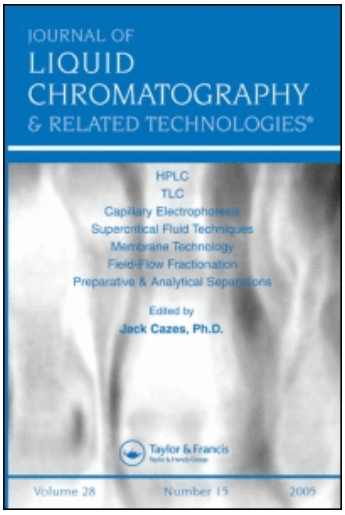

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF BACITRACIN METHYLENEDISALICYLATE

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ABSTRACT

Our previously described isocratic RP HPLC procedure was convenient for monitoring and quality control of bacitracin production and Zn - bacitracin feed grade preparations. Separation and quantitative determination of the main active bacitracin components (A, B_1 and B_2) are possible and those elution is not interrupted by other ingredients in this type of samples. But when the methylenedisalicylic salt of bacitracin was tested some modification of method were necessary for the correct separation of bacitracin components. Mobile phase had to be modified and polystyrene based packing was an alternative and useful complement to octadecylated silica gel packings.

INTRODUCTION

Polypeptide antibiotic bacitracin, produced by certain strains of Bacillus licheniformis and Bacillus subtilis appears as the mixture of structurally similar poly peptides / 1 - 4 /. Bacitracins : A, B_1 and B_2 are the main microbiologically active components and about 90 % off all activity could be attributad to them / 2 , 5 /.

Asa growth promotor in veterinary use , bacitracin stimulates biomass production of different animals.

For the preparation of bacitracin feed grade products, direct precipitation of bacitracin from the fermentation broth by salts of divalent cations is commonly used. Among them, bacitracin zinc salt is the most usual form of feed grade aplication. Another usual form of the bacitracin feed grade preparation is the salt of methylenedisalicylic acid. It is prepared by the precipitation of bacitracin with methylenedisalicylic acid / 3 /. This form has some specific advantages in animal feeding / 6 /.

For the HPLC determination of bacitracin gradient elution method has been described by the authors Tsuji and Robertson/ 2 /. On the basis of this method, isocratic reverse phase procedure for the quick and precise separation and determination of bacitracin was modified and described / 5 /. In this report, mentioned isocratic method was modified for the appropriate HPLC separation of bacitracin components , when the bacitracin methylenedisalicylate was tested.

MATERIAL AND METHODS

Chemicals

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 : 68 i.u. / mg (Institution for Drugs Control and Testing Zagreb , Yugoslavia). All samples of bacitracin methylenedisalicylate feed grade preparations were supplied by Krka Pharmaceuticals (Novo mesto , Yugoslavia).

Chromatographic Conditions

The HPLC system consisted of an LKB pump 2150,LKB Variable wavelength monitor 2151, LKB Rapid spectraldetector 2140, LKB Computing integrator 2220 (LKB ,Bromma, Sweden) and Rheodyne loop injector 7125 with 20 ul fixed loop (Rheodyne , Inc., Cotati, CA). Separations were made on ambient temperature and detection was (UV) 220 nm. The following type of chromatographic columns were used.

Silica gel supported columns :

Guard column, Chrompack reverse phase 75 X 2.1 mm I.D.Columns: Chrompack Chromspher C18 5 um 150 X 4.6 mm I.D. (Chrompack, Middelburg, Netherlands), UltroPac TSK ODS - 120 A , 5 um 250 X 4.6 mm I.D., (LKB, Bromma, Sweden).

Polystyrene supported column :

PLRP - S 8 um, 100 A 250 X 4.6 mm I.D. (Polymer Labs.,Church Stretton, U.K.).

For the bacitracin separation on the used silica gel supported RP C 18 columns,

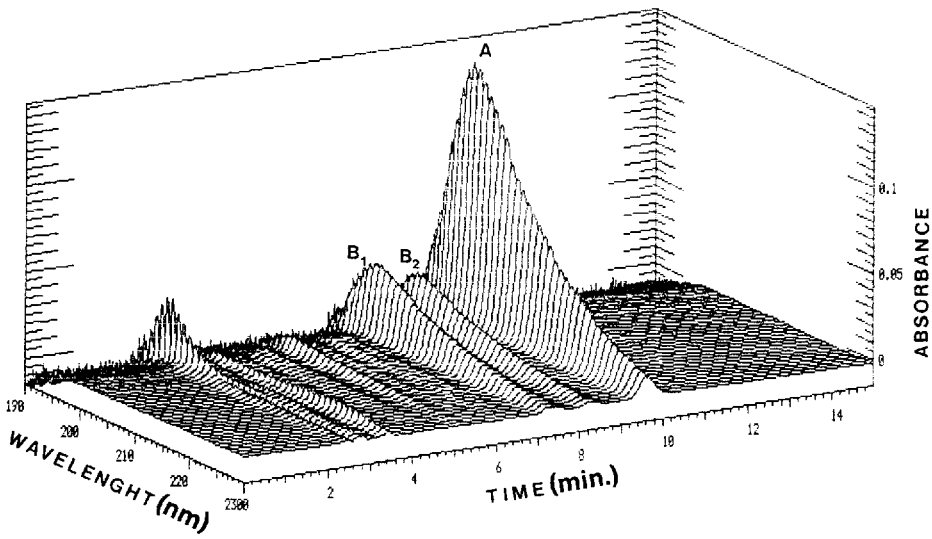

Fig. 1. Topogram print out from diode array detector. Sample ; Zn - bacitracin reference standard. Separation was made on the : TSK ODS 120 A 5 um column (250 x 4.6 mm I.D.). Chromatographic conditions ; mobile phase 0.05 M KH_2PO_4 : CH_3CN : CH_3OH46 : 27 : 27 (v/v), flow rate: 1.4 ml/min, detection (UV) 220 nm.

mobile phase 0,05 M KH_2PO_4 : CH_3CN : CH_3OH - 46 : 27 : 27 (v/v) and flow rate 1.4 ml/min. were used.

