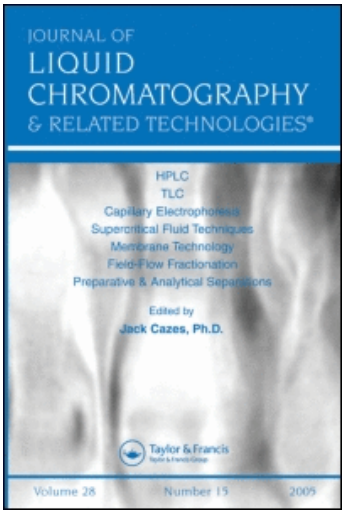

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Semiquantitative Estimation of Enrofloxacin and Ciprofloxacin by Thin-Layer Chromatography–Direct Bioautography

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ABSTRACT

Fluoroquinolones, relatively new chemotherapeutics, are widely used in the treatment of both human and veterinary diseases because of their broad spectrum of antibacterial activity and good pharmacokinetic properties. Enrofloxacin is used in veterinary medicine, while its main metabolite, ciprofloxacin, is one of the most frequently used human antibiotics in the world. Hence, there is a need for assays capable of determining and distinguishing between these drugs. Thin-layer

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chromatography–bioautography (TLC–B) combines TLC with microbiological detection. In the present paper, semiquantitative determination of enrofloxacin and ciprofloxacin standards by TLC–B using Chrom Biodip® Antibiotics Test Kit is presented. The optimal conditions for bioautographic detection were established. The exponential relations were proposed for approximation of dependencies between areas of zone inhibitions and logarithm of the amounts applied. The detection limit for standards of both enrofloxacin and ciprofloxacin was 0.01 ppm, lower than maximum residue limits (MRLs) established for various food products.

Key Words: Enrofloxacin; Ciprofloxacin; Direct bioautography; Chrom Biodip® Test Kit.

INTRODUCTION

Fluoroquinolones are a relatively new group of synthetic antimicrobial agents derived from 3-quinolone carboxylic acid.[1,2] The predecessor of the class was nonfluorinated nalidixic acid with a narrow spectrum of activity that was mainly limited to treating urinary tract infections. The spectrum of antibacterial activity was considerably enhanced by introducing a fluorine atom on the number 6-carbon atom. The new 6-flouroquinolone chemotherapeutics have become very useful in a variety of infections, especially caused by pathogens resistant to older antibiotics. They are now widely used in the treatment of both human and veterinary diseases. Fluoroquinolones have a broad spectrum of antibacterial activity that is excellent against Gram-negative bacteria and good against Gram-positive and anaerobic species. The drugs have very good pharmacokinetic properties, are generally well tolerated, and can be easily administered. These favorable properties give them great potential for both use and misuse. The misuse of these antibiotics in human and veterinary medicine has led to the loss of their efficacy and to the emergence of drug-resistant bacteria. Therefore, there is a need for developing analytical methods to monitor the levels of fluoroquinolone residues in biological fluids and edible animal tissues.[3–5] The biggest risk is associated with the drugs used both in human and in veterinary medicine (flumequine, norfloxacin, or ofloxacin, for instance) and with those that produce similar metabolites. Enrofloxacin is used in veterinary medicine on cattle, pigs, poultry, fish, dogs, and cats. The major metabolite of enrofloxacin is its human counterpart, ciprofloxacin, one of the most popular human antibiotics in the world. Hence, the assays have to be capable of distinguishing between these drugs.[6–9]

High-performance liquid chromatography (HPLC) is the most popular method in fluoroquinolones analysis.[3,5–12] Thin-layer chromatography (TLC), not so frequently used in the analysis of antibiotics, mainly because of generally lower sensitivity, possesses at least one great advantage over HPLC—the possibility of applying many samples in one run. It is possible to enhance the sensitivity of TLC by combining the method with mass spectrometry[13,14] or, in a less expensive way, using derivatization[14,15] or bioautography.[16–26]

