A Radioimmunoassay Method to Screen for Antibiotics in Liquid Waste at Confined Livestock Operations, with Confirmation by Liquid Chromatography/Mass Spectrometry

By M.T. Meyer, J.E. Bumgarner, E.M. Thurman, Kenneth A. Hostetler, and J.V. Daughtridge

ABSTRACT

Approximately one-half of the 50 million pounds of antibiotics produced in the United States are used in agriculture. Because of the intensive use of antibiotics in the management of confined livestock operations, the potential exists for the transport of these compounds and their metabolites into the Nations's water resources. A commercially available radioimmunoassay method, developed as a screen for tetracycline antibiotics in serum, urine, milk, and tissue, was adapted to analyze water samples at a detection level of approximately 1.0 part per billion. This method has a semiquantitative analytical range of 1 to 20 parts per billion. Six liquid waste samples from hog lagoons, 14 surface water samples, and 3 ground-water samples were used to test the radioimmunoassay method as a screen for tetracycline antibiotics. The radioimmunoassay tests yielded positive results for tetracycline antibiotics in samples from all six hog lagoon samples and one surface-water sample. Dilutions of 10 to 100 fold of the hog lagoon samples indicated that tetracycline antibiotic concentrations ranged from approximately 1 to several hundred parts per billion in liquid hog waste. A new liquid chromatography/mass spectrometry method was used to confirm the radioimmunoassay and also to identify the tetracycline antibiotics to which the radioimmunoassay test was responding. The new liquid chromatography/mass spectrometry method with online solid-phase extraction and a detection level of 0.5 microgram per liter confirmed the presence of chlortetracycline in one hog lagoon sample and in one surface-water sample.

INTRODUCTION

A wide variety of pharmaceutical compounds has been detected at low concentrations in some of the lakes, rivers, and ground water in Europe (Holm and others, 1995; Buser and others, 1998; Raloff, 1998). These findings have raised concern, not only that pharmaceutical compounds are present in our water systems but that they may have detrimental effects on ecological and human health. In a recent literature review of studies on pharmaceutical compounds in the environment, virtually no studies were found on the potential effects of the presence of these compounds on the environment (Halling-Sorenson and others, 1998).

Natural and synthetic antimicrobials are one class of prescribed pharmaceutical compounds that are of human health concern because of increased bacterial resistance. More than 50 million pounds of antibiotics are produced in the United States per year, and about one-half of these are used for agriculture (Levy, 1998). Approximately 40 percent of the antibiotics that are produced are used for livestock (for example, swine, poultry, and cattle) and the majority of these antibiotics are given in subtheraputic doses as feed additives to enhance growth (Levy, 1998). Antibiotics are also used in aquaculture and sprayed on fruit trees to inhibit fungal growth. The interest in pharmaceutical compounds in the environment is new, and for the most part, methods have not been developed to concentrate and analyze these compounds at levels at which they probably occur in the environment (Raloff, 1998).

Immunoassay is a screening technique developed for use in the clinical health sciences that has been readily adopted for use by

environmental scientists over the last decade primarily for pesticides in water and sediment. However, immunoassays for pharmaceutical compounds currently are used in biological media where the concentrations are relatively high. To assess the use of these tests on environmental samples where the concentrations are usually low, the techniques must be modified to lower their limits of detection. In the same way, quantitative extraction and identification techniques, such as solid-phase extraction (SPE), liquid chromatography (HPLC), and liquid chromatography/mass spectrometry (LC/MS), for pharmaceutical compounds were developed for biological samples. Because many of these compounds are at much lower concentrations in the environment and are often polar, the challenge for environmental chemists is to modify or develop new extraction and analysis methods for the identification of pharmaceutical compounds in water and sediment.

