Comparison of Microbial Receptor Assay and Liquid Chromatography for Determination of Penicillin G and Amoxicillin in Milk Powder

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A microbial receptor assay (Charm II Tablet Beta-Lactam Test) and liquid chromatography (LC) were compared for determination of penicillin G (PG) and amoxicillin (AMOX) in reconstituted milk powder. Nonfat dry milk and whole dry milk were reconstituted (10%, w/v) to concentrations of 0, 2.5, 5, 7.5, and 10 ppb PG; nonfat dry milk was reconstituted (10%, w/v) to 0, 7.5, 10, and 15 ppb AMOX. Reconstituted samples were analyzed blindly by each method. Concentrations determined by both methods demonstrated good agreement. A significant difference between methods (p ≤ 0.05) was observed only for 7.5 ppb PG in defatted dry milk. Significant differences were not observed between known concentrations and concentrations determined by the Charm II assay for PG or AMOX in defatted dry milk and PG in whole dry milk. Results by LC showed significant differences (p ≤ 0.05) between known and measured concentrations at 10 ppb PG in both milks and 0 ppb AMOX in defatted dry milk. These results suggest that both the microbial receptor assay and LC may be useful for determination of PG and AMOX near safe level and tolerance, respectively, in reconstituted milk powder.

Production of quality, residue-free products is a priority of the dairy industry. Testing of fluid milk for beta-lactam antibiotics prior to processing has been required by the U.S. Food and Drug Administration (FDA) since 1992 (1). Several screening tests have been evaluated and approved for such testing (1). Although this testing and evaluation was specifically applied to commingled milk, several studies compared various screening tests for detection of beta-lactams in milk from individual cows (2–7). A microbial receptor assay has been used to quantitate selected beta-lactams, oxytetracycline, and spectinomycin in milk (2, 3, 8); however, little work has been reported for other dairy products, including milk powder. An extraction procedure/enzyme-linked immunosorbent assay (ELISA) was developed to determine sulfamethazine in unreconstituted milk powder (9).

According to the manufacturers, some milk residue test kits will detect beta-lactams in reconstituted milk powder [10; and personal communications (1996): Gist-Brocades, Menomonee Falls, WI, and Idetek, Inc., Sunnyvale, CA]. In one study, penicillin-contaminated milk was spray-dried to form milk powder, and reconstituted samples were tested by Delvotest SP and LacTek B-L test kits to determine concentrations of remaining antibiotic (11). Charm Sciences has a procedure for quantitatively determining beta-lactams in fluid milk with the Charm II Tablet Beta-Lactam Test (Charm II Test; 12).

The purpose of this study was to compare results of the Charm II Test and liquid chromatography (LC) on reconstituted defatted and whole dry milk samples spiked with penicillin G (PG) near the FDA safe level for fluid milk (5 ppb) and reconstituted defatted dry milk samples spiked with amoxicillin (AMOX) near the FDA tolerance for fluid milk (10 ppb).

Experimental

Milk Powders

(a) Nonfat dry milk.—Obtained as Extra Grade Low Heat Nonfat Dry Milk from Mid-America Farms (Springfield, MO).

(b) Dried whole milk.—Obtained as Extra Grade Whole 28.5% Spray Processed Dry Milk from Dietrich’s Milk Products, Inc. (Middlebury Center, PA).

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Antibiotics

(a) LC.—AMOX and PG were used as received (Sigma Chemical Co., St. Louis, MO), with appropriate corrections for purity, if known. Stock solutions of antibiotic standards were prepared in LC grade water at 1 mg/mL or less and were stored frozen at –20°C until needed. Working dilutions of 100, 10, and 1 μg/mL were prepared. PG is reportedly stable for several days at refrigeration temperatures (13). It has been recommended that AMOX be used <6 h after preparation or thawing (14).

(b) Charm II Test.—AMOX and potassium PG were obtained from Sigma Chemical Co.

Reagents

(a) Charm II Test.—The Charm II Tablet Beta-Lactam Test Kit for PG, AMOX, ampicillin, cefotiofur, and cephrapine (Competitive Assay) was obtained from Charm Sciences, Inc. (Malden, MA). Scintillation fluid was Opti-fluor (Packard BioScience BV, Groningen, The Netherlands).

(b) LC.—Acetonitrile was LC grade (EM Omnisolv, Gibbstown, NJ), or equivalent. Tetraethyl ammonium chloride; 1-decanesulfonic acid, sodium salt (98%); and dodecyl sulfate, sodium salt (98%) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Apparatus

(a) Charm II Test.—Glassware included 600 mL and 1 L beakers, 15 mL plastic disposable conical graduated centrifuge tubes, and disposable borosilicate test tubes (13 and 16 mm). All glassware was cleaned with a detergent (MICRO; International Products, Trenton, NJ) in a 70°C water bath for 30 min. This was followed by rinsing in tap water, soaking in acid bath (ca 0.01N H₂SO₄), and rinsing in deionized water.

