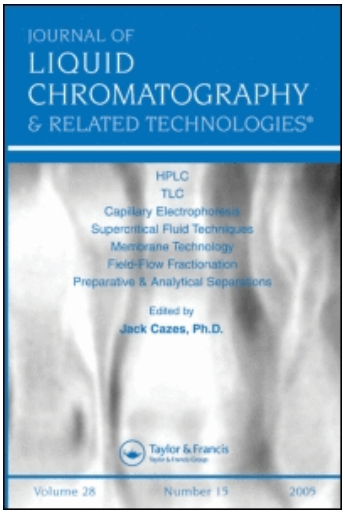

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Abstract: Cephalosporins, relatively new antibiotics related to penicillins, are widely used in the treatment of both human and veterinary diseases because of their broad spectrum of antibacterial activity and good pharmacokinetic properties. Cefacetril, belonging to cephalosporins, is commonly used in treating mastitis in cows. In the present paper, cefacetril excretion with milk was examined by means of the TLC-DB and HPLC method. Thin-layer chromatography-direct bioautography is the technique which combines TLC with microbiological detection. Semi-quantitative determination of cefacetril in milk by TLC-DB was compared with quantitative HPLC analysis. An exponential relationship was proposed for calibration curves in bioautography.

Keywords: Cefacetril, Cephalosporins, Excretion in milk, HPLC, TLC-DB

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INTRODUCTION

The main cause for inhibitors in bulk tank milk is associated with intramammary treatment of dairy cows due to clinical mastitis. The reasons for contamination are milking of treated cows, not regarding prescriptions for dosage and withholding periods. Positive results are mainly obtained when treated cows are milked within 12 hours after treatment with antibiotics.

The β-lactam antibiotics are the most frequently found residues contaminating milk of lactating cows.[1,2] The class of β-lactams is divided into subclasses that are penicillins, cephalosporins, cephamycins, carbacephems, clavams, carbapenems, and monobactams.

The cephalosporins are bactericidal semisynthetic antibiotics with both Gram-positive and Gram-negative activity. Their structure is derived from 7-aminocephalosporanic acid (7-ACA), while the penicillins are derived from 6-aminopenicillanic acid (6-APA).

Cephalosporins are classified in generations by their antimicrobial properties. Cefacetril belonging to first-generation cephalosporins has excellent activity against Gram-positive cocci, such as *Staphylococcus aureus* (Fig. 1).[3] It is commonly used for treatment of mastitis in lactating cows caused by *Streptococcus agalactiae, S. dysgalactiae, S. uberis, Staphylococcus aureus, Escherichia coli, and Klebsiella.* Cefacetril shows high resistance against β-lactamase activity and has a low toxicity. The maximum residue level (MRL) in milk was established at 125 ppb. The withdrawal period for milk is 4 days. Cefacetril, like other cephalosporins, can be detected by microbiological or receptor tests. However, these methods lack selectivity and give, at most, semiquantitative results.[4] High-performance liquid chromatography (HPLC) is sensitive and selective enough and, therefore, is usually used

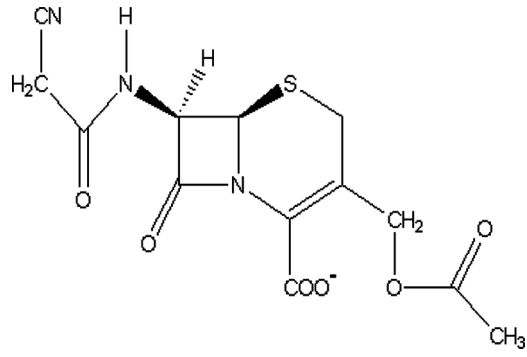

Figure 1. Structure of cefacetril.

as a confirmatory method after positive microbiological test to determine, quantitatively, a given drug or as a multiresidue method to distinguish among various drugs.[5–7] In spite of that, when many samples are analyzed, thin-layer chromatography (TLC) seems to be more convenient.[8–11]

Thin-layer chromatography-direct bioautography (TLC-DB) combines TLC with microbiological detection.[12–15] The method is more sensitive than conventional TLC and, additionally, gives information about antibiotic properties of the analytes. This is its great advantage over an HPLC method. In direct bioautography, the developed TLC plates are dipped in a bacterial growth medium seeded with an appropriate bacterial strain. The location and size of growth inhibition zones allows for the information about the kind and quantity of antibiotics.

