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Brief communication

Stability and bioactivity of tetracycline hydrochloride water medication in a swine production unit

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Summary

Tetracycline hydrochloride-medicated water samples taken from commercial farms were found to darken to yellow or brown. Tetracycline hydrochloride from collected samples was quantified by high performance liquid chromatography and assessed for its biological activity. Samples were biologically active in the expected range, but safety could not be determined.

Keywords: swine, tetracycline hydrochloride, water medication, bioactivity, degradation

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Resumen - Estabilidad y bioactividad de la medicación en agua con hidrocloruro de tetraciclina en unidades de producción porcina

Las muestras de agua medicada con hidrocloruro de tetraciclina tomadas de granjas comerciales se oscurecieron a amarillo ó café. Mediante cromatografía líquida de alto desempeño se cuantificó el hidrocloruro de tetraciclina de las muestras recolectadas y se valoró su actividad biológica. Las muestras estaban biológicamente activas, dentro del rango esperado, pero no se pudo determinar su seguridad.

Résumé - Stabilité et bioactivité d'eau médicamentée avec de l'hydrochlorure de tétracycline dans des unités de production porcine

On nota dans des échantillons d'eau médicamentée avec de l'hydrochlorure de tétracycline prélevés dans des fermes commerciales une coloration jaune à brune. On quantifia par chromatographie liquide à haute performance l'hydrochlorure de tétracycline dans les échantillons prélevés et on détermina son activité biologique. Les échantillons étaient biologiquement actifs à l'intérieur de l'écart attendu, mais la sécurité n'a pu être déterminée.

Tetracycline hydrochloride (HCl) soluble powder is indicated for use in swine for the treatment of *Escherichia coli* diarrhea and susceptible respiratory pathogens. Some tetracycline HCl product labels suggest that the stock solution be mixed daily for use in swine facilities; however, mixing of fresh stock solutions is often performed only after the batch of medication has been completely used. Prepared tetracycline HCl water solutions may change color after standing. During two recent studies, we observed that the stock solution of tetracycline HCl-medicated water began to change color 24 hours after mixing.[1,2] Two reports state that tetracycline HCL is degraded by a combination of high humidity and temperatures > 37°C.[3,4] Tetracycline has also been shown to degrade after exposure to ultraviolet and fluorescent light.[3]

Mason et al[2] took water samples every 24 hours over the course of a week from 24 individual nipple drinkers from which pigs were provided with tetracycline HCl-medicated water at three different concentrations: the recommended label dose (250 ug per mL), twice the label dose (500 µg per mL), and one-half the label dose (125 µg per mL). The concentrations of tetracycline HCl in the samples were measured via high performance liquid chromatography (HPLC). Those results were compared to minimum inhibitory concentrations (MIC) measured via micro broth dilution assay to test the bioactivity of the tetracycline HCl contained in the water samples. This study was prompted by the notable color change of the medicated water samples 24 hours after mixing and the ensuing concern over the stability and bioactivity of the medication despite the manufacturer's claim for stability at 24 hours. Water samples analyzed in this study were collected just prior to a new batch of medication being prepared, which was approximately 24 hours after mixing of the original stock solution, but may have been slightly longer. For the purposes of the study, samples times are considered to be 24 hours after initial mixing. We proposed that tetracycline HCl in water would be adequately stable and biologically active, despite its color change, for 24 hours in the study period.

Materials and methods

The North Carolina State University (NCSU) animal care and use committee approved the protocol for this study, which was performed in a commercially designed research swine facility at NCSU in accordance with Institutional Animal Care and Use Committee procedures.

Twenty-four Yorkshire-Landrace barrow pigs approximately 8 weeks old were housed individually in pens that were each provided with a single water-nipple drinker. Tetracycline HCl was added to water sources at the manufacturer's

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recommended label dose, at half the label dose, and at twice that dose, with six pigs per treatment group. In addition, for six pigs, tetracycline HCl was not added to the drinking water (control group). All pigs were randomly assigned to treatment groups. Individual water containers were filled with water from the farm. Medicated water was prepared by the addition of preweighed tetracycline HCl soluble powder (AmTech, Des Moines, Iowa) to each pig's water source. No other sources of tetracycline HCl were available to pigs prior to or during the study. For the purposes of this study, on two occasions, samples were collected from each drinker 24 hours after initial mixing of the soluble powder into the water source. Water samples were analyzed by chromatography and microbial analysis consistent with Clinical Laboratory Standards Institute (CLSI) specifications.[5,6]

