Pathways of Chemical Degradation of Polypeptide Antibiotic Bacitracin

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We described the main pathways of bacitracin (Bc) decomposition, chromatographically set the position of its major degradation products and evaluated microbiological activity of isolated components of Bc and its degradation products. All processes of Bc decomposition under stress and accelerated test conditions were monitored with HPLC, performed mainly on a new type reversed-phase (RP-18e) monolithic silica column (Chromolith®) enabling fast separation times and some of them also on conventional HPLC columns. Diode array detection, preparative HPLC and FAB mass spectrometry were used for identification of individual Bc components. We found that the major decomposition mechanism in water solutions of Bc is oxidation, and in alkaline solutions, deamidation. In oxidation process the components B1, B2 and B3 and A are oxidized into their corresponding oxidative products H1, H2, H3 and F respectively by the same mechanism. A detailed study of oxidative degradation products revealed that HPLC separation with an acid mobile phase caused splitting of peaks of components H2, H3 and F into two peaks but the peak of component H1 did not split due to its special structural properties. For the component A we confirmed gradual formation of desamido product through an intermediate. FABMass spectra of oxidative degradation products of Bc to be relatively stable, and desamido degradation products to be rather unstable. The estimation of kinetics of Bc decomposition was presented with a semi-quantitative model. Microbiological activity of individual isolated active components of Bc was established and the negligible antimicrobial activity of the degradation products was confirmed.

Key words bacitracin; stability; oxidation; deamidation; degradation product; monolithic silica reversed-phase column

Bacitracin (Bc) belongs to the group of cyclic polypeptide antibiotics with a complex composition and structure and is produced by certain strains of Bacillus licheniformis and Bacillus subtilis. It is mainly active against Gram-positive bacteria. Bc consists of a mixture of structurally similar polypeptides from 12 amino acids (AA). The most important and known component is Bc A (Fig. 1). Other important components are B1, B2 and B3 which together with component A, represent more than 96% of the total antimicrobial activity in commercial products. Bc is often available in the form of a more stable complex with Zn (ZnBc).

The basic structure of Bc consists of seven AA membered ring and five amino acids in the side chain. The terminal part of the side chain ends with thiazoline ring, consisting of 1-cysteine and 1-isoleucine or of L-cysteine and L-valine (Fig. 1). Ikai and co-workers determined the structure, sequence of amino acids and molecular weights of 15 major and minor Bc components using FAB, LC/MS and MS/MS.

The above mentioned polypeptide components are structurally very similar and they differ from each other only in the substitution of one to three of L-isoleucines by L-valine in the side chain or in the ring of 7 AA or in the terminal thiazoline ring (Fig. 1). Recently the nomenclature for three minor components D1, D2 and D3 has been changed to C1, C2 and C3 respectively in the PharmEuropa and in the Ph.Eur. 5th.

Bacterial resistance against antibiotics has become a serious health issue in recent years. However, bacterial resistance to bacitracin is still scarce despite its great and wide use in human and veterinary medicine, as well as in animal feed additives over the past several decades. In general, it is true that resistance to peptide antibiotics occurs

Fig. 1. Structure of Known Bacitracins and Transformation of the Amino-Thiazoline Ring (a), (b) to the Keto-Thiazole Ring (c), (d) in the Molecule of Bc by Oxidation, (Asn - Asparagine, Asp - Aspartic Acid, Glu - Glutamic Acid, His - Histidine, Ile - Isoleucine, Leu - Leucine, Lys - Lysine, Orn - Ornithine, Phe - Phenylalanine, Val - Valine)
rarely compared to the other antibiotics.\textsuperscript{5,9,13} Thus, bacitracin can serve as a potential lead molecule for a design of a new potent peptide antibiotic and analogues.\textsuperscript{9} Due to the therapeutic significance of peptide antibiotics we have set our goal to study the stability of Bc focusing on the mechanism of its decomposition.

Up to now stability studies of Bc or its formulations have only been performed by microbiological methods,\textsuperscript{14—18} therefore it is very little known about its degradation products which could be clearly and doubtlessly chromatographically defined.

