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Fast separation of bacitracin on monolithic silica columns

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Abstract

The development of isocratic and gradient stability indicating HPLC methods for bacitracin (Bc) and bacitracin zinc (BcZn), which are complex mixture of several related polypeptides, is described. The methods are based on a new type of reversed phase (RP-18e) monolithic silicagel stationary phase. Chromatographic experimental conditions used on conventional column with microparticles were adopted and further modified to achieve efficient separation of Bc. The influence of methanol and acetonitrile in combination with phosphate buffer was thoroughly studied to separate microbiologically active components A, B1, B2, B3 and their oxidative degradation products F, H1, H2 and H3. Chromatographic peaks of all the mentioned components were identified using compounds isolated previously by preparative HPLC. Applying isocratic or gradient approach, highly efficient separation was achieved together with drastically reduced analysis times (ca. 6 min) compared to all published HPLC methods up to date. With thus developed HPLC methods, it is possible to evaluate not only the main degradation product F, but for the first time also several other oxidative degradation products of Bc (H1, H2 and H3). Such methods are also suitable for routine quality control and stability testing. Validation of both isocratic and gradient methods confirmed the selectivity and efficiency comparable to that on microparticulate columns, yet contrary to conventional columns with highly reduced analysis time.

Keywords: Bacitracin; Silica monolithic column; Reversed-phase chromatography

1. Introduction

Bacitracin (Bc) is a cyclic polypeptide antibiotic produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* and is mainly active against Gram-positive bacteria. Bc is a mixture of several structurally related polypeptides composed of 12 amino acids (Fig. 1). The most important components known as A, B1, B2 and B3 are mainly responsible for microbiological activity of the drug. Among several degradation products, found in Bc, only Bc F, which is microbiologically inactive and claimed as nephrotoxic, is usually mentioned in literature. It is the main degradation product formed by oxidative deamination of the aminothiazoline moiety of Bc A to the corresponding kethotiazole ring (Fig. 1) [1–3].

Not long ago, Ikai et al. published structural characterization of the major and minor Bc components and their molecular weights using fast atom bombardment (FAB) LC/MS and MS/MS [2,3]. They confirmed that the main Bc peptides differ from each other in replacement of one to three of L-isoleucines, including the N-terminal one, by L-valines. Accordingly, a new nomenclature of some components of Bc was proposed (Fig. 1) [3–5]. Additionally in Supplement 4.5 of European Pharmacopoeia 4th, the component F is designated as E [4].

Microbiological method for quantitative assay of Bc is still official [4,6]; however, the HPLC method for quality control of Bc and for the evaluation of component F was currently introduced in Supplement 4.5 of European Pharmacopoeia 4th [4]. So far, HPLC separations of Bc were rather time consuming and unpractical for daily routine quality control. Analysis time needed for Bc separations is usually in the range of 25 min to more than 1 h [2,4,5,7–12] and rarely a

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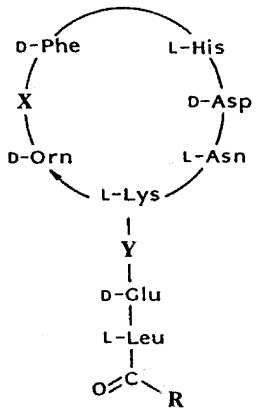
Bacitracin	X	Y	R
A	L-Ile	L-Ile	(A)
B1	L-Ile	L-Ile	(B)
B2	L-Val	L-Ile	(A)
B3	L-Ile	L-Val	(A)
C1	L-Val	L-Ile	(B)
C2	L-Ile	L-Val	(B)
C3	L-Val	L-Val	(A)
E	L-Val	L-Val	(B)
F	L-Ile	L-Ile	(C)
H1	L-Ile	L-Ile	(D)
H2	L-Val	L-Ile	(C)
H3	L-Ile	L-Val	(C)
I1	L-Val	L-Ile	(D)
I2	L-Ile	L-Val	(D)
I3	L-Val	L-Val	(C)



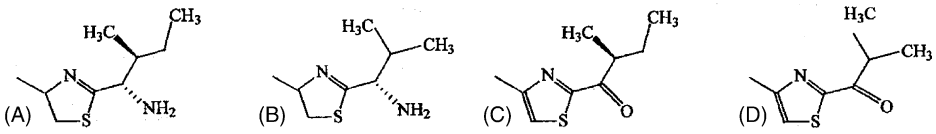


Fig. 1. Structures of known bacitracins. Asn—asparagine, Asp—aspartic acid, Glu—glutamic acid, His—histidine, Ile—isoleucine: Leu—leucine, Lys—lysine, Orn—ornithine, Phe—phenylalanine; Val—valine.

bit shorter [13]. The aim of this study was to develop an effective, stability-indicating and faster HPLC separation of Bc for daily routine quality control.