Thin-layer chromatography–bioautography (TLC–B) combines TLC with microbiological detection. The developed TLC plates are placed on, or dipped in, a bacterial growth medium seeded with an appropriate bacterial strain. The location of zones of growth inhibition allows for the information about the kind and quantity of antibiotics. The typical bioautography procedure is based on agar-diffusion technique, i.e., antibacterials migrate by diffusion from the TLC plate to an inoculated agar plate.[19,20] In so-called direct bioautography, bacteria grow directly on the TLC plate, so that not only separation but also incubation and visualization are performed on the plate. Merck has recently developed a ready-to-use direct bioautographic test kit, Chrom Biodip® Antibiotics.[19,23,24] In our laboratory we used it for determination of doxycycline and/or flumequine in milk after chromatography of the antibiotics on TLC or high-performance TLC (HPTLC) plates.[25,26] However, at least according to our knowledge, there are no other reports on the test application. There is also a lack of examples on TLC–B of enrofloxacin and ciprofloxacin. In this paper, semiquantitative determination of enrofloxacin and ciprofloxacin by TLC–B using Chrom Biodip® Antibiotics Test Kit is presented.

EXPERIMENTAL

Equipment and Reagents

DS sandwich chambers[27] were purchased from Chromdes, Lublin, Poland. Pre-coated silica gel TLC plates $Si60F_{254}$ 10×20 cm, $Si60F_{254}$ 10×20 cm with concentrating zone, HPTLC $Si60F_{254}$, and HPTLC $Si60F_{254}$ with concentrating zone, were purchased from E. Merck KGaA, Darmstadt, Germany. Hexane, dichloromethane, methanol, HPLC grade, were purchased from Merck while acetone, 25% aqueous ammonia, and 2-propanol, all analytical grade, were purchased from P.O.Ch. Gliwice, Poland. Enrofloxacin and ciprofloxacin were supplied by Sigma (St. Louis, MO). Chrom Biodip® Antibiotics Test Kit was purchased from Merck.

Methods

Preparation of Antibiotic Solutions

The stock solutions of enrofloxacin and ciprofloxacin at 1 mg mL^{-1} were prepared in 0.03 M aqueous solution of NaOH. The working solutions were the mixtures, prepared by the dilution of stock solutions with methanol.

Sample Spotting and Development

The standard solutions were applied to the TLC plate using a Linomat 5 Camag applicator (Muttenz, Switzerland). The mobile phase was dichloromethane/methanol/2-propanol/25% aqueous ammonia, 3 : 3 : 5 : 2.

Bioautography

Bioautography was performed according to Chrom Biodip® Antibiotics Test Kit recipe. 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 hr at 37°C.

The developed TLC plates were dried successively in air and in a vacuum desiccator. Then, they were immersed briefly in the microorganism (MO) solution and incubated overnight at 28°C. After incubation, the plates were sprayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-solution and incubated for about 30 min. Cream-white inhibition zones were seen against a purple background. The plates were dried in air and scanned for documentation. The areas of the inhibition zones were then measured with a planimeter.

RESULTS

The Chrom Biodip® Test Kit contains *B. subtilis* spore suspension, nutrient medium, and tetrazolium salt MTT detection reagent. The developed chromatographic plate is immersed in inoculated nutrient medium and incubated to permit the growth of bacteria. After spraying with MTT, dehydrogenases of living bacteria convert tetrazolium salt into purple formazan forming cream-white inhibition zones in place of antibiotic spots. There are only scarce reports on the application of this test.[19,23–26] Eymann and Hauck present TLC–B of feed extracts and standards of chlortetracycline, oxytetracycline,

penicillins, and monensine.[24] It seems that these antibiotics can be determined to 1ng per spot, despite monensine, for which the limit of detection was about 10 ng. However, it was not clearly demonstrated. According to our previous papers it was possible to determine flumequine and doxycyline in milk at the level of about 1 ng per spot.[25,26]

