Research Note

Stability of Tetracycline Antibiotics in Raw Milk under Laboratory Storage Conditions

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ABSTRACT

Raw milk samples collected from bulk milk tankers may be screened for the presence of tetracycline antibiotics using rapid screening tests. If tetracycline residues are detected, the milk may be shipped to a laboratory for high-pressure liquid chromatography (HPLC) analysis. Because the milk may be shipped on ice blocks, it is important to know whether tetracycline residues are stable at that temperature and for how long. Control raw milk samples fortified with 50 ppb each chlortetracycline, demeclocycline, methacycline hydrochloride, minocycline, oxytetracycline, and tetracycline were incubated at 4°C or 25°C, then analyzed using a metal chelate affinity chromatography extraction and HPLC. No loss of tetracycline was observed after 48 h of storage at 4°C or 24 h at 25°C. Losses ranging from 4 to 13% and 0 to 18% were noted after 72 h at 4°C and 48 h at 25°C, respectively.

Tetracycline antibiotic drugs are commonly administered to food-producing animals to control diseases. Veterinary uses in such animals are approved for chlortetracycline, oxytetracycline, and tetracycline. The U.S. Food and Drug Administration (FDA) has set tolerances for these residues in milk at 300 ppb. In addition, human drugs such as minocycline, demeclocycline, and methacycline may also be used in animals.

An array of analytical tests is used to assure the safety of the milk supply. Rapid multiresidue microbiological or immunoassay screening tests are used to test milk collected from tanker trucks. If the screening test detects drug residues, milk samples may be shipped to a laboratory where very accurate and specific regulatory tests are used to confirm that drug residues are present in the milk. The milk is usually shipped in an insulated container with ice blocks. Raw milk is unstable, containing many enzymes and a microbial population that may lead to the destruction of the antibiotic residues. For example, in order for raw milk to be accepted for processing as grade A in the USA, it must contain no more than 750,000 somatic cells and 300,000 bacterial cells per milliliter.

A review of the literature yielded no information on the stability of tetracycline antibiotic residues in milk. Therefore, we studied the stability of six tetracycline residues in raw milk stored at 4°C and 25°C.

MATERIALS AND METHODS

Materials. Chlortetracycline, demeclocycline, methacycline, minocycline, oxytetracycline, and tetracycline reference standards were obtained from the U.S. Pharmacopeial Convention (Rockville, Md.). Stock standard solutions (100 μg/ml) of each tetracycline were prepared in methanol and stored in actinic glass volumetric flasks at −10°C for up to 2 months. A mixed intermediate standard containing all six tetracyclines (1.0 μg/ml in methanol) was prepared from stock standards and stored at −10°C for up to 5 days.

Mixture tetracycline working standards (30, 50, 75, or 100 ng/ml in McIlvaine–EDTA–NaCl buffer) were prepared from the intermediate standard and used immediately, since they are unstable. Two sets of working standards were prepared for each high-pressure liquid chromatography (HPLC) run. One set was prepared and used at the beginning of the run, and a second set was prepared and used at the end.

HPLC-grade methanol and acetonitrile were obtained from EM Science (Gibbstown, N.J.). Deionized water was obtained from a Milli-Q Water System (Millipore Corp., Milford, Mass.). Chelating Sepharose Fast Flow was obtained from Pharmacia LKB (Uppsala, Sweden). Metal chelate resin, sodium succinate buffer, copper sulfate solution, and McIlvaine–EDTA–NaCl buffer were prepared as previously reported (1–3).

Control raw milk was obtained from the FDA Center for Veterinary Medicine and the U.S. Department of Agriculture Beltsville Agricultural Research Center, both in Beltsville, Md.

Apparatus. An ultrafreezer capable of maintaining −70°C was used to store milk samples. The refrigerated centrifuge was a Sorvall RC-3 with fixed-angle rotor holding 18-mm diameter tubes. Disposable polypropylene minicolumns were obtained from Bio-Rad (Hercules, Calif.). Centricon 30 centrifugal ultrafilters were obtained from Amicon, Beverly, Mass.

The HPLC system consisted of a series 410 LC pump (Perkin Elmer, Norwalk, Conn.) set at 1.0 ml/min, a loop injector with 1.0-ml loop (Rheodyne, Berkeley, Calif.), and a Model 383A pro-
TABLE 1. Spiked tetracycline levels found in milk incubated at 4°C for 24, 48 and 72 h or at 25°C for 24 and 48 h compared with milk stored in an ultrafreezer at −70°C (0 h incubation).

