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High-performance thin-layer chromatography–bioautography for multiple antibiotic residues in cow's milk

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Abstract

An analytical method to identify and quantify multiple antibiotic residues (chloramphenicol, ampicillin, benzylpenicillin, dicloxacillin and erythromycin) in cow's milk by high-performance thin-layer chromatography (HPTLC) combined with bioautography was developed. The test microorganism used for bioautography was *Bacillus subtilis* ATCC 6633. Antibiotic residues were extracted with acetonitrile, fat eliminated with petroleum ether and residues isolated with dichloromethane The sensitivity of the method guarantees the detection of the above-mentioned antibiotics at levels below maximum residue limits (MRL) allowed for milk. Percentage recoveries ranged between 90 and 100%, with coefficients of variation between 7.2 and 21.3%. Some advantages of this methodology over thin-layer chromatography (TLC)/bioautography are also discussed.

Keywords: Bioautography; Antibiotics

1. Introduction

Antibiotics are widely used in dairy cattle for the treatment of diseases involving bacterial infections, especially mastitis. Its use may produce residues in milk and subsequently, the induction of allergic reaction in humans, as well as resistance in pathogen bacteria, which may result in health problems [1,2].

In many countries, governmental authorities have established monitoring programs to determine the antibiotic levels in food as well as the highest allowable levels of these residues.

Various methods have been described to determine antibiotic residues in milk. Chemical techniques like high-performance liquid chromatography (HPLC), gas–liquid chromatography, radioimmunoassay, thin-layer chromatography (TLC), electrophoresis, as well as microbiological and immunological assays [1–6] are the most commonly used methods to detect antibiotic residues in milk.

The coupling of thin-layer chromatography with

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microbiological detection (bioautography) has been used for the identification and quantification of several antibiotics [7,8]. It is considered a simple, cheap and quite sensitive and specific method [2]. The application of bioautography combined with TLC in antibiotic residues detection in milk has been demonstrated previously by Choma et al. [2], Noa and colleagues [3,9] and Keskin [10].

The development of high-performance thin-layer chromatography (HPTLC) has improved resolution and sensitivity over TLC [11]. Echterhoff and Petz [12] have used it in the analysis of nitrofuran residues in milk and egg, with chemical developing for detection. No reports that combined HPTLC with bioautography were found in the literature, so the aim of this work was to develop an antibiotic multiresidue methodology in milk, combining HPTLC with bioautography.

2. Experimental

2.1. Materials

- H-separating chamber for 50×50 mm HPTLC plates and microcapillary tubes (1- to 5-μl volume) were obtained from DESAGA (Sorstedt Gruppe, Germany).
- Disposable Petri dishes 10 cm I.D. were purchased from SyM Laboratories (Mexico).
- Vernier callipers (Scala, USA).

2.2. Chemicals

Acetonitrile, petroleum ether, chloroform, methanol, acetone, glycerin, dichloromethane, hydrochloric acid and sodium chloride were pesticide grade from J.T. Baker (Phillipsburg, USA).

Chloramphenicol (CAP, 94.7% purity), benzylpenicillin (PEN, 96%), dicloxacillin (DIC, 94.7%) and erythromycin (ERY, 96%), were purchased from Cosufar Mexico and ampicillin (AMP, 98%) from Sigma, USA.

HPTLC silicagel 60 pre-coated plates (10×10 cm) on pre-scored glass were supplied by Merck (Darmstadt, Germany).

2.3. Antibiotic solutions

2.3.1. Stock solutions

All antibiotics were individually dissolved in methanol at a concentration of 1 mg/ml and kept at 4 °C.

2.3.2. Working solutions

Each stock solution was diluted with methanol to a final concentration of: 100 μg/ml for chloramphenicol, 15 μg/ml for benzylpenicillin, 25 μg/ml for ampicillin, 50 μg/ml for dicloxacillin and 25 μg/ml for erythromycin.

2.4. Fortified milk

Fresh milk (3.3% fat) from known origin, free from antimicrobial residues, was adjusted to pH 5–6 with hydrochloric acid. An aliquot of 50 ml milk sample was fortified with each antibiotic working standard solution to a level of 0.1 μg/ml.

