measurement solutions

Standard solution. About 12 mg of reference standard ZnBc (67.9 IU/mg) was accurately weighed into a 10-ml volumetric flask. The solvent was added to the volume and the flask was shaked in an ultrasonic bath to dissolve bacitracin. The solution was then filtered through a membrane filter with pore diameter of 0.45 μm (concentration of ZnBc ≈ 80 IU/ml).

Sample solution. About 1 g of sample ZnBc feed grade (10% f.g.) was accurately weighed into a 50-ml volumetric flask and the solvent was added to the volume. The flask was shaken well on a vortex mixer, then agitated for further 10 min on an ultrasonic bath. The solution was then filtered through a membrane filter with pore diameter of 0.45μm (concentration of ZnBc ≈ 80 IU/ml).

3. Results and discussion

An effective and reproducible separation of the main microbiological active components of bacitracin A, B_1 and B_2 could be achieved by isocratic HPLC method published previously [10] using a Nucleosil 5 μm C-18, 100 Å (150 × 4.6 mm ID) column and a mobile phase A without pH adjustment. The objective of the present work was therefore to increase the selectivity of chromatographic separation not only for above-mentioned components but for impurities, degradation products and ballast materials of ZnBc feed grade product as well.

We kept the isocratic elution of the main components A, B_1 and B_2 and added the step gradient elution to separate later on eluted related substances. Such modification gave a positive impact on separation efficiency and on detection limits of minor signals. Impurities and degradation products of bacitracin could therefore be detected at lower levels and at shorter retention times resulting in faster analyses as a whole.

In order to achieve the best possible selectivity of analytical method, HPLC separations were conducted on extremely complex samples e.g. on ZnBc feed grade material. In these analyses one unidentified peak X originating from ballast material was considerably interfering with the peaks of bacitracin B_1 and B_2 as a result of minor changes in chromatographic conditions. UV spectra of a compound X was quite different compared to bacitracin spectra A, B_1 and B_2 enabling differentiation of this compound from bacitracin. We tried to solve this problem by using Eurospher 5 μm C-18 and Kromasil 5 μm C-8 and longer columns packed with Nucleosil 5 μm C-18, achieving a partial separation of the peak X (Fig. 2). Furthermore we confirmed that the peak B_1 was not homogenous as previously mentioned [10]. As Kromasil 5 μm C-8 column during screening procedure proved to be sufficiently ro-

bust with good efficiency and promising selectivity we chose it for our further investigations. Based on our previous findings that the ionic strength of 0.05 M KH_2PO_4 water solution was optimal for separation of A, B_1 and B_2 [10], only the pH of the buffer was changed from 2 to 8 in further mobile phase adjustments. We found that at lower pH the ballast component X was shifted strongly to the beginning of the chromatogram and the main components of bacitracin in ZnBc f.g. were also eluted at much shorter retention times. They were not resolved completely. On the other hand the bacitracin peak B_1 was clearly resolved into three subunits, probably isomers (B_1' B_1'', B_1''') in the sample of ZnBc f.g. and in the reference standard ZnBc (Fig. 3(a)). This has not been observed in others studies yet. Their UV spectra were identical and peak purity was confirmed (Fig. 3(b)).

The peak of the degradation product F, however, was split under these conditions into at least two peaks probably isomers (F′ and F″) (Fig. 3(a) and (b)). This separation which was evidently achieved only on Kromasil column also proved that the degradation product F is in fact not homogenous. At higher pH values the retention times of A, B_1 and B_2 increased, reaching the optimal separation performance at pH 6 (Fig. 4(a) and (b)). Actually, using the same mobile phase and step gradient elution, a similar separation profile but with lower resolution factors was obtained on Nucleosil 5 μm C-18 column (Fig. 5). In both cases the unidentified component X was well resolved and considerably shifted to the beginning of chromatograms (Fig. 4(a) and (b)). Increasing pH of the mobile phase buffer to 8, caused a further shift of bacitracin peaks towards longer retention times. However, variations of pH had only slight or no impact on the retention time of