The use of monolithic stationary phase was therefore taken into account for reduction of separation times. Monolithic organic polymer materials for HPLC columns have been known for some time but the general drawback of this material was swelling or shrinking in organic solvents, lack of mechanical stability and usually lower performance characteristics. A quite different situation is with the newly developed material, which is based on silicagel skeleton and prepared by sol–gel process, the result being formation of bimodal pore structure macropores and mesopores. Macropores with a diameter of approximately 2 μm and mesopores with a diameter of approximately 13 nm in the skeleton show a higher porosity compared to conventional microparticulate columns. Macropores serve as flow through pores and enable analytes to be transported to the active surface for subsequent separation. A high porosity and permeability of stationary phase enable high flow rates, high efficiencies due to the better mass transfer and low column backpressures [14,15].

In this paper, we present the application of a new type of monolithic silicagel Chromolith column showing efficient separation of Bc with concomitant significant reduction in analysis time and better samples throughput. It is also shown that the developed HPLC method is more suitable for quality control and stability testing of Bc compared to other known methods.

2. Experimental procedures

2.1. Materials and reagents

KH_2PO_4 p.a. (J.T.Baker, NY, USA); water: HPLC grade (Milli-Q-185 Plus); acetonitrile, HPLC grade (Rathburn, Wakerburn, Scotland); methanol, Lichrosolv (Merck, Darmstadt, Germany); H_3PO_4 (85%) supra pure (Merck, Darmstadt, Germany); KOH p.a. (Merck, Darmstadt, Germany); bacitracin batch no.: 424923/141901 and bacitracin zinc (BcZn) batch no.: 413810/1 (Fluka, Buchs, Switzerland), dried before use in vacuum at 60 °C for 3 h [16]. Pure components of Bc: A, B1, B2, B3, F, H1, H2 and H3 were isolated by preparative HPLC method (in press). Phosphate buffer 0.05 M, pH 6.0: KH_2PO_4 was dissolved in water and pH was adjusted to pH 6.0 with 20% KOH.

2.1.1. Solutions for HPLC measurements

Solvent for BcZn: phosphate buffer (0.05 M, pH 6.0): H_3PO_4 (85%) = 99.5:0.5 (v/v).

Solvent for Bc and isolated components (A, B1, B2, B3, H1, H2, H3 and F): water, HPLC grade.

All solutions with Bc, BcZn or with isolated components of Bc have been filtered through a membrane filter with pore diameter of 0.45 μm before HPLC measurements.

Sample solutions of Bc (BcZn): About 20 mg of bacitracin (22 mg bacitracin zinc) were accurately weighed into a 10-ml volumetric flask. The solvent was added to the volume and the flask was shaken for 10 min in an ultrasonic bath

to dissolve (concentration 2 mg/ml for Bc, 2.2 mg/ml for BcZn).

Stock solution of Bc: 220.0 mg of Bc was accurately weighed into a 50-ml volumetric flask, added solvent to the volume and dissolved (concentration 4.4 mg/ml).

Stock solution of component F: 5 mg of isolated component F was accurately weighed into a 10-ml volumetric flask, added solvent to the volume and dissolved (concentration 0.5 mg/ml).

2.1.2. Validation

The methods were validated for linearity, repeatability of injections, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The selectivity was proved by diode array detector and by chromatographic checking with above-mentioned isolated components of Bc [16,17].

Linearity of Bc was determined on seven levels of concentration with three injections for each level from 25% (0.5 mg/ml) to 150% (3 mg/ml). Solutions were prepared by diluting the stock solution of Bc.

Linearity of F was determined on six levels of concentration with two injections for each level from 0.5% (0.01 mg/ml) to 15% (0.3 mg/ml) with regard to the concentration of Bc in sample solution. Solutions for linearity were prepared by diluting the stock solution of component F.

Repeatability of injections for Bc was determined by six injections of sample solution of Bc (concentration 2 mg/ml).

Repeatability of injections for F was determined by six injections of two concentrations for 1% (0.02 mg/ml) and 5% (0.1 mg/ml) solutions prepared by diluting the stock solution of component F.

Accuracy for determination of Bc and of component F was tested by examining the linearity (response versus amount) of the procedures and evaluated by least-squares regression analysis.

