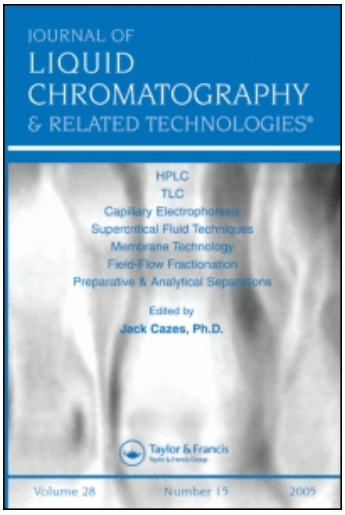

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Isolation of Peptide Components of Bacitracin by Preparative HPLC and Solid Phase Extraction (SPE)

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ABSTRACT

This paper describes isolation procedure of antimicrobially active components of bacitracin (Bc) and its main oxidative degradation products needed for further stability investigations. The procedure of isolation consisted of chromatographic separation of components and degradation products, purification of isolates using solid phase extraction (SPE), and lyophilisation of pure samples. A gradient preparative HPLC separation on Kromasil 5C8 (250 × 16 mm I.D.) column was based on our previously developed analytical method, increasing the ratio of methanol against acetonitrile in the mobile phase and adjusting the flow rate, gradient profile, Bc concentration, and injection volume to achieve an efficient separation of compounds of interest under scale-up conditions. Eluates

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collected after chromatographic separations were concentrated and purified using SPE procedure, simultaneously removing buffer salts derived from the mobile phase. Pure substances eluted from the SPE cartridges were then dried by lyophilisation and used for identification purposes and for further stability experiments. Identity and purity of all substances were checked and confirmed chromatographically, using diode array detection on fast monolithic silica gel Chromolith RP-18e (100 × 4.6 mm I.D.) column after each step of the isolation procedure. Finally, the lyophilisates of active Bc components (A, B1, B2, and B3) and their corresponding oxidative degradation products (F, H1, H2, and H3) were analysed, and their identity confirmed by fast atom bombardment (FAB) mass spectrometry.

Key Words: Bacitracin; Preparative HPLC separation; Scale up; Solid phase extraction; Monolithic silica gel RP-18e (endcapped) column.

INTRODUCTION

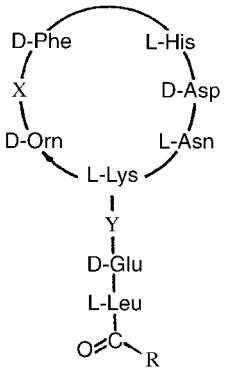
Bacitracin (Bc) is a cyclic polypeptide antibiotic produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* and is mainly active against Gram-positive bacteria. Bc is a mixture of several structurally related polypeptides (Fig. 1).[1,2]

The basic structure of Bc consists of seven amino acids membered ring and five amino acids in the side chain. The terminal part of the side chain ends with thiazoline ring, composed of L-cysteine and L-isoleucine or L-valine. Not long ago, Ikai and co-workers published structural characterisation of 15 major and minor Bc components[3,4] named A, B1, B2, B3, C1, C2, C3, E, F, H1, H2, H3, I1, I2, and I3.[5,6] They determined the molecular weights of the mentioned compounds using fast atom bombardment (FAB) LC/MS and MS/MS, confirming 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 (Fig. 1).[3,4] The most known and microbiologically important components of Bc are A, B1, B2, and B3 (Fig. 1).[1–4] In general, Bcs A and B represent about 96% of the total microbiological activity in commercial products.[2,7]

Among several degradation products found in Bc, only Bc F, which is microbiologically inactive and claimed to be nephrotoxic, is usually mentioned in literature. It is the main degradation product formed by oxidative deamination of the aminomethylene–thiazoline moiety of Bc A to the corresponding ketothiazole moiety (Fig. 1).[1–4]

Published preparative HPLC methods for isolation of active components of Bc[8,9] are not efficiently enough for isolation of the main individual

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)



