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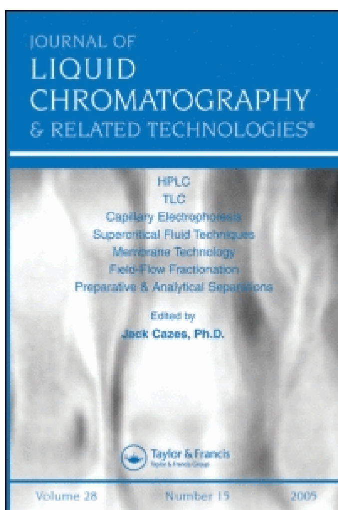

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COMPARATIVE DETERMINATION OF BACITRACIN BY HPLC AND MICROBIOLOGICAL METHODS IN SOME PHARMACEUTICALS AND FEED GRADE PREPARATIONS

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ABSTRACT

In this text, reverse phase HPLC method for separation of bacitracin is described. By selection of optimum chromatographic parameters in isocratic elution, the procedure for quick simple and precise determination of the most important active bacitracin components A, B_1 and B_2 was obtained. In relation to microbiological determination it was concluded that selective HPLC method gave considerably more useful analytical information on properties and origin of bacitracin samples. Intermethod differences in analysing bacitracin feed grade preparations, can be explained by specific ingredients in this type of samples and less selective microbiological method. Described HPLC method expresses certain advantages over some other published HPLC methods and it is suitable for quantitative and qualitative determinations of bacitracin in pharmaceuticals and feed grade preparations. In stability studies, it is possible to observe, in this way, the appearance of bacitracin F, the main degradation product of bacitracin. It was also found out that component of bacitra-

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cin B_1 is not homogeneous. Under specific conditions it can be separated in two different subunits, and we sign them as B_1' and B_1''.

INTRODUCTION

Antibiotic bacitracin, produced by certain strains of Bacillus licheniformis and Bacillus subtilis is not a substance having a definite and unique structure but it appears as the mixture of structurally similar polypeptides. In reference literature, there is not unique designation of separate polypeptide components of bacitracin. The following componentes are mentioned: A, A_1, B, C, D, E, F_1, F_2, F_3, G, and B_1, B_2, X, |1 - 6|. Bacitracin A and B (B_1, B_2) are the major microbiologically active components. Sequence of amino acids in bacitracin A is indicated in Fig. 1. In bacitracin B (B_1, B_2), one of amino acids of L-isoleucine was replaced by L-valine. Differences between B_1 and B_2 are not defined completely. The main degradation product of bacitracin is bacitracin F resulting by transformation of bacitracin A and B |3,5|.

Pure bacitracin has an usual activity between 60 and 80 international units per milligram. In animal feed and feed grade preparations the potency of bacitracin is expressed in term of its weight. Then, 1 (one) gram of activity is equivalent to 42000 units. (23,8 µg ≘ 1 unit) |7|.

In water solutions, bacitracin is setlled with addition of bivalent metal cations. In the form of zinc salt bacitracin shows the best stability and better microbiological activity. Commercial products of Zn-bacitracin feed grade contain 4-6 % of zinc |3,5|.

Out of all analytical methods, microbiological methods |4,8,9| have been up to now used mostly for quantitative determination of bacitracin. In 1974, reverse phase HPLC method for separation of bacitracin with gradient elution was first applied by Tsuji at al. |6|. Subsequently, this method was improved by Tsuji and Robertson |2|. Using microbiological and HPLC methods, the

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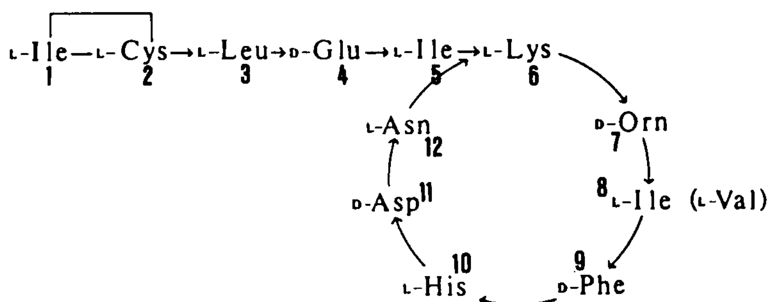

FIGURE 1. Sequence of amino acids of bacitracin A (B). In bacitracin B, one of L-isoleucine is substituted with L-valine.