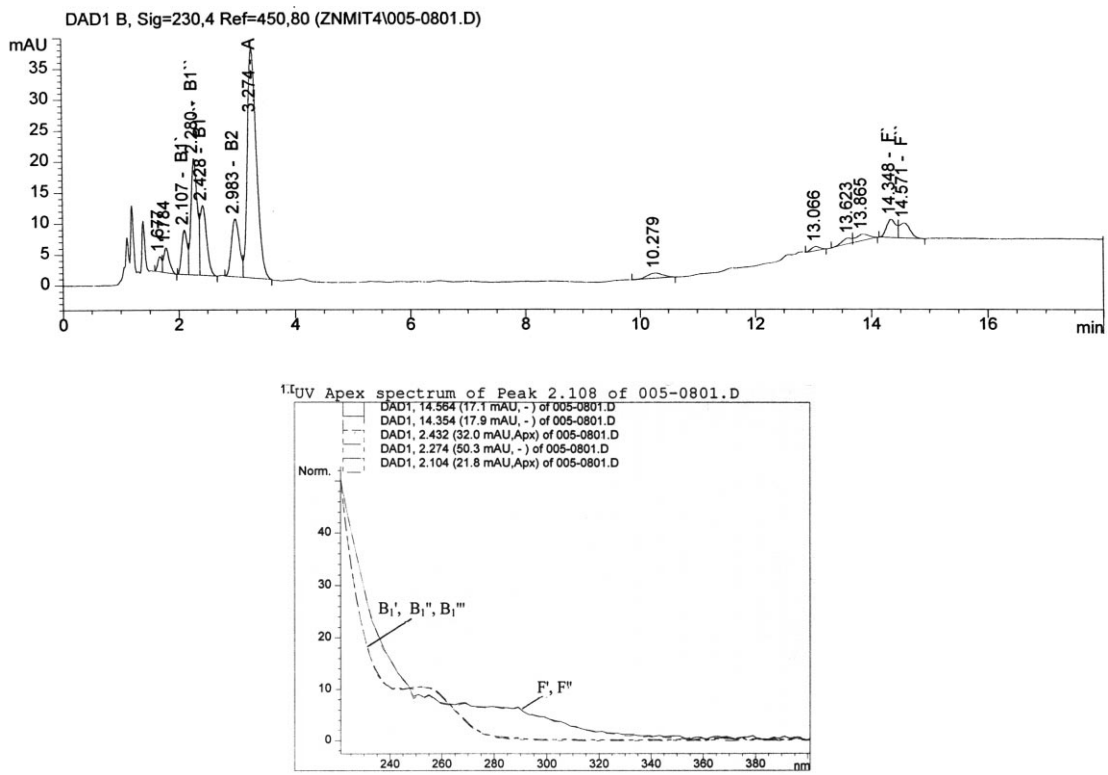

Fig. 3. The influence of pH 2 of phosphate buffer in mobile phase on HPLC separation of ZnBc on Kromasil 5 μm C-8, 100 Å (150 × 4.6 mm ID) ($\lambda = 230$ nm) (a). UV spectra of isomers B_1', B_1'', B_1''' and F′, F″ are scanned by diode array detector (b).

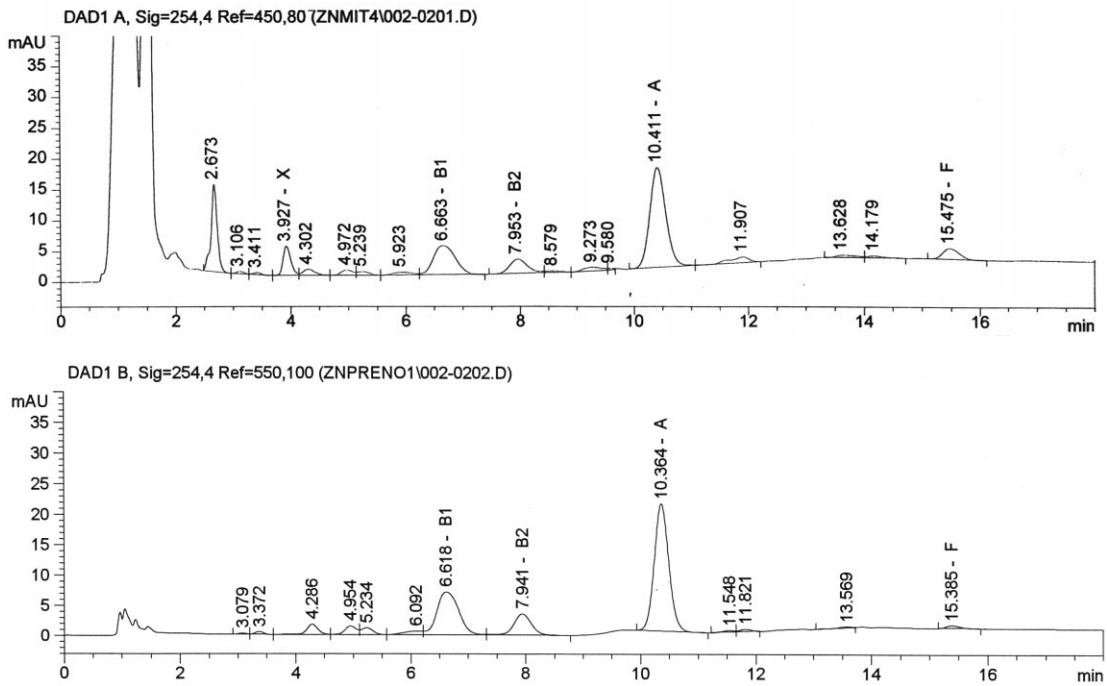

Fig. 4. The influence of pH 6 of phosphate buffer in mobile phase on HPLC separation of sample ZnBc feed grade (a) and standard bacitracin (b) on Kromasil 5 μm C-8, 100 Å (150 × 4.6 mm ID) ($\lambda = 254$ nm).