| Temperature (°C) | Time (h) | n | Minocycline [ppb (CV)] | Oxytetracycline [ppb (CV)] | Tetracycline [ppb (CV)] | Demeclocycline [ppb (CV)] | Chlorotetracycline [ppb (CV)] | Methacycline [ppb (CV)] |
|-----------------|----------|---|------------------------|-----------------------------|-------------------------|---------------------------|-----------------------------|--------------------------|-------------------------|
| −70             | 0b       | 10| 35.7 (10.9)            | 38.7 (11.8)                | 35.1 (10.2)             | 32.8 (8.6)                | 30.2 (16.5)                | 29.2 (11.1)              |
|                 | 24       | 9 | 36.7 (8.3)             | 38.0 (7.5)                 | 37.4 (3.8)              | 34.3 (6.5)                | 31.8 (5.5)                 | 28.4 (6.5)               |
|                 | 48       | 15| 35.7 (13.6)            | 41.5 (11.2)                | 34.1 (14.5)             | 32.6 (11.8)               | 29.3 (15.4)                | 30.1 (6.7)               |
|                 | 72       | 12| 31.2 (5.9)             | 35.9 (12.7)                | 30.9 (6.7)              | 29.8 (5.0)                | 28.6 (9.0)                 | 28.0 (11.0)              |
| 25              | 24       | 5 | 36.1 (5.0)             | 37.6 (4.2)                 | 36.2 (1.8)              | 31.1 (1.9)                | 29.0 (3.5)                 | 27.5 (3.2)               |
|                 | 48       | 3 | 32.9 (6.8)             | 39.2 (7.6)                 | 34.0 (7.8)              | 26.8 (3.6)                | 25.4 (6.8)                 | 27.1 (6.5)               |

a CV, coefficient of variation.

b Zero-hour milks were fortified milks that had been removed from the ultrafreezer and analyzed concurrently with the incubated samples.

grammable absorbance detector (ABI, Ramsey, N.J.) set at 355 nm. The HPLC column was a PLRP-S, 5 μm, 100 Å, 150-× 4.6-mm column equipped with a guard column of the same packing material (No. 111–3500, Polymer Labs, Amherst, Mass.). The HPLC mobile phase consisted of a 0.01 M oxalic acid-acetoni-trile–methanol gradient (1–3).

Procedures. Fresh control raw milk samples were obtained and fortified as follows: Approximately 100 ml of milk was added to a 500-ml volumetric flask. Intermediate standard (25.0 ml) was added to the milk and mixed by swirling. Milk was added and mixed by inverting the flask for at least 1 min. Aliquots of the fortified milk were put into polypropylene culture tubes and stored at −70°C.

For the stability study, tubes of fortified and control milk were removed from the freezer and thawed. A control and a fortified milk sample were analyzed at this time. The rest of the tubes were placed in the refrigerator (4°C) or incubator (25°C). Milk samples were removed from the incubator and refrigerator after 24 h and analyzed immediately. This was repeated after 48 h for the 4°C and 25°C milk and after 72 h for the 4°C milk.

Milk samples were analyzed by AOAC International official metal chelate affinity–liquid chromatographic method (Method 995.04 (1–3)) for multiple tetracycline residues in milk. Briefly, defatted milk samples were mixed with sodium succinate buffer (pH 4) and centrifuged. The supernatants were eluted through metal chelate affinity columns (MCAC), and the tetracyclines were eluted with McIlvaine–EDTA–NaCl buffer. The MCAC column eluates were deproteinized by ultrafiltration through Centricon 30 centrifugal ultrafilters. They were analyzed by gradient HPLC.

Data analysis. Standard curves were prepared for each of the six tetracyclines from standard chromatograms. Sample concentrations were determined by linear regression, using the formula \( y = mx + b \), where \( x \) = peak area and \( y \) = concentration of extract injected in nanograms per milliliter. Correlation coefficients were routinely >0.99. Mean tetracycline levels found in the milk samples and coefficients of calculation were calculated using Quattro-Pro for Windows, version 6.0 (Novell Inc., Orem, Utah).

RESULTS AND DISCUSSION

Milk samples fortified at 50 ppb with six tetracyclines were analyzed by HPLC after being stored at −70, 4, or 25°C. Using the official AOAC International method (3), we were able to resolve all six antibiotics without any interferences from milk matrix peaks.

This stability study was repeated three times. The results (Table 1) show that no loss of tetracycline residues in the milk was observed after 48 h storage at 4°C or 24 h at 25°C. After 72 h at 4°C and 48 h at 25°C, the tetracycline levels are generally somewhat lower, suggesting that there may be slight losses.

Carson (1) reported that tetracyclines are not stable at room temperature in McIlvaine–EDTA–NaCl buffer. We found that the HPLC standards prepared in this buffer were stable for only a few hours. Conversely, the ultrafiltered sample extracts, also in McIlvaine–EDTA–NaCl buffer, were much more stable. For example, there was only a 10% loss of tetracyclines in sample extracts that were stored for a week at 4°C. The extra stability of the sample extracts may arise from Cu\(^{2+}\) ions, which resulted from the elution of the sample from the metal chelating agarose columns. Consequently, the sample extracts were stored at 4°C before injection, and fresh working standards in McIlvaine–EDTA–NaCl buffer were prepared at the beginning and the end of each HPLC run.

The results of this study indicate that shipping raw milk on ice blocks (at 4°C) probably will not result in degradation of tetracycline residues when analyses are conducted within 48 h.

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REFERENCES