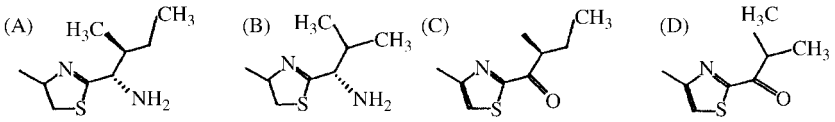


Figure 1. Structures of known components of Bc. Asn—asparagine, Asp—aspartic acid, Glu—glutaminic acid, His—histidine, Ile—isoleucine, Leu—leucine, Lys—lysine, Orn—ornithine, Phe—phenylalanine; Val—valine. R: the end group of the side chain; (A): L-cysteine and L-isoleucine; (B): L-cysteine and L-valine; (C) and (D) are oxidative forms of their corresponding end groups.

active peptide components of Bc or for isolation of their oxidative degradation products. In the present paper, we describe a procedure for isolation of the main components of Bc by preparative HPLC, followed by solid phase extraction (SPE) and lyophilisation. Each step of isolation and SPE purification was checked by fast HPLC methods on reverse-phased monolithic silica gel columns recently introduced into practice.[10–12] FAB mass spectrometry was used for positive identification of isolated microbiologically active peptides of Bc (A, B1, B2, and B3) and their oxidative degradation products (F, H1, H2, and H3). The results of our study show that the mentioned procedure yields excellent purity of isolated substances, enabling further studies on stability and microbiological activity of these compounds.

EXPERIMENTAL

Chemicals and Reagents

KH_2PO_4 p.a., (J.T. Baker, NY, USA); Water: HPLC grade (Milli-Q-185 Plus); acetonitrile (ACN), HPLC grade (Rathburn, Wakerburn, Scotland); methanol (MeOH), Lichrosolv (Merck, Darmstadt, Germany); H_3PO_4, (85%) suprapure (Merck, Darmstadt, Germany); KOH, p.a. (Merck, Darmstadt, Germany); Bc (Fluka, Buchs, Switzerland); phosphate buffer 0.05 M, pH 6.0: the required amount of KH_2PO_4 was dissolved in water and its pH adjusted to pH 6.0 with 20% KOH; hydrogen peroxide solution 30%, Ph.Eur. (Merck, Darmstadt, Germany). Reagent for detection of potassium (K^+ ions): sodium tetraphenylborate (calignost) (Merck, Darmstadt, Germany); reagent solution was prepared according to Ph.Eur. 4.[13]

HPLC Columns and SPE Cartridges

Chromolith column RP-18e (100 × 4.6 mm I.D.) endcapped (Merck, Darmstadt, Germany); Kromasil C-8, 5 μm, 100 Å (150 × 4.6 mm I.D.), (250 × 4.6 mm I.D.), and (250 × 16 mm I.D.) (Ekka Nobel, Surte, Sweden, packed in BIA d.o.o. Ljubljana, Slovenia).

SPE cartridges: (packed with reverse-phased bonded silica) Supelclean LC-18; 12 mL, 2000 mg (Supelco, PA, USA).

Instrumentation

HPLC quarternary pump mod. Series 1050, variable UV detector mod. Series 1050 or diode array detector HP, an autosampler with column heater mod. AS3000 (Thermo Separations, USA), Integrator mod. HP 3396 Series II (Hewlett Packard, USA).

HPLC instrument for preparative separation: gradient pump; constametric 4100 (Thermo Separations), injector: mod. 7125 with 2 mL loop (Rheodyne, USA). Column heater (Thermo Separations), variable UV detector (Knauer, Germany). Integrator mod. HP 3396 Series II (Hewlett Packard), Rotary evaporator (Elektromedicina, Slovenia). Lyophiliser (Heto, Hetosicc Type CD 2.5, Denmark).

SPE apparatus: SPE Vacuum Manifolds with 12 position manifolds (Macherey-Nagel, Germany).

Preparative HPLC Isolation of Bc Components

Chromatographic Conditions for Preparative Gradient HPLC Method

Kromasil C8, 5 μm (250 × 16 mm I.D.); flow: 9 mL/min; detection: UV, $\lambda = 254$ nm; injection: 2 mL; temperature: 35°C; mobile phase A (MFA): (acetonitrile (ACN)/methanol (MeOH) = 30/70, v/v)/(phosphate buffer 0.05 M KH_2PO_4, pH 6.0) = 55/45, v/v; Mobile phase B (MFB): (ACN/MeOH = 30/70, v/v)/(phosphate buffer 0.05 M KH_2PO_4, pH 6.0) = 70/30, v/v.