2.5. Milk extraction

The Malisch [13] multiresidue method for the determination of residues of chemotherapeutics was used to extract the analytes. Fortified milk samples (50 ml) were extracted by thoroughly mixing in a commercial blender at high speed with acetonitrile (150 ml) for 1 min. The liquid phase was decanted into a 500-ml separating funnel. Petroleum ether (100 ml) was added and shaken for 1 min, after which the upper phase (ether) was discarded. Sodium chloride (10 g) was carefully poured into the separating funnel and shaken gently to dissolve the salt as much as possible. After that, 100 ml dichloromethane was added to the acetonitrile phase and shaken again for 1 min. The dichloromethane phase (lower) was drained into a round-bottomed flask and evaporated to dryness at 40 °C in a rotary evaporator. The

residue was reconstituted in 1 ml of methanol and used for spotting on HPTLC plates.

A non-fortified milk sample was treated in the same way for negative control.

2.6. Solvent system

The developing solvent used for HPTLC was a mixture of dichloromethane–acetone–methanol–glycerin (64:20:15:1 v/v).

2.7. Culture media

2.7.1. Tripticase soy agar

Dissolve 15 g pancreatic digest of casein, 5 g papaic digest of soybean meal, 5 g sodium chloride and 15 g agar in water and dilute to 1 liter. Adjust final pH to 7.3±0.2 if necessary (BBL trypticase soy agar was found satisfactory).

2.7.2. Brain heart infusion

Dissolve 6 g brain heart infusion (solid), 6 g peptic digest of animal tissue, 5 g sodium chloride, 3 g dextrose, 14.5 g pancreatic digest of gelatin and 2.5 g disodium phosphate in water and dilute to 1 liter. Adjust final pH to 7.4±0.2 if necessary (BBL Brain Heart Infusion was found satisfactory).

2.7.3. Mueller–Hinton agar

Dissolve 2 g beef extract, 17.5 g acid hydrolysate of casein, 1.5 g starch and 17 g agar in water and dilute to 1 liter. Adjust final pH to 7.3±0.3 if necessary (Merck Mueller–Hinton agar was found satisfactory).

2.8. Inoculum

Bacillus subtilis ATCC 6633 was grown in tripticase soy agar for 24 h at 37 °C. One or two well isolated colonies were transferred into 2 ml BHI and mix thoroughly. After a 24-h incubation at 37 °C, turbidity was adjusted with peptonated sterile saline, using a 0.5 McFarland turbidity standard [14,15], to produce an inoculum containing 1×10^8 cfu per ml.

2.9. Inoculated media

Molten Mueller–Hinton agar (100 ml of sterile water containing 3.8 g of media) was inoculated with 0.3 ml of the bacterial suspension. The inoculated medium was cooled to 46 °C and poured (12 ml) into 100-mm disposable Petri dishes. Gel solidification should occur on a level surface. Petri dishes should be stored at 4 °C and used within 48 h.

2.10. HPTLC

The pre-scored HPTLC plates were simply cut into four 5×5 cm pieces by pressing against a hard surface. Antibiotic standard working solutions [CAP and ERY (1 μl), PEN, AMP and DIC (2 μl)]; milk blank and fortified milk sample extracts (5 μl), were spotted onto HPTLC plates, using calibrated capillaries. Optimal application volumes of standard working solutions for each antibiotic under study were previously determined by applying 1, 2 and 5 μl of 50, 25 and 10 ng/μl solutions of AMP, DIC and ERY (total drug amount applied 50 ng) and 100, 50 and 20 ng/μl solutions on CAP (total drug amount 100 ng). For PEN: 1, 2 and 4 μl of 40, 20 and 10 ng/μl solutions were tested (total drug amount 40 ng).

Each 5×5 cm HPTLC was suitable for spotting four test solutions. During application, spot size should not exceed 2–3 mm, to avoid inhibition zones overlapping during bioautography. After spot drying, plates were developed in the solvent system to a distance of 4.8 cm from the application zone and carefully dried with a blow dryer for complete solvent removal.

