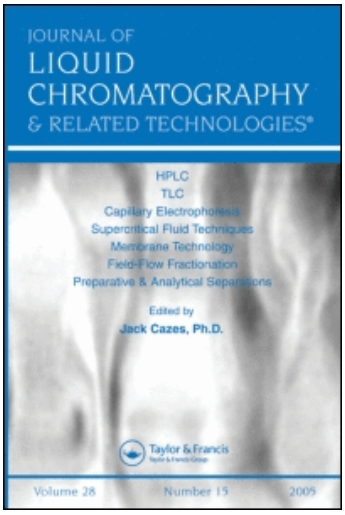

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DETECTION OF BENZYLPENICILLIN IN MILK BY HPLC

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ABSTRACT

A high-performance liquid chromatography method for the determination of benzylpenicillin (Penicillin G) in milk is presented. The samples were extracted with acetone, the organic layer then being evaporated to dryness and cleaned-up using C_2 solid phase extraction column. The method is simple and robust.The lower limit of quantification was 4 μg/l and the limit of detection close to 2 ppb. The average recovery was 82%.

INTRODUCTION

Beta-lactam antibiotics, especially benzylpenicillin (BZP), are commonly used in veterinary medicine. Their wide application

represents a potential hazard to consumers due to the persistence of residues in milk. Despite their low toxicity, beta-lactam antibiotics are nevertheless a group of drugs which have been reliably documented to harm human health, residues of BZP in food inducing allergic reactions (1).

A number of sensitive screening tests have been described for the detection of β-lactam antibiotic residues in milk. These include microbiological tests (2), immunoassays (3,4), competitive binding (2, 5, 6) and enzyme inhibition (7). These are all capable of detecting residues at levels of 10 μg/l or less. With the possible exception of immunoassay, none of the screening procedures can differentiate the various β-lactam antibiotics from one another. False positive tests may occur. A capillary gas chromatographic method has been described (8), with a sensitivity of <1 μg/l, which requires lengthy partitioning clean-up and derivatization steps. Several analytical methods for the determination of BZP based on high-performance liquid chromatography have been published (9-14). The methods are, however, time-consuming and either require the use of large quantities of chemical reagents, or do not achieve the required sensitivity.

The purpose of the present study was to develop a rapid, simple and sufficiently sensitive method (15), for the determination of BZP, which required only small quantities of chemical reagents. The fact that many β-lactam antibiotics cannot be partitioned between buffers and organic solvents had to be taken into consideration(10).

MATERIALS AND METHODS

Chemicals and Reagents

Samples of cows milk were used.

All chemicals and solvents were of analytical or HPLC grade.

BZP was supplied by Sigma Co. (St. Louis, MO, USA). Stock

solutions (1mg/ml) and working standards were prepared fresh weekly by dilution with distilled water. The solutions were stored in the refrigerator.
Extraction columns Bond Elut (1cc/100 mg) C_{18}, C_8 and C_2, were purchased from Varian (Harbor City, CA, USA).

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (12°c) from Messgeräte Werk Lauda, (Lauda Köningshafen, Germany), and a LC 235C Diode Array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 200 nm. The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).
The analytical column (stainless steel, 25 cm x 4.6 mm. ID) and guard column (stainless steel, 2 cm x 4.6 mm. ID), were packed with 5-µm particles of Supelcosil LC-C_{18} DB (Supelco, Bellefonte, PA, USA), and operated with a constant column temperature of 35°c.
The mobile phase was a mixture of two solutions, A and B (66:34). Solution A was 0.02 M heptanesulphonate-0.01 M $Na_2HPO_4 \cdot 2H_2O$, made by dissolving 4.45 g/l 1-heptane sulphonic acid sodium salt (Supelco) and 1.78 g/l di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany) in c. 750 ml of water. The pH was then adjusted to 2.15 with 5 M phosphoric acid, and the solution made up to volume (1l) with water. Solution B was acetonitrile.
The flow-rate was 0.9 ml/min for 3 min, 0.6 ml/min for 10 min, followed by 2 ml/min for 2 min.The samples were injected at intervals of 17 min. Aliquots of 125 µl were injected onto the column for the determination of BZP.

Sample pretreatment

To 1 ml milk was added 200 µl H_2O (or standard) and 10 ml acetone. The sample was mixed for approx. 10 sec. and then centrifuged for 3 min. (3000 rpm.). The supernatant was transferred to a clean glass-stoppered centrifuge tube. The organic layer was evaporated to c. 600 µl at 45 °c under a stream of nitrogen and then 1 ml hexane was added.The sample was shaken vigorously for 5 sec., and centrifuged for 2 min. The upper layer (hexane) was discarded and the water layer washed again with hexane, and the water was evaporated to dryness. The dry residue was dissolved in 350 µl H_2O-CH_3CN (8 : 2) and the solution loaded onto a conditioned C_2 column.

Clean-up on SPE-column. The column was activated with 1 ml CH_3CN and 2x1 ml water and suctioned to dryness for 5 sec. The extract of milk was loaded into the column and slowly suctioned through (c. -1 in. Hg.). The column was suctioned to dryness for 5sec., and with vacuum -5 in. Hg. washed with 2x50 µl H_2O-CH_3CN (9:1), suctioned to dryness for 5 sec., and then eluted with 4x100 µl H_2O-CH_3CN (8:2) with full vacuum using a VacMaster system (International Sorbent Technology). The collected eluate was centrifuged for 3 min. at 3000 rpm. prior to injection into the HPLC.