Programme

The isocratic elution with 100% of MFA was kept for 35 min. Then the content of MFB was linearly increased to 100% in the time interval of 35–49 min. Isocratic elution of MFB was kept from 49 to 58 min, then the system was equilibrated again with 100% MFA between 58 and 65 min.

Sample Solution of Bc for Isolation of Components A, B1, B2, and B3

About 750 mg of Bc was weighed into a 50-mL volumetric flask. Water was added to the volume and the flask was shaken in an ultrasonic bath for 10 min (conc. ~15 mg Bc/mL).

Sample Solution of Oxidised Bc for Isolation of Components H3 and F

A water solution of Bc was placed into a climatic chamber at 60–70°C for 1 week or more to oxidise to their corresponding oxidative products. The alternative procedure used was an accelerated oxidation of Bc by H_2O_2 (1–2 drops of 0.3% H_2O_2 added to 10 mL of Bc solution).

Sample Solution of Oxidised Component B1 and B2 for Isolation of Components H1 and H2

The components B1 and B2 were isolated by preparative HPLC. Then the volume (ca 100 mL) of each eluate containing B1 and B2 was reduced (to ca 70 mL) by rotary evaporation at lower pressure and at the temperature of about 60°C. The water was added (ca 30 mL) and the oxidation of components B1 and B2 to the corresponding oxidation products H1 and H2 was performed according to the above described procedure. Both solutions (H1 and H2) were concentrated on SPE cartridges before injection of samples on the preparative HPLC column.

Procedure for Isolation of Individual Components B1, B2, B3, A, H1, H2, H3, and F

Bc solutions (B1, B2, B3, and A), oxidised Bc (H3 and F), oxidised B1 (H1), and oxidised B2 (H2) were injected on a preparative HPLC column. The eluates for individual components were collected during the time when the individual peak was clearly separated from the others. Altogether, about 75–225 mL of eluates were obtained for each individual component in the mobile phase with concentrations in the range of 0.5–1 mg/mL. All solutions used for analytical and preparative separations were filtered through a membrane filter with pore diameter of 0.45 μm before being injected on the suitable HPLC column.

SPE

Bc Sample Solution for Determination of Capacity of SPE Cartridge Packed with Sorbent

Bc was dissolved in a mixture of (ACN/MeOH = 30/70, v/v)/(phosphate buffer 0.05 M KH_2PO_4, pH 6.0) = 40/60, v/v and all stages of procedure were performed as described.

Sample Solutions of Individual Components (Isolated by Preparative HPLC)

About 30% of organic solvent from the collected eluates of each microbiologically active component (B1, B2, B3, and A) and about 40% of organic solvent from the collected eluates of each degradation product (H1, H2, H3, and F) were evaporated on a rotary evaporator at reduced atmospheric pressure and at the temperature of 35°C for active components and at about 60°C for degradation products. Before applying the collected eluates of individual components of Bc on SPE cartridges, the evaporated part of organic solvent was replaced by water.

The SPE Extraction Procedure[14,15]

Activation

The sorbent was activated by passing methanol (two times of SPE volume tube) and then water (two times of SPE volume tube).

Adsorption

Solutions of isolated components of Bc were passed through the packed SPE cartridges using a gentle vacuum (applied at the end of the column). The flow rate was reasonably constant. The last fractions of eluates were chromatographically tested on Chromolith column in the presence (breakthrough) of each corresponding component.

Washing

The salt of the buffer was eluted by water (100 mL). The last millilitres of water were collected in test tubes and tested with a drop of calignost in the presence of K^+ ions (buffer) in comparison with the standard potassium solution (10 ppm).

Drying

SPE tubes were dried with vacuum (3–5 min).

Elution

Each individual component of Bc was eluted with methanol by portions: (4×3 mL).