2.11. Bioautography

Each HPTLC plate was gently placed face down on a Petri dish, contacting the silica layer with the inoculated media during 25 min (an initial test was carried out to determine the optimal contact time between HPTLC plate and the inoculated media; 15-, 20-, 25- and 30-min periods were studied).

The Petri dish was then inverted and pressed

slightly to separate the HPTLC plate from the agar. Inverted Petri dishes were then incubated for 18–24 h at 37 °C.

Inhibition zone diameters from fortified milk extracts were measured with Vernier callipers and compared with antibiotic standards inhibition zones. R_f values were also determined.

2.12. Antibiotic calibration standard curves

Increasing concentrations of each antibiotic were spotted on the HPTLC plate as indicated in Fig. 1. For this test, plates were not developed in the solvent system. Bioautography was carried out as described above. The concentration ranges tested were: PEN: 8, 16, 24 and 32 μg/ml; AMP: 10, 20, 30 and 40 μg/ml; DIC and ERY: 25, 50, 75 and 100 μg/ml; CAP: 50, 100, 150 and 200 μg/ml (in all cases, application volume was 1 μl). After bioautography, the inhibition diameters were measured using Vernier callipers and the means, standard errors, correlation coefficients and dose–response lines calculated. Each antibiotic was tested with 10 replicates.

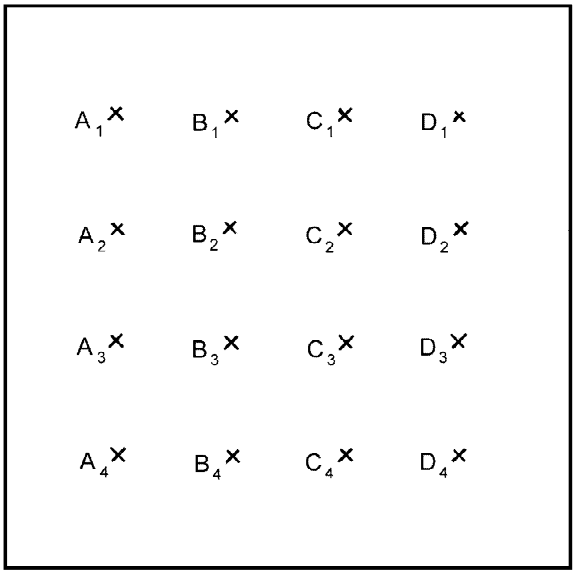

Fig. 1. General scheme of antibiotic application on 5×5 cm HPTLC plate for calibration curves. A, B, C, D: antibiotics; 1, 2, 3, 4: increasing concentrations; ×, application points.

2.13. Detection and quantification limits

The standard deviation (SD) of the response and the slope of the dose–response lines from the calibration curves of each antibiotic were used to estimate the detection and quantification limits [16]. The following formulae were used:

$$\mathrm{LOD} = \frac{3\ \mathrm{SD}}{\mathrm{Slope}}; \qquad \mathrm{LOQ} = \frac{10\ \mathrm{SD}}{\mathrm{Slope}}$$

2.14. Recovery

Antibiotic residue recoveries were determined from 10 replicate analyses of fortified milk samples run under identical conditions of HPTLC-bioautography. Antibiotic standard solutions and milk extracts were applied alternately on the HPTLC plates. The inhibition diameters were measured using Vernier callipers and the values obtained were interpolated in the calibration curves. Precision was estimated by calculating coefficients of variation for each drug recovery for the 10 replicates.

3. Results and discussion

Preliminary experiments to determine the optimal volume of application for each antibiotic tested showed that inhibition zone diameter was independent of applied volume (1, 2 or 5 μl); it was only dependent on drug amount (Table 1).

The chromatography developing time was shorter in HPTLC (5 min) than in TLC (45 min) [3,18]. Even though it would be necessary to run three HPTLC plates (four spots in each) to analyze the same number of samples as in 20×20 cm TLC plates (10–11 spots) [3,18], the overall developing time would still be smaller (3 plates×5 min = 15 min) than in TLC (45 min).

Less developing solvent volume would also be necessary for analyzing the same number of samples by HPTLC (3 runs×10 ml = 30 ml) compared to 280 ml in TLC [3,17,18]. The use of smaller quantities of expensive, toxic solvents that can be harmful to the environment is another advantage of the method proposed.