The tetracyclines are a widely used class of antibiotics in the United States. Currently chlortetracycline, and oxytetracycline are 2 of only 10 antibiotic compounds licensed for use as growth promoters for livestock in the United States (Swick, 1996). It has been shown that tetracycline-resistant bacteria in swine outflow can pass this resistance on to bacteria commonly found in soil (Haack and others, in press). Because little is known about the occurrence, fate, and transport of antibiotics in the environment, it is important to begin assessing where and at what levels they may occur in our water resources. This paper presents the results for a modified radioimmunoassay (RIA) procedure for screening tetracycline antibiotics in water, evaluates the method as a screening tool for source areas and surface and ground water, and compares the results of the RIA to a newly developed LC/MS method with online SPE.

SAMPLING AND ANALYTICAL METHODS

Sampling and analytical methods were tested and documented during this investigation. The following sections describe the sampling and analytical procedures developed for the RIA and LC/MS methods.

Sampling

Water samples were collected as part of investigations being conducted for the U. S. Environmental Protection Agency (USEPA) Neuse River Research project and the U.S. Geological Survey (USGS) Toxic Substances Hydrology, Emerging Contaminants Program. Two to four liters (L) of water or liquid hog waste were collected at each site packed in ice, and shipped in coolers to the USGS North Carolina District Research Laboratory (NCDRL) in Raleigh. The samples were filtered either through a 0.2-micron glass fiber filter in the field or in the NCDRL. All of the samples are stored at 3 degrees Celsius (^{0}C) in refrigerators at the NCDRL and the USEPA National Exposure Research Laboratory (NERL) Research Triangle Park, N.C. The hog-waste samples were centrifuged at 3,000 revolutions per minute (RPM) for 40 minutes in a swing bucket centrifuge and then prefiltered through a paper filter under vacuum before being filtered through a glass-fiber filter.

Radioimmunoassay

Radioimmunoassay analyses were conducted by USGS and USEPA researchers at NERL. Charm II[1] RIA tests (Charm Sciences Inc.; Malden, Mass.) for tetracycline antibiotics were used to analyze the samples. This tetracycline RIA test recently was approved by the Food and Drug Administration for use in determining safe levels of tetracycline antibiotics in milk (Smucker, 1998). The procedure used for the RIA analyses was modified from the company-supplied instructions for urine analysis in the following manner: (1) the powdered MSU extraction buffer supplied by the manufacturer with the tests was diluted into 100 milliliters (mL) of ultrapure water (10X buffer) instead of 1 L of water (normal-strength buffer), (2) instead of adding 13.3 microliters (µL) of sample to 2 mL of MSU normal-strength buffer, 0.5 mL of the 10X buffer was added to 4.5 mL of sample, and (3) the

[1] The use of firm, trade, or brand names is for identification only and does not imply endorsement by the U.S. Geological Survey.

chlortetracycline standard pellet supplied by the manufacturer was diluted in 200 mL of ultrapure water instead of 10 mL of water to make a 200-parts per billion (ppb) standard solution. Serial dilutions then were made to produce 100-, 50-, 20-, 10-, 5-, 1-, and 0.5-ppb solution standards.

Chlortetracycline has less affinity for the RIA test antibodies than the other commonly used tetracycline antibiotics, oxytetracycline and tetracycline (Smucker, 1998). Thus, the level of detection established with the chlortetracycline standard ensures that the other tetracyclines also will respond to the RIA at that level or less.

The procedure that was used is as follows: 4.5 mL of sample, standard, or ultrapure water and 0.5 mL of 10X buffer was added to a borosilicate test tube. A binder tablet, containing the antibody bound to a microbial cell was then added to the sample and vortexed for 10 seconds (s). Next, the tablet containing the tritium (^{3}H)-labeled chlortetracycline was added to the sample and vortexed for 15 s. The sample was incubated at 35 ^{0}C for 5 minutes (min) and centrifuged at 3,300 RPM for 5 min. Following this procedure, the liquid was decanted and 0.3 mL of water was added to the test tube and gently vortexed for 2 s to break up the pellet at the bottom of the test tube. Next, 3 mL of scintillation fluid was added to the test tube and vortexed. The sample then was allowed to sit undisturbed for 1 min before being analyzed in the Charm 6600[1] scintillation counter. The ^{3}H activity was analyzed for 1 min and the average count per minute (CPM) was recorded.