It is well known that Bc-F is the oxidation product of Bc-A containing ketothiazole instead of amino-thiazoline moiety (Fig. 1). It is microbiologically inactive and claimed to be nephrotoxic.\textsuperscript{1,6,9,19,20} Other oxidative degradation products of microbiologically active components B1, B2 and B3 have almost not been mentioned, although Ikai and co-workers determined their molecular weights and proposed their structures and names H1, H2 and H3 respectively.\textsuperscript{9} In our recently published HPLC method for Bc, the mentioned oxidative degradation products H1, H2 and H3 were chromatographically clearly defined for the first time.\textsuperscript{21,22} We used mainly the new type monolithic silica reversed-phase Chromolith RP-18e (100×4.6 mm I.D.) column which dramatically reduced analysis time.\textsuperscript{21} In the present paper we more clearly confirmed the pathway of Bc (ZnBc) oxidation by performing additional stress tests on its isolated microbiologically active components. Non-oxidative desamido degradation products of Bc formed by deamidation of free amide group of AA L-asparagine are mentioned in publications even less frequently than oxidative ones.\textsuperscript{2,6,9} With the present study we chromatographically defined for the first time the most important Bc desamido degradation products obtained in the range of pH 5 to 12.

Since data concerning microbiological activity of individual components of Bc are relatively scarce,\textsuperscript{5,24} we also determined the antimicrobial activity of isolated components A, B1, B2 and B3. In addition activity of isolated oxidative degradation products F, H1, H2 and H3 and main non-oxidative hydrolytic degradation products of Bc-A was evaluated for the first time.

MATERIALS AND METHODS

\textbf{Materials and Reagents} \(\text{KH}_2\text{PO}_4\) p.a., (J. T. Baker, N.Y., U.S.A.); Water: HPLC grade (Milli-Q-185 Plus); Acetonitrile (ACN), HPLC grade (Rathburn, Wakerburn, Scotland); Methanol (MeOH), LiChrosolv (Merck, Darmstadt, Germany); \(\text{H}_2\text{PO}_4\) (85\%) supra pure, \(\text{K}_2\text{HPO}_4\) p.a., \(\text{Na}_2\text{HPO}_4\) p.a., \(\text{NaH}_2\text{PO}_4\) p.a., HCl 32\%, p.a., \(\text{H}_2\text{O}_2\) 30\%, p.a., KOH, p.a. and NaOH, p.a. (Merck, Darmstadt, Germany); Bacitracin Batch No: 413810/1 (Fluka, Buchs, Switzerland), dried at 40\% (samples were taken off at initial, after 2, 4, 7 and 8 d) and at 50±2\% (samples were taken off at initial, after 1, 2, 4, 5, 7 and 8 d) up to 8 d, filtered and analysed by HPLC.

\textbf{Preparation of buffers for mobile phases (MF):} Phosphate buffer 0.05 m, pH 6.0: \(\text{KH}_2\text{PO}_4\) was dissolved in water and pH was adjusted to pH 6.0 with 20\% KOH. Phosphate buffer 0.05 m, pH 2.0: \(\text{KH}_2\text{PO}_4\) was dissolved in water and pH was adjusted to pH 2.0 with \(\text{H}_2\text{PO}_4\) (ca. 40\%).

Nessler reagent was prepared according to Ph.Eur. \textsuperscript{5th}. ToSpec Q hybrid tandem mass spectrometer (EBE-qQ geometry Micromass, Manchester, U.K.). Fast atom bombardment (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\textsuperscript{+} ions produced by typically operating the Cs\textsuperscript{+} 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 s per decade and mass resolution of 2000.

\textbf{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, U.K.). Fast atom bombardment (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\textsuperscript{+} ions produced by typically operating the Cs\textsuperscript{+} 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 s per decade and mass resolution of 2000.

\textbf{Stress Testing of Bc in Buffer Solutions with Different pH} Solutions of 0.05 m \(\text{Na}_2\text{HPO}_4\) were adjusted to pH of 5 and 7 with NaOH (20\%) and solutions of 0.05 m Na\textsubscript{2}H\textsubscript{PO\textsubscript{4}} were adjusted to pH 10 and 12 with NaOH (20\%). Bc was dissolved in prepared phosphate buffers (10 mg/ml). Sample

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solutions of Be were placed at controlled room temperature (25 ± 2°C) and at stress condition in thermostatic cabinet at 50 ± 2°C up to 6 d and then diluted to conc. 2 mg/ml with water, filtered and analysed by HPLC.