peak F. We found that by increasing the pH of the mobile phase we, as a rule, obtained longer retention times for the main active polypeptides A, B_1 and B_2. This can be explained by the fact that we approached the bacitracin isoelectric point at pH 8.5–8.8 [4]. Chromatographic peaks, however, became broader thus reducing the method sensitivity. Considering also the unfavourable alkaline medium, pH 6 of the buffer was chosen as the most appropriate.

Through the validation procedure, the developed method proved to be accurate, precise and linear in the range between 80% and 155% of the stated potency of ZnBc feed grade – 10%. The recovery obtained was 96.4% with RSD = 4.3% ($n = 6$), by the method of standard addition and a better intermediate precision for the sum of A + $B_1 + B_2$ than 1.5%. The square correlation coefficient of the regression line was 0.999 based on 8 concentration points. The matrix of our feed grade samples were not available because ZnBc f.g. is an extremely complex material consisting of bacitracin and fermentation broth. The selectivity of the method was therefore based on peak purity approach with diode array detection, which confirmed that there was no interference between bacitracin peaks A, B_1 and B_2 and impurities, decomposition products or ballast materials in the entire UV range. Appropriate selectivity of separation of bacitracin at declared conditions was

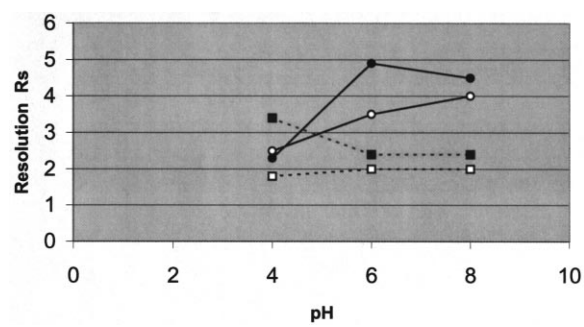

Fig. 5. The graph of resolution factors (Rs) of bacitracin components obtained on Kromasil 5 μm C-8, 100 Å (150 × 4.6 mm ID) and Nucleosil 5 μm C-18, 100 Å (150 × 4.6 mm ID) columns with step gradient elution; (■) B_1/B_2 Kromasil; (●) B_2/A Kromasil; (□) B_1/B_2 Nucleosil; (○) B_2/A Nucleosil.

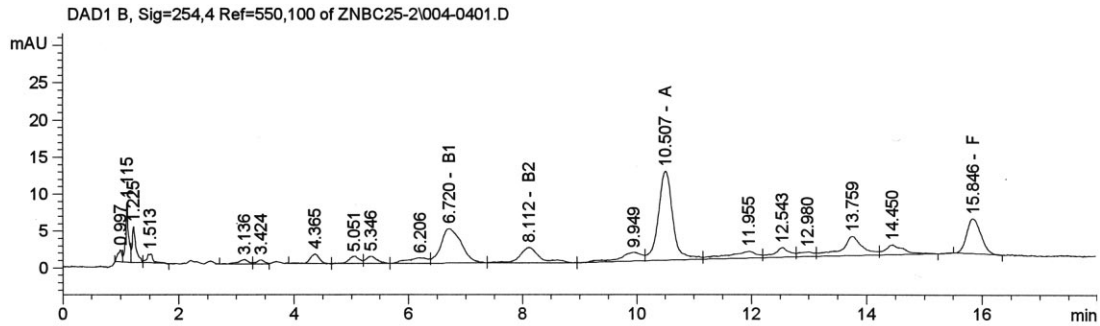

Fig. 6. HPLC chromatogram of bacitracin in water solution exposed to stress condition (3 days at 50°C).

additionally proven on the samples of bacitracin exposed to stress conditions (Fig. 6) showing no interference with related compounds.

4. Conclusions

The obtained analytical procedure is highly selective and effective for the simultaneous determination of active polypeptide A, B_1 and B_2, known complex of F and unknown degradation products of bacitracin. It therefore enables the determination of microbiologically significant active polypeptide components A, B_1 and B_2 and their relative ratio, as well as the evaluation of degradation complex F and other related substances. This method is consequently much more acceptable compare with Ph. Eur. 3rd and BP 99 [12] spectrophotometric procedure for determination of bacitracin F and related substances, especially in pharmaceutical finished dosage forms and in bacitracin feed grade products.

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