Lyophilisation

Isolated components purged with MeOH from SPE were evaporated to some millilitres on a rotary evaporator at lower pressure and at a temperature upto 35°C. Then water was added to volume of 5–6 mL, the solutions were frozen under the liquid N_2, and the process of lyophilisation was run to the constant weight of each component.

Identity and Purity of Isolated Components

Each eluate of the separated component was chromatographically checked on identity and purity immediately after conclusion of each phase of isolation procedure: after isolation, after evaporation, after SPE, and after lyophilisation.

Chromatographic Conditions

Chromolith RP-18e (100×4.6 mm I.D.) endcapped column; flow rate: 5 mL/min, detection: UV, $\lambda = 230$ nm, injection volume: 20 μL, temperature: 40°C. MFA: (ACN/MeOH = 10/90, v/v)/phosphate buffer (0.05 M, pH

6.0) = 54/46, v/v. MFB: (ACN/MeOH = 10/90, v/v)/phosphate buffer (0.05 M, pH 6.0) = 65/35, v/v.

Programme

The isocratic elution with 100% of MF A was kept for 3.0 min. Then the content of MFB was linearly increased to 100% in the time interval of 3.0–5.6 min. After isocratic elution of MF B from 5.6 to 6.0 min, the system was equilibrated again with 100% MF A between 6.0 and 6.5 min.

FAB Mass Spectrometry Analysis

All mass spectral data were acquired in the positive-ion mode by using an AutoSpec Q hybrid tandem mass spectrometer (EBE-qQ geometry Micromass, Manchester, UK). FAB ionisation was used for mass spectrometric determination of Bc components in glycerol matrix samples at acceleration voltage 8000 V. FAB ionisation used a beam of Cs^+ ions produced by typically operating the Cs^+ ion gun at 40 kV. The conventional mass spectra were obtained by scanning the magnet in the mass range of 50–1500 mass units, at a scan speed of 3 sec per decade and mass resolution of 2000.

RESULTS AND DISCUSSION

When optimizing the chromatographic conditions for preparative HPLC separation, we considered the published article on analytical separations of Bc using Kromasil C8, 5 μm (150 × 4.6 mm I.D.) column.[16] In order to improve separation of components B1 and B2, we used a longer column (250 mm) (Fig. 2). Figure 2 shows an appropriate efficiency (N) of a longer column with the same mobile phase but an inadequate selectivity ($\alpha \approx 1$) for the separation of components B1 and B2, which are difficult to separate.[5,12]

By increasing the MeOH part with regards to ACN in the mobile phase from 50 : 50 (v/v) to 30 : 70 (v/v), we significantly increased the selectivity of separation ($\alpha > 1$) between the mentioned components (B1 and B2). We, thus, transferred altered mobile phase from the analytical column to the preparative column of the same length (scale up) but with a bigger diameter and with the same stationary phase (Kromasil C8, 5 μm, 250 × 16 mm I.D.), and we finally optimised the method as well (Fig. 3).

We increased the flow in the preparative method from 1.4 to 9 mL/min. According to a theoretical calculation [Eq. (1)],[17,18] we could have chosen a bigger flow (≈17 mL/min) in ideal separation conditions, nevertheless,

