Determination of Antibiotic Residues in Milk Samples

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[1] This work was summarised from same named PhD thesis

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Summary

In this study, antibiotic residues in raw milk and pasteurized milk products sold in Ankara- Turkey investigated. For this purpose; a total of 240 milk samples, which contains 10 raw and 10 pasteurized milk samples per month collected from various markets in Ankara between April 2003 and March 2004 were analyzed in terms of penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin by using TLC (Thin Layer Chromatography)/Bioautographic method, in which Bacillus subtilis ATCC 6633 was used as test microorganism. The minimum detectable concentrations for penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin, as μg/L were 4, 100, 200, 100 and 1000, respectively and recovery rate as percentage were 75.6, 79.7, 80.9, 84.7 and 73.5, respectively. The concentrations found among pasteurized samples were 150.4 μg/L oxytetracycline and 33.5 μg/L penicillin G and 7688.4 μg/L of neomycin among raw samples, which are higher than the maximum residue limits in milks accepted in Turkey and European Countries. According to the total number of samples analysed, the ratio of contamination with antibiotics was detected as 1.25%.

Keywords: Milk, Antibiotic, Residue, TLC/Bioautographic method

INTRODUCTION

The first use of antimicrobials for treatment of infections in veterinary medicine was in the late 1940s, shortly after their development. In the treatment bovine mastitis, antibiotics are widely used and improper application can lead to the contamination of milk at farm level. Nowadays, beta lactam (penicillin G etc), aminoglycoside (streptomycin, neomycin, etc) and tetracycline (oxytetracycline, etc) antibiotics are the most frequently used antimicrobials for treatment of mastitis in dairy cows and consequently, the most
commonly found type residues in milk. Residues are of concern due to their possible adverse effects on people allergic to antibiotics, potential buildup of antibiotic-resistant organism in humans and inhibition of starter cultures used to produce cultured milk products such as yogurt and cheeses. These reasons make it important to effectively control antibiotic residues in milk and therefore, regulatory authorities have enacted maximum residue limits (MRLs) for a number of antiinfective agents in milk. National monitoring programs to control the veterinary drug residues in various animal origin foods, including milk, are compulsory in all EC countries and Turkey. Detectable concentrations of antibiotic residues in milk supplies higher than the MRLs are illegal.

Running effective monitoring programs requires specific, sensitive and reliable analytical methods that can detect all drug residues below regulated levels. The overall objective is to develop and validate multiresidue methods in order to support the implementation of both existing as well as future regulations in the area of food control.

Various analytical methods have been described to determine antibiotic residues in milk, such as microbiological, chromatographic, immunochemical, receptor and enzyme-based tests. Microbiological tests are commonly applied in dairy and in survey studies. For the detection of antimicrobial residues in milk, microbiological screening tests are used that utilize bacterial test strains such as Bacillus stearothermophilus var. calidolactis, Streptococcus thermophilus and Bacillus subtilis ATCC 6633.

The coupling of thin layer chromatography with microbiological detection (TLC/bioautography) has been used for the identification and quantification of several antibiotics. It is considered a simple, cheap and quite sensitive and specific method. The application of bioautography combined with TLC in antibiotic residues detection in milk and animal tissue has been demonstrated previously by Choma et al. and Neidert et al.

The purpose of this study was to analyse residues of streptomycin, penicillin G, oxytetracycline, gentamicin, and neomycin by using TLC/bioautography; that are frequently seen in raw and pasteurized milk products sold in Ankara-Turkey.

**MATERIAL and METHODS**

**Apparatus and Reagents**

Antimicrobial standards penicillin G potassium, oxytetracycline, streptomycin sulphate, gentamicin and neomycin sulphate were purchased from Sigma-Aldrich Ltd. HPLC grade acetone, glycerine, chloroform, potassium hydrogen phthalate, n-propanol, Whatman cellulose TLC plates (200X200 mm) (Merck), Standard Plate Count Agar and Tryptose Soy Agar (Oxoid) were purchased. Bioplates (sterilized glass plates-245x245 mm) prepared privately.

**Impregnation liquid:** Mix 0.1 N phthalate buffer pH 3.75 and glycerine (19+1).

Stock solutions of standards were prepared by dissolving each compound in milli-Q water at a concentration of 1 mg/ml and stored in amber vials in a freezer (-20°C). Fresh stock solutions were made every month. Working standard solutions (different concentrations) were made by serially diluting the stock solutions with milli-Q water every time just before use and stored at 4°C in amber glass vials. Method validation samples were prepared at levels of ½, 1 and 2 or 4 times MRL values. All spiked samples (standards and method validation samples) were assayed 6 times in duplicate spread over three working days. The variation coefficient of the within-laboratory reproducibility for method validation samples was calculated.