As mentioned in the Introduction there is no information on TLC–B of enrofloxacin (E) and its active metabolite ciprofloxacin (C). It is not easy to separate these compounds by TLC because their structures are very similar (Fig.1). The conditions for TLC of enrofloxacin and ciprofloxacin were reported earlier.[28] In this paper, we attempted to find optimal conditions for semiquantitative determination of these antibiotics by TLC–direct bioautography. Some preliminary experiments were done first. We found that:

1. The incubation time should be prolonged from that advised by Merck (2 hr at 35°C) to 4 hr at 37°C. Under these conditions bioautograms are of high contrast and legible. The prolonged time was also proposed by Botz et al.[19]
2. Spots applied with an applicator gave more compact inhibition zones compared with spots applied at the same quantity manually with a syringe. The more compact spots led to a higher, by one order of magnitude, sensitivity.
3. It was impossible to obtain lower detection limits using HPTLC plates or the plates with concentrating zones, instead of common TLC plates.

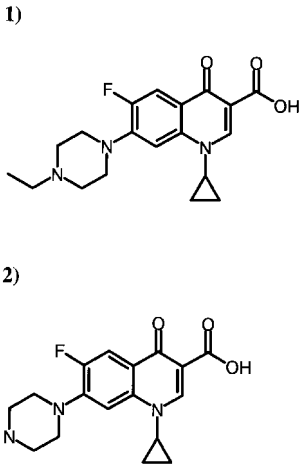

Figure 1. Structures of (1) enrofloxacin (E) and (2) ciprofloxacin (C).

4. The direction of development (along or cross the plate) did not influence the size of the inhibition zones.
5. The widths of the applied bands did not influence the areas of inhibition zones but only the shapes of the zones. However, limits of detection were lower for narrow bands, e.g., 1 mm, probably because the spots were more compact.

It is well known that the inhibition zone area depends on the volume of injected samples. Figure 2 presents this phenomenon for given amounts of antibiotics. As can be seen, the larger the volume applied, the larger the area obtained, for the same amount of antibiotic in the spot. The greatest difference occurred between areas of ciprofloxacin spots, which were obtained by applying 1 and 10 μL of the antibiotic solutions. Thus, standards of enrofloxacin and ciprofloxacin were applied at various concentration levels, and at a fixed volume, for a given plate. The TLC–B of standards applied at 1 μL is presented in Fig. 3. The limit of detection was at the level of 1 ppm, i.e., minimum detectable dose (MDD) equaled 1 ng of enrofloxacin and ciprofloxacin. For 10 μL spotted volume, the limit of detection of enrofloxacin was 10 times lower, i.e., it equaled 0.1 ppm while the detection limit of ciprofloxacin was 100 times lower and equaled 0.01 ppm (MDD equaled 0.1 ng) (Fig. 4). When the spots were applied as 5 mm wide bands instead of 1 mm wide bands, the level of detection was the same for

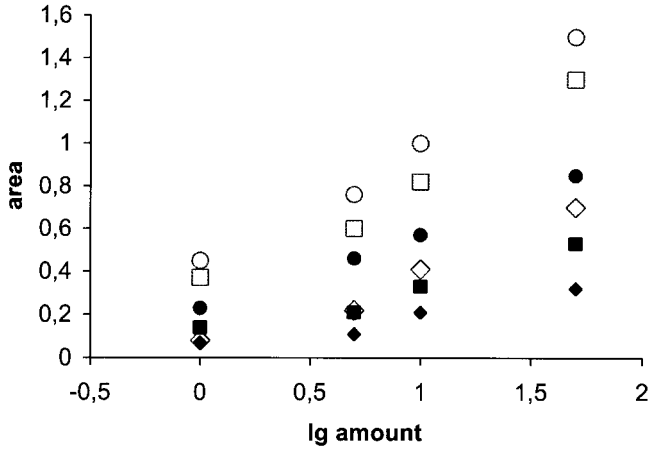

Figure 2. Areas of inhibition zones (cm^2) versus logarithm of amounts of antibiotic standards applied onto the plate (ng) in given volume. Rhombus, 1 μL; square, 10 μL; circle, 50 μL. Black points for enrofloxacin, white points for ciprofloxacin.

