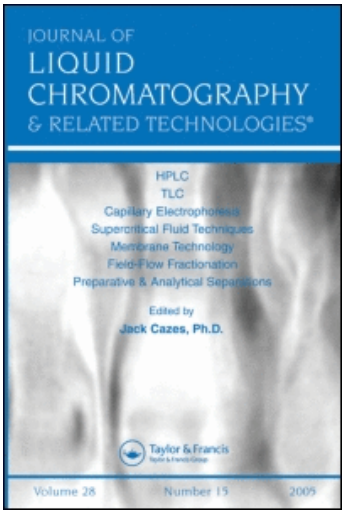

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 04 October 2000

To cite this Article Hormazábal, Víctor and Yndestad, Magne(2000) 'RAPID ASSAY FOR THE DETERMINATION OF ZINC BACITRACIN IN FEED BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY', Journal of Liquid Chromatography & Related Technologies, 23: 7, 1083 — 1088
To link to this Article: DOI: 10.1081/JLC-100101509
URL: http://dx.doi.org/10.1081/JLC-100101509

J. LIQ. CHROM. & REL. TECHNOL., 23(7), 1083–1088 (2000)

RAPID ASSAY FOR THE DETERMINATION OF ZINC BACITRACIN IN FEED BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A simple method for the determination of zinc bacitracin in feed is presented. The samples were extracted with methanol-hydrochloric acid and 1 mL of the supernatant was evaporated to c. 50 μL. After adding water and mixing, the water layer was cleaned up using LMS solid phase extraction columns, and injected to the liquid chromatography-atmospheric pressure ionization ion spray. The lower limit of quantification was 4 mg/kg in chicken and swine feed.

INTRODUCTION

Bacitracin is a polypeptide antibiotic with various active components. These similar polypeptides may differ by only one amino acid.[1]

Bacitracin is to some extent used in human medicine.[2] Stabilized by reaction with zinc or methylene disalicylate, it is added to animal feed for growth promotion, feed efficiency, and disease control.[3]

The concentration of zinc bacitracin (ZnB) in feed varies in different countries, mostly between 5 to 50 mg/kg.

Traditionally, microbiological assays[4,5,6] have been used for qualitative and quantitative determination of bacitracin. Several methods based on UV-detection have also been reported for analysis of the drug.[2,3] These methods are, however, time-consuming or require relatively large amounts of reagents.

The purpose of the present study was to develop a rapid, simple, and specific LC-MS method for the determination of ZnB in chicken and swine feed, with a sensivity which would at least meet the quantitative detection requirement of 5mg/kg set by the Norwegian Feed Control Authority.

EXPERIMENTAL

Materials and Reagents

Samples of chicken feed free of ZnB produced by Felleskjøpet , Oslo, Norway, were used as control material and for spiking with ZnB, to conduct the recovery experiments.

All chemicals and solvents were of analytical or HPLC grade. ZnB was donated by Alfarma A.S. (Oslo, Norway). Stock solution (1mg/mL) was made by dissolving 25 mg ZnB in 2.5 mL HCl (37%)-MeOH (1 + 99) and diluting to 25 mL with MeOH. The working standards were prepared by dilution with MeOH. The standard solutions were stored in a refrigerator at +4°C. Solid phase extraction (SPE) columns Bond Elut (1cc 25 mg) LMS, were purchased from Varian (Harbor City, CA, USA).

Bond Elut adaptors, reservoirs of 125 mL (Analytichem), were connected to the columns when large volumes were applied.