The limit of detection for component F was determined by diluting stock solution F of known concentration until the responses were three–four times the noise.

The limit of quantification for component F was determined by diluting stock solution F of known concentration until the responses were 10 times the noise.

2.1.3. HPLC columns

Chromolith column RP-18e (100 mm × 4.6 mm i.d.)—endcapped (Merck, Darmstadt, Germany); Synergi Hydro-RP (C18) 4 μm, 80 Å (250 mm × 4.6 mm i.d.)—endcapped (Phenomenex, Torrance, CA, USA); Hypersil BDS (C18) 5 μm, 130 Å (250 mm × 4.6 mm i.d.) (Thermo Hypersil-Keystone, Runcorn, Great Britain).

2.1.4. Apparatus

- HPLC system Hewlett Packard model Series 1100 controlled with Chemstation software consisted of a binary gradient pump, a diode array detector, an autosampler and a column heater.
- HPLC quarternary pump Hewlett Packard model Series 1050, variable UV detector model Series 1050, an autosampler Thermo Separation Products with column heater model AS3000, Integrator model HP 3396 Series II.

2.2. Chromatographic conditions for Chromolith RP-18e (100 mm × 4.6 mm i.d.) endcapped column

2.2.1. Isocratic method

Flow rate: 5 ml/min, detection: UV λ = 230 nm, injection volume: 20 μl, temperature: 40 °C; mobile phase (MF): acetonitrile–methanol (10:90, v/v)–phosphate buffer (0.05 M, pH 6.0) (56:44, v/v).

2.2.2. Gradient method

Flow rate: 5 ml/min, detection: UV λ = 230 nm, injection volume: 20 μl, temperature: 40 °C, MFA: acetonitrile–methanol (10:90, v/v)–phosphate buffer (0.05 M, pH 6.0) (54:46, v/v), MFB: acetonitrile–methanol (10:90, v/v)–phosphate buffer (0.05 M, pH 6.0) (65:35, v/v).

Programme: The isocratic elution with 100% of mobile phase A was kept for 2.8 min. Then, the content of mobile phase B was linearly increased to 100% in the time interval from 2.8 to 3.8 min. After isocratic elution of mobile phase B from 3.8 to 5.0 min, the system was equilibrated again with 100% mobile phase A between 5.0 and 5.7 min.

2.3. Chromatographic conditions for conventional columns: Synergi-Hydro-RP 4 μm, Hypersil BDS 5 μm with dimensions (250 mm × 4.6 mm i.d.)

2.3.1. Gradient method

Flow rate: 1.4 ml/min, detection: UV, λ = 230 nm, injection volume: 20 μl, temperature: 35 °C, MFA: acetonitrile–methanol (10:90, v/v)–phosphate buffer (0.05 M, pH 6.0) (56:44, v/v), MFB: acetonitrile:methanol (10:90, v/v)–phosphate buffer (0.05 M, pH 6.0) (65:35, v/v).

Programme: The isocratic elution with 100% of mobile phase A was kept for 20 min. Then, the content mobile phase B was linearly increased to 100% in the time interval from 20 to 38 min. After isocratic elution of mobile phase B from 38 to 52 min, the system was equilibrated again with 100% mobile phase A between 52 and 57 min.

3. Results and discussion

At the beginning of our study, known experimental conditions for HPLC separation of Bc on the conventional Kromasil C8, 5 μm column [13] were slightly modified and transferred to the monolithic silicagel Chromolith RP-18e (100 mm × 4.6 mm i.d.) column. Nearly the same composition of mobile phase (acetonitrile–methanol (50:50, v/v)–phosphate buffer (0.05 M, pH 6.0) (50:50, v/v)) was used; however, because of the better mass transfer properties of a monolithic column, the speed of mobile phase was