Conditions for HPLC Separations
Chromolith RP-18e (100×4.6 mm I.D.) Endcapped Column: Flow rate: 5 ml/min, detection: UV λ = 230 nm, injection volume: 20 μl, temperature: 35°C; MFA: ACN–MeOH (30:70, v/v)–phosphate buffer (0.05 M KH₂PO₄, pH 6.0) (50:50, v/v), MFB: ACN–MeOH (30:70, v/v)–phosphate buffer (0.05 M KH₂PO₄, pH 6.0) (70:30, v/v).

Gradient Programme: The isocratic elution with a 100% mobile phase A was kept for 30 min. Then the content of mobile phase B was linearly increased to 100% in the time interval of 3.0 to 5.5 min. After isocratic elution of mobile phase B from 5.5 to 5.8 min, the system was equilibrated again with 100% mobile phase A between 5.8 and 6.5 min.

Kromasil C8 5 μm (250×4.6 mm I.D.) Column—Acid Mobile Phase (pH 2): Flow rate: 1.4 ml/min, detection: UV, λ = 230 nm, injection volume: 20 μl, temperature: 30°C; MFA: ACN–MeOH (30:70, v/v)–phosphate buffer (0.05 M KH₂PO₄, pH 2.0) (52:48, v/v), MFB: ACN–MeOH (30:70, v/v)–phosphate buffer (0.05 M KH₂PO₄, pH 2.0) (60:40, v/v).

Gradient Programme: The isocratic elution with 100% mobile phase A was kept for 20 min. Then the content of mobile phase B was linearly increased to 100% in the time interval of 20 to 38 min. After isocratic elution of mobile phase B from 38 to 40 min, the system was equilibrated again with 100% mobile phase A between 40.1 and 44 min.

Hypersil BDS 5 μm (250×4.6 mm I.D.) Column: Flow rate: 1.4 ml/min, detection: UV, λ = 230 nm, injection volume: 20 μl, temperature: 35°C; MFA: ACN–MeOH (10:90, v/v)–phosphate buffer (0.05 M KH₂PO₄, pH 6.0) (56:44, v/v), MFB: ACN–MeOH (10:90, v/v)–phosphate buffer (0.05 M KH₂PO₄, pH 6.0) (65:35, v/v).

Gradient Programme: The isocratic elution with 100% of mobile phase A was kept for 20 min. Then the content of mobile phase B was linearly increased to 100% in the time interval of 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.

Microbiological Assay of Isolated Bc-A, B1, B2, B3, H1, H2, H3, F and Hydrolytic Products of Component A (Intermediate Xa1 and Desamido Product Xa)
Preparation of phosphate buffer pH 6.0: 2.0 g of K₂HPO₄ and 8.0 g of KH₂PO₄ were dissolved in 1000 ml of water and pH was adjusted to pH 6.0 with H₃PO₄ (18 N) or KOH (10 N). The basic test solutions of isolated components Bc-A, B1, B2, B3, H1, H2, H3, F, Xa1 and Xa were prepared at the concentration of about 0.5 mg/ml, each, with phosphate buffer pH 6.0.

Microbiological activities of each individual component were determined by procedure described in Ph.Eur. 5th ed. on the microorganism Micrococcus luteus ATCC 10240.

RESULTS AND DISCUSSION

The antibiotic Bc is of a complex and variable peptide composition. The study of decomposition mechanism in such systems is tricky and often unsuccessful due to numerous possible interferences. In order to firmly establish and confirm the major degradation pathways we isolated microbiologically active components of Be (A, B1, B2, B3), their oxidative degradation products (H1, H2, H3, F) and hydrolytic desamido degradation products (Xa1, Xa) of BeA. The isolation was done with a preparative fast gradient HPLC separations (ca. 6 min) on the new type of monolithic silica reversed phase column.22)

Using stress stability tests on the isolated Bc components we were able to identify the decomposition processes and individual oxidative and nonoxidative desamido degradation products. The peak that was eluted before each microbiologically active component (B1, B2, B3, A) corresponded to hydrolytic-desamido degradation product (Fig. 2). The retention times of these degradation products of Be (Xa, Xb1, Xb2, Xb3) were shorter while those of oxidative ones (H1, H2, H3, F) were longer compared to their parent intact components. A pronounced increase of oxidative products H1 is illustrated in Fig. 2b showing the decomposition pattern of isolated components B1 in water solution at 50°C. In water solutions containing Be or its isolated active components at elevated temperatures the peaks of hydrolytic desamido products increased very slowly during time (Fig. 2).

