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Determination of flumequine and doxycycline in milk by a simple thin-layer chromatographic method

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Abstract

Tetracycline and quinolone antibiotics have for many years served as important classes of veterinary drugs. Two representatives of both classes: doxycycline from tetracyclines and flumequine from quinolones are often administered together. When the withdrawal periods are not obeyed, the antibiotic residues may be present in edible products, e.g., in meat, eggs or milk. In the present paper a simple thin-layer chromatography (TLC) screening method is established for determining these drugs in milk. Only two developments of the plate with concentrating zone are needed: one as a clean-up procedure, the other as a proper analysis. The spots were detected both by UV lamp with dual wavelength (254 and 366 nm) and by densitometry. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Flumequine; Doxycycline

1. Introduction

Antibiotics are widely employed in animal husbandry. They are used both for the prophylaxis and the treatment of diseases (e.g., mastitis in cows) and as feed additives to promote mass gain. Antibiotics can be added directly to food products (mainly to milk) to prolong their freshness.

All these cases raise the occurrence of antibiotic residues in animal tissue and milk intended for human consumption. The occurrence of antibiotic residues in the food supply has been widely documented [1,2]. The detected concentrations can range from 0.01 ppm to even 10 ppm. In many countries, government authorities have established monitoring programs for antibiotic determination in food [3,4]. In most European countries the legislation and use of veterinary drugs are regulated both on a national and European Union (EU) level [1,2,5–8]. In the United States safe levels for residues of antibiotics are set by the Center for Veterinary Medicine of the US Food and Drug Administration [9].

Many methods have been described for determination of antibiotics in various edible products [10–12]. High-performance liquid chromatography (HPLC) is one of the most popular and sensitive methods [13–15]. However, it usually requires tedious sample pre-treatments such as: protein precipitation [16,17] ultrafiltration [18,19], partitioning [20], metal chelate affinity chromatography (MCAC) [21–23], solid-phase dispersion (SPD) [13,24] or solid-phase extraction (SPE) involving ion-exchange [25],

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adsorption [26], immuno [27] or reversed-phases (C_{18}) [14,18,21,28]

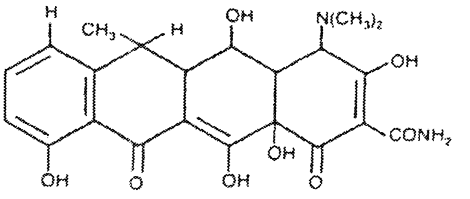
Sample purification can be omitted or reduced when screening methods are applied. There are many screening tests available, e.g.,: microbial inhibition (STOP, LAST, CAST, FAST, BR-Test, Delvotest P [11,29]), enzyme (Penzyme [29]), immunoassay [enzyme-linked immunosorbent assay (ELISA) [30], Cite Probe [31]] or radioimmunoassay (Charm [32,33]), which can be applied with some limitations.

Maximum residue levels (MRLs) for antibiotics are primarily based on microbiological data. Microbial inhibition tests are cheap and simple but they lack specificity and sensitivity. For most antibiotics, except for β-lactams, the detection limits they offer are higher than 100 ppb. The MRL value recommended by the EU for tetracyclines in meat and milk is just 100 ppb [1,13].

Enzyme and radioimmunoassay tests are more sensitive, though quite expensive. Detection limits for the Charm II test, for instance, do not exceed 100 ppb (100 ppb for doxycycline and only 10 ppb for tetracycline) and this test has been evaluated as a confirmatory method for positives from microbial screening assay [34].

This problem can be solved by thin-layer chromatography (TLC), which is a simple, cheap and quite sensitive and specific method. The screening TLC method is usually coupled with bioautography [35], but UV or fluorescence [36,37] detection can also be applied.