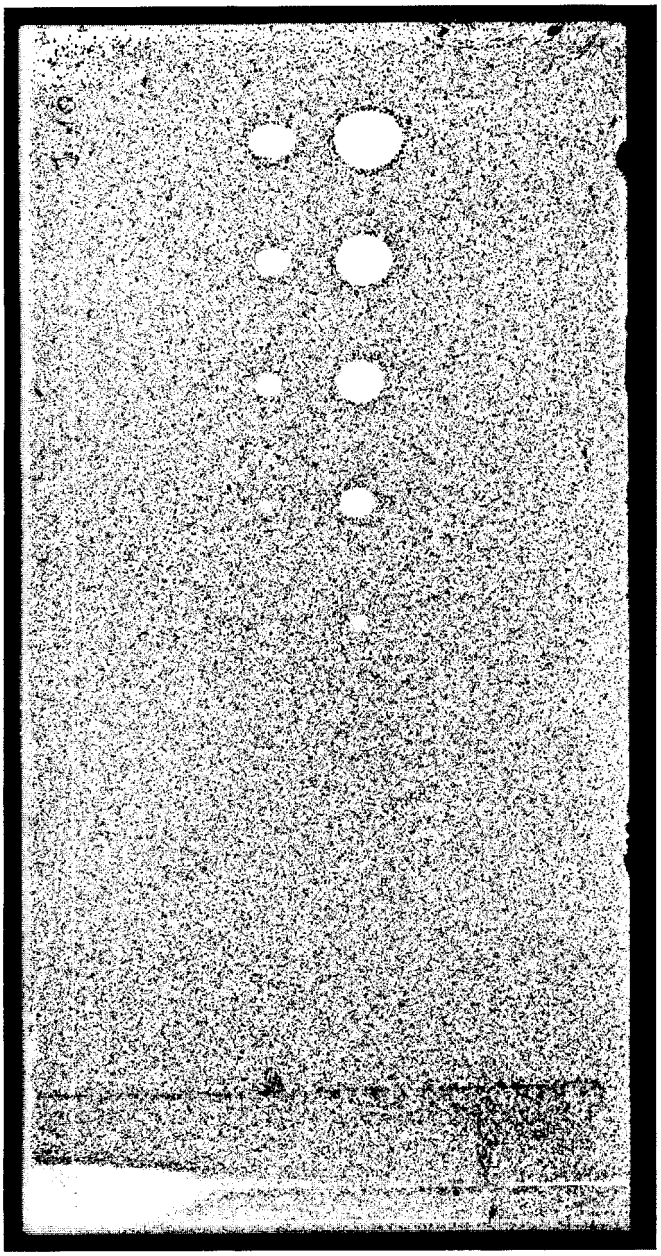

Figure 3. TLC–B of enrofloxacin and ciprofloxacin standards on TLC Si60F_{254} plate. The applied volume equaled 1 μL, bandwidth was 1 mm. The mobile phase was dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2, developed to the end of the TLC plate and then continuously for about 2 hr (cross the plate). From left to right the concentrations of antibiotic standards applied: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 ppm (μg mL^{-1}), i.e., 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 ng of each antibiotic per spot. Upper spots, enrofloxacin; lower spots, ciprofloxacin.