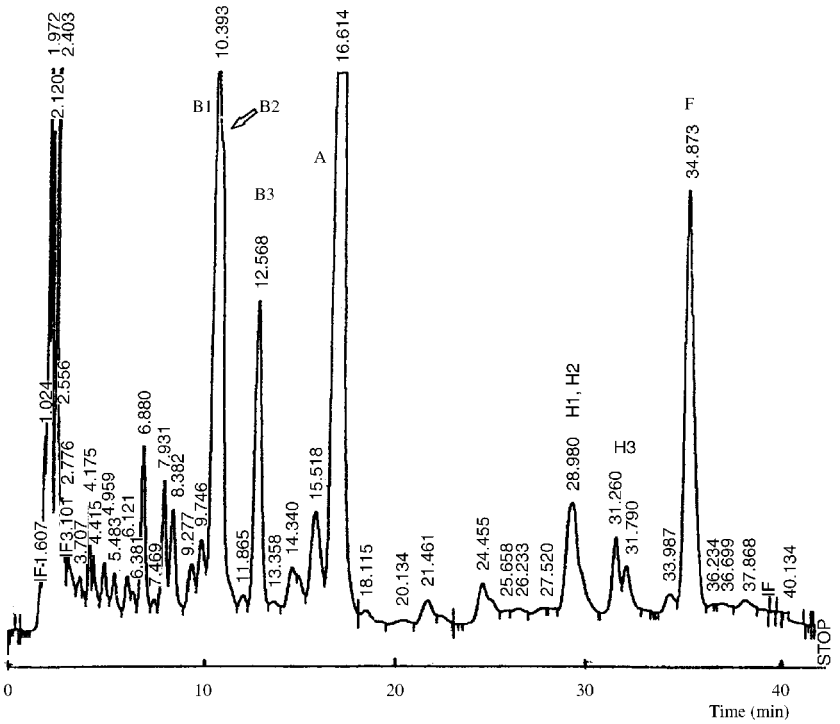


Figure 2. HPLC separation of Bc on Kromasil C8, 5 μm (250 × 4.6 mm I.D.) column, with ratio of ACN : MeOH = 50 : 50 (v/v) in the mobile phase with phosphate buffer (0.05 M KH_2PO_4, pH 6.0). (B1, B2, B3, A—microbiologically active components; H1, H2, H3, F—oxidative degradation products). Experimental conditions: column: Kromasil C8, 5 μm (250 × 4.6 mm I.D.); flow: 1.4 mL/min; detection: UV, $\lambda = 230$ nm, injection: 20 μL; temperature.: 30°C, MF A: (ACN/MeOH = 50/50, v/v)/(phosphate buffer 0.05 M KH_2PO_4, pH 6.0) = 49/51, v/v; MF B: (ACN/MeOH = 50/50, v/v)/(phosphate buffer 0.05 M KH_2PO_4, pH 6.0) = 60/40, v/v. *Programme:* The isocratic elution with 100% of MF A was kept for 18 min. Then the content of MF B was linearly increased to 100% in the time interval of 18–35 min. Isocratic elution of MF B was kept from 35 to 40 min, then the system was equilibrated again with 100% MF A within 40–43 min.

the chosen flow proved to be quite appropriate due to a very complex composition of Bc.

$$F_p = \frac{F_a \cdot (D_p)^2}{(D_a)^2} \tag{1}$$

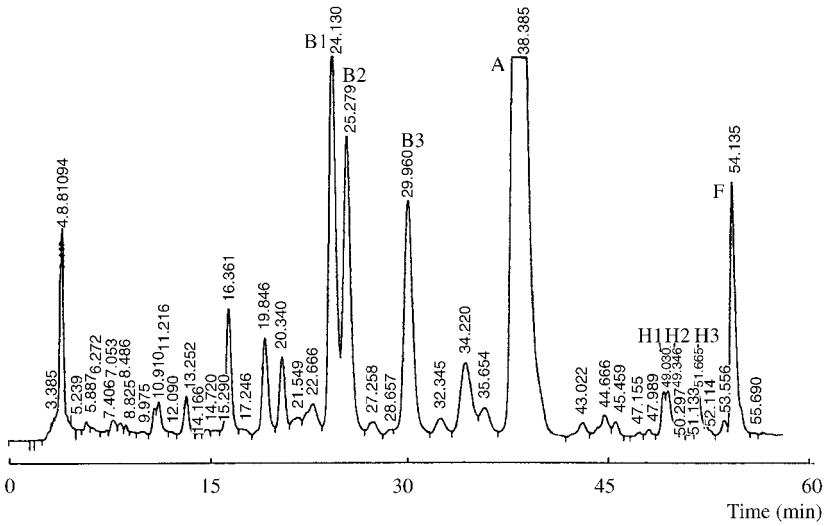


Figure 3. Preparative gradient HPLC separation of Bc on Kromasil C8, 5 μm (250 × 16 mm I.D.) column, with the ratio of ACN/MeOH = 30/70, v/v in the mobile phase with phosphate buffer (0.05 M KH_2PO_4, pH 6.0). (B1, B2, B3, A: microbiologically active components; H1, H2, H3, F: oxidative degradation products). Experimental conditions are described in Experimental: Preparative HPLC Isolation of Bc Components.

where F_p, flow rate of the preparative column; F_a, flow rate of the analytical column; D_p, diameter of the preparative column; D_a, diameter of the analytical column.