Tetracyclines are a group of broad-spectrum antibiotics widely used in veterinary practice. They are usually combined with other drugs, such as β-lactam or quinolone antibiotics. Doxycycline, belonging to tetracyclines, as well as flumequine, from quinolones, are very often prescribed together. Their structures are shown in Fig. 1. According to the Polish regulations neither doxycycline nor flumequine is allowed to be present in food supply but still they may be available commercially [38]. Similarly in the United States doxycycline is not approved for animal use and so cannot be present in food [23,34,39] while the EU has set a MRL value for doxycycline in milk of 100 ppb [13,34] and the World Health Organization (WHO) allows residues of flumequine of 500 ppb in meat [40]. In any case, both doxycycline and flumequine are available for



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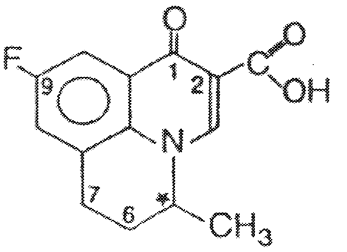

b

Fig. 1. Structures of (a) doxycycline, (b) flumequine.

veterinary use all over the world and they can be detected in meat or milk when waiting periods are not abided by. High concentrations of those antibiotics can be found in milk when they are purposefully added to prolong its freshness.

In the present paper a simple, screening method of isolation and determination of these antibiotics in milk is described. Only two developments of TLC plate are needed: one as a clean-up procedure, the other as a proper analysis. The sensitivity is not very high and similar to that of microbiological tests, so application of the present method is limited to milk samples purposefully contaminated with antibiotics. Work on this method are to be continued in order to obtain better sensitivity.

2. Experimental

2.1. Materials

Soczewiński's glass sandwich chambers with glass distributors [41,42] were purchased from Polish Chemical Reagents (P.O.Ch.), Lublin, Poland.

Pre-coated silica gel TLC Si60 and Si60 $F_{254,}$

10×20 cm, with and without concentrating zone were purchased from E. Merck, Darmstadt, Germany.

2.2. Chemicals

Methanol (MeOH), chloroform ($CHCl_3$), acetonitrile (MeCN), citric acid, 1-propanol, 2-propanol, isopropyl ether, hexane (p.a.) were purchased from P.O.Ch. Gliwice, Poland.

Tetracycline (TC), oxytetracycline (OTC), chlorotetracycline (CTC), doxycycline (DC), as their hydrochlorides and flumequine (FL) were supplied by Polfa, Tarchomin, Poland.

2.3. Preparation of antibiotics solutions

0.1-g portions of each of tetracyclines and flumequine were weighed accurately into a 10-ml volumetric flask. The flumequine was dissolved in 1% Na_2CO_3 and then diluted to volume with water; tetracyclines were diluted to volume only with water, all at a concentration of 10 mg/ml. The working solutions were the mixtures, prepared by the dilution of 100 μl of each stock solution in 9.9 ml of methanol (tetracyclines) or water (flumequine) to produce 0.1 mg/ml standards. Milk (2% fat) was fortified with one of the stock solutions of antibiotics (usually 5 μl of a stock solution per 1 ml of milk). Volumes (1–5 μl) of standard solutions or milk spiked with antibiotics were applied on the TLC plates using a Hamilton microsyringe (Bonaduz, Switzerland).

2.4. Solvent system

The developing solvents were: $CHCl_3$–MeOH (1:1), MeCN–MeOH (1:1), MeCN, MeOH, 0.1 *M* citric acid–MeOH (1:9; 1:4), 0.05 *M* citric acid–MeOH (1:4, 1:3, 1:2), 0.01 *M* citric acid–MeOH (1:4, 1:3, 1:2), 0.05 *M* citric acid–MeOH–2-propanol (1:2:2), 0.05 *M* citric acid–MeOH–1-propanol (1:2:2), 0.05 *M* citric acid–MeOH–isopropyl ether (1:2:3), 0.05 *M* citric acid–MeOH–2-propanol (1:3:1).

2.5. Detection

After air drying, spots were visualized by a UV lamp (HA-05 Haland, Warsaw, Poland) with dual-wavelength: 254 nm and 366 nm. Flumequine absorbs 254 nm light, so the use of plates with fluorescent indicator is necessary, while tetracyclines' fluorescent spots are visible at 366 nm.