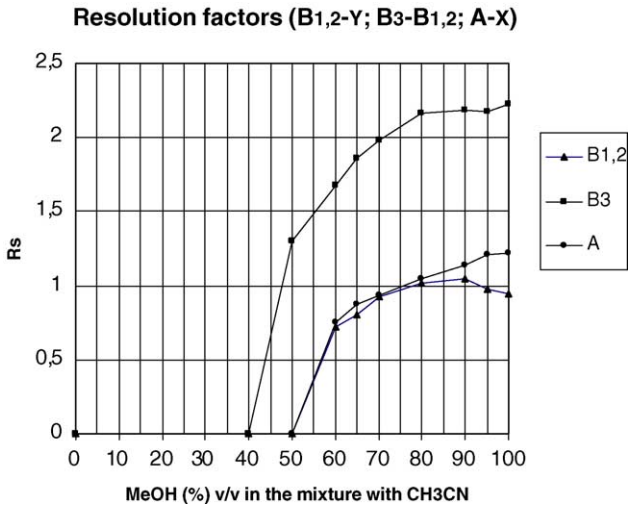


Fig. 2. The graph of resolution factors (Rs) for components of Bc B1,2, B3 and A to their nearest neighbouring peaks on Chromolith RP-18e (100 mm × 4.6 mm i.d.) column with different ratio of acetonitrile–methanol in mobile phase: (acetonitrile–methanol):(phosphate buffer 0.05 M, pH 6.0) = 50:50 (v/v), ratio between organic phase and phosphate buffer is constant (50:50, v/v); flow rate: 5 ml/min; detection: λ = 230 nm; injection volume: 20 μl; temperature: 40 °C.

increased from 1.4 to 5 ml/min. The temperature of the column was increased from 30 to 40 °C to decrease the viscosity of mobile phase resulting in lower backpressure. Detection of Bc was performed at 230 nm because at this wavelength, we achieved better sensitivity for impurities and degradation products. Only a partial resolution of the main components of Bc was obtained at above-mentioned conditions due to interferences of some smaller neighbouring peaks.

For positive identification of several peaks and definite confirmation of selectivity, we used isolated pure active components A, B1, B2, in B3 and their corresponding main oxidative degradation products F, H1, H2 and H3. All above-mentioned substances were isolated by preparative HPLC method, purified by solid phase extraction, lyophilised and finally confirmed by FAB mass spectrometry (in press).

The selectivity of the methods was further confirmed by the application of diode array detection.

As optimal concentration and pH of phosphate buffer in the mobile phase had already been determined previously on conventional columns [10,13], we tried to improve the selectivity of Bc separation by changing the ratio between methanol and acetonitrile in the mobile phase. The ratio between organic phase and buffer was kept constant 50:50