Considering Eqs. (2)[9,17] and (3)[19] and specificity of our sample (a possibility of interference with peptide impurities, low selectivity of components B1 and B2, and poor solubility of Bc in the used solvents), we chose optimum concentration of 15 mg Bc/mL and injection volume of 2 mL.

$$\mathrm{Sl_p} = \frac{\mathrm{Sl_a} \cdot (D_p)^2 \cdot L_p}{(D_a)^2 \cdot L_a} \tag{2}$$

where $\mathrm{Sl_p}$, sample load of the preparative column; $\mathrm{Sl_a}$, sample load of the analytical column L_p, length of the preparative column; L_a, length of the analytical column.

$$V_s < \frac{1}{2} F_p (t_y - t_x) \tag{3}$$

where V_s, maximum sample volume that can be injected on the preparative HPLC column; F_p, flow rate of the preparative column; t_x, t_y, retention times (for a small sample) of bands (x, y).

With the exception of component *F*, the position of oxidative degradation products of Bc in the HPLC chromatogram was not known at the beginning and we could not identify components H1, H2, and H3 from the published HPLC chromatogram of Ikai et al.[3,4] either. We used the accelerated degradation of individual isolated components B1, B2, and B3 (at elevated temperatures) to determine exact position (retention time) of their oxidative degradation products H1, H2, and H3 in the HPLC chromatograms.

In spite of gradient elution, the separation of both oxidative degradation products of components H1 and H2 arising from B1 and B2 also represented a considerable problem, since they did not separate (Fig. 2) or only the traces of separation were observed (Fig. 3). This might be explained with the structure of terminal chain of both components—H1 and H2, which have a keto group on the thiazole ring instead of amino group (Fig. 1), the latter being known to strongly interfere with silanol groups (–SiOH) of the stationary phase, thus differing from the keto group. A direct isolation of H1 and H2 from Bc with preparative HPLC methods was not possible. Prior to this, we had to carry out oxidation of the isolated components B1 and B2 by loading both sample solutions at elevated temperature, and then we were able to isolate both components H1 and H2 at the repeated chromatography on the preparative HPLC.

Another chromatographic problem was the peak of component H3, which demonstrated itself as a cleft or deformed peak on both the analytical as well as the preparative column (Figs. 2 and 3). We found out that this resulted from epimerisation of component H3.[20]

We did not notice the effects of overloaded columns at our experimental conditions (Fig. 3). An excellent separation of Bc components on the preparative column with Kromasil 5C8 stationary phase, justified the choice of the phase and the experimental conditions, since the resolutions were comparable to those obtained on the analytical column.

Kromasil 5C8 stationary phase proved to be the stationary phase of choice, due to its excellent mechanical and chemical stability, easy transit from analytical separation to preparative one (scale up), good repeatability, possibility of regeneration, high capacity yielding big recovery of peptides, as well as due to relatively low costs.

A part of organic solvent was evaporated on a rotary evaporator from eluates of isolated components obtained with preparative HPLC. Since we used gradient elution profile, we evaporated about 30% of organic phase from eluates B1, B2, B3, and A, which were collected in more polar solvent, and about 40% of organic solvent from components H1, H2, H3, and F, which were collected in a relatively less polar solvent. The evaporated