In general, the decomposition of all isolated microbiologically active components of Be, as well as that of the antibiotic Be itself followed the same analogy. Oxidative and nonoxidative degradation profile of Be in water solution at 40 and 50°C for up to 8 d is presented graphically in Fig. 3.

We detected some additional unknown degradation products in the samples stored at higher temperatures (e.g. 50°C) when chromatographing the isolated Bc components. These degradation products were eluted between the peak of the active component and the peak of the corresponding oxidative degradation product (Fig. 2b).

In order to re-check and confirm these findings we also performed separations of Be and its components on conventional HPLC columns requiring, however, at least five times longer separation times.21,22)

As found by our experiments the favoured pathway of degradation of Be stored at elevated temperatures in neutral water solutions is oxidation (Fig. 2). This was further verified in stress testing by adding an oxidant (H₂O₂) to the water solution of Be, since practically identical HPLC chromatograms were obtained. Addition of alkali (NaOH) or acid (HCl) in boiling water for 15 min was very stressful for Be which was practically completely decomposed. In general Be in diluted alkaline aqueous medium was more sensitive to oxidation than in diluted acidic aqueous medium. The decomposition of ZnBc was significantly slower under all stress conditions.

Using HPLC separations, DAD detection, HPLC preparative isolations and FAB mass spectrometry we firmly established that oxidative degradation of all microbiologically active components is governed by the same mechanism (from aminomethylene-thiazoline moiety into a keto-thiazole moiety) (Fig. 1).26,9 Each active component of Be is oxidized into corresponding derivative A into F, B1 into H1 (Figs. 1, 2), B2 into H2, B3 into H3 and (Fig. 1).21,22

In several papers on HPLC separation of Be carried out up to the present we frequently pointed out that under certain experimental conditions (especially when mobile phases with
lower pH were used) the peaks of degradation products may be asymmetric or may even split into two subunits (e.g. F splits into F\textsuperscript{H11032} and F\textsuperscript{H11033}). A pronounced splitting of peaks of oxidative degradation products H2, H3 and F, with the exception of H1, was observed on conventional reversed phase HPLC columns when acidic mobile phase with pH 2 was used (Fig. 4).

The splitting phenomenon can be explained with epimerisation at the terminal side chain of Bc (Fig. 1). The thiazole ring in the side chain of components H2, H3 and F is condensed from L-cysteine and L-isoleucine while in components H1 it is condensed from L-cysteine and L-valine.\textsuperscript{6,9} The H1 molecule at the mentioned side chain does not have a chiral centre while each of other oxidative degradation products (H2, H3 and F) has one (Fig. 1).

Using DAD detector we confirmed identical UV spectra for all mentioned oxidative pairs of degradation products H2–H2\textsuperscript{H11032}, H3–H3\textsuperscript{H11032} and F–F\textsuperscript{H11032} (Fig. 4) and identical molecular masses by FAB mass spectrometry.

Some smaller peaks with longer retention times were eluted after the mentioned major oxidative degradation products. They most probably indicate decomposition of oxidative products F, H1, H2 and H3 and have similar characteristic UV spectra (\(\lambda_{max} = 290\) nm) (Fig. 4). All significant oxidative degradation products in HPLC chromatograms have recently been identified.\textsuperscript{21,22} In the articles on HPLC separations published by other authors up to the present, the degradation products have hardly been mentioned—with the exception of component F. We presume that the reason lies in the complexity of HPLC separation of Bc itself as well as in asymmetric and splitted peaks of oxidative products (more frequently on the peak H3 and partially on the peak F), which have caused certain dilemmas and ambiguities. Also the Ph.Eur.\textsuperscript{5th} does not require degradation products of Bc with exception of component F to be identified and evaluated in HPLC chromatograms.