Samples were run in sets of six, which included one negative control, one standard, and four samples. The average negative control was calculated after all the samples were run. The negative control point (NCP), the cutoff point for separating a positive and negative response, was set at three standard deviations below the average CPM. A standard linear regression curve was developed for the 1 through 20-ppb standards by using the natural log (ln) of the CPM and the ln of the concentration of the standards. Samples that tested at concentrations of 20 ppb or more were diluted with ultrapure water at factors of 10, 20, and 100 and reanalyzed until their CPM was in the working range of the standard curve.

Liquid Chromatography/Mass Spectrometry

The LC/MS analyses were performed in the USGS Organic Geochemistry Research Laboratory (Lawrence, KS) by using a Hewlett Packard[1] 1100 series LC and a diode array detector (DAD) interfaced with an electrospray MS and on-line SPE. A 250 x 3 millimeter (mm) LC column packed with 5-micrometer (µm) diameter C_{18} was used to separate the tetracycline compounds. The extraction conditions were as follows: 50 mL of sample was automatically pumped through a C_{18} SPE disk and eluted with the mobile phase solvents described next. The LC mobile phase was 80 percent channel A containing 0.1 percent trifluoroacetic acid (TFA) in a 2:7:91 mixture of methanol/acetonitrile/water (MeOH/ACN/H_2O) and 20-percent channel B containing 100 percent MeOH. This mixture was held 1 min and then ramped to 25 percent channel A and 75-percent channel B over 15 min at a flow rate of 0.4 mL/min. The DAD monitoring wave length was 355 nanometers (nm). The electrospray MS parameters that were used were positive ion mode, a fragmentor ion voltage of 70 volts, and selected ion monitoring (SIM) for the following ions: 444, 445, 446, 461, 462, 479, 481, and 501. The purity of the quantitative standards of chlortetracycline, oxytetracycline, and tetracycline was 79, 97, and 98 percent respectively. The standard solutions of chlortetracycline concentrations were corrected for purity.

RESULTS AND DISCUSSION

Results of the modified RIA procedure for tetracycline antibiotics are presented in the following sections. Also presented is a comparative analysis of a subset of samples analyzed by RIA and a new LC/MS method.

Radioimmunoassay Methodology

In order to analyze water samples with a lower level of detection than the procedures offered by the manufacturer, three parameters were considered -- the size of the test tube required for the analyzer, the need to increase the amount of sample to be analyzed, and the amount

and strength of the buffer to add to the sample to maximize the CPM of the negative control samples. It was found that some sample spilled during vortexing if more than 5.5 mL of sample and buffer combined was added to the 10-mL test tube. Based on the detection levels for the urine procedure, it was determined that 4 to 4.5 mL of sample would be required to achieve a detection level of 1 to 10 ppb for water samples. From this information it was deduced that 0.5 mL of buffer and 4.5 mL of sample was the maximum amount that could be added to the sample test tubes without losing any sample while vortexing.

Based on these requirements, four sets of negative control samples with different buffer strengths were analyzed in duplicate. For this experiment 4.5 mL of ultrapure water was added to eight test tubes. Next, each set of two test tubes received 0.5 mL of one of the following: water, normal-strength, 10x, or 100x buffer. The negative controls with the 10x buffer had the highest CPM.

The modified RIA procedure for the tetracycline antibiotics resulted in an average negative control CPM of 1,934 ± 106.5 (one standard deviation (sd); n = 26) and an established NCP of 1,614 CPM; three standard deviations below the average CPM of the negative controls. The average CPM for the 1 ppb standards was 1,337 ± 90.6 CPM (1 sd; n = 3) and was always more than 180 CPM less than the NCP. The CPM for the 0.5-ppb standards were less than the NCP in 50 percent of the samples. These data indicate that the RIA procedure can be reliably used to detect samples with 1 ppb of chlortetracycline, but the procedure will give a false negative 50 percent of the time in samples containing 0.5-ppb chlortetracycline.