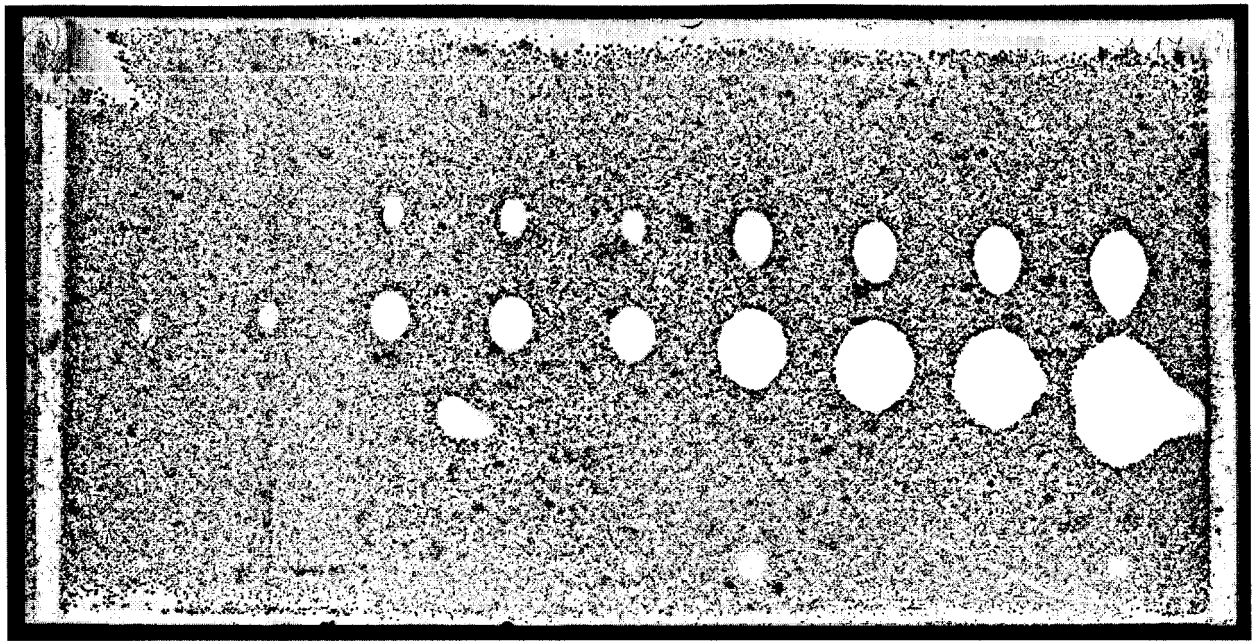

Figure 4. TLC–B of enrofloxacin and ciprofloxacin standards on TLC $Si60F_{254}$ plate. The applied volume equaled 10 μL, bandwidth was 1 mm. The mobile phase was dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2, developed to the end of TLC plate and then continuously for about 2 hr (cross the plate). From left to right the concentrations of antibiotic standards applied: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 ppm ($\mu g\ mL^{-1}$), i.e., 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng of each antibiotic per spot. Upper spots, enrofloxacin; lower spots, ciprofloxacin.

enrofloxacin and ciprofloxacin and it amounted to 0.1 ppm (Fig. 5). The level of 0.01 ppm for both enrofloxacin and ciprofloxacin was obtained when 50 μL volumes of antibiotic standards were spotted (Fig. 6). It means that it is possible to obtain MDD at 0.5 ng for both enrofloxacin and ciprofloxacin. The limit of detection of 0.01 ppm obtained for standards is lower than maximum residue levels (MRLs) established by the European Union for various species and matrices.[5] In view of the size of the smallest spots visible in Fig. 5 or in Fig. 6, it seems possible to detect at least twice as low quantities of antibiotics, especially of ciprofloxacin, which gives larger spots. In spite of repeated experiments it was impossible to detect lower amounts.

It seems to be a characteristic feature of the TLC–B method that below a certain amount of the antibiotic applied onto the plate, the inhibition zone is not formed. Because of the accidental character of bacterial growth and local variability of growth conditions, it happens that the same small dose may or may not give a clearly visible inhibition zone on a plate. Therefore, it is necessary to be very careful in determining the detection level of the method. The lowest detectable amount must be applied many times and the zone must be visible in most of the cases. It is impossible to predict this value on the basis of the calibration curve obtained at higher concentrations. In particular, the prediction of the detection limit based on linear calibration curve (in semi-log scale), as presented in the paper by Ramirez et al.,[20] could be erroneous.