Equipment for the Charm II Test included a centrifuge (Baxter S/P Brand Multifuge Centrifuge, McGaw Park, IL) and a Vortex mixer (Maxi Mix II, Barnstead/Thermolyne Corp., Dubuque, IA). A Charm II 6600 Autoanalyzer and a block incubator (85°C) were obtained from Charm Sciences, Inc.

(b) LC.—Glassware included 25 and 50 mL graduated cylinders, 15 mL conical graduated centrifuge tubes (calibrated to 1 and 4 mL), 250 mL glass-stoppered sidearm flasks, and 125 mL conical flasks. All glassware was cleaned in a detergent (MICRO; International Products, or equivalent) at about 60°C for 30 min, followed by rinsing in deionized water, 0.01N HCl or H₂SO₄, and deionized water.

LC equipment-cleanup system included a pump (Model 9012, Varian Instrument Group, Sugarland, TX), an autosampler (WISP Model 712) with 2000 μL loop (Waters Chromatography Division, Milford, MA), a fraction collector (FOXY, ISCO, Inc., Lincoln, NE), a detector-data system, 990 diode array detector (Waters), and a column (Supelcosil LC-18, Supelco, Bellefonte, PA), 4.6 x 150 mm, 5 μm particle size.

LC analysis equipment included a pump (Varian Model 9012), an autosampler (Varian Model 9090) with a 200 μL loop, a detector (Waters Model 481 UV-VIS), data system.

Figure 1. Mean ± SD penicillin G (ng/mL = ppb) in reconstituted defatted dry milk determined by Charm II and LC versus known concentrations, as measured in blinded analysis (n = 5–6 per concentration).

Figure 2. Mean ± SD penicillin G (ng/mL = ppb) in reconstituted whole dry milk determined by Charm II and LC versus known concentrations, as measured in blinded analysis (n = 5–6 per concentration).
Additional equipment included a Vortex evaporator (Buchler Instruments, Fort Lee, NJ), a thermostatted hot plate with shallow tray, and plastic-coated lead rings to weight side-arm flasks during evaporation.

**Spiked Samples**

Reconstituted nonfat dry milk, diluted to 10% (w/v) with LC grade water, was spiked to 0, 2.5, 5, 7.5, and 10 ppb PG or 0, 5, 7.5, 10, 15, and 20 ppb AMOX. Whole dry milk, reconstituted nonfat dry milk was also spiked to 0, 5, 7.5, 10, 15, and 20 ppb AMOX. For all dry milk and drug concentration combinations, solutions were divided into 10 tubes of 8 mL each and frozen at −70°C. Twice a day for 5 days, a tube of each milk and drug concentration combination was quick-thawed and analyzed by the Charm II assay.

The mean value in counts per minute (cpm) of the 10 replicates of each concentration, as read directly from the Charm II analyzer (B), was divided by the mean reading (cpm) of the tubes of antibiotic-negative milk (B₀). This ratio, B/B₀, was plotted against the known concentrations of the spiked samples to form a standard curve (12).

The useable portion of the curve was that portion between the concentration determined to be at the limit of quantitation (LOQ; i.e., the lowest tested concentration whose B value was statistically different from the mean B for 0) and the concentration after which the curve was no longer linear. Any sample that produced a reading outside those values was either diluted with negative milk to bring it within the useable portion of the graph or was considered below the LOQ. To determine the original concentration of antibiotic in the unknown samples, concentrations read off the standard curve were multiplied by the dilution factors that were used to bring them within the useable portion of the curve (12).

For example, the standard curve for PG in reconstituted whole dry milk is no longer sufficiently linear at concentrations >6 ppb. The mean B for 6 ppb was 668 and the B₀ was 1207. A reconstituted whole dry milk sample of unknown concentration of PG and a B value (mean of 3 replicates) of 513 cpm would have a concentration >6 ppb because, the lower the B value, the higher the concentration. Diluting the sample 1:1 with negative milk brings the reading to 729. B/B₀ would be 729/1207, or 0.60. The corresponding concentration, read off the standard curve, is 5.5 ppb. Multiplying by 2 (the dilution factor) gives a final concentration of 11 ppb.

**Determination of Level of Quantitation**

For the purposes of this study, LOQ was defined as the mean cpm of the lowest concentration tested that was significantly different from the mean cpm for 0 ppb. Spiked samples, 0, 0.75, 1.5, and 2.0 ppb PG (n = 4-6 per concentration) and 0, 1.25, 2.5, and 5 ppb AMOX (n = 6 per concentration), were tested by the Charm II Test. The Wilcoxon 2-Sample Test was used to evaluate the potential difference in cpm 14C between each concentration and cpm 14C for 0 ppb for penicillin in re-

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**Figure 3. Mean ± SD amoxicillin (ng/mL = ppb) in reconstituted defatted dry milk determined by Charm II and LC versus known concentrations, as measured in blinded analysis (n = 5–6 per concentration).**

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(Varian Model 654), and columns (Supelcosil LC-18 and Supelcosil LC-18-DB), both 4.6 × 150 mm, 5 μm particle size.