Chromatographic Conditions

The analysis were performed on a Perkin Elmer LC-MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler. The acquired data were entered into a Model 8500 Apple Power Macintosh and processed with either Multiview 1.4 or MacQuan 1.6 software packages, (Perkin Elmer) for spectral information and quantification data processing, respectively. An API 100 LC/MS system (PE SCIEX) single quadruple mass spectrometer with a Turbo-Ion Spray Inlet for the API LC/MS was employed for this study. The turbo probe was adjusted to 150°C and the air flow was 6 L/min. The LC/MS was set to collect single-ion data in positive ion mode for the ions at m/z 705.1 for ZnB. The entrance electrode voltages were adjusted to provide the optimum intensity for the molecular ion. This was obtained with N_2 nebulizer gas at 5 L/min. and curtain gas at 10 L/min. The ion source was 4500V, the orifice 10V, and the ring 330V, while the quadrupole 0 was -10. The analytical column

(stainless steel, 5cm x 4.6 mm I.D.) and guard column (stainless steel, 2.0 cm x 4.6 mm I.D.) were packed with 5-µm particles of Supelcosil LC-ABZ+Plus (Supelco, Bellefonte, PA, USA). The guard column was connected to an A. 318 precolumn filter with a A-102X frits (Upchurch Scientific, USA).

The mobile phase was a mixture consisting of 55% 0.01M (0.77g/L) ammonium acetate (channel A) and 45% methanol (channel B) for 1 min., 100% methanol for 7 min., and 55% 0.01M ammonium acetate and 45% methanol for 7 min. The samples were injected at intervals of 15 min. The flow rate was 1 ml/min. The LC eluent was split post-column approximately 1:20 so that c. 50 µL flowed into the Ion-Spray ion source.

Sample Pretreatment

Exactly 2 g of feed was weighed into a 50 mL graduated centrifuge tube with screw cap (Nunc, Roskilde, Denmark), and volumes of 1 mL methanol, or standard (the total volume added in this step should be 1 mL) and 7 mL MeOH - (37%) HCl (99 + 1) were added. The mixture was homogenized for approximately 6 sec. in an Ultra-Turrax TP 18/10 (Jake & Kunkel KG, Ika Werk, Staufen, Germany) and then left in the extraction fluid for 5 min. After mixing and centrifugation for 5 min (5000 rpm.), a 1 mL volume of the supernatant (corresponding to 0.2 g feed) was pipetted into a conical centrifuge tube. The sample was evaporated to approx. 50 µL under a stream of air, using a Reacti-Term heating module at 40°C and a Reacti -Vap evaporating unit (Pierce, Rockford. IL, USA). After adding 10 mL water, the sample was shaken for 5 sec. and loaded into a conditioned 1cc/25 mg LMS column.

Clean-up on SPE-Column

The column was conditioned with 1 mL methanol, followed by 2 x 1 mL water. The aqueous extract was applied into the column. Conditioning took place under gravity flow and application of the sample with slow flow (1 mL/min.). The column was washed at a vacuum of -5 inches Hg. with 3 x 1 mL water and 0.5 mL 20% methanol in water, and then suctioned to dryness for c. 10 sec. (at a vacuum of -10 in. Hg.) using a Vac Master system from International Sorbent Technology. The column was then eluated with 1.5 mL methanol (vacuum -5 in. Hg.), after which a volume of 1 mL water was added to the eluate. The mixture was blended and c. 0.5 mL was filtered through a Costar Spin-X centrifuge filter unit with 0.2 m nylon membrane, and centrifuged for 2 min. at 10000 rpm. (5600g). Aliquots of the filtrate (35 L) were injected into the LC/MS at intervals of 15 min. for the determination of ZnB.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for ZnB were determined by spiking chicken feed samples with standard solutions to yield 4, 5, 25, 35, and 50 μg ZnB per gram of sample, respectively. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked feed with those of pure standard solutions.

The linearity of the standard curves for ZnB in feed was calculated using peak height measurements.

RESULTS AND DISCUSSION

The standard curve were linear in the investigated area 4 - 50 μg/g for ZnB in chicken feed, while the corresponding correlation coefficients were r = 0.998. The recovery and repeatabilities for ZnB from feed are shown inTable 1.

The recovery varied from 71 to 72 %. The precision of the recovery studies was 0.9 and 1.2 % for 5 and 50 μg/kg ZnB in feed, respectively.

The limit of quantification for ZnB was 4 μg/g and the limit of detection 2 ug/g. The detection limit of the assay was calculated to be three times the baseline noise from a drug-free chicken feed. The method presented in this paper is selective, robust, and accurate.