Quantitative bioautographic analysis is usually done by the regression analysis of the inhibition zones sizes. According to some papers, the relationship between the diameter or area of inhibition zone plotted against the logarithm of the concentration of the antimicrobial applied is linear.[19–22] Our experiments show that this approximation is possible only for a narrow range of concentrations (one or two orders of magnitude). For a wider range of concentration, exponential relation fits better. The dependencies between the areas of inhibition zones and amounts applied of enrofloxacin and ciprofloxacin are plotted in Fig. 7. Figure 7(b) differs from (a) only in the *x*-axis scale—linear in (a) and logarithmic in (b). Exponential curves, plotted in Fig. 7(c), were obtained with a higher correlation coefficient than for linear regression in semi-logarithmic scale [Fig. 7(b)]. The usefulness of the exponential plot is well-illustrated in Fig. 8, where the relations between area and logarithm of the amount spotted were established on the basis of four bioautograms and for a wide range (covering four orders of magnitude) of concentration. Because of a large scattering of points, which is due to a character of microbial detection, correlation coefficients were not as good as those obtained for a single plate. Still, the exponential shape of the curves was evident.

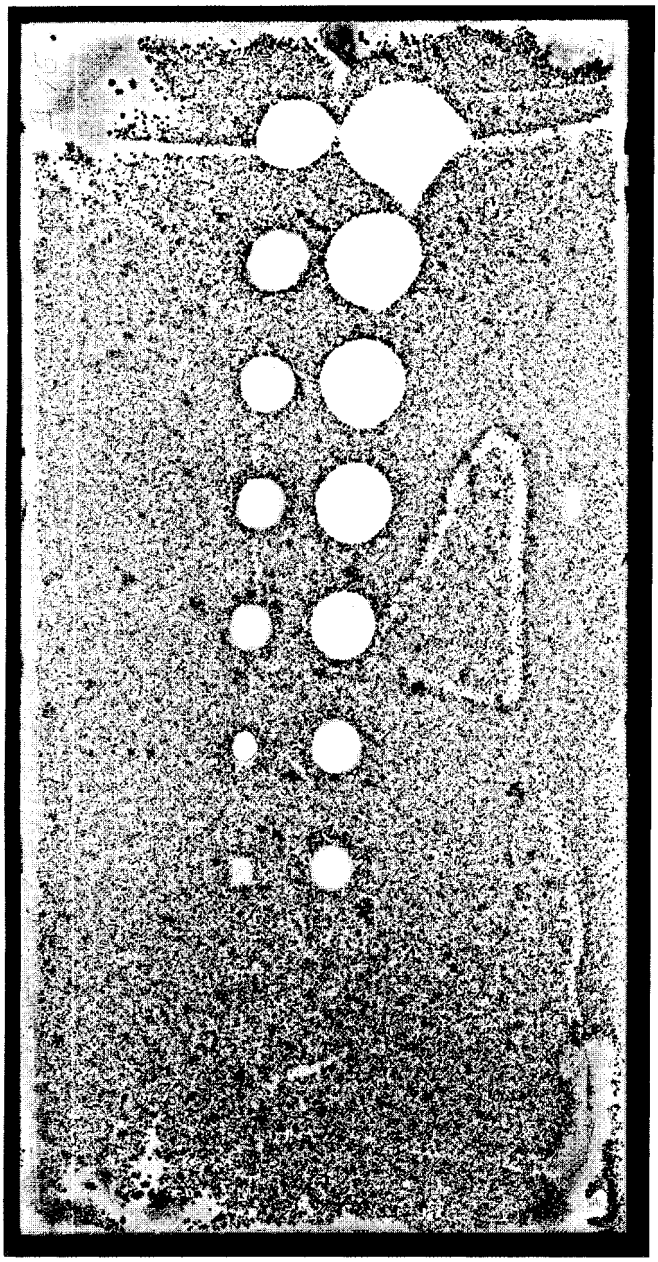

Figure 5. TLC–B of enrofloxacin and ciprofloxacin standards on TLC Si60F_{254} plate. The applied volume equaled 10 μL, bandwidth was 5 mm. The mobile phase was dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2, developed to the end of TLC plate and then continuously for about 2 hr (cross the plate). From left to right the concentrations of antibiotic standards applied: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 ppm (μg mL^{-1}), i.e., 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng of each antibiotic per spot. Upper spots, enrofloxacin; lower spots, ciprofloxacin.