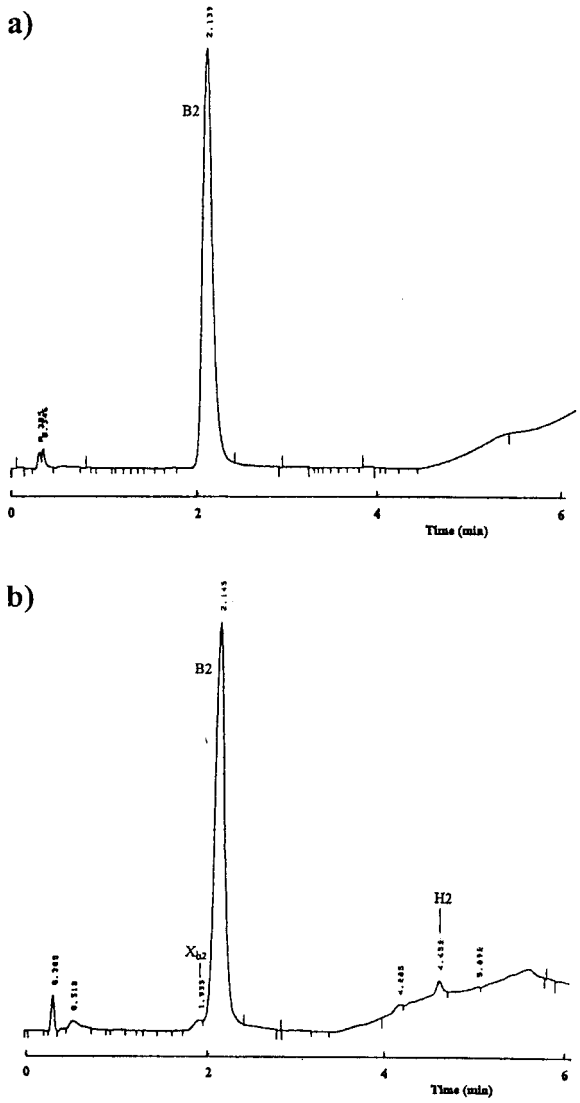


Figure 4. An example of comparison between HPLC chromatograms of component B2 of Bc immediately after preparative HPLC isolation (a), immediately after lyophilisation (b). (B2—microbiologically active component, Xb_2—unknown degradation product, H2—oxidative degradation product.) Separation was performed on monolithic silica gel Chromolith RP-18e (100 × 4.6 mm I.D.) column. Experimental conditions are described in Experimental: Identity and Purity of Isolated Components.

portion of organic phase was then replaced with water to increase polarity and then applied to the reverse-phased SPE cartridges.

Prior to the beginning of concentrating and cleaning of individual peptide components on SPE cartridges, we established adsorption capacity of a cartridge. This was done with Bc antibiotic, which is a natural complex mixture of numerous above mentioned peptide components (Fig. 1). The ratio between significant microbially active components in our sample was the following: B1 : B2 : B3 : A = 16.6 : 14.4 : 12.8 : 56.2 (%). The capacity of the used SPE cartridges (Superclean LC-18, 2000 mg) for Bc was found to be between 285 and 295 mg.

We needed pure Bc components without buffer salts for identification on a mass spectrometer, and also for other research purposes, therefore, we had to carry out cleaning of the adsorbed peptide components on the SPE cartridge by rinsing them with water. The quantity of water needed for removal of buffer salts was determined by using calignost—a sensitive reagent for potassium determination.

In all stages of our research work—in preparative HPLC extraction, in SPE extraction, the purity of isolated Bc components was controlled with a rapid monolithic silica gel Chromolith RP-18e (100 × 4.6 mm I.D.) column (Fig. 4a and b). The control of a possible breakthrough of individual components after application of sample solutions on SPE cartridges, and the control of quantitative recovery of individual components after rinsing from SPE cartridges were carried out in the same way.

The monolithic silica gel Chromolith RP-18e column proved to be perfect for a rapid and effective separation of isolated Bc components and their possible degradation products. The use of conventional columns for testing the

Table 1. Chromatographic purity of some isolated components of Bc immediately after preparative HPLC isolation and after lyophilisation. Between isolation and lyophilisation several phases of concentration and purification were performed.