2.6. Methods

The standards of antibiotics were applied on the TLC plates with fluorescent indicator F_{254}. The samples of milk fortified with flumequine and/or doxycycline stock solutions were injected on the TLC plates with fluorescent indicator F_{254} into the middle of special regions of trapezoidal shape created by the incision into the plate's concentrating zone. The TLC plates were set into Soczewiński's sandwich chamber. The construction of this chamber imposes the removal of about 0.8 cm of adsorbent layer from the TLC plate – the zone denoted A at the chromatograms. The plates, prepared as described, were pre-developed with hexane to remove a lipid fraction from the milk samples. Then they were developed to a distance of 15 cm with a proper solvent system.

2.7. Calibration

Flumequine and doxycycline standards were injected on the Si60 plates without fluorescent indicator by use of a AS-30 Desaga (Heidelberg, Germany) applicator. The developed plates were placed under Shimadzu CS-9001 (Kyoto, Japan) scanning densitometer working in the reflection mode. We also chose the linear scanning mode instead of the zigzag one on account of its being more sensitive and less time-consuming. The wavelengths were: 360 nm for doxycycline and 325 nm for flumequine.

2.8. Recoveries

Milk samples (5 μl) containing 0.1, 0.25 and 0.5 μg of both flumequine and doxycycline (0.02, 0.05, 0.1 μg/μl of each antibiotic in milk) were injected on the concentration zones of Si60 plates. Addition-

ally, the standards containing the same amounts of the antibiotics were injected. After the development with 0.05 *M* citric acid–MeOH–2-propanol (1:3:1) the plates were placed under a Shimadzu CS-9001 (Kyoto, Japan) scanning densitometer (the reflection and linear modes). The measurements were repeated four times. Then mean values and standard deviations were calculated.

3. Results and discussion

The idea of the presented method was very simple: (1) to inject a sample of milk containing antibiotics onto concentrating zone of a plate, (2) to defat the sample by developing with lipophilic solvent, and (3) to separate two antibiotics choosing proper solvent system (it is assumed that proteins of milk should sorb onto widepore silica gel of a concentrating zone).

However, it was much more difficult to make the method work. There were basically three tasks to deal with.

Firstly, how to obtain a regular front of developing solvent having spots of milk at the start? We found that a regular front was formed when samples were injected into the middle of the specially prepared regions of trapezoidal shape created by incision into the plate's concentrating zone. The borders of the concentration zone are the top and the bottom of the trapeze edge, other edges are formed by the skew incisions made with a scalpel (see chromatograms).

Secondly, it was essential to pre-develop the plate with a lipophilic solvent to remove milk lipids. The tailing of the spots of the analyzed substances occurred when we omitted this clean-up stage. Among several tested lipophilic solvent hexane seemed to be the best one.

The third and the most important task was to find a proper developing system for the separation of the standards and then for the separation of milk antibiotics. Doxycycline, as other tetracyclines, has the propensity to form chelation complexes with metal ions, to bind with sample matrix proteins and to adsorb onto silica gel. As it is known from the literature [11] it is essential to use chelating agent as ethylenediamine tetraacetate (EDTA), citric or oxalic acid for efficient extraction of tetracyclines from milk and for good separation of them. We checked that solvents of high elution strength as $CHCl_3$–MeOH (1:1), MeCN–MeOH (1:1), MeCN or even MeOH (no chelating agent added) are unable to elute doxycycline from the start. The simplest solvent system, which solved the problem, was methanol in a mixture with citric acid water solution. We tested different concentrations of citric acid and obtained the best separation with 0.1 *M* citric acid–MeOH (1:9) (Fig. 2). We obtained good separation of standard antibiotics' spots and separation of doxy-