**Sample Collection**

As part of the study, between 03.04.2003 and 30.03.2004 (for one year), a total of 240 milk samples, 10 of which were raw milk and 10 were pasteurized milk every month were collected from different points of sales in Ankara-Turkey. Attention was paid to ensure that the milk samples analysed were of different brands and were collected from different locations. Milk samples were kept in the refrigerator (4°C) until analysis and were analysed within two days at most.

**Assay organism:** Bacillus subtilis ATCC 6633 Difco spore solutions were obtained from The Ministry of Agricultural and Rural Affairs, Central Veterinary Control and Research Institute. It cultured on Tryptone Soya Agar (European Pharmacopeia).

**TLC solvent system:** Acetone-chloroform-n-propanol-impregnation liquid (16 + 20 + 27 + 16)

**Preparation of Milk Samples**

For extraction of the antibiotic residues of milk samples, methods specific to liquid chromatography developed by Tyczkowska et al. were used. Accordingly, it was extracted 1 ml from each milk sample and placed in centrifuge tubes. In order to precipitate the proteins in milk 1 ml of acetonitrile - methanol, deionized water
(40:20:20) mixture was added on it. After stirring with hand thoroughly, it was centrifuged at 3000 rpm for 10 min and the portion remaining on supernatant after proteins were precipitated was used for analysis.

**Thin Layer Chromatography**

Cellulose plates were divided into 10 equal channels. And then the 10 μl extracts were applied to the channels. During application, air current was applied on the plate to prevent the sample from decomposing. The plate was left to rest for 5 min in room temperature to dry them. Afterwards, the plates were placed in the development tanks prepared at least one hour in advance, in order to help the ambiance reach saturation. Then it is waited for the solution to rise up to 15 cm on the plate. Plate was removed from the tank and dried. The dried plate was then applied to the newly prepared bio-plate.

**Bioautography**

Seventeen and a half grams of Standard Plate Count Agar was measured and 1 L distilled water was added and was melted in 100°C water for 2 h and was sterilized by keeping for 16 min under 1 atmosphere pressure at 121°C. And by adjusting its pH to 7.2, its temperature was decreased by helping it contact water occasionally up to the temperature (38-40°C) required for *B. subtilis* to reproduce. For every 100 ml agar, 400 μl from *Bacillus subtilis* ATCC 6633 spore solution was added and as a result, 4 ml was added to 1 L agar and it was shaken for 15 sec. to help it diffuse homogenously. 300 ml agar was added to every bio-plate sterilized in advance for one h at 121°C under 1 atmospheric pressure. After waiting for agar to become solid (for around 20 min), the TLC plates that were developed by applying sample extracts or antibiotics standards and dried, were placed on the food-lot surface and was allowed to contact agar for 20 min. At the end of contact period, plates were removed and bio-plates were left for incubation for 16 h at 37°C. The inhibition zones diameters that developed at the end of the period were measured using calliper 16.

**RESULTS**

The Rf means obtained by preparing and using the standards, at certain concentrations, for detecting the streptomycin, penicillin G, oxytetracycline, gentamicin and neomycin residues using the TLC/bioautography method as well as the their low impact densities, their recovery rates and their minimum detectable concentrations are given in the Table 1.

As a result of the analysis in one of the pasteurized milk samples collected in April 2003, 150.4 μg/l oxytetracycline and 33.5 μg/l penicillin in another company’s sample was detected, whereas in the raw milk sample obtained from Ankara-Ayaş Gökyayla village, 7688.4 g/l neomycin was detected. Based on the total number of analysis samples, the ratio of contamination with antibiotics was detected as 1.25%.

**DISCUSSION**

TLC/bioautography method developed by Neidert et al.16 for detecting antibiotic residues in animal tissue was used. The method developed by Tyckowska et al.17 for extraction and detection of penicillin G from cow milk by HPLC was used to extract the antibiotic in milk. As a result of the recovery procedure carried out using these methods, 75.6% for penicillin G, 79.7% for oxytetracycline, 80.9% for streptomycin, 84.7% for gentamicin, and 73.5% for neomycin were calculated as recovery rates.