The analysis of standards showed that the CPM of the 1-, 5-, and 10-ppb standards varied but did not overlap and that the CPM values of the 10- and 20-ppb standards were separated in approximately 75 percent of the analyses. The CPM range from approximately 1,400 to 410 for the 1 through 20-ppb standards, respectively. A log-log linear regression of the CPM and concentration of the 1- through 20-ppb standards had a correlation coefficient (r) of 0.96 ($n = 16$; $p < 0.1$). The CPM data from the analysis of the 50-, 100-, and 200-ppb standards were less than that of the 20 ppb standards; however, the CPM of these standards overlapped with each other. The CPM for these standards ranged from 390 to 290. These data indicate that the samples contained more standard than the ^{3}H-labeled antibodies could effectively compete with to give scintillation readings reliable enough to estimate concentrations greater than 20 ppb.

Radioimmunoassay Results

The concentrations determined from the tetracycline RIA for samples with CPM less than the NCP are shown in figure 1. The data from the hog waste samples indicate that the concentrations ranged from approximately 1 to nearly 800 ppb (fig. 1). These data also indicate that the tetracycline antibiotics are commonly used in confined livestock operations for swine and that the tetracycline compounds are able to withstand very active microbial environments.

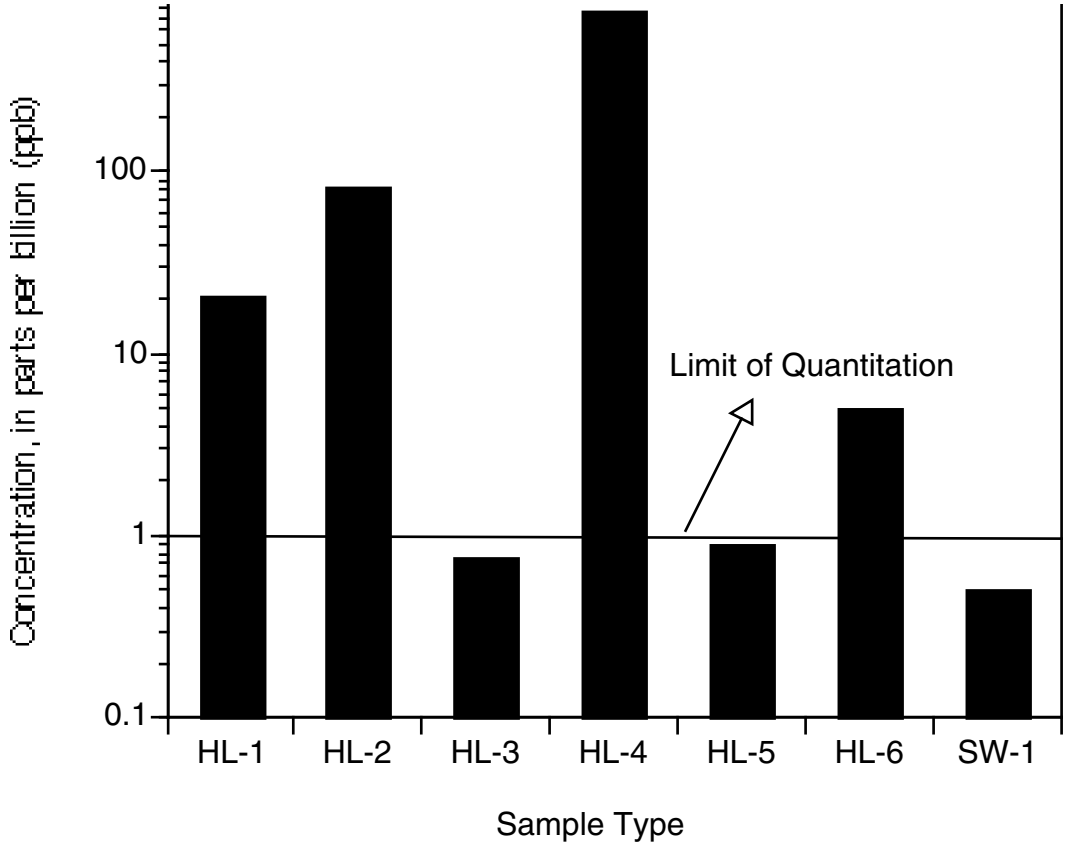

Figure 1 : Graph showing tetracycline radioimmunoassay concentrations for selected samples from hog lagoons (HL) and surface water (SW) with positive responses.