B. subtilis proved to be sensitive enough to all five

Table 1
Inhibition diameters (mm) produced with different application volumes (1, 2 and 5 μl) of each antibiotic (total drug amount was the same)

Antibiotic	Inhibition diameter (mm) for different application volumes ($n=10$)		
	1 μl	2 μl	5 μl
Ampicillin[a]	9	8.5	8.5
Dicloxacillin[a]	6	5.5	5.5
Erythromycin[a]	11	10.5	10.5
Chloramphenicol[b]	7	6.5	6.5
Benzylpenicillin[c]	11.5	11	11

All means in the same row do not differ ($P>0.05$).
[a] 50 ng/μl×1 μl=50 ng; 25 ng/μl×2 μl=50 ng; 10 ng/μl×5 μl=50 ng.
[b] 100 ng/μl×1 μl=100 ng; 50 ng/μl×2 μl=100 ng; 20 ng/μl×5 μl=100 ng.
[c] 40 ng/μl×1 μl=40 ng; 20 ng/μl×2 μl=40 ng; 10 ng/μl×4 μl=40 ng.

antibiotics tested. Well defined inhibition zones were produced by all of them in fortified milk extracts. No antibiotic activity was observed in control milk extracts.

The preliminary experiments conducted to set the optimal contact period (10, 15, 25 and 30 min) between HPTLC plates and inoculated media showed that a contact period of 25 min was necessary to produce a good response, in contrast with 15 min reported previously for TLC-bioautography [3,17,18]. Inoculated media should be perfectly solidified, otherwise adsorbent detachment of the HPTLC plate may occur. Lesser amounts of inoculated media would be necessary for analyzing the same number of samples by the method proposed (3 plates×12 ml=36 ml) compared to 100 ml in TLC-bioautography [3,17,18].

The extraction method used in the present work allowed high purity milk extracts, which produced clear and measurable inhibition zones. It has been recommended to use high purity extracts [2,18] because fat residues can cause spot tailing with the consequent production of elongated inhibition zones, which are very difficult to measure. Also matrix components may exert a significant effect on the mobilities of antibiotics [18]. The use of petroleum ether during extraction to defat milk samples and the isolation of analytes with dichloromethane proved to be useful, because no spot tailing was observed and R_f variability was lower than 16%, except for AMP (Table 2). No antibiotic activity was detected in the petroleum ether extract, which indicated that no analytes were extracted in this phase.

Fig. 2 presents the calibration curves for all antibiotics tested. As it can be seen, inhibition diameters presented a logarithmic correlation with antibiotic concentration, with determination coefficients (R^2) between 0.96 and 0.99. Fig. 3 shows a real bioautogram for two milk samples, an erythromycin standard and a milk sample fortified with this antibiotic.

The R_f values, percentage recoveries, detection and quantification limits for each antibiotic under study are shown in Table 2. The R_f values obtained were comparable with those reported by Noa et al. [3]. Ampicillin and chloramphenicol were readily

Table 2
R_f means and coefficients of variation (C.V.); % recoveries means and coefficients of variation; detection and quantification limits for HPTLC/bioautography determination of chloramphenicol, ampicillin, benzylpenicillin, dicloxacillin and erythromycin

Antibiotic	R_f[a]		% Recovery		Detection limit (μg/ml)	Quantification limit (μg/ml)	MRL (μg/ml)
	Mean ($n=10$)	C.V.	Mean ($n=10$)	C.V.			
Chloramphenicol	0.75	3.0	100	9.2	0.010	0.030	0[b,c]
Ampicillin	0.11	23.0	100	7.7	0.002	0.007	0.004[c]
Benzylpenicillin	0.18	14.0	90	14.3	0.005	0.014	0.004[c]
Dicloxacillin	0.19	3.5	100	21.3	0.009	0.027	0.03[c]
Erythromycin	0.20	16.8	95.3	7.2	0.004	0.013	0.05[c]

[a] Mobile phase: dichloromethane–acetone–methanol–glycerin (64:20:15:1).
[b] CODEX (1996).
[c] EEC (1992), Maximum Residues Limit (MRL).