High performance liquid chromatography analysis of water samples was performed on a Waters Alliance HPLC 2695 with autosampler (Waters Corporation, Milford, Massachusetts) coupled to a photodiode array. Peak integration at 354.4-nm wavelength by Empower software (Waters Corporation) was used for tetracycline HCl quantification for each medicated water sample. A Waters Atlantis 4.6 × 150-mm C18 column was held at 35°C during a 4-minute sample analysis cycle using 28% acetonitrile, 72% 0.05 M oxalic acid buffer mobile phase. All samples were kept in the dark at 4°C and were initially processed within 14 days of collection. Two hundred µL of water was added to an equal volume of releasing agent (78% water, 20% acetonitrile, 2% o-phosphoric acid) in a YM 10,000 Ultracel kit (Millipore Corporation, Milford, Massachusetts), placed in an Eppendorf tube, and centrifuged at 7840*g* for 30 minutes at 22°C. The lower limit of quantification (the smallest concentration that can be reproducibly quantified) for tetracycline HCl-medicated water samples was 0.1 µg per mL, and the limit of detection was consistently ≤ 0.05 µg per mL. A standard curve was made for both the commercial tetracycline HCl standard (≥ 97% pure; Sigma-Aldrich, Milford, Massachusetts) and the water medication product. The standard included values from 80 to 800 µg per mL (including 125, 250, and 500 µg per mL) and had an R^2 of 0.99, and intra-day variability was < 15%. The sample processing method was based on Dorr et al,[1] and the HPLC method was a modified version of Cheng et al[7] and Santosa et al.[8] This technique was identical to the procedure used by Mason et al.[2] Samples were processed initially within 14 days of collection and then processed again to verify that concentrations on HPLC were unchanged when the bioactivity assay was performed. A set of tetracycline HCL stock-concentration samples stored at 4°C were reanalyzed after 6 months to show chromatographic changes over time.

Mueller Hinton (MH) broth (Sigma-Aldrich, St Louis, Missouri) was prepared and $CaCl_2$ and $MgCl_2$ stock solutions were added to the MH broth to meet CLSI specifications.[5,9] Two stock solutions of 250 µg per mL tetracycline HCl water medication and tetracycline HCl standard (Sigma-Aldrich, Milford, Massachusetts) were prepared and filter-sterilized (0.45 µm) under vacuum. These two standards were analyzed via HPLC to confirm concentrations.

Escherichia coli ATCC 25922 was cultured at 37°C on Luria-Bertani agar plates. Clinical Laboratory Standards Institute procedures[5,6,9] were used for media preparation and for MIC testing. Briefly, a suspension of *E coli* ATCC 25922 was prepared in phosphate buffered saline at a concentration equivalent to a 0.5 McFarland turbidity standard. This suspension was diluted 1:100 in MH broth to give approximately 10^6 colony forming units (CFU) per mL for use in bacterial susceptibility testing. The bacterial concentrations of the test inocula were confirmed by enumerating CFU in duplicate dilution series. Sterile Costar round-bottom, 96-well plates with lids (Thermo Fischer Scientific, Milford, Massachusetts) were used for all experiments. Serial dilutions of both the medicated water samples and the commercially available tetracycline HCl standard were made in the plates following CLSI methods. Plates were incubated for 20 to 24 hours at 37°C. The MIC was defined as the lowest dilution that demonstrated no visible growth. Each daily sample and all controls were tested in duplicate. Any daily sample test for which the two MIC results varied by more than one dilution was repeated to verify the MIC. The MIC ranges are reported for the combined duplications of one pen's medicated water sample.

Sample results collected on separate days from the same drinker were averaged and a coefficient of variation across days is reported. An average percentage of the HPLC-measured tetracycline HCl concentrations to the expected concentration (based on the dose) of each drinker is included in the calculation of the medicated-water group average. A standard deviation is included with this average. Finally, all groups were averaged to determine a grand mean.