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above authors found out that practically complete antimicrobial activity of bacitracin could be attributed to components A, B_1 and B_2 |2|. Gallagher et al. have developed HPLC method with isocratic elution for determination of bacitracin in animal feed additives |10| which has been also accepted by AOAC as official procedure |11|. Recently, Oka et al. have described isocratic HPLC procedure for separation of bacitracin components on polymer type RP-HPLC columns |12|.

In our experimental study we have guided ourselves with findings by Tsuji and Robertson |2|, and on this base we have developed isocratic reverse phase method for determination of bacitracin |13|. Later, the method was improved |14| and is presented in more details in this report. In this study, particularly we were interested in comparison of results of microbiological and HPLC methods in determination of bacitracin as well as in ratio of the most important active components of bacitracin: A, B_1 and B_2. Described method is convenient both for quantitative and qualitative determination of bacitracin in pharmaceuticals and feed grade preparations as well as for stability studies of bacitracin.

MATERIAL AND METHODS

Reagents and Materials

Acetonitrile, methanol (Merck, Darmstadt, F.R.G.). Water was deionised and distilled. KH_2PO_4 p.a., H_3PO_4 (85 %) p.a. (Kemika, Zagreb, Yugoslavia). Standard Zn-bacitracin: 69 i.u./mg (Institution for Drugs Control and Testing, Zagreb, Yugoslavia). Samples of Zn-bacitracin feed grade 10 % and 20 % were supplied by Krka (Novo mesto, Yugoslavia), and Zn-bacitracin feed grade 10 % and 15 % were supplied by Apothekarnes, (Oslo, Norway). Pharmaceutical preparation of bacitracin (powder and globules) were supplied by Lek (Ljubljana, Yugoslavia).

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Chromatographic Conditions and Methods

The HPLC system consisted of a Varian LC-8500 gradient system (Varian Assoc. Palo Alto, CA), a Spectroflow monitor SF 770 (Kratos Analytical, Remsey, NJ), an LKB pump 2150, an LKB Variable wevelenght monitor 2151 (LKB, Bromma, Sweden), a Rheodyne Model 7125 loop injector with a 20 μl fixed loop (Rheodyne, Inc.,Cotati, CA). Integration was based on peak area measuring and an HP Model 3390 A Recording Integrator (Hewlett-Packard, Avondale, PA). External standard procedure was used for quantitative determination of bacitracin, and it was expresed by summing of neighboring peak areas of bacitracin A, B_1 and B_2.

Mobile phase was delivered at 1,4 ml/min. and consisted of 0,05 M KH_2PO_4 in water and (methanol - acetonitrile, 1 : 1) in the ratio 51/49 (v/v) for column Nucleosil 5 C 18, 5 μm (150 x 4,6 mm I.D.) (Macheray - Nagel, Düren, F.R.G.) and 54/46 (v/v) for column ChromSpher C 18 5 μm (150 x 4,6 mm I. D.) (Chrompack, Middelburg, Netherlands), respectively. Separations were made on ambient temperature and detected at 220 nm.

Microbiological determination of bacitracin was tested according to BP 80.

Preparation of Standard and Samples of Bacitracin

"Acid mobile phase" for dissolving of bacitracin was mixture of mobile phase and H_3PO_4 (85 %) in the ratio 99/1 (v/v). Bacitracin standard and different bacitracin samples were dissolved in "acid mobile phase" by mixing on the vortex mixer for few minutes and subsequently agitating in an ultrasonic bath for additional 3 minutes. Crude particles were removed by centrifugation. By further dilution with "acid mobile phase", dilutions of approximately 10 i.u./ml of bacitracin were prepared. Before injecting, these solutions were filtered through 0,45 μm millipore membrane filters.

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RESULTS AND DISCUSSION

Optimization of Chromatographic Parameters

Described isocratic HPLC procedure for separation of bacitracin on reverse phase was developed from the gradient HPLC method by Tsuji and Robertson |2|, so that single chromatographic parameters were changed gradually. It was found out that out of all parameters for separation of bacitracin the concentration of KH_2PO_4 was the most important (Fig. 2).