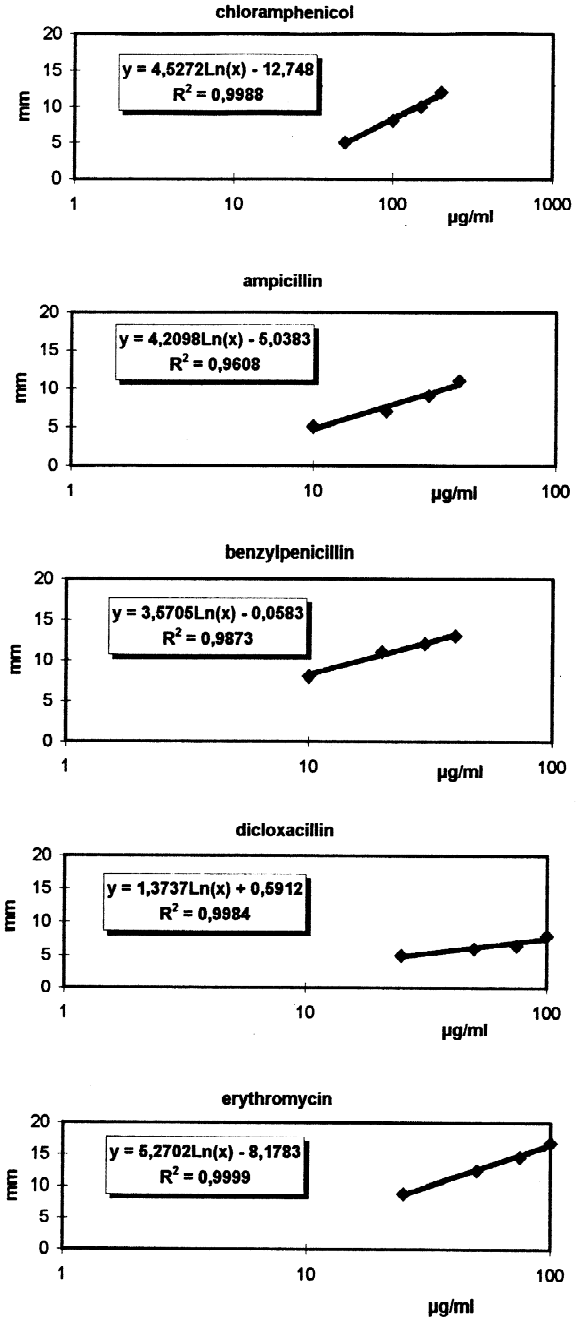

Fig. 2. Calibration curves of chloramphenicol, ampicillin, benzylpenicillin, dicloxacillin and erythromycin (application volume 1 μl).

identifiable by their R_f, however, dicloxacillin, benzylpenicillin and erythromycin R_f values were too close to clearly identify these three antibiotics. The insufficient separation of these antibiotics, if present in milk samples, would make their identification difficult, with the possibility of misinterpreting the bioautograms. Percentage recoveries ranged between 90 and 100% and were similar to those reported with TLC-bioautography [3,17]. The precision of the method was below 21.3%, which is lower than the coefficients of variation (<32%) attained by Echterhoff and Petz [12] in the HPTLC determination of a mixture of four nitrofurans.

Detection limits for all antibiotics investigated (Table 2) were below or near maximum residues levels established for these antibiotics by the CODEX Alimentarius for Veterinary Medicine Residues in Food [19] and the EEC [20]. Detection limits for CAP (0.010 μg/g) and ERY (0.004 μg/g) were lower than those achieved by Noa et al. (0.040 and 0.020 μg/g, respectively) with TLC-bioautography [3], which proves the higher sensitivity of HPTLC over TLC. Quantification limits of three (AMP, PEN and CAP) of the five antibiotics studied were above the MRL.