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**Sample Preparation for Comparison of LC and Charm II Assays**

Aliquots (6 × 50 mL) of each concentration of PG and 0, 7.5, 10, and 15 ppb AMOX were randomly numbered, frozen
constituted defatted dry milk and whole dry milk and AMOX in defatted dry milk.

**LC Testing**

LC testing was performed according to the method of Moats and Harik-Khan (15).

**Charm II Assay**

The Charm II Tablet Beta-Lactam Test (Competitive Assay) was performed according to the manufacturer’s instructions (16). On the day of testing, samples were quick-thawed in warm water and centrifuged 5 min at 1200 g. The liquid between the fat layer and the precipitate was removed, pipetted into a clean test tube, and cooled on ice for 10–15 min before testing.

**Statistics for Comparing the Two Tests**

Concentrations of decoded samples were compared using a 2-sample, 2-sided t-test. Significant differences were considered present at \( p \leq 0.05 \). Because values for the Charm II Test results for all 0 concentrations of both drugs were not quantitatable (i.e., below LOQ), analysis was not performed for 0 ppb concentrations.

**Statistics for Comparing Results of Each Test to Known Concentrations**

Charm II and LC measurements were compared with the known concentrations by individual 2-sided t-tests. Differences were considered significant at \( p \leq 0.05 \). Because values for the Charm II Test results for all 0 concentrations of both drugs were not quantitatable (i.e., below LOQ), analysis was not performed for 0 ppb concentrations.

**Results and Discussion**

Based upon our results, use of the Charm II Tablet Test for Beta-Lactams allowed quantitation of beta-lactam residues in reconstituted dry milk powder at concentrations above a calculated LOQ. Shah et al. (17) define LOQ as “the lowest concentration on the standard curve which can be measured with acceptable accuracy, precision, and variability.” If the mean determined value is acceptably close to the true value, and if replicates are sufficiently close to each other, the value is considered quantitatable. We added the requirement that the value be significantly different from readings (in cpm) at 0 ppb. Based on the values we tested, LOQs were determined to be 1.5, 0.75, and 2.5 ppb for PG in reconstituted defatted dry milk, PG in reconstituted whole dry milk, and AMOX in reconstituted defatted dry milk, respectively.

Variability of cpm \(^{14}\)C for the samples was evaluated by daily testing of milk samples at each concentration for each antibiotic and milk powder. CVs for 0, 2.5, 5.0, 7.5, and 10 ppb PG in defatted dry milk were 8.1, 7.0, 6.1, 7.9, and 5.8%, respectively, and, for PG in whole milk were 4.8, 5.5, 4.1, 5.3, and 10.2%, respectively. CVs for 0, 5, 7.5, 10, 15, and 20 ppb AMOX in defatted dry milk were 7.1, 4.4, 2.0, 6.3, 7.6, and 5.7%, respectively. Except for the CV for 10 ppb PG in whole milk (10.2%), all CVs were below our acceptance level of 10%. Analysis of variance showed no day effect (\( p \leq 0.05 \)) on cpm and B/B\(_{0}\) (measured cpm/average cpm of blank milk samples) for each antibiotic and milk at each concentration.

Comparisons of the LC and Charm II testing of PG in defatted and whole dry milk and AMOX in defatted dry milk are shown in Figures 1–3. Only the result at 7.5 ppb for PG in defatted dry milk showed a significant difference (\( p \leq 0.05 \)) between tests (Figure 1). No significant (\( p \geq 0.05 \)) differences were observed between measured and known concentrations of drugs as determined by the Charm II assay (Figures 1–3). Results by LC showed significant differences at 10 ppb PG in both milks (Figures 1 and 2) and 0 ppb AMOX in defatted dry milk (Figure 3). Significant differences were not detected at other concentrations.

Results of this study suggest that the microbial receptor assay may be useful for determination of concentrations of PG in defatted and whole dry milk powder and AMOX in dry milk powder. Concentrations determined by microbial receptor assay and LC were in good agreement. Testing was performed on milk powder spiked to various concentrations of PG and AMOX. Spiked samples may not perfectly represent behavior of antibiotics in milk powder which is prepared by heating milk; however, there is no practical way to prepare milk powders with a specific known final concentration of antibiotic in milk powder. A limitation of the microbial receptor test is the lack of ability to specifically identify individual beta-lactam antibiotics. It would be necessary to determine the identity of the compound as penicillin or AMOX for the microbial receptor assay to determine concentrations. Quantitation at levels near the tolerance or safe level for fluid milk (5 ppb for penicillin and 10 ppb for AMOX) is possible. Samples containing higher concentrations can be analyzed if they are diluted appropriately to fall within the useable portion of the standard curve.

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**References**


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