With an incrise of concentration of phosphate buffer in mobile phase by factor 2,5 in relation to concentration in procedure of Tsuji and Robertson |2|, the components of bacitracin A, B_1 and B_2 were separated more efficiently in approximately 6 times shorter period. Change of pH value for a few tenths of value has not been substantial for separation quality. When concentration of KH_2PO_4 increase in the range of 0,01 to 0,08 M it was found out that up to concentration of 0,05 M value of separation factor (α) and resolution (R_s) had been increased, while the factor of capacity (k') for single components of bacitracin had been decreased. By increase of concentration of KH_2PO_4 above 0,05 M there was no effect in separation improvement while on the con-

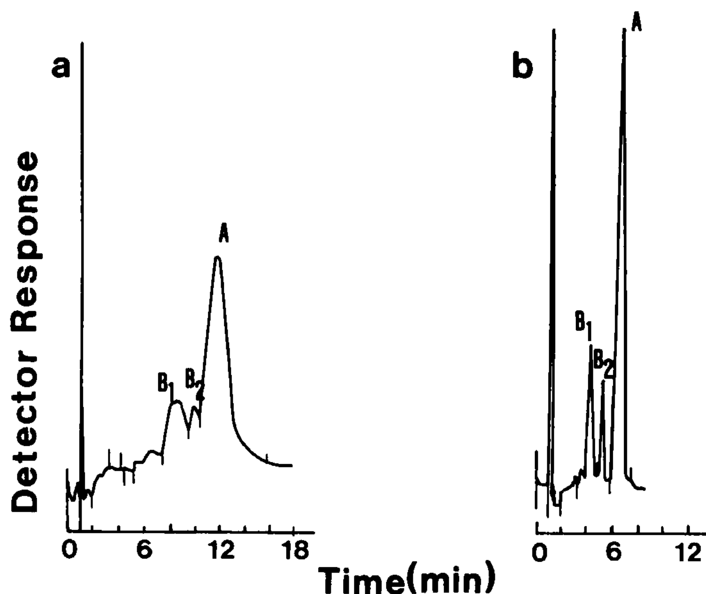

FIGURE 2. Influence of KH_2PO_4 concentration in mobile phase, on the HPLC separation of Zn-bacitracin working standard on the column: Nucleosil 5 C 18 5 µm (150 x 4,6 mm I.D.).
a) 0,01 M KH_2PO_4 : (CH_3OH : CH_3CN = 1 : 1)/51 : 49 (v/v)
b) 0,05 M KH_2PO_4 : (CH_3OH : CH_3CN = 1 : 1)/51 : 49 (v/v)
Other experimental conditions are the same as those mentioned in the text.

trary risk of salts chrystallization off was much increased.Thus 0,05 M KH_2PO_4 was selected as optimal concentration in mobile phase.

In connection with preparation of mobile phase, it should be noted that even minimal deviation from optimal ratio between water phase (0,05 M KH_2PO_4) and organic phase (acetonitrile - methanol) strongly affecting separation.

Appearance of shortening retention time in components A,B_1 and B_2 in reverse stationary phase could be explained by incre-

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ase of ionic strength of mobile phase, this leading to increased solubility of peptides (bacitracin) and shortening of their retention timeson stationary phase. Also KH_2PO_4 in mobile phase, decreases interaction between polar group of bacitracin and silanole groups of stationary phase, thus also resulting in shortening of retention time and contributing to good symmetry of peaks. In relation to statement made by Oka at al. |12|, in application of suggested procedure, no tailing effects in separation of bacitracin components occured on reverse phase columns. In such a way of separation of bacitracin, application of polymer RP-HPLC columns is not necessary in order to avoid appearance of non symmetric peaks.

During preparation of sample and standard of bacitracin according to Tsuji and Robertson |2|, it was noticed that negative peak appeared in chromatograms occassionally. So it is recommended that "acid mobile phase" for dissolving bacitracin is used. Such way of sample preparation provides application of the same system of solvent both in sample and mobile phase and more efficient dissolving of bacitracin.