Of the 17 surface- and ground-water samples analyzed, only one sample had a CPM less than the NCP. The tetracycline concentration of this sample was less than 1 ppb. These data indicate that although tetracycline antibiotics commonly are used in both swine and poultry, they generally are not transported into surface and ground water at concentrations greater than 1 ppb. In this sense the modified RIA method is not sensitive enough to detect antibiotics at the levels in which they may occur in streams or in ground water. However, these data also suggest that this class of antibiotics does occur in high concentrations at sources where transport can occur.

The presence of antibiotics in hog lagoons is a potentially important finding because of increased antibiotic resistance in bacteria. Currently it is not known whether bacteria that are present in the hog lagoons are developing resistance to the antibiotics introduced into these microbially active environments. Also it is uncertain to what extent resistant bacteria or their plasmids may be transported from these areas into surface and ground water where resistance may be transferred to other bacteria. To confirm the RIA results a new LC/MS was used to analyze a subset of samples.

Comparison of Radioimmunoassay and Liquid Chromatography/Mass Spectrometry

Table 1 shows 4 samples analyzed by LC/MS for conformational analysis, identification of the tetracycline compounds detected by the RIA tests, and for comparison of the RIA and LC/MS. The LC/MS analyses show that chlortetracycline was present in the hog lagoon and one of the surface-water samples in which a positive response was obtained from the RIA and that tetracycline compounds were not detected by RIA in the samples where it was not detected by LC/MS (table 1). These data indicate that the modified RIA procedure was an effective screen for the presence of tetracycline antibiotics in water and liquid waste samples.

Table 1. Concentrations of selected samples analyzed by radioimmunoassay (RIA) and liquid chromatography/mass spectrometry (LC/MS) as

chlortetracycline in (ppb, parts per billion; µg/L, micrograms per liter; HL, hog lagoon; SW, surface water; and GW, ground water sample; nd, not detected).

Sample Name	RIA (ppb)	LC/MS µg/L
HL_5	5	5.0
SW_1	(+)[1]	0.5
SW_2	nd	nd
GW_1	nd	nd

[1]CPM greater than the NCP and less than 1.0 part per billion (ppb)

These initial RIA and LC/MS comparison data indicate that there is good agreement between the two methods; however, a larger data set needs to be established before this is confirmed. For example, research has shown that tetracycline compounds form epimers as they pass through an animal and that this results in isomers being excreted that are different from the predominant isomer of the tetracycline administered to the animal (Blanchflower and others, 1997; Kennedy and others, 1998).

In the LC/MS analyses, the mass spectra obtained from the hog lagoon sample was the same as for the chlortetracycline standard, but the retention time was different, due to the difference in the isomeric composition of the chlortetracycline. Currently the affinity of these excreted chlortetracycline isomers to the RIA test antibodies is not known. It may be that these excreted isomers do not bind with the same affinity to the antibodies in the RIA test as the predominant isomer of which the chlortetracycline standard is mostly composed. This is an area that warrants further investigation.

CONCLUSIONS

A modified procedure was developed to analyze water samples at a detection level of approximately 1 ppb for a commercially available RIA used for testing the tetracycline class of antibiotics in biological media. The modified RIA test detected tetracycline compounds at concentrations ranging from 1 to several hundred ppb in all hog lagoon samples that were tested. A positive response of less than 1 ppb was elicited from the modified RIA tests in only 1 of 17 surface- and ground-water samples. The level of nondetection among the water samples indicates that antibiotics, if present, in surface and ground water are generally at concentrations less than 1 ppb.