Validation of the pretreatment procedure

The precision, recovery and linearity of the pretreatment procedure were determined by analyses of spiked milk samples in the concentration range 4-300 ng/g.
The spiked samples were extracted using the above described procedure. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked milk samples with those of standard solution. The linearity of the

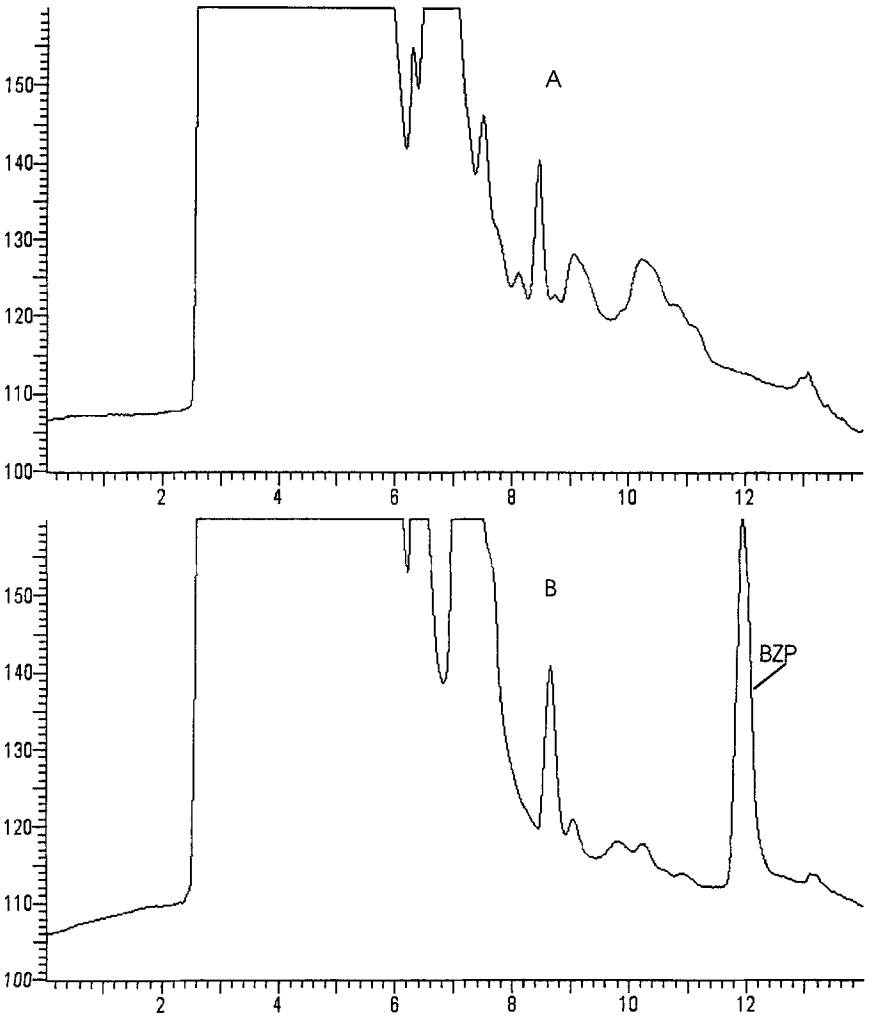

FIGURE 1

Chromatograms of extracts from milk.

A: drug-free milk, **B**: "real" sample of milk contains 200 ng/ml BZP.

TABLE 1

Recovery and repeatability for benzylpenicillin from spiked samples of milk.

Sample	No. of Samples	Amount of drug added (ng/ml)	Recovery % BZP Mean	SD*
Milk (1 ml)	8	10	82	0.8
	8	100	82	0.7

SD*= standard deviation

standard curves for BZP in milk was tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of extract of blank samples and real samples are shown in Figure 1.

The chromatographic system appeared to be efficient for the determination of BZP in milk, the limit of quantification being 4 ng/ml and the limit of detection close to 2 ng/ml. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies. The extraction procedures were validated, and the results are shown in Table 1. The average recovery for BZP over the concentration range of the standard curve was 82%.

The precision of these recovery studies varied from 0.7 to 0.8% for BZP in milk. The linearity of the standard curves was 0.999 for milk, when using the external standard method of calculation. The results also show that the precision and accuracy of the quantification of BZP are good.

The retention and elution properties of BZP were studied on the bonded-phase extraction columns. The drugs were retained on C_8 and C_2 and only partly retained on the C_{18} column using water-acetonitrile (8:2). C_8 columns seem to retain a little more impurities that C_2 columns. A large amount % CH_3CN in the elution mixture gave more impurities. Moreover c. 15% of BZP remains in the column when using water-acetonitrile (8:2) as elute. A suitable pH in the mobile phase is important if good separation from residues of endogenous compounds is to be obtained.

This study has shown that residues of the antibiotic compound BZP in milk may be determined without being partitioned between buffers and organic solvents. The cost of chemicals and the manual work-up procedures are also reduced compared to previously published methods. An experienced technician can carry out sample clean-up of about 24-27 milk samples per day. The assay shows good precision when using the external standard method. The method is robust, simple, and sufficiently sensitive. The quantification is linear over a wide concentration range. The chromatographic system was specific with regard to BZP.

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