Results

Results of the HPLC analysis of the standard tetracycline HCl water medication solutions were within the expected range of area under the curve (AUC) integration. These control concentrations exhibited the expected inhibition for the low end of the MIC range of *E coli* ATCC 25922 (MIC of 0.5 to 1.0 µg per mL).[5] High performance liquid chromatography analysis results (Table 1) show that in the three medicated-water groups, tetracycline HCl concentrations were within the assay variability of their true values. However, there was some degradation of tetracycline-medicated water over a 24-hour period. Table 1 shows the average concentration (by HPLC) of each medicated water sample on two different days (24 hours after mixing on each day) as the percentage of the concentration that should be present (ie, compared to the standard curve), with a corresponding coefficient of variation across days. Means of each medicated water group were 89%, 86%, and 77% for the 125 µg per mL, 250 µg per mL, and 500 µ per mL groups, respectively. The mean for all medicated water samples (not including the control samples) was 84% of the expected concentration. Of all of the medicated water treatment groups, tetracycline HCl at twice the manufacturer's label concentration (500 µg per mL) had a numerically lower relative concentration than the two other medicated groups after 24 hours.

Figure 1 shows comparative chromatographs of tetracycline HCl degradation over time. Chromatograph A shows the commercial tetracycline HCl standard, while chromatograph B shows a representative medicated water sample at the same concentration (125 µg per ml). Sample A is a standard curve chromatograph and therefore shows the purity of the standard. Chromatograph tracing C demonstrates the change in a commercial tetracycline HCl standard at 125 µg per ml after refrigeration at 4°C for 6 months. This

Table 1: Comparison of results of the HPLC assay for tetracycline hydrochloride (HCl) water concentration in samples from individual drinkers in a swine nursery, and the antimicrobial activity of the same water samples*

HPLC assay of tetracycline HCl concentration		MIC assay
Average (µg/mL) (% of expected value)	**CV (%)**	**Range (µg/mL)†**
0 µg/mL		
0.00 (100)	173.21	0
125 µg/mL		
100.01 (80)	33.39	125-250
104.49 (84)	10.31	125
114.46 (92)	7.58	125-250
121.96 (98)	19.85	125-250
107.91 (86)	ND‡	125-250
118.87 (95)	18.50	125-250
250 µg/mL		
220.48 (88)	3.33	125-500
205.54 (82)	6.43	250-500
238.66 (95)	4.73	250-500
211.84 (85)	9.24	125-500
182.56 (73)	20.03	250-500
233.14 (93)	6.13	250-500
500 µg/mL		
398.66 (80)	6.90	250-500
337.40 (67)	43.43	250-500
376.06 (75)	14.58	250-500
372.30 (74)	28.24	250-500
400.41 (80)	13.14	250-500
428.58 (86)	2.75	250-500

* Stock solutions of tetracycline HCl soluble powder were prepared for 24 individual nursery pens (one pig per pen), including concentrations of 0 µg/mL, 125 µg/mL, 250 µg/mL (manufacturer's label dose), and 500 µg/mL tetracycline HCl (six pigs per treatment). On two occasions, samples were collected from each drinker 24 hours after the stock solution had been mixed. Samples were assayed in duplicate by HPLC and the results were compared to the expected water concentrations (determined using standard curve samples); CVs were calculated for these samples. In addition, microdilution assays were performed using *Escherichia coli* ATCC 25922. Assays of positive and negative control samples (not included in the table) were performed for each sample plate and were of acceptable consistency and accuracy to calculate HPLC integration and MIC ranges.

† Water concentration ranges were back-calculated from the MIC found for the bacteria using standard curve control assays.