In the recovery study in meat conducted by Neidert et al.,16 these values were as follows; 95% for penicillin G, 95-98% for oxytetracycline, 80% for streptomycin, 100% for gentamicin, and neomycin. Accordingly, recovery levels in this study were lower for antibiotics other than streptomycin. The difference is believed to be due to differences between samples. Besides the fact that, TLC/bioautography is not a costly method compared to the other analysis methods, which can be adapted to laboratory conditions and applied easily; it has also certain advantages such as enabling distinction between

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Tested Concentrations (μg/L)</th>
<th>Minimum Detectable Concentrations (μg/L)</th>
<th>Recovery Rates (%)</th>
<th>Rf Values</th>
<th>MRLs * (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>2, 4, 8, 16</td>
<td>4</td>
<td>75.6</td>
<td>0.84</td>
<td>4</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>50, 100, 200, 400</td>
<td>100</td>
<td>79.7</td>
<td>0.34</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100, 200, 400, 800</td>
<td>200</td>
<td>80.9</td>
<td>0.50</td>
<td>200</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>50, 100, 200, 400</td>
<td>100</td>
<td>84.7</td>
<td>0.24</td>
<td>100</td>
</tr>
<tr>
<td>Neomycin</td>
<td>750, 1500, 3000, 6000</td>
<td>1000</td>
<td>73.5</td>
<td>0.07</td>
<td>1500</td>
</tr>
</tbody>
</table>

*a: Maximum Residues Limits (EC directive and Turkish Food Codex)*
antibiotics when appropriate plate and development systems are selected, leading more frequent use.

The degree of contamination of milk and dairy products with anti-bacterial additive residues differs, depending on the level of education, legislations and effectiveness of food inspection in different countries. According to the results of the technical report prepared for milk hygiene, by World Health Organization and Joint Expert Committee on Food Additives (JECFA), the rate of contamination of milk and dairy products with anti-bacterial additives in developed countries such as USA, Australia, UK and Scotland was 7-10% until 1969, after that year, the rate of contamination of the same products decreased to 0.5% in USA, 2.1% in Australia, 1.5% in UK and 3.4% in Scotland due to the precautions taken after the given date. The same report indicates that, in underdeveloped and developing countries, which fall behind in terms of increasing the level of awareness of stock breeders, improvement of hygienic conditions and in terms of inspection effectiveness, the rate of contamination for milk and dairy products might be higher.

In Turkey, there are only a limited number of antibacterial residue studies in milk. The most comprehensive is the one related to detection of veterinary medicine residues in foods carried by 5 Veterinary Control and Research Institutes in various cities in Turkey and by the 9 Provincial Control Laboratories, under the leadership of the Ministry of Agriculture and Rural Affairs. In this study, 3084 milk sample were analysed using intertest method for the determination of antibiotic and other inhibitor substances, using intertest and agar diffusion tests with the goal of detecting antibiotic residue in 2 of the 150 raw milk samples collected in the surrounding region, the following rates were obtained; with the Intertest method, 78 positive (17.56%), 65 suspicious (14.63%), 301 negative (67.79%); with the triple plate method, carried out using B. subtilis, 24 (5.40%) positive, 1 suspicious (0.22%), 419 (94.36%) negative. It is indicated that this difference is due to the method used, sampling time and the establishments.

As a result of the study conducted by Şanlı et al., for the detection of chloramphenicol residues on 444 raw and pasteurized milk samples collected from public and private sector establishments in Ankara and the surrounding region, the following rates were obtained; with the Intertest method, 78 positive (17.56%), 65 suspicious (14.63%), 301 negative (67.79%); with the triple plate method, carried out using B. subtilis, 24 (5.40%) positive, 1 suspicious (0.22%), 419 (94.36%) negative. It is indicated that this difference is due to the method used, sampling time and the establishments.

Chloramphenicol is an additive that was banned for use in livestock used for milk production and breeding, with the circular dated 19 April 1993 and numbered 419, issued by the Ministry of Agriculture and Rural Affairs due to its certain unwanted affects. However it is noteworthy and frustrating that it was detected in milk in various residue detection studies conducted after that date.

Even though the contamination rates discovered in milk samples as a result of residue detection studies carried in Turkey in previous years are so high, the contamination level detected in this study was only 1.25%. This can be explained by the increased public awareness about food safety and healthy nutrition and efforts of producers to market high quality products after the media started emphasizing the issue.

As a result the recovery values of the residues of penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin detected using the TLC/Bioautography method in milk were found to be satisfactory. Based on the results of this study and previous studies, it was determined that the probability of detecting antibiotic
in milk during spring and autumn is higher than the probability of detecting it in other months. The values detected are higher than the MRLs accepted in European Countries and Turkey. However, the fact that there is less antibiotic contamination in the milk produced in Turkey, compared with the residue rates obtained in previous studies, is considered to be a positive sign in terms of food safety.

REFERENCES