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Determination of Bacitracin in Different Samples

Results of microbiological and HPLC determination of bacitracin in two different pharmaceutical products are shown in Table 1. It is evident that there is a very good correlation between microbiological and HPLC determination.

Results of microbiological and HPLC analysis of bacitracin in feed grade preparations of two different manufacturers (Krka and Apothekarnes) are indicated in Tables 2 and 3, and typical chromatograms in Fig. 3. Results of HPLC determination of bacitracin activity for Krka samples are on average by 16,0 % lover than results of determination by microbiological method (Table 2). For Apothekarnes samples, results of HPLC determination are on average by 13,8 % lower than results of microbiological determination

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TABLE 1

Results of Bacitracin Determination in Antibiotic Powder with Bacitracin and Globules with Bacitracin

sample[A]	HPLC	microbiological BP 80
bacitracin powder[B]	content of bacitracin (i.u./g)	
sample No 1		
$\bar{X}$	243,6	248,3
% of declared	97,3	99,3
RSD	± 1,1	± 2,1
sample No 2		
$\bar{X}$	234,7	242,0
% of declared	93,9	96,8
RSD	± 1,8	± 2,4
bacitracin globules[C]	content of bacitracin (i.u./glob.)	
$\bar{X}$	2356	2410
% of declared	95,1	96,4
RSD	± 2,3	± 2,6

[A] Each result ($\bar{X}$) represents average of two separate determinations of the same batch No (each injected two times)

[B] Antibiotic powder with bacitracin: declared contents of bacitracin is 250 i.u./g

[C] Globules with bacitracin: declared contents of bacitracin is 2500 i.u./glob.

TABLE 2

Results of Bacitracin Determination in Krka 10 % Zn-bacitracin Feed Grade Preparations

sample No[A]	HPLC	microbiological BP 80
	content of bacitracin i.u./mg (g/kg)	
1	3,61(86)	4,50(107)
2	3,61(86)	4,58(109)
3	3,82(91)	4,37(104)
4	3,99(95)	4,45(106)
$\bar{X}$	89,5	106,5
SD	± 4,36	± 2,08
RSD	± 4,87	± 1,95

[A] Each result represent average of two separate determination of the same batch No (each injected two times). Declared content of bacitracin is: 4,2 i.u./mg (100 g/kg)

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(Table 3). With HPLC method, also a simultaneous determination of ratio of microbiologically active components of bacitracin is possible. Ratio of bacitracin A, B_1 and B_2 for both groups of samples as well as for reference standard of bacitracin are indicated in Table 4. From these results, it is clear that ratio of components A, B_1 and B_2 in Krka samples is very similar to ratio in reference standard. In group of Apothekarnes,amount of bacitracin A is somewhat lower but amount of component B_1 is higher. However, from Table 4 it is also evident that Apothekarnes samples show better homogeneity of components A, B_1 and B_2 in relation to Krka samples. Statistical parameters of SD and RSD for single components in Apothekarnes samples are lower than in Krka samples, thus pointing out probably more stable technological process of biosynthesis of this manufacturer.

TABLE 3

Results of Bacitracin Determination in Apothekarnes 15 % Zn-bacitracin Feed Grade Preparations

sample No[A]	HPLC	microbiological BP 80
	content of bacitracin i.u./mg (g/kg)	
1	6,01(143)	6,85(163)
2	5,92(141)	6,72(160)
3	5,88(140)	7,02(167)
4	5,97(142)	6,76(161)
5	5,46(130)	6,72(160)
6	5,62(141)	6,72(160)
$\bar{X}$	139,5	161,8
SD	± 4,76	± 2,76
RSD	± 3,40	± 1,71

[A] Each result represents average of two separate determinations of the same batch No (each injected two times). Declared content of bacitracin is: 6,3 i.u./mg (150 g/kg)

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In relation to microbiological analysis by which total activity of antimicrobial components of bacitracin are determined quantitatively, by HPLC method, outlined in this article, it is possible, besides quantitative determination, to conduct also qualitative determination of single components as well as identification and determination of substance origin. It is important to point out that microbiological determination has been nonselective and that in such a way, simultaneously with active components, degradation products and other ingredients, which may have certain antimicrobial activity or act sinergistically, should be expected. This relation probably results in deviations of HPLC and microbiological determination in analysis bacitracin in the