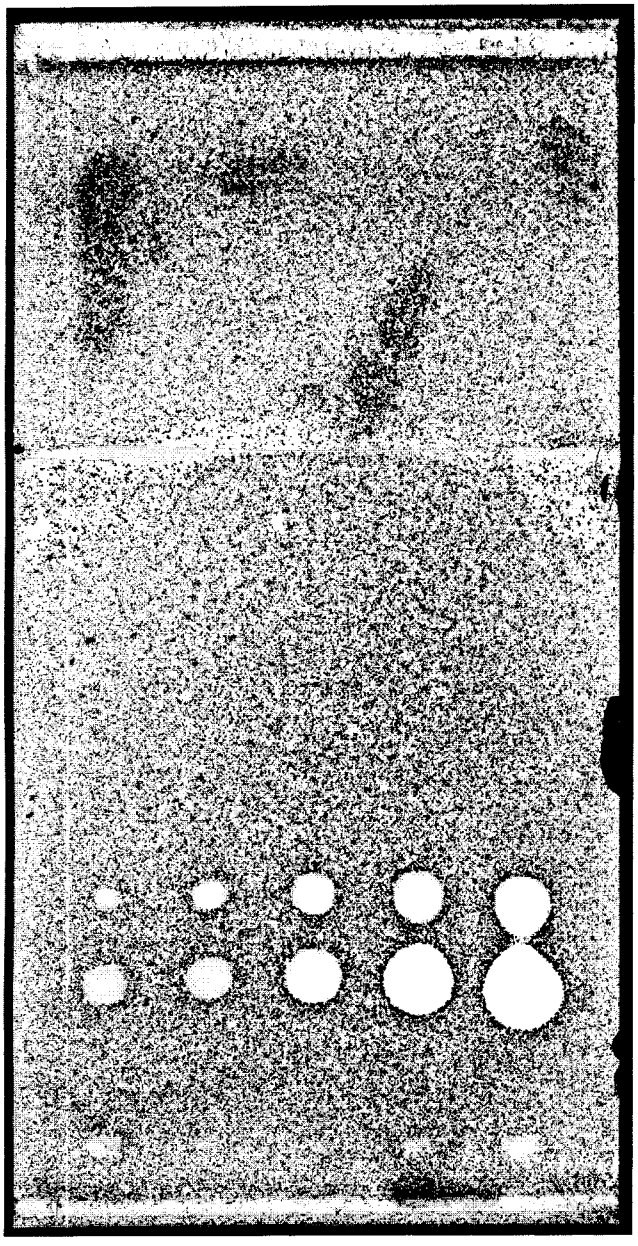

Figure 6. TLC–B of enrofloxacin and ciprofloxacin standards on TLC Si60F$_{254}$ plate. The applied volume equaled 50 µL, bandwidth was 5 mm. The mobile phase was dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2, developed to the distance of about 12 cm (along the plate). From left to right the concentrations of antibiotic standards applied: 0.01, 0.05, 0.1, 0.5, 1 ppm (µg mL^{-1}), i.e., 0.5, 2.5, 5, 25, 50 ng of each antibiotic per spot. Left-hand spots, enrofloxacin; right-hand spots, ciprofloxacin.