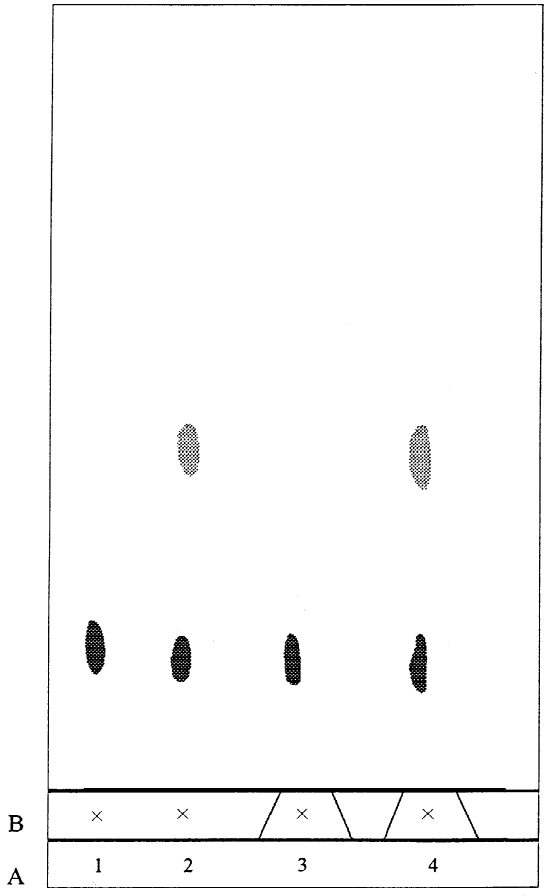

Fig. 2. TLC chromatogram of: (1) doxycycline (0.5 μg in the spot); (2) doxycycline and flumequine (0.5 μg+0.5 μg); (3) milk spiked with doxycycline (0.25 μg of DC in the spot); (4) milk spiked both with doxycycline and flumequine (0.25 μg of DC and 0.25 μg of FL). Solvent system: 0.1 *M* citric acid–MeOH (1:9). The plate was pre-developed with hexane. (A) About 0.8 cm part of the plate without sorbent; (B) concentration zone, about 2/3 part (~1.7 cm) of the original one.

cycline from flumequine in milk. For this solvent we could reliably identify 0.25 μg of doxycycline and flumequine.

Comparable results were obtained when three component mixtures were applied, e.g.,: 0.05 *M* citric acid–MeOH–2-propanol (1:2:2), 0.05 *M* citric acid–MeOH–1-propanol (1:2:2) and 0.05 *M* citric acid–MeOH–isopropyl ether (1:2:3). The former gave the best separation, so this mixture was used to separate doxycycline and flumequine from fortified milk. The detection limit of flumequine was the same as previous, while doxycycline at this concentration was overloaded.

We tested other proportions of mixtures of solvents and found that the proportion 1:2:2 can be replaced by 1:3:1 of 0.05 *M* citric acid–MeOH–2-propanol (Fig. 3). The chromatogram shows good separation of the antibiotics. Portions of milk were fortified with different amounts of doxycycline and flumequine. The sharp spot of 0.05 μg of flumequine in milk was obtained but doxycycline at this level was invisible. However, it was possible to detect spots of 0.1 μg of doxycycline and flumequine in milk. The separation of 0.25 μg of the antibiotics was also good while at the level of 0.5 μg the spot of doxycycline was overloaded.

Fig. 4 shows densitometric profiles of doxycycline and flumequine separated from milk on silica gel Si60 without fluorescent indicator using the above described solvent system. The presence of the indicator is necessary when spots of flumequine are detected under the UV lamp, while it hinders detection by means of densitometer.

Fig. 5 presents calibration curves of doxycycline and flumequine. As can be seen there is a linear dependence between integrated areas and amounts of antibiotics in the range of 0.1 to 0.5 μg.