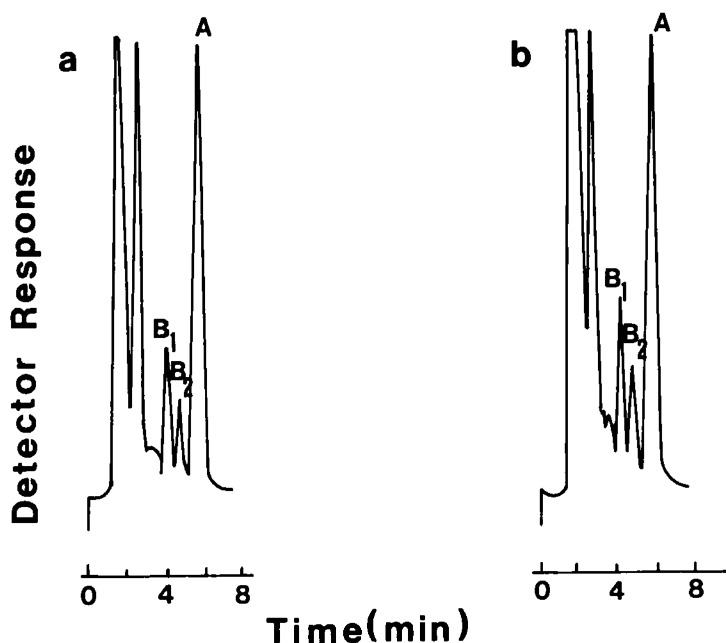

FIGURE 3. Chromatograms of 10 % feed grade preparations of two different manufacturers. Column : ChromSpher C 18 5 µm (150 x 4,6 mm I.D.). Other experimental conditions are the same as those mentioned in the text.

a) Krka preparation

b) Apothekarnes preparation.

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samples of feed grade preparation. In this type of samples, in addition to bacitracin, also a series of other substances originating from biotechnological process of bacitracin production are appeared (Fig. 3). Among this ballasting agents some of them can be even toxic, like bacitracin F actualy is |4|. In the purity testing of bacitracin on bacitracin F and related supstances, BP 80 recommend spectrofotometric method |8|. This method is less selective in relation to HPLC method, and couldn't be applied

TABLE 4

Comparison of Zn-bacitracin Feed Grade Preparations of Krka and Apothekarnes with Bacitracin Standard According to Ratio of Components A, B_1 and B_2

sample No[A]	percentage ration		
	A	B_1	B_2
Krka			
1	70,6	20,6	8,7
2	76,6	12,4	10,8
3	70,6	20,2	9,1
4	80,1	10,2	9,8
5	78,2	11,4	10,5
$\bar{X}$	75,2	15,0	9,8
SD	± 4,27	± 5,03	± 0,92
RSD	± 5,8	± 33,0	± 9,4
Apothekarnes			
1	65,1	23,5	11,4
2	65,3	24,4	10,2
3	65,7	23,7	10,5
4	64,0	25,7	10,9
5	64,4	25,7	9,9
$\bar{X}$	64,9	24,6	10,4
SD	± 0,96	± 1,05	± 0,59
RSD	± 1,06	± 4,30	± 5,64
Reference[B] standard			
$\bar{X}$	75,7	15,9	8,4
SD	± 1,37	± 0,92	± 0,33
RSD	± 1,37	± 5,83	± 3,92

[A] Each result represents average of two separate determinations of the same batch No (each injected two times)

[B] $\bar{X}$ represents mean of four separate determinations of the same batch refference standard (each injected two times)

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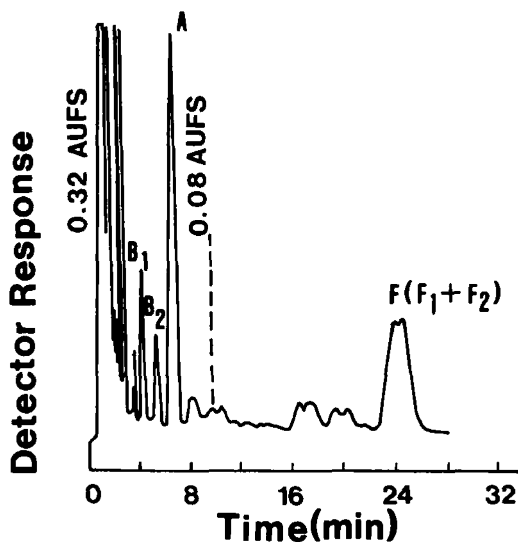