Besides the prevailing oxidative decomposition of Bc, a minor, non-oxidative decomposition is going on in aqueous solutions. The general mechanism of non-oxidative decomposition is deamidation of AA L-asparagine (–NH\textsubscript{2} group is replaced by –OH), which is the only AA with a free amide group in Bc. With deamidation, L-asparagine is converted into aspartate or isoaspartate according to more general scheme presented by M. D. Dibiase and M. K. Kottke\textsuperscript{26} and somewhat more detailed by B. Li and R. T. Borchardt \textit{et al.}\textsuperscript{27}

We established that Bc in water solutions is relatively slowly decomposed at room temperature (25 °C) through the mechanisms of deamidation. This reaction is faster at room temperature in alkaline medium especially at pH 12 as can be seen from Fig. 5. Desamido products of Bc which are eluted just
before corresponding active components (Figs. 2, 5, 6) have UV spectra (λ_{max} \approx 254 nm) identical to their parent intact microbiologically active compounds (Fig. 6).

With Bc separation on a conventional HPLC column we demonstrated that desamido product of component A is forming indirectly—through the intermediate Xa1, which is slowly converted into final desamido degradation product Xa (Fig. 5). Our finding has also been confirmed by the study of...
deamidation of the isolated Bc component A in the alkaline media (Fig. 6). UV spectra of the component A, intermediate Xa1 and that of the end desamido degradation product Xa are identical (Fig. 6).

The hydrolytic degradation process of other components of bacitracin was not studied in details, nonetheless we presume that it follows the similar mechanism as that of the component A. In our studies we marked hydrolytic degradation products of the components B1, B2 and B3 as Xb1, Xb2, Xb3, which is a uniform denomination of a possible hydrolytic intermediate and of the final desamido form (Figs. 2, 5). The intermediate (Xa1) and final desamido product (Xa) were partly separated on the conventional Hypersil BDS column (Figs. 5, 6), but were not separated under conditions used on a short monolithic silica Chromolith RP-18e column resulting in a joint peak of a slightly distorted form (Fig. 2).

In addition to HPLC analyses, we proved the deamidation of Bc by the Nessler reagent which reacted with released ammonia. The colour of the alkaline solution turned to yellow-brown of various intensity, depending on the pH, and on the amount of the released ammonia.

Comparison of the pH related rate of Bc A deamidation shows process to be very rapid at higher pH (especially at pH 12) and at elevated temperature (50 °C) (Fig. 7). At these conditions, also the hydrolytic degradation products Xa1 and desamido product Xa are extremely unstable (Fig. 7).

We found that desamido products at temperatures exceeding 40 °C decompose first, most probably into corresponding relevant oxidative products; furthermore we presume that under alkaline conditions deamidation is going on also on oxidative degradation products probably resulting in a large number of minor degradation products. Contrary to desamido products of Bc (Xb1, Xb2, Xb3, Xa) the oxidative ones (H1, H2, H3, F) are significantly more thermally stable.

Even in fresh Bc water solutions the desamido degradation products may already be present. Chromatographically this can be clearly seen with component A and partially also with components B1 and B2, since desamido products are usually eluted in one peak on a short monolithic silica Chromolith RP-18e (100×4.6 mm I.D.) column (Fig. 2) or only partially separated on conventional columns (Figs. 5, 6).

The portion of component B3 is relatively small (10—13%) compared to other microbiologically most significant components of Bc, consequently the amount of its degradation desamido product (Xb3) in neutral water solutions is usually small and poorly visible also due to its flat form. In addition, it can be sometimes hidden in the peak of B3 or can interfere with the previous peak of the component B2-depending on the conditions of HPLC separation.