Excretion of cefacetril in milk, after intramammary application at different time after injection, was examined by means of TLC-DB and HPLC. The aim of the paper was to compare results obtained by both methods.

EXPERIMENTAL

Equipment and Reagents

DS sandwich chambers were purchased from Chromdes, Lublin, Poland.[16] Pre-coated silica gel TLC plates $Si60F_{254}$ were purchased from E. Merck KGaA, (Darmstadt, Germany).

Cefacetril was supplied by Sigma (St. Louis, MO, U.S.A). Methanol and acetonitrile HPLC grade were from Merck (Darmstadt, Germany), while trifluoroacetic acid (TCA) and KH_2PO_4 from POCh (Gliwice, Poland). The Chrom Biodip® Antibiotics Test Kit was purchased from E. Merck, (Darmstadt, Germany). Filter units with PTFE membranes were supplied by Chromacol Ltd, (UK). Masticef (4 syringes filled with a suspension of cefacetril in a dose of 250 mg) was produced by Biowet Drwalew S.A., Poland.

Methods

Administration of Cefacetril

Cefacetril (Masticef), in a dose of 250 mg, was administered into a teat orifice of one quarter of the udder by means of a syringe containing 5 mL of the drug suspension. Then, the udder was thoroughly massaged in

order to distribute and absorb the drug faster and more effectively by the mammary gland. Samples of milk containing the antibiotic were taken after 2, 3, 6, 8, 10, 24, 36, and 48 h from Masticef administration.

Preparation of Standards

The stock solution of cefacetril was prepared in water at 1 mg ml^{-1}. The standard solutions were prepared by diluting the stock solution with water and TCA to obtain standards at concentrations from 0.3 to 150 ppm in water-TCA (3:1). All solutions were stored at −18°C.

Extraction of Cefacetril from Milk Samples

3 mL of milk were mixed with 1 mL of 70% TCA and centrifuged at 5,000 g for 6 minutes. The supernatant was filtered through a 0.2 μm PTFE membrane and subjected to chromatography.

High-Performance Liquid Chromatography

The HPLC system consisted of Gilson pump-305 and UV/VIS-155 detector (Gilson, Middleton, USA), a sample injector model 7125 with 20 μL loop (Rheodyne, Cotati, USA), CSW32 acquisition system (DataApex, Prague, Czech Republic). LiChrocard RP18 125 × 3 mm, 5 μm column (Merck, Darmstadt, Germany) was used. HPLC mobile phase was prepared by mixing 0.1 M KH_2PO_4, pH 3 with acetonitrile in proportion 90 to 10. Flow rate equaled 1 ml min^{-1}.

Bioautography

Bioautography was performed according to the Chrom Biodip® Antibiotics Test Kit recipe.[17] One bottle of nutrient medium was mixed with 200 mL of 0.5 M TRIS buffer in a 300 mL Erlenmeyer flask, adjusted to pH 7.2 with 1 M hydrochloric acid and autoclaved for 20 min. The sterile medium was then inoculated by pipetting in the *Bacillus subtilis* spore suspension and incubated for 4 h at 37°C (incubation time was prolonged compared to that proposed by Merck). The developed TLC plates were dried successively in air and a vacuum desiccator. They were then immersed briefly in the microorganism (MO) solution and incubated 20 h at 37°C. After incubation, the plates were sprayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution and left at room temperature for about 30 min. Cream-white inhibition zones were

observed against a purple background. The plates were dried in air and scanned for documentation. The inhibition zone areas were then measured with a planimeter.

RESULTS AND DISCUSSION

Deproteinized milk samples were analyzed in parallell by means of HPLC and TLC-DB. Because concentrations of cefacetril in the samples were not known, three different volumes, i.e., 1, 5, and 10 μL were applied on a TLC plate. Then, the plates were dried and subjected to bioautography without development, similarly as was done in the method established by Dhenasar.[18,19] However, Dhenasar after application of antibiotic samples quantified them using densitometry, while, in this case, bioautography was carried out. Figure 2 presents a typical bioautogram for extracts obtained from milk samples applied in volumes of 5 and 10 μL. The values of cefacetril concentrations in milk samples were calculated from the calibration curves, linear for HPLC (Fig. 3) and exponential for TLC-DB (Fig. 4). Our previous experiments showed that linear approximation for calibration plot in TLC-DB is possible only for a narrow range of concentrations (one or two orders of magnitude).[20] For a wider range of concentrations, exponential relations fit better. Therefore, exponential dependencies between the areas of inhibition zones and the logarithm of concentration of cefacetril were used as calibration curves. As was also proved earlier,[20] the size of the inhibition zone depends on the applied volume of the antibiotic solution. Thus, the concentration of cefacetril in a given sample was