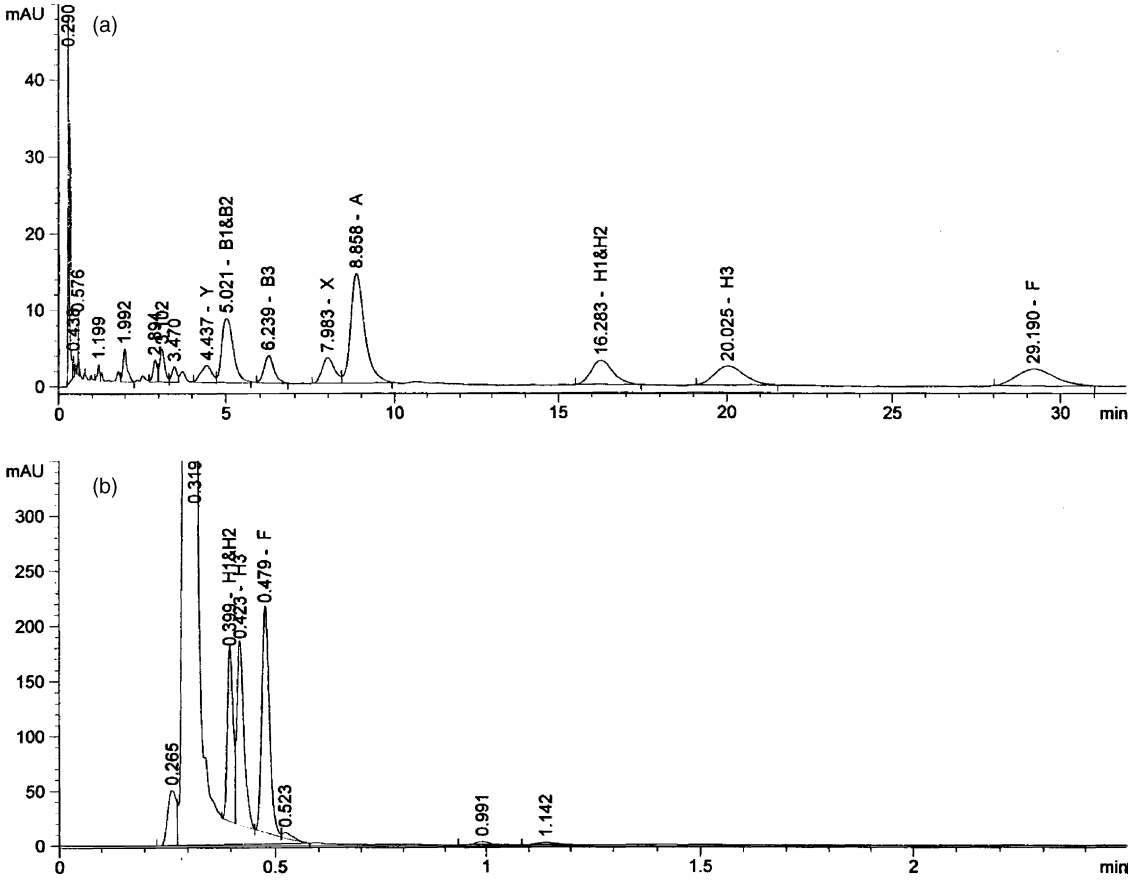


Fig. 3. Influence of the composition of mobile phase with the same strength on resolution of bacitracin on Chromolith RP-18e (100 mm × 4.6 mm i.d.) column: (a) methanol–phosphate buffer (0.05 M, pH 6.0) in the ratio of 50:50 (v/v), (b) acetonitrile–phosphate buffer (0.05 M, pH 6.0) in the ratio of 40:60 (v/v); flow rate: 5 ml/min, λ = 230 nm, temperature: 40 °C, injection volume: 20 μl. Solution of Bc is spiked with components H1, H2, H3 and F. (B1,2, B3 and A—microbiologically active components of Bc; H1,2, H3 and F—oxidative degradation products of Bc; Y, X—unknown related substances of Bc).

(v/v). We stepwise changed the ratio between acetonitrile and methanol till the complete replacement of acetonitrile with methanol (Figs. 2 and 3). Fig. 2 shows that these modifications resulted in better separation of Bc. In each case, however, components B1 and B2 eluted together in one peak (=B1,2) and similar co-elution of their corresponding oxidative degradation products H1 and H2 (=H1,2) was observed. In front of the peaks B1,2 and A, interferences of minor neighbouring peaks which were assigned *Y* and *X* can be seen (Fig. 3a). Degradation products H1,2, H3 and F were efficiently separated. In the case of complete replacement of acetonitrile with methanol, however, much higher pressure in a HPLC system and extremely long retention times were observed, particularly for the component F (approximately 30 min) (Fig. 3a).

We found that methanol had an important impact on improving the selectivity of Bc separation but on the other hand, acetonitrile was needed as organic modifier. Acetonitrile increased the strength of the mobile phase, shortened the retention times especially of oxidative degradation products and lowered the viscosity of mobile phase and consequently the pressure in the HPLC system. It is known from the solvent strength nomograph for reversed-phase HPLC that exactly 50 vol.% methanol has the same strength as 40 vol.% acetonitrile in respect of the water as a weak solvent [18]. A big difference in selectivity between acetonitrile and methanol on separation of Bc can be seen from the comparison of the chromatograms, obtained with the mobile phases of the same strength: once with methanol only and once with acetonitrile

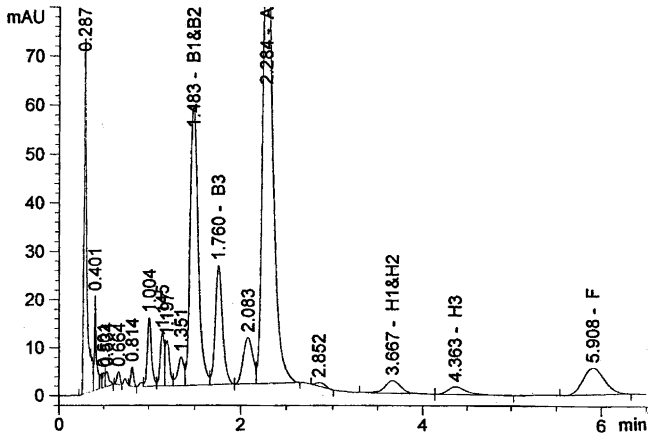


Fig. 4. HPLC chromatogram of bacitracin (2 mg/ml) stored for one day at 25 °C on Chromolith RP-18e (100 mm × 4.6 mm i.d.) column with isocratic elution; see Section 2.2.1; B1,2, B3 and A—microbiologically active components of Bc; H1,2, H3 and F—oxidative degradation products of Bc.