Component of Bc	Purity after preparative HPLC isolation (%)	Purity after the last stage of isolation–lyophilisation (%)
B1	99.2	94.5
B2	98.9	93.7
B3	97.8	94.5
A	99.4	93.0
F	95.7	95.3

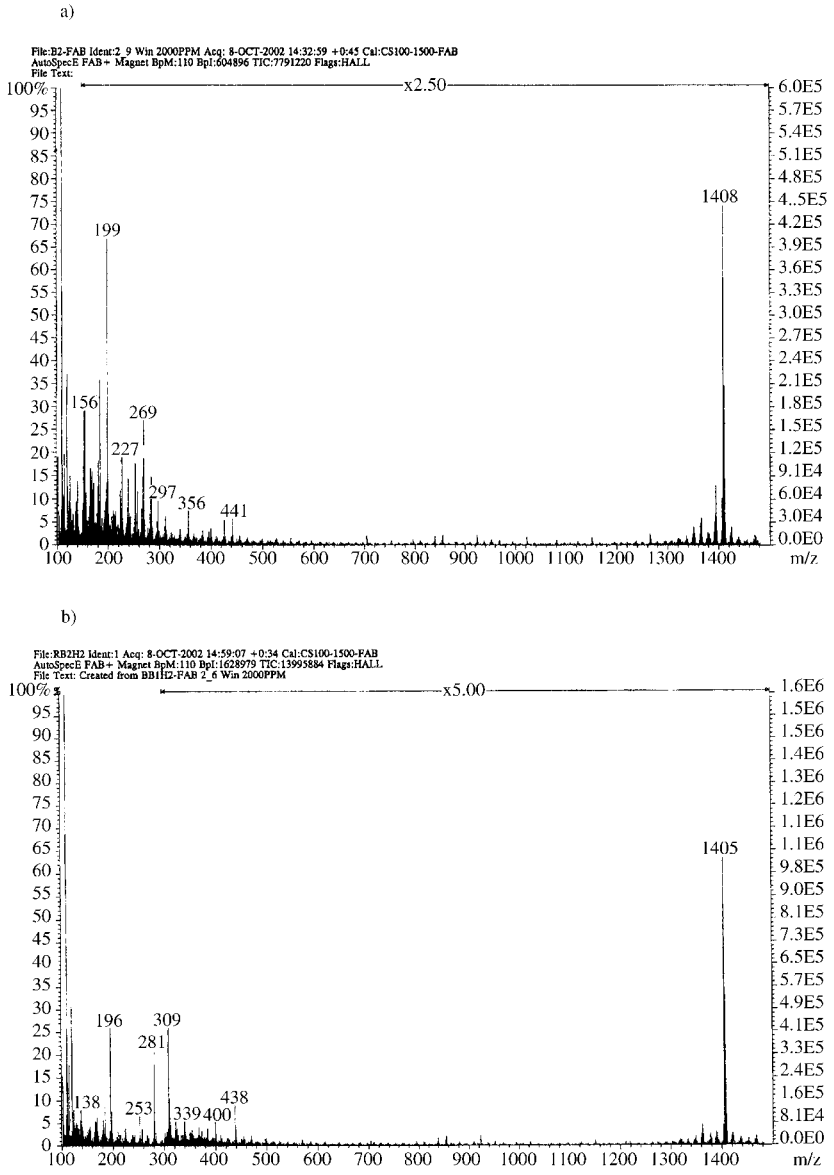


Figure 5. An example of FAB mass spectra: component B2 (a) protonated molecular mass: 1408 and its corresponding oxidative degradation product H2 (b) protonated molecular mass: 1405.

purity and isolation of stability sensitive peptide components is not suitable, and could not be used due to a time consuming separation.

The purity of isolated components was high immediately after preparative HPLC isolation (Table 1, Fig. 4a), but during further treatment including the above stated isolation phases, a minor decomposition of the active Bc components (A, B1, B2, and B3) was observed. This shows a problematic stability of the mentioned peptide components (Table 1, Fig. 4b). We did not observe further degradation of oxidative degradation products (F, H1, H2, and H3). This has been confirmed also by a similar purity of component *F* immediately after preparative HPLC isolation, and at the end after SPE extraction and lyophilisation (Table 1).

Final identification of individual active components Bc A, B1, B2, and B3 and their oxidative degradation products F, H1, H2, and H3, were done by determining the molecular masses with FAB mass spectrometry. Molecular masses corresponded to those proposed by Ikai et al.[4] (A: 1421, B1: 1407, B2: 1407, B3: 1407, H1: 1404, H2: 1404, H3: 1404, F: 1418). We hereby present two FAB mass spectra of components B2 and H2 (molecular masses have been protonated) (Fig. 5).

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