It can be easily predicted that, contrary to off-line methods, the presented one should give 100% recoveries. Table 1 shows the mean recoveries and the standard deviations of doxycycline and flumequine isolated from milk fortified at three concentration levels. Almost all recoveries are close to 100%. It was checked that no amount of antibiotics remained after development at the start and between the injection points and the developed spots. So all injected amounts should be found in the developed spots and the recovery error is probably connected

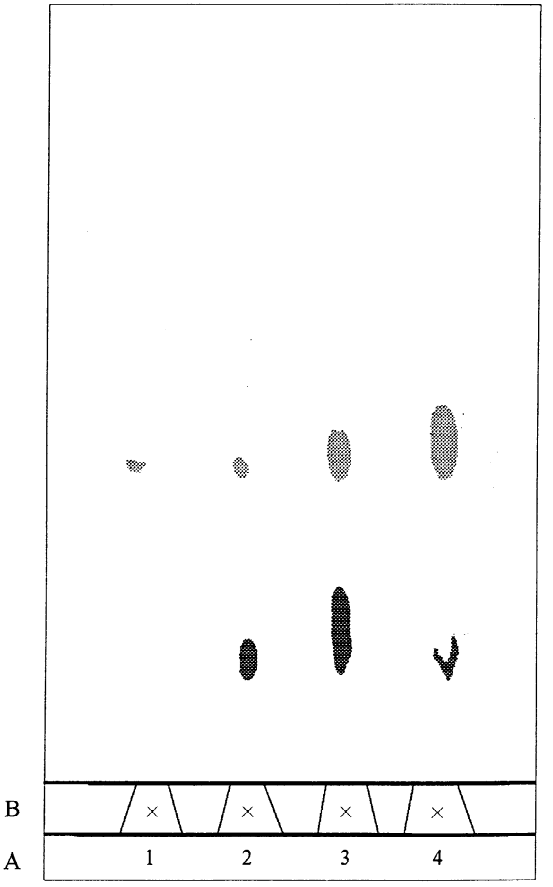

Fig. 3. TLC chromatogram of milk spiked both with doxycycline and flumequine: (1) 0.05 μg; (2) 0.1 μg; (3) 0.25 μg; (4) 0.5 μg of each. Solvent system: 0.05 *M* citric acid–MeOH–2-propanol (1:3:1). The plate was pre-developed with hexane. (A) About 0.8 cm part of the plate without sorbent; (B) concentration zone, about 2/3 part (~1.7 cm) of the original one.

solely with the densitometric measurements. As can be seen from Fig. 4 the peak of flumequine is strongly tailing which may cause a substantial error during the integration process especially when a small amount of flumequine is injected.

It seemed to be interesting whether tetracyclines, other than doxycycline, could also be separated from flumequine. Fig. 6 presents chromatogram developed with 0.05 *M* citric acid–MeOH–2-propanol (1:3:1). This time the milk was spiked successively by doxycycline alone, doxycycline and flumequine mixture and the mixture of four tetracyclines: doxycycline, tetracycline, oxytetracycline and chloro-

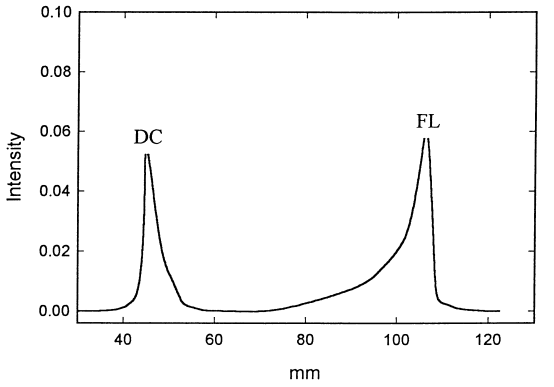

Fig. 4. Densitometric profiles of doxycycline and flumequine (each of 0.25 μg) separated from milk on silica gel Si60 without fluorescent indicator (λ=325 nm). Solvent system: 0.05 *M* citric acid–MeOH–2-propanol (1:3:1). The plate was pre-developed with hexane.

tetracycline (all at the level of 0.25 μg). Additionally, at the first position, the pure milk was injected as a blank sample. As it is seen all tetracyclines migrated in one spot and could be separated as a group from flumequine.