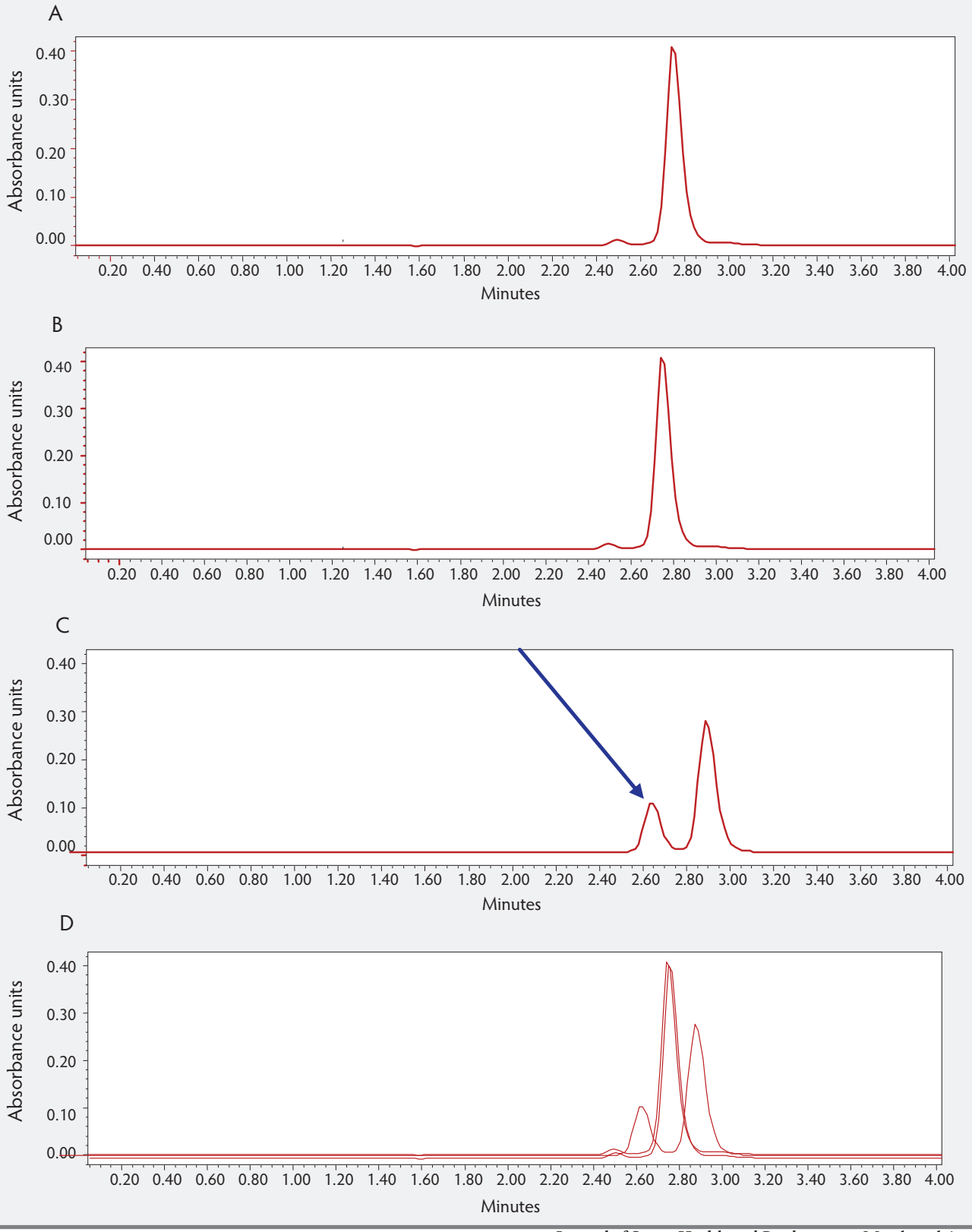
‡ Only one sample MIC analysis was acceptable and therefore CV was not calculated.

HPLC = high performance liquid chromatography; CV = coefficient of variation; MIC = minimum inhibitory concentration; ND = not done.

chromatograph exaggerates the smaller peak that can be seen in chromatograph B, and possibly in chromatograph A. The first visible peak in chromatograph A changes gradually from almost non-existent to a slight but noticeable peak on chromatograph B; however, on chromatograph C, this initial peak is very large (indicated by the arrow). These differences show that the second and tallest peak represents tetracycline HCl, while the smaller peak represents an epimerization product.[10,11] The small peak enlarges over time and becomes very large in the oldest tetracycline HCl standard, as seen in chromatograph C.

The MIC assay results were used to determine the bioactivity of the three medicated water concentrations and were within one dilution of the calculated HPLC value. Both control stock solutions of the water medication product and the commercial tetracycline HCl standard inhibited bacterial growth at between 0.5 and 1 µg per mL (consistent with CLSI standards[9]), while bacteria grew in all of the unmedicated (control) water samples. Samples from the half-label doses (125 µg per mL) and the label doses (250 µg per mL) of tetracycline HCl-medicated water were consistently at or above the expected MIC concentrations, according to CLSI standards.[5,6,9] However, bioactivity of the 500 µg per mL tetracycline HCl-medicated water was less than that of the other two treatment concentrations.