Conformational analysis of a subset of the samples by LC/MS supported the RIA analyses and proved that the RIA was responding to chlortetracycline. To more fully determine the extent to which the RIA and LC/MS methods agree a larger set of samples needs to be analyzed.

The LC/MS retention time data indicate that the chlortetracycline isomer in the samples is different from the predominant isomer of the standard. This study indicates that the RIA method presented is a reliable screen for tetracycline compounds in liquid waste from hog lagoons and in water samples at concentrations as low as 1 ppb. The data from the hog lagoons indicate that antibiotics may be present in high levels in areas where they may be transported into surface and ground water The data also suggest that a more sensitive method should be developed for analyzing antibiotic compounds in water that is located away from the contributing contaminant sources.

REFERENCES

Blanchflower, W.J, McCracken, M.P., Haggan, S.H., and Kennedy, D.G, 1997, Confirmatory assay for the determination of tetracycline, oxytetracycline, chlortetracycline and its isomers in muscle and kidney using liquid chromatography-mass spectrometry: Journal of Chromatography B, v. 692, p. 351-360.

Buser, Hans-Rudolf, Muller, M.D., and Theobald, N., 1998, Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea: Environmental Science, and Technology, v. 32, no. 1, p. 188-192.

Haack, B.J., Andrews, R.E, Jr., and Loynachan, T.E., in press, Isolation of Enterococcus faecalis exhibiting conjugal tetracycline resistance from swine lot outflow, *in* Biotechnology risk assessment: USEPA/USDA/Environment Canada, College Park, ML. University of Maryland Biotechnology Institute.

Halling-Sorenson, B., Nielson, S.N., Lanzky, P.F., Ingerslev, F., Lutzheft, H.C.H., and

Jorgensen, S.E., 1998, Occurrence, fate and effects of pharmaceutical substances in the environment-A review: Chemosphere, v. 36, no. 2, p. 357-393.
Holm, J.V., Kirsten, Rugge, Bjerg, P.L., and Christensen, T.H., 1995, Occurrence and distribution of pharmaceutical compounds in the groundwater downgradient of a landfill: Environmental Science and Technology, v. 29, no. 5, p. 1415-1420.
Kennedy, D.G., McCracken, M.P., Carey, M.P., Blanchflower, W.J., and Hewitt, S.A., 1998, Iso- and epi-iso-chlortetracycline are the principal metabolites in the hen's egg: Journal of Chromatography A, v. 812, p. 327-337.
Levy, S.B., 1998, The challenge of antibiotic resistance: Scientific American, v. 278, p. 46-53.
Raloff, Janet, 1998, Drugged water: Does it matter that pharmaceuticals are turning up in our water supplies?: Science News, v. 153, p. 187-189.
Smucker, J.E., 1998, Chief, Milk and Safety Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration: accessed February 8, 1999, at URL http://vm.cfsan.fda.gov/~ear/IMS-A36.html
Swick, R.A., 1996, Role of growth promotants in poultry and swine feed: American Soybean Association, Technical Bulletin AN04-1996, p. 10.

AUTHOR INFORMATION

M.T. Meyer, Research Geochemist, U.S. Geological Survey, Raleigh, North Carolina (mmeyer@usgs.gov)

J.E. Bumgarner, Research Biologist, and J.V. Daughtridge, Research Technician, U.S. Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

E.M. Thurman, Research Geochemist, and Kenneth A. Hostetler, Research Chemist, U.S. Geological Survey, Lawrence, Kansas

ACKNOWLEDGMENTS

The authors wish to acknowledge Herb Buxton of the Toxics Substances Hydrology Program and the U.S. EPA (IAG #DW14938589-01-0) that funded this research. We also wish to thank those who have joined and helped the new and loosely formed Emerging Contaminants Groups by volunteering to collect the samples needed to do this research.