Thus the problem of determining and separating

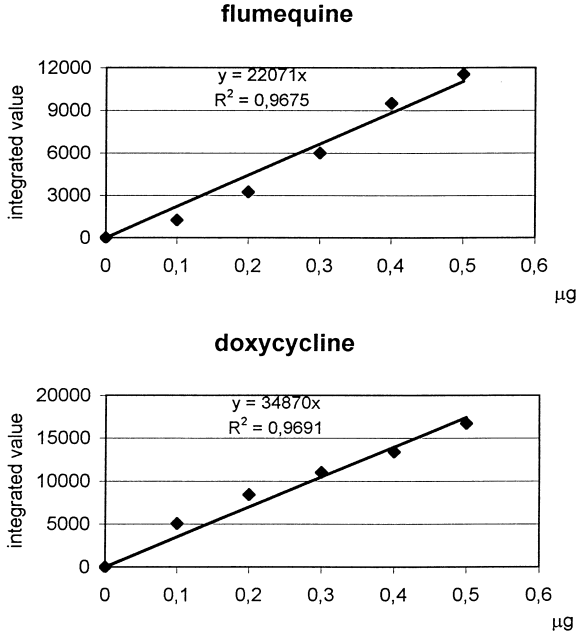

Fig. 5. Calibration curves of doxycycline and flumequine. Solvent system: 0.05 *M* citric acid–MeOH–2-propanol (1:3:1).

Table 1
Mean recoveries and standard deviations of doxycycline and flumequine isolated from fortified milk (n=4 at each concentration level, mobile phase: 0.05 *M* citric acid–MeOH–2-propanol, 1:3:1)

Concentration level (μg/μl)	Doxycycline (%)	Flumequine (%)
0.02	98.2±7.7	149.3±8.2
0.05	93.2±6.4	96.4±8.0
0.10	101.0±2.3	105.9±9.5

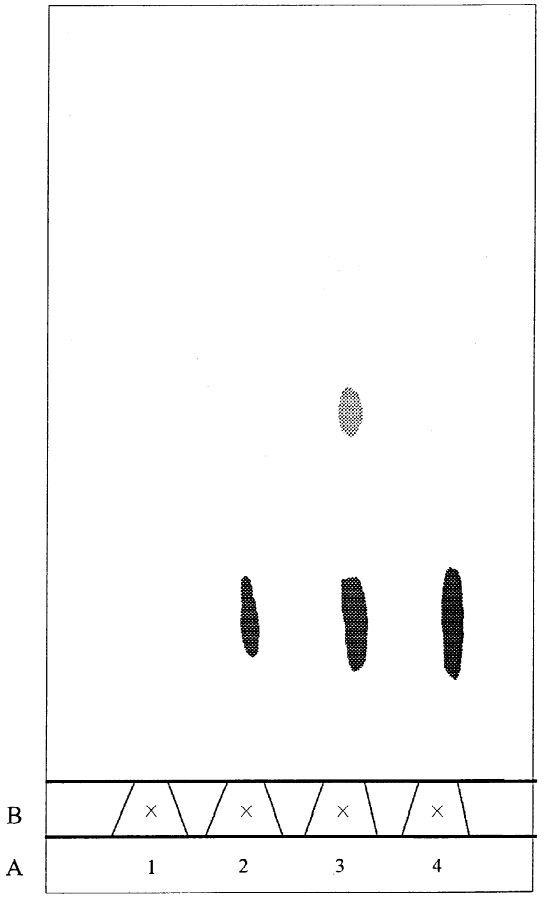

Fig. 6. TLC chromatogram of: (1) pure milk; (2) milk spiked with doxycycline (0.25 μg of DC in the spot); (3) milk spiked both with doxycycline and flumequine (0.25 μg of DC and 0.25 μg of FL); (4) milk spiked with doxycycline, tetracycline, oxytetracycline and chlorotetracycline (0.25 μg of each). Solvent system: 0.05 *M* citric acid–MeOH–2-propanol (1:3:1). The plate was pre-developed with hexane. (A) About 0.8 cm part of the plate without sorbent; (B) concentration zone, about 2/3 part (~1.7 cm) of the original one.

doxycycline from flumequine in milk was solved: we checked that the proper solvent system containing the chelating agent could elute the antibiotics from milk injected onto the concentrating zone of a plate.