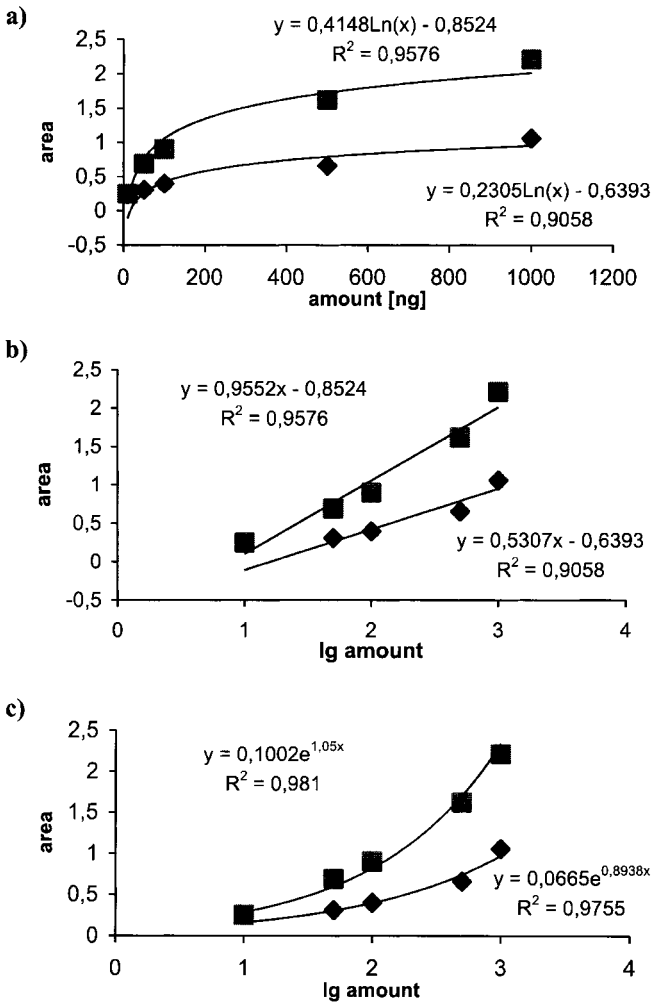

Figure 7. Areas of inhibition zones (cm^2) plotted against amounts (a) and against logarithm of amounts of antibiotic standards (b and c) applied onto the plate (ng) in 1 μL volume. ◆, enrofloxacin; ■, ciprofloxacin.

CONCLUSIONS

Conditions of TLC–B of enrofloxacin and ciprofloxacin were established. The size of inhibition zone depends on the applied volume of the antibiotic solution. The MDD for the standards of the antibiotics equaled 0.5 ng.

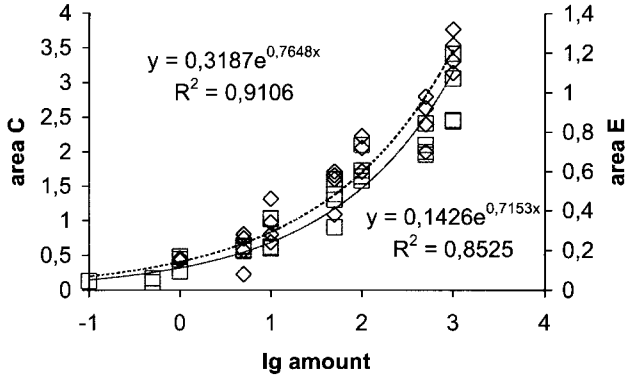

Figure 8. Areas of inhibition zones (cm^2) plotted against logarithm of amounts of antibiotic standards applied onto the plate (ng) in 10 μL volume. --◇--, for enrofloxacin, —□—, for ciprofloxacin. The plots are established on the basis of four bioautograms. The range of concentrations and applied amounts are the same as for Figs. 4 and 5.

Limit of detection was at the level of 0.01 ppm for 50 μL of antibiotic standard solutions applied onto the plate. Exponential dependence of the area of the inhibition zone versus logarithm of amount applied was determined for a wide range of concentrations—four orders of magnitude. It is impossible to establish the MDD from the linear calibration curve in a semi-log scale obtained for the higher amounts of applied antibiotic standards.

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