The detection limit of the assay depends mainly on the sensitivity of the LC/MS. This in turn could be influenced by such factors as the position of the ion spray inlet, the composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

Table 1

Recovery and Repeatability for ZnB from Spiked Samples of Chicken Feed

Sample	n	Amount of Drug Added (μg/g)	Recovery of ZnB SD[a]	Rec.[b]
Feed 2g	8	5	0.9	71
	8	50	1.2	72

[a] SD = standard deviation. [b] Rec. = recovery.

Normally, commercial ZnB-medicated chicken and swine feed will contain about 5 to 50 μg/g. The assay shows good precision in this area, when using the external standard method.

Chromatograms of clean samples of chicken feed and spiked samples with ZnB are shown in Figure 1.

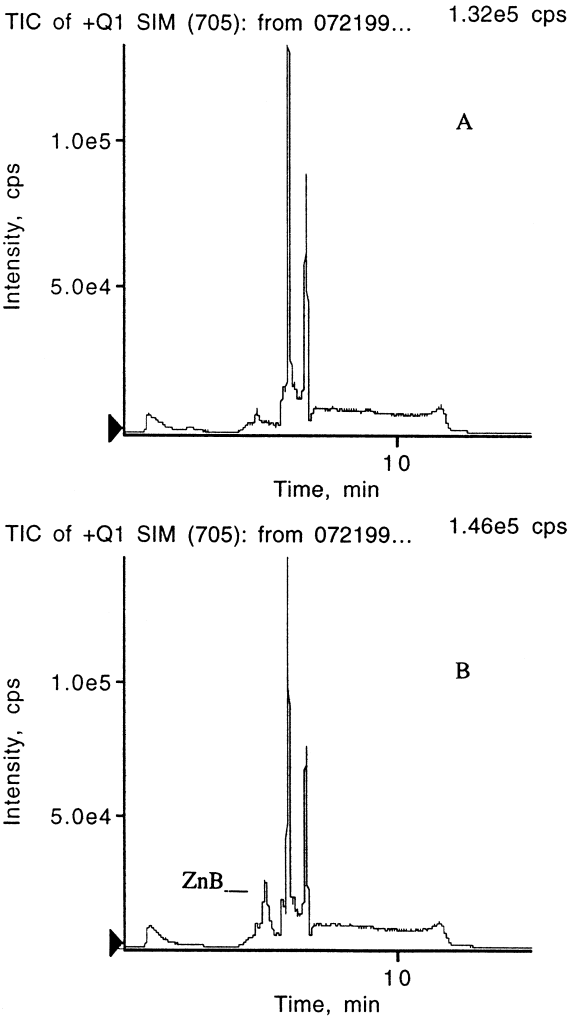

Figure 1. Chromatograms of extracts from chicken feed. A: drug-free feed, B: feed spiked with Zinc Bacitracin (25 μg/g).

The ion spray mass spectrum of ZnB detected two components with strong signals, representing two different arginine residues. The compounds had a base peak at m/z 705.1 and m/z 712.1 corresponding to the doubly charged ion, [M+2H]2+. Only a weak singly-charged species was observed at m/z 1409.5 and m/z 1423.2.

We preferred to validate the precision, recovery, linearity, detection, and quantification limits for ZnB based on ion m/z 705.1. The m/z 712.1 yields a stronger peak than m/z 705.1 but unfortunately there was an interference in the chromatogram.

The advantage of the LC/MS technique lies in a combination of the separation capabilities of HPLC and the power of MS as an identification and confirmation method. Quantification using selected ion monitoring has high selectivity, sensitivity, and broad dynamic range. Thus LC/MS seems to provide a better alternative than HPLC.

ZnB is still allowed for use as a feed additive in many countries. However, such use has now been banned within the European Union, and the issue is being debated in international forum such as Codex. Nevertheless there will still be a need to have methods available for controlling the content of ZnB in feed, whether it is permitted or not.

ACKNOWLEDGMENT

We are grateful to the Norwegian Research Council for financial support.

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Received September 3, 1999 Author's Revisions November 22, 1999
Accepted September 14, 1999 Manuscript 5154