For the separation of bacitracin on polystyrene packed column, the mobile phase 0.05 M KH_2PO_4 : CH_3CN : CH_3OH 60 : 20 : 20 (v/v) and flow rate 1.0 ml / min. were used.

Preparation of Samples

Bacitracin standard and samples of bacitracin methylenedisalicylate were prepared under the same conditions as previously described for Zn - bacitracin / 5 /.

RESULTS AND DISCUSSION

From the topogram of diode array detection (three dimensional chromatogram projection in time, wavelength and absorbance) it is evident that the most sensitive

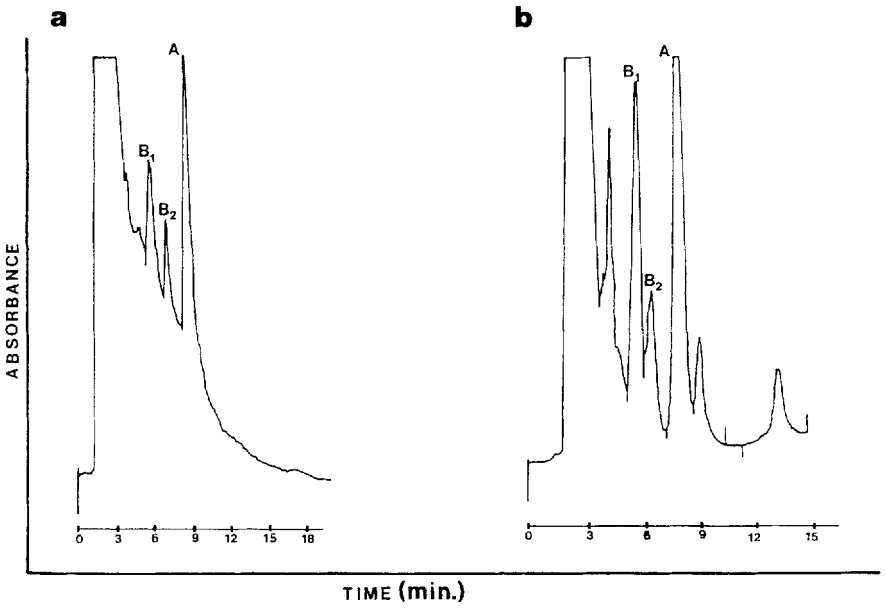

Fig. 2. Separation of bacitracin feed grade preparationin in the form of methylenedisalicylic salt. a) silica gel based column : Chromspher C 18 5 um 150 X 4.6 mm I.D. mobile phase ; 0.05 M KH_2PO_4 : CH_3CN : CH_3OH in ratio 46 : 27 : 27 (v/v), flow rate : 1.4 ml/min, detectiom (UV) 220 nm. b) polystyrene based column (PLRP - S 8um,100 A 250 X 4.6 mm I. D.), mobile phase 0.05M KH_2POP_4 :CH_3CN : CH_3OH in ratio 60 : 20 : 20 (v/v), flowrate : 1.0 ml/min, detection (UV) 220 nm.

detection is at : 198 - 200 nm (Fig. 1). But when the high enough sensitive spectrophotometric detectors were used, satisfactory detector responses were obtained at 220 or even 230 nm detection level. In that way high noise disturbances at the maximum absorbance range were avoided. During the development of isocratic HPLC method / 5 / it was found out that the ratio between 0.05 M aqueous sodium dihydrogenphosphate solution and organic phase (acetonitrile - methanol) was strongly affecting retention and separation of the bacitracin components ; A , B_1 (B_1' + B_1'), and B_2 / 5 /. All these findings from previous work , try to be included in the separation of bacitracin methylenedisalycilate. But if this form of bacitracin was tested on the columns with octadecilated silica gel packing in the same way, as previously described / 5 / the separa-tion of bacitracin components A , B_1 and B_2 was not successful. Bacitracin components are superimposed on the tailing part of strong solvent peak (Fig. 2 a). To avoid this problem without gradient elution,

changes in mobile phase preparation were performed. By the increasing part of aqueous 0.05 M KH_2PO_4 solution in relation to organic phase (acetonitrile - methanol) retention time of bacitracin components was prolonged. But however tried, bacitracin components were not resolved from solvent peak completely , while on the other hand sensitivity of the system was strongly decreased. For the final resolving of this problem polystyrene based column has to be used.

It is well known that polys tyrene based packed RP columns express certain very useful properties in peptides / 8 - 10 / and also bacitracin separation / 7 /. According to these facts modification of the HPLC method was conducted to test methylene-disalicylic salt of bacitracin. When PLRP-S column was used, increase of aqueous part in the mobile phase preparation caused prolonged bacitracin separation , while tailing effects on the solvent peak were strongly reduced. The best separation of bacitracin in the form of methylenedisalicylate salt,was achived on the PLRP - S column with mobile phase 0.05 M KH_2PO_4 : CH_3CN : CH_3OH n ratio 60 : 20 : 20 (v/v) in aproximately 10 minutes (Fig. 2b). In this way the influence of interfering agents on the separation of bacitracin components were successfuly avoided.

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