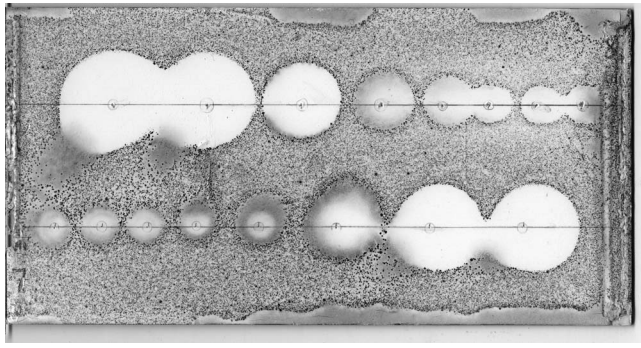

Figure 2. Typical bioautogram for extracts obtained from milk samples collected 2, 3, 6, 8, 10, 24, 36, and 48 hours after Masticef administration to cow; I. Lower trial – 5 μL upper trial 10 μL.

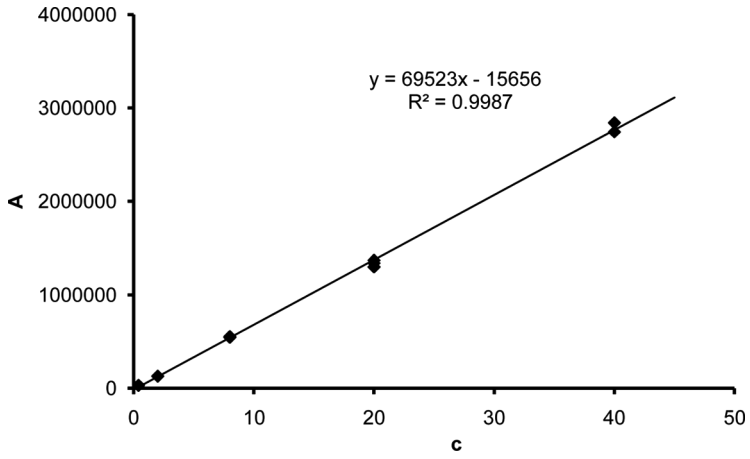

Figure 3. Calibration curve for HPLC.

calculated from the calibration curve plotted on the basis of inhibition zone areas of standards applied in the same volume as the analyzed sample (milk extract). The final concentration for a given sample was calculated as an average of three values obtained on the basis of three calibration curves. As seen in Fig. 4, the calibration curve for 5 and 10 μL volumes spotted overlap while for 1 μL it runs lower.

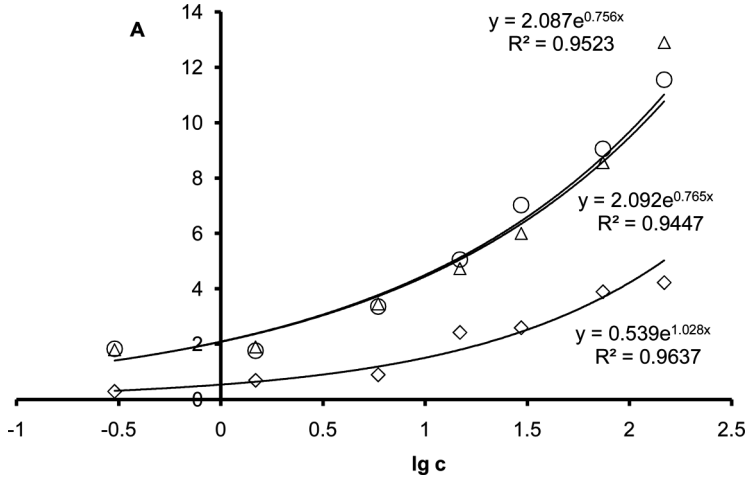

Figure 4. Calibration curves for TLC-DB obtained for different volumes applied onto the plate: rhombus – 1 μL, circle – 5 μL, triangle – 10 μL.