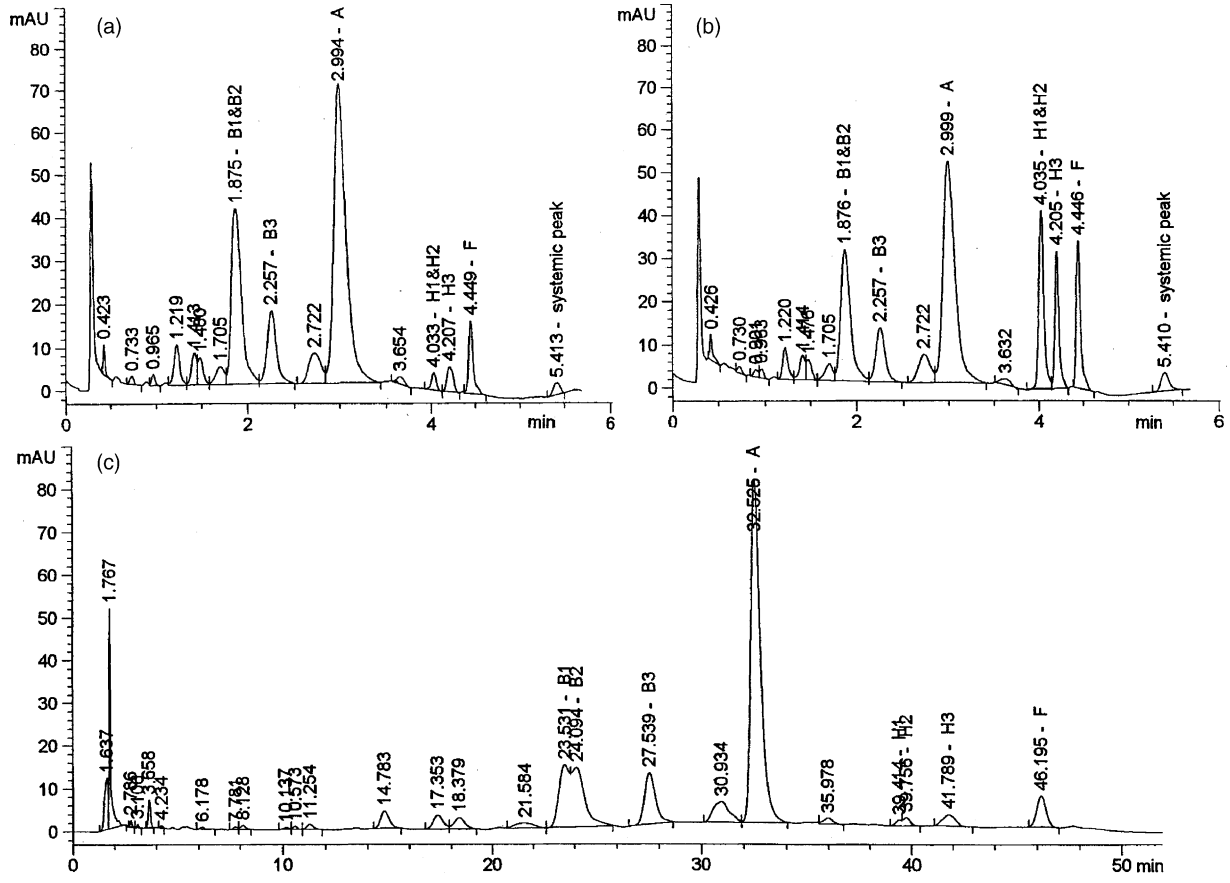


Fig. 5. HPLC chromatograms using gradient elution: Bc (2 mg/ml) (a) and Bc spiked with components H1, H2, H3 and F (b) on Chromolith RP-18e (100 mm × 4.6 mm i.d.) column; see Section 2.2.2; Bc (2 mg/ml) on Synergi Hydro-RP, 4 μm (250 mm × 4.6 mm i.d.) column (c); see Section 2.3.1; B1,2, B3 and A—microbiologically active components of Bc; H1,2, H3 and F—oxidative degradation products of Bc.

only (Fig. 3a, b). It is evident that in the case of acetonitrile alone, the separation is unsuitable because of co-elution of all active components in the solvent peak (Fig. 3b).

From the practical standpoint, the best compromise among the acceptable chromatographic performance, duration of the analysis and the pressure in the HPLC system was reached, when the ratio between acetonitrile and methanol was in the range of 30:70–10:90 (v/v) (Fig. 2). However, the optimum performance was achieved with the ratio of acetonitrile to methanol 10:90 (v/v). Simultaneously, the proportion between organic phase and phosphate buffer was increased from 50:50 (v/v) to 56:44 (v/v) for isocratic elution in favour of organic phase, thus reducing significantly the retention times for degradation products (Fig. 4). Isocratic method is practical for daily routine quality control of Bc; however, for low levels Bc degradation products, higher injection volumes are preferred (e.g. 50 μl).