Figure 1: Chromatographs (absorbance units versus time) for changes in tetracycline for samples collected from drinkers in individual pens in a swine nursery. Tetracycline hydrochloride (HCl) was added to the water source for each pen at the manufacturer's recommended label dose (250 μg/mL), at half that dose (125 μg/mL), and at twice that dose (500 μg/mL), with six pigs per treatment group. Tetracycline HCl water medication was mixed at concentrations of 80, 125, 250, 500, and 800 μg/mL for a standard curve for high performance liquid chromatography (HPLC) analysis to qualify analyzed water samples. A: HPLC commercial standard containing 125 μg/mL tetracycline HCl; B: Water sample from farm containing 125 μg/mL tetracycline HCl water medication; C: Water sample in A after being stored for 6 months at 4°C. As the tetracycline ages, the first visible peak (arrow) begins to enlarge in relation to the major (tetracycline) peak. This peak appears to correspond to the increase in 4-epitetracycline formation as described in the literature.[10,11] D: Overlay of peaks to show relative heights and placements.



These MIC results were consistent with the results of the HPLC analyses performed shortly after collection of water samples, ie, after 24 hours, the amount of tetracycline in each medicated sample was approximately 77% of the original concentration.

Discussion

The HPLC analysis and MIC assays show that overall, tetracycline HCl, despite its change in color, was still biologically active against *E coli* ATCC 25922 at the susceptible concentrations reported by CLSI. Bacteria were inhibited at tetracycline concentrations consistent with HPLC analysis despite freezing and thawing of the water samples. Higher variability in the MIC dilution assay may have been associated with the delay in processing of samples, which included freezing and thawing. The control MIC assays for tetracycline HCl for both the freshly prepared water medication standard and chemical standard solutions were consistent across plates and days. The HPLC results for tetracycline HCl standard chemical and soluble powder solutions were consistent with the MIC values for *E coli* ATCC 25922, and thus these solutions are likely to inhibit the growth of susceptible bacteria. Our results show that, under the study conditions, there is no observable difference in microbial inhibition for approximately the first 24 hours, ie, the stability of the drug is adequate for at least the first 24 hours after mixing in water. However, there appeared to be a slightly greater drop in stability and bioactivity of the water samples medicated with tetracycline HCl at 500 µg per mL than in the samples containing either the label dose (250 µg per mL) or half-label dose (125µg per mL). A drop in stability at 500 µg per mL may be consistent with the decreased buffering capacity of the water at pH 6.5 (the pH of the water on farm) when large amounts of drug are present.[3,4] It has been reported that tetracycline HCl is more stable in acidic conditions (approximately pH 4) than at normal to alkaline pH.[4] In acidic conditions, the greater number of hydrogen atoms may prevent epimerization of the drug, thus maintaining antimicrobial ability.[3]

In relation to this stability concern, it was noted on HPLC analysis that the chromatographs contained a small peak that was larger when the samples were analyzed 2 weeks after they had been thawed. This peak placement is consistent with peaks associated with tetracycline epimerization products called epitetracyclines,[10,11] and corresponds to the epitetracycline peak shown in detail on HPLC chromatographs by Naidong et al[10] and Stephens et al.[11] Epimerization products are not necessarily responsible for the color change of the medicated water. It is possible that a carrier or other molecule present in the tetracycline HCl formulation is responsible for the color change.

It is well known that expired or aged tetracycline products may cause nephrotoxicity: specifically, a reversible Fanconi's syndrome of proximal tubular necrosis with proteinuria, reportedly caused by anhydro-4-epitetracycline.[12,13] The enlarged initial chromatographic peak is not consistent with the chromatographic elution of peaks reported for epimerization products that result in renal damage. However, absolute identification and toxicological evaluation of the potential epimerization products are beyond the scope of this study, and further evaluation of these epimerization products should be performed in the future.

Implications

- Under the conditions of this study, tetracycline HCl water medication is effective in inhibiting the growth of susceptible bacteria as assessed by a minimum inhibitory concentration assay, even after a mild to moderate color change.
- The stability of tetracycline HCl when mixed according to label directions is within the margin of error for the liquid chromatography assay during the first 24 hours.

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