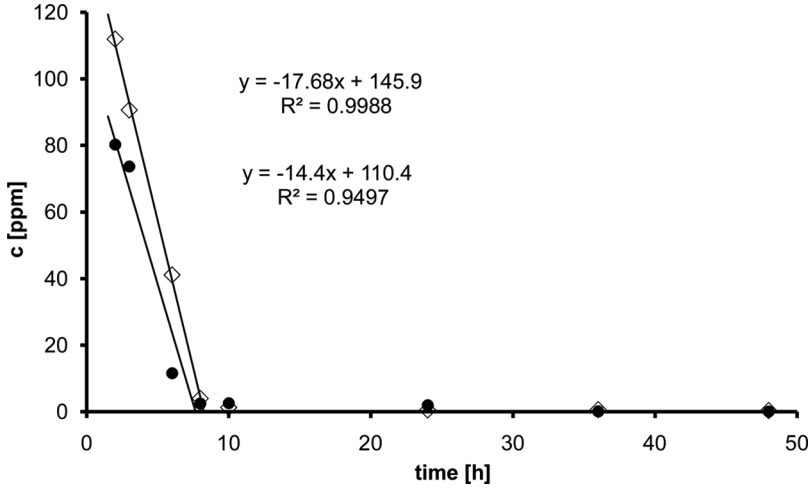

Figure 5. Concentration of cefacetril (ppm) in milk as a function of time after administration of the drug to cow I. Circle – HPLC data, rhombus – TLC data. Upper equation and R^2 for TLC-DB, lower for HPLC.

It is known that the larger the volume applied, the larger the area should be obtained for the same amount of antibiotic in the spot.[20] In the present work, this rule was not as evident, since the samples were applied on the plate and were not developed. Probably, in this case,

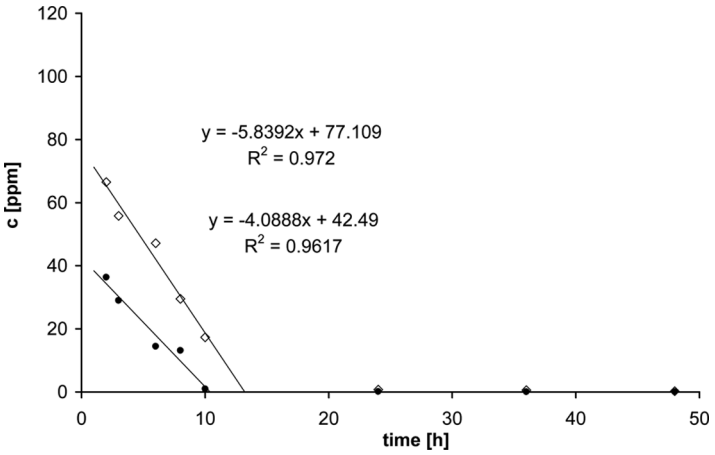

Figure 6. Concentration of cefacetril (ppm) in milk as a function of time after administration of the drug to cow II. Circle – HPLC data, rhombus – TLC data. Upper equation and R^2 for TLC-DB, lower for HPLC.

Table 1. Activity of cefacetril against some bacteria causing mastitis represented by MIC [$\mu g ml^{-1}$] values

Staph. aureus	Str. agalactiae	Str. dysgalactiae	Str. uberis	E. coli	Klebsiella
0.5–1	0.4–0.7	0.1–0.3	1–2	4–8	4–8

spot diameters for the samples applied in 5 and 10 μL did not differ essentially.

Figures 5 and 6 present results obtained by means of TLC-DB and HPLC for two different cows treated with Masticef. The maximum concentration of cefacetril in milk was observed two hours after administration to cows. As the cefacetril in the first ten hours was excreted very quickly and with a constant rate, the linear curves (both for TLC-DB and HPLC) can be plotted for points belonging to this range. Cefacetril concentration exceeded the MIC values for the microorganisms causing mastitis (despite *Klebsiella and E. coli*) for up to at least ten hours (Table 1). Cefacetril level in milk from both cows was close to the MRL value 24 hours after administration. The TLC-DB results coincide well with the HPLC ones.

CONCLUSIONS

Thin-layer chromatography-direct bioautography can be used as a method for semi-quantitative determination of cefacetril residues in milk. The screening of these drugs is possible, even at the MRL level imposed by the European Union. The method enables testing of many samples in one run, which is a great advantage over the HPLC methods. The method gives similar information as HPLC in respect to excretion of the drug to milk after intrammamary administration.

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