FIGURE 4. HPLC separation of Zn-bacitracin feed grade preparation, (exposed to accelerated aging) on the column: Nucleosil 5 C 18 5 µm (150 x 4,6 mm I.D.). Other experimental conditions are the same as those mentioned in the text.

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in bacitracin feed grade preparations because of their interfering ballast substances.

In preparation of pharmaceuticals products of bacitracin, the substance of bacitracin has very similar composition profile as bacitracin standard (Fig. 2 b). (Both have been refined mixture of components A, B_1 and B_2). This is the most probable reason why in these preparations significant differences in results of microbiological and HPLC determination of bacitracin is not occured (Table 1).

Unlike time consuming microbiological analysis lasting up to 18 hours |8,9|, HPLC method for determination of bacitracin, outlined in this report, provides possibility to separate the main active bacitracin components (A, B_1 and B_2) within 6 to 7 minu-

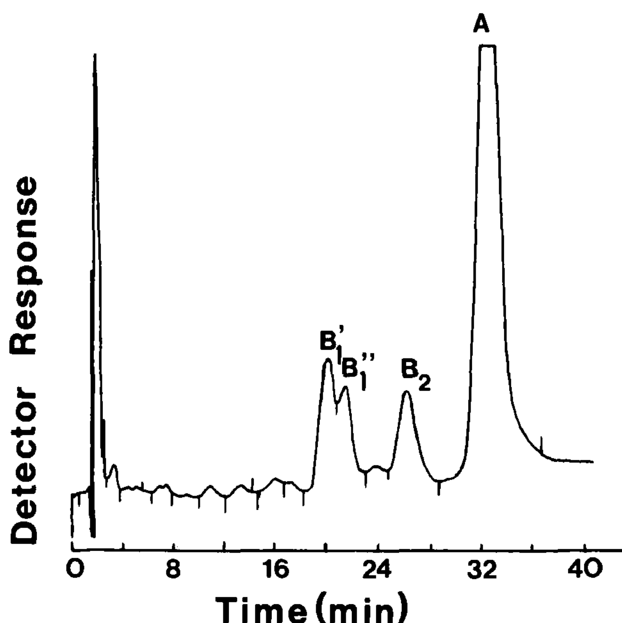

FIGURE 5. HPLC separation of bacitracin B_1' and B_1'' in Zn-bacitracin working standard on the column: Nucleosil 5 C 18 5 µm (150 x 4,6 mm I.D.). Mobile phase composition is 0,05 M KH_2PO_4: CH_3OH, in ratio 47/53 (v/v). Other experimental conditions are the same as those mentioned in the text.

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tes. These components may be precisely quantitatively determined since their peaks are sharp and symmetric. Quality of separation does not stay behind long lasting and complex method of Tsuji and Robertson |2|. In relation to AOAC method |11|, it is concerned that the method described here is also more suitable for stability studies and observing of bacitracin F. On such a way it is possible to monitoring appearance of more polar components, for instance bacitracin F. In our suggested HPLC method, bacitracin F is eluted in significantly shorter time and in peak of bacitra-

cin F, components F_1 and F_2 are eluted together (Fig. 4). According to AOAC method |11|, bacitracin F is bounded much more intensively to reverse stationary phase and eluted with long retention time, thus enabling precise determination of this substance under these conditions.

We also found out that in all samples as well as in reference standards, component B_1 is not homogeneous, but consists of two different subunits, we sign them as B_1' and B_1'' (Fig. 5).Partial separation of these new appearing components were obtained by complet replacing of acetonitrile with methanol in mobile phase. Separation of component B_1 in two subunits is probably caused by interaction of remaining silanole groups on the surface of stationary phase and basic amino acids (lisine, histidine) of eluting bacitracin components. This type of interactions are known in peptide separation |15|.

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