

Extraction and Determination of Tetracyclines in Chicken Meat by Solid Phase Extraction and High Performance Liquid Chromatography

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Abstract— Antibiotics are powerful substances used to treat bacterial infection. When used properly, they have the capacity to save lives. However, it can also pose as a dangerous threat if taken regularly, even in trace amounts. It acts as a trigger for antibiotic resistance in bacteria, which renders common antibiotics useless. In this study, two tetracycline drugs, namely doxycycline and oxytetracycline were analyzed in locally sourced chickens from Bulacan, Philippines for antibiotic residues. HPLC analysis was done to determine the concentrations of the antibiotics. Standard calibration curves from concentrations of 100 ppm, 80 ppm, 40 ppm, 10 ppm, 1 ppm, 100 ppb and 10 ppb of the tetracyclines produced a linearity of 0.995 and 0.9994 for doxycycline and oxytetracycline, respectively. 19 chicken samples were tested: 2 spiked samples, 2 blank samples and 15 unknown samples. The spiked samples were able to confirm the antibiotic peak in the samples. However, the blank samples of doxycycline emitted the same chromatogram as the unknown samples which led to two variations in the computation for the concentration, uncorrected and corrected. The known samples had an average retention times of 13.9845 minutes for doxycycline and 7.090 minutes for oxytetracycline. Average uncorrected concentration for doxycycline is 1.1722 ppm and the average corrected concentration is 0.8384 ppm. Oxytetracycline was not found in the meat samples. LOD and LOQ is 0.2651 ppm and 0.8033 ppm for doxycycline, respectively and 2.821 ppm and 8.854 for oxytetracycline. Doxycycline concentrations were found to be above the regulation standards set by the EU.

Keywords: antibiotic resistance, tetracycline, chicken meat, HPLC analysis

I. INTRODUCTION

In the year 1929, the antibiotic Penicillin was discovered by a bacteriologist named Alexander Fleming. Penicillin was able to cure illnesses that were incurable at the time, such as gonorrhea, pneumonia, tuberculosis and urinary tract infection. Since its discovery, antibiotics have been exploited for human consumption, especially towards the field of livestock growth and wellness. Due to this unmonitored use of antibiotics, resistance build-up towards certain antibiotic drugs has become more evident throughout the years (Castro, J. 2014).

According to Chopra, I. & Roberts, M. “there has been an emergence of microbial resistance towards antibiotics, Tetracycline, due to excessive use in clinical practice”. Resistance build-up could be rooted from the medicine and food people intake on a regular basis. According to the World Health Organization (WHO), “the global production and consumption of chicken has risen from 58.9 million tons in

2000 to 74.2 million tons in 2007”. This spike in poultry production and consumption, may give rise to certain concerns for the maintenance and the upbringing of chicken meat.

In usual poultry preparations, the chicken is given chicken feed and most importantly, is given antibiotics. Tetracyclines are used for inhibiting tumor growth and inflammation; they are also growth promoters (Terin, M., Yildirim, I., & Ciftci, K., n.d.). It is ideal that the poultry is completely ridden off of all tetracycline residues; however, even through controlled usage of the substance, there could still be residues found in some chicken products.

Consuming small amounts of antibiotics on a daily basis could increase the risk of antibiotic resistance, making certain bacteria immune to future treatment. Attaining results, positive or negative, from testing chicken samples could allow consumers and farmers a better understanding of what they are consuming and distributing. According to Alexander Fleming, “*the time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.*” It is only a matter of time before our lack of understanding on self-medication with antibiotics harms the human species.

A. Resistance to Antibiotics

Antibiotic resistance is caused by bacteria adapting and changing in ways that gives them immunity towards the of antibiotics (Read, A., *et al.* 2014). Development of these antibiotic resistance bacteria is a direct result of the overuse of antibiotics. (CDC, 2013)

In bacteria, evolution of antibiotic resistance can begin by horizontal gene transfer (HGT), which allows trait transfer from different species of bacteria. Spontaneous mutation is also one factor in the development of antibiotic resistance. Antibiotics leave the drug-resistant bacteria and eliminate the drug sensitive bacteria leaving space for reproduction for the drug-resistant bacteria as a result of natural selection. (Bartlett, Gilbert, & Spellberg, 2013)

An in-depth understanding as to how antibiotic resistant bacteria can impact the human species is explained by Chopra, I & Roberts, M. (2001). During the 1950s a scientific study was done to evaluate the susceptibility of pathogenic and commensal bacteria towards tetracyclines. It was stated that only 2% of the 433 different types of *Enterobacteriaceae* was drug-resistant to tetracyclines. However, in the year 1953 the emergence of a tetracycline-resistant bacteria, *Shigella*

dysenteriae, was discovered (Akiba, T., K. Koyama, Y. Ishiki, S. Kimura, and T. Fukushima 1960). In the year 1955, certain strains of the bacteria, *Shigella*, became the world's first multiple drug resistant bacteria; it cannot be fought with tetracyclines, chloramphenicol and streptomycin. The rapid change in genetic mutation for certain bacteria, not only affects humans but animals, specifically livestock, as well.

In livestock, antibiotics are used primarily to promote growth and health by preventing infections. Continuous use of antibiotics in livestock is said to produce larger yields and higher quality products.

Treated livestock are ingested by humans when they consume these meat products. 35 years ago, the first case of transfer of resistant bacteria to humans from farm animal was discovered. Molecular detection methods have traced the cause to the consumption of meat products by humans.

One way to slow