4. Conclusions

The HPTLC-bioautography method described was suitable for the detection of ampicillin, benzylpenicillin, dicloxacillin, chloramphenicol and erythromycin residues in milk samples at the tolerance levels, which would allow its use in routine monitoring of these antibiotics in milk. Major improvements of this methodology over TLC/bioautography [3,17,18] were: better sensitivities for CAP and ERY, easier to handle materials, smaller amounts of bacteriological media and developing solvents, and reduced spotting amounts of standard and milk extracts. Further work would be necessary to improve PEN, DIC and ERY resolution. In addition, considering that a very small fraction of the final extract is actually spotted on the 5×5 cm HPTLC plate, it would be worthwhile to try to reduce milk sample volume. In this way, highly expensive organic solvents used during extraction would also be reduced, which could make the methodology less

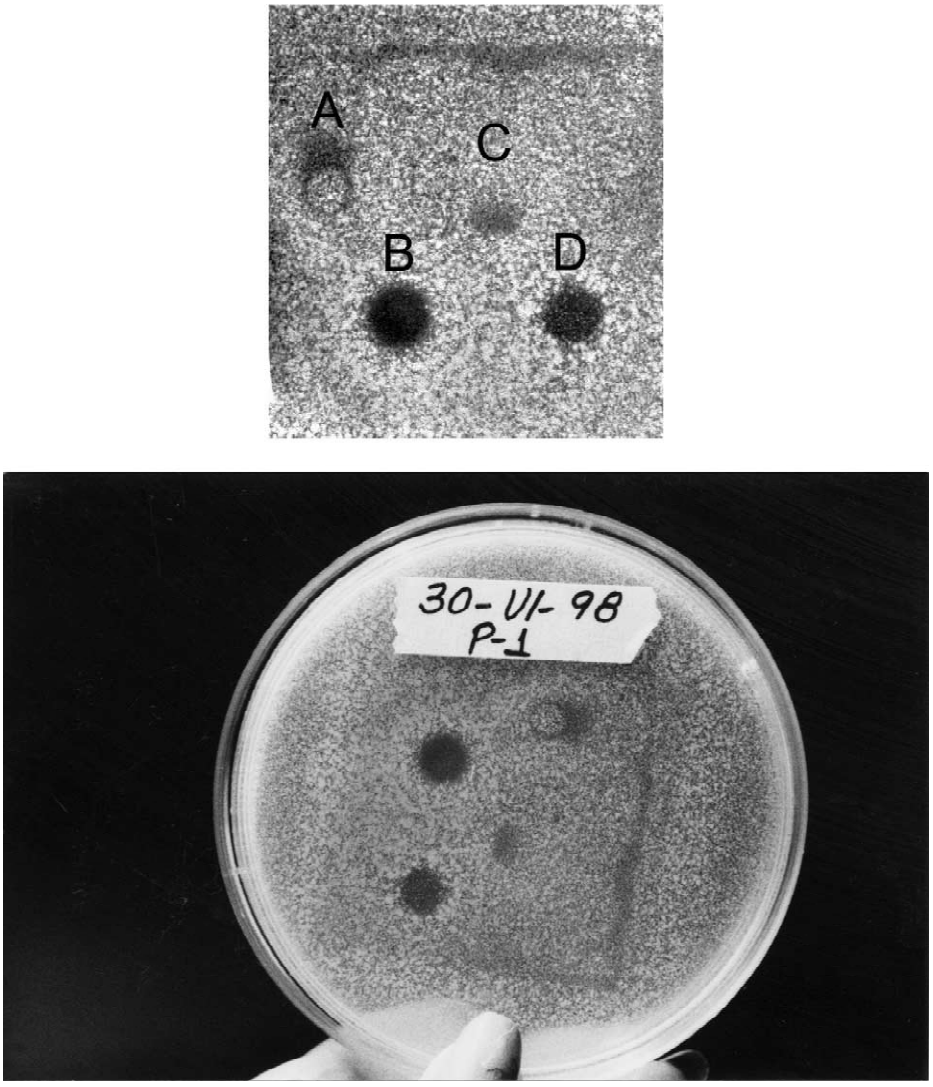

Fig. 3. Real bioautogram of: (A, C) milk samples (application volume 5 μl), unidentified antibiotics; (B) erythromycin working standard solution (25 μg/ml, application volume 1 μl); (D) control milk fortified with erythromycin (0.1 μg/ml, application volume 5 μl).

expensive. The concentration of the final extract and higher spotting volumes should also be tested to improve sensitivity.

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