The last two problems we had to solve focused on two questions: (1) whether impregnation of a plate with some chelating agent could improve separation and (2) whether it was possible to separate antibiotics on a plate without concentrating zone.

It is known from the literature that impregnation of a plate (with EDTA, for instance) is often applied for separation of tetracyclines [37,43]. We impregnated plates with different media, e.g., 5% Na_2EDTA, 0.01 *M* citric acid and 0.01 *M* citric acid–MeOH (1:4) and found that in the case of separation of doxycycline from flumequine impregnation was useless because it resulted in much worse separations.

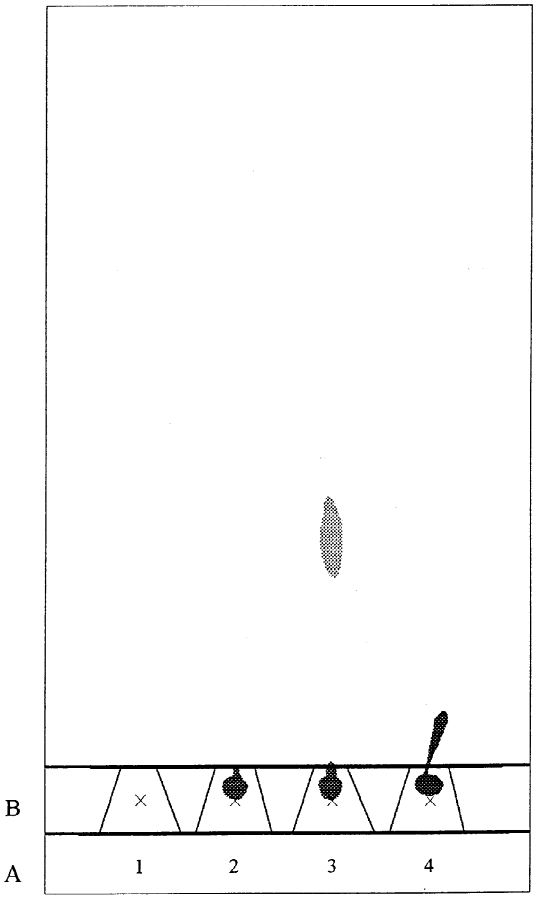

Fig. 7. As in Fig. 6 but this time on the plate without concentrating zone. (A) About 1 cm part of the plate without sorbent; (B) injection zone (note, that B does not refer to the concentration zone).

To obtain the answer to the second question we injected the same samples and developed them with the same phase as in Fig. 6, but this time using a plate without a concentrating zone (Fig. 7). The spots of tetracyclines remained at the start. This was probably caused by very strong adsorption of tetracyclines onto silanol groups, so strong that even the addition of complexing agent (e.g., citric acid) in the developing solvent was not sufficient to elute them. Such strong adsorption probably does not occur on widepore silica gel of concentrating zone. It seems that only milk proteins can sorb on the surface of the concentrating zone while tetracyclines, separated in this way from the milk matrix, can freely migrate.

4. Conclusions

The TLC screening method described above is very simple and cheap since only two developments of the same plate with concentrating zone are needed. The method allows both the separation from the milk matrix and the determination of two antibiotics from different classes: flumequine and doxycycline. As was shown other tetracyclines can be isolated as a single spot from milk and can be easily separated from flumequine. The authors' experience points to the possibility of employing the presented method (using different mobile phases) to analysis of other drugs. The sensitivity of the method is not high and similar to that of microbiological tests. In the future we plan to address ourselves to the problem of improving upon the sensitivity of the TLC method as well as of extending its applications.

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