In this as well as our previous studies[21,22] we performed chromatographic separations by using the mixture of MeOH, ACN, and 0.05 M phosphate buffer pH 6.0 in proper ratios for mobile phases. For an effective separation of Bc it is important to use a mobile phase not only with an appropriate ratio of non-polar to polar solvent, which is in general similar for all described HPLC procedures, but also with an appropriate ratio of ACN to MeOH. Identification of final degradation products (Xa1, Xa) was established using a two step preparative HPLC. First we used gradient preparative HPLC to isolate the mixture of both hydrolytic degradation product (Xa1+Xa) followed (Fig. 8a),22 in the next step by isolation of individual Xa1 and Xa using preparative isocratic HPLC separation (Fig. 8b). With FAB mass spectrometry we confirmed that the actual deamidation of AA L-asparagine in the
Bc molecule occurred as late as in transformation from Xa1 to Xa. Protonated molecule mass increased for one unit from 1422.6 for component A to 1423.6 for desamido product Xa, the intermediate Xa1 still having the same protonated molecular mass as A (Fig. 9).

On the basis of kinetic study of Bc in water solution at additional stress and accelerated storage conditions it was established that the degradation of Bc can be presented by the first order reaction kinetics with the degradation rate constant \( k \) of 0.0133 \( \text{d}^{-1} \) at room temperature (25 °C) and of 0.0040 \( \text{d}^{-1} \) in refrigerator (5 ± 3 °C) (personal communication).

The kinetics and main pathways of decomposition of Bc in water solution can be summarized in a semi-quantitative model (Fig. 10), in which the reaction rates constants of decomposition follow the order: \( k_1 \) (oxidation) > \( k_3 \) (deamidation) > \( k_4 \) (desamido degr. products) > \( k_2 \) (oxidized degr. products) (Fig. 10).

We set antimicrobial activity of individual isolated components of bacitracin B1, B2, B3 and A, and their corresponding oxidative degradation products H1, H2, H3 and F as well as the desamido degradation products of the component A (Xa1, Xa). Pharmacopeial method was applied obtaining the results given in Table 1. As we can see the component A has the highest antimicrobial activity which is at least 25% higher than those of the components from B group. Oxidative degradation products (H1, H2, H3, F) have very low activity, as can be said also for the hydrolytic degradation products of the component A (Xa1, Xa). We believe by analogy that this applies to all other desamido degradation products of Bc (Xb1, Xb2, Xb3) as well. Because Bc decomposes in practically inactive components during storage, HPLC analy-
sis was performed of each active component before microbiological assay and its peak area (%) was considered into corrected activity (Table 1).

Referring to the degradation products we think it would be useful to check the credibility of the old and frequently quoted information about nephrotoxicity of the component F if Bc is used parenterally.\(^1,6,19,20\) If this information proves to be accurate, we believe that there is a great certainty that this may be true for all other oxidative degradation products of Bc-H1, H2 in H3, as well, since there are very slight structural differences between them. There is also the possibility that nephrotoxicity could be ascribed to the multicomponent nature of Bc itself. With regard to the antimicrobial inefficiency of Bc degradation products we believe that the key groups responsible for activity of Bc are amide group on AA L-asparagine and a free N-terminal amino group representing therefore the pharmaco-forming groups of the drug. We presume that the mentioned groups play a key role by the indirect, as well as by direct interaction of Bc with a lipid pyrophosphate C-55 in the bacterial cell and as such in the antimicrobial activity—i.e. inhibition of the biosynthesis of the bacterial wall.

On the basis of microbiological analyses we can conclude that the microbiological method is a relatively selective one, since the contribution of antimicrobial activity of oxidative and desamido degradation products is practically nil. A small contribution to the total antimicrobial activity of the major components of Bc is made by some microbiologically less important and characteristic components of Bc which are generated during the fermentation (e.g. a group of Bc components C).

CONCLUSION

Using HPLC chromatography we followed the main pathways of decomposition of the peptide molecules of Bc in water solution under different conditions and found two major pathways of decomposition—oxidation and deamidation. Based on the knowledge of Bc decomposition processes we can expect a similar, yet significantly slower decomposition of the Bc solid substance itself when not protected from humidity. Under normal conditions oxidation proved to be the highest priority pathway of decomposition leading to degradation products showing negligible antimicrobial activity.

A full and complementary picture of Bc quality is achieved only with a combination of both analytical methods—microbiological and HPLC. Among the latter methods we consider HPLC on monolithic silica Chromolith RP-18e column especially suitable since it gives exceptionally quick results with good separation performance, reducing the risk of decomposition of potentially sensitive peptide molecules during measurements to the minimum.

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