The retention times of degradation products can be further reduced by the use of gradient elution (Fig. 5a, b). It is evident that great reduction of analysis time was achieved compared to conventional endcapped column (Synergi Hydro-RP 4 μm (250 mm × 4.6 mm i.d.)) (Fig. 5c). Gradient method is preferred for more selective separation and enhanced sensitivity for detection of degradation products H1,2, H3 and F. In the case of gradient elution, the systemic peak can be observed.

As mentioned in Section 2, 40 °C was used for column heating because the separation on monolithic column deteriorated at higher temperature.

The isocratic elution lasted less than 7 min (Fig. 4) and gradient elution lasted less than 6 min (Fig. 5a, b), which are exceptionally short analysis times compared to all currently known separations of bacitracins.

It must be pointed out that all components of Bc were efficiently separated on Chromolith column with the exception of pair B1 and B2 and their degradation products H1 and H2, respectively. The separation of B1 and B2 was not achieved even with the mobile phase composed only of methanol and phosphate buffer (Fig. 3a), which proved to be effective for separation of mentioned peak pairs in our previous study performed on conventional column [8,10]. It is worth mentioning that the separation of peaks B1 and B2 is most difficult to achieve as recognised by other authors, too [5]. Nevertheless, peaks B1 and B2 could be separated quite effectively on conventional columns, e.g. Hypersil BDS (Fig. 6) using practically the same composition of mobile phase as used for separations on Chromolith column. For the quality evaluation of Bc, unresolved peaks are not problematic, since the active components A, B1, B2 and B3 have identical UV spectra and equal response factors (personal communication). According to these findings, the quality of Bc or BcZn could be expressed for microbiologically active components as A, as the sum (B1 + B2 + B3), as the ratio of A:(B1 + B2 + B3) and as the total sum (A + B1 + B2 + B3) using normalization procedure as proposed by Supplement 4.5 of European Pharmacopoeia 4th [4]. Among known related peptides, only determination of the component F (designated as E) is prescribed by Supplement 4.5 of European Pharmacopoeia 4th [4]. Evaluation of other known degradation products, e.g. H1,2 and H3 besides F as well as unknown related peptides, is possible by using our isocratic or gradient method. All mentioned impurities are oxidative degradation products of Bc B1, B2, B3 and A with related structures and identical UV spectra strongly suggesting the equality of their response factors. In our separate study, it was established that all degradation products H1, H2, H3 and F are microbiologically inactive (personal communication).

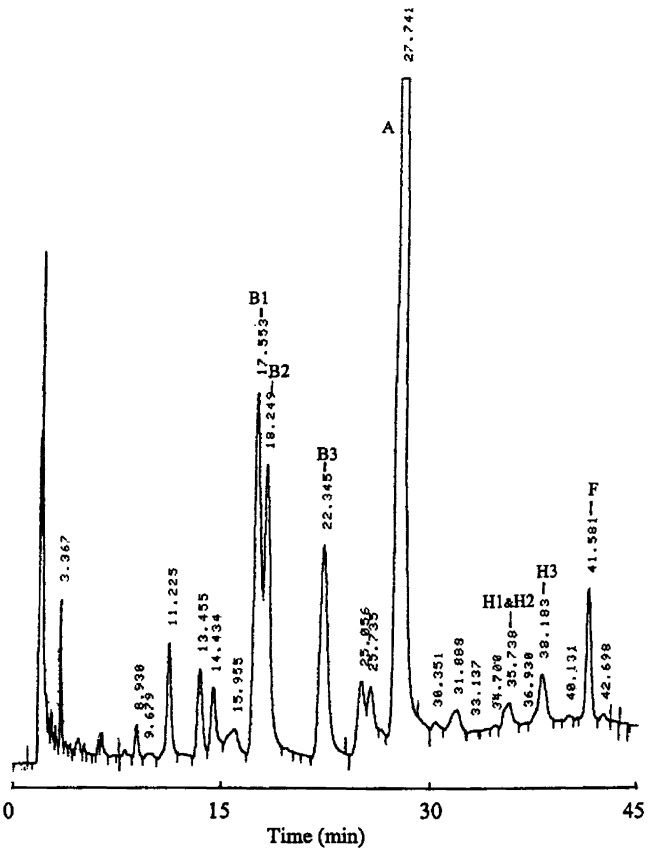


Fig. 6. HPLC separation of components of bacitracin (2 mg/ml) on Hypersil BDS, 5 μm (250 mm × 4.6 mm i.d.) column with gradient elution; see Section 2.3.1; B1,2, B3 and A—microbiologically active components of Bc; H1,2, H3 and F—oxidative degradation products of Bc.

It is surprising that in USP 25th [16], neither monographs for Bc and BcZn active substances nor monograph for bacitracin for injection prescribe the requirements for related substances.

The validation of the developed methods proved their suitability for Bc separation (Table 1). Because the chromatographic processes of separation on monolithic column are carried out very fast, some experimental factors have a slightly more pronounced impact on separation. It was established that variations of different factors such as composition of mobile phase (even for only 1%) or different pumping systems for gradient elution (regarding the delay of programmed composition of mobile phase) influence fine tuning of resolution and retention times of Bc components.

Table 1
Validation parameters of HPLC analyses of Bc (BcZn) on monolithic silicagel Chromolith RP-18e (100 mm × 4.6 mm i.d.) column

Validation parameters	HPLC method	
	Isocratic	Gradient
Repeatability of injections for Bc, $\sum$(A + B1,2 + B3)[a] (n = 6)	RSD < 1.5%	RSD < 1.5%
Linearity of Bc, $\sum$(A + B1,2 + B3) (seven concentration points)	25–150%	25–150%
Determination coefficient	r^2 = 0.9993	r^2 = 0.9998
Slope	k = 620.0	k = 655.2
Confidence intervals of k (α = 0.05)	612.2–627.9	650.8–659.6
Y-intercept	b = −4.632	b = −3.357
Confidence intervals of b (α = 0.05)	−17.56 to 8.30	−12.01 to 5.29
Accuracy of Bc (seven concentration points)		
Y-intercept (%) (bias) (relative to the detector response at the working concentration of Bc (about 2 mg/ml))	b = 0.38%	b = 0.26%
Repeatability of injections for component F[b] (n = 6)		
Concentration F (5%)	RSD = 3.5%	RSD < 1%
Concentration F (1%)	Not performed	RSD < 2.5%
Linearity of component F (six concentration points)	0.5–15%	0.2–15%
Determination coefficient	r^2 = 0.9994	r^2 = 0.9998
Slope	k = 2097.2	k = 886.6
Confidence intervals of k (α = 0.05)	2060.2–2134.2	874.7–898.5
Y-intercept	b = −6.186	b = 1.433
Confidence intervals of b (α = 0.05)	−11.47 to −0.907	−0.146 to 3.012
Accuracy of component F (six concentration points)		
Y-intercept (%) (bias) (relative to the detector response at the working concentration of component F (about 5%))	b = 3.30%	b = 0.92%
Limit of detection (LOD) for F	0.2% (0.004 mg/ml)	0.05% (0.001 mg/ml)
Limit of quantification (LOQ) for F	0.5% (0.01 mg/ml)	0.2% (0.004 mg/ml)

[a] Working concentration: 2 mg/ml Bc = 100%
[b] Concentration of component F in water 1% (=0.02 mg/ml); 5% (=0.1 mg/ml).

The validation procedures proved both isocratic and gradient HPLC methods for qualitative control of Bc and BcZn on Chromolith column to be linear, precise, accurate, selective and also sensitive for determination of oxidative degradation products as shown in Table 1. It is recommended, however, that sample solutions should be analysed within 3 h after preparation due to instability of Bc water solutions.

4. Conclusion

By using monolithic reversed-phase silica Chromolith column, we were able to shorten the analysis time of Bc dramatically. Because of the exceptionally high speed and effectiveness of separation, we see the advantage of the analysis used not only in the every day routine quality control of Bc, but also in stability studies. The latter allows for the increase in sample throughput, what is particularly important for stability sensitive samples needed to be analysed in a very short time. With described isocratic or gradient HPLC method, we can get complete information about the amount of microbiologically active components B1, B2, B3 and A and their ratio as well and about their corresponding oxidative degradation products H1, H2, H3 and F.

In the future, numerous analyses performed on this kind of columns can be expected not only because of the speed, but also because of the selectivity which is similar to that achieved on conventional columns with microparticles as demonstrated in our work. Furthermore, the method transfer from conventional column to monolithic silica column is not problematic as shown by our work. In some cases, however, especially when dealing with a very complex mixture, the optimisation of the fine tuning resolution is needed because of a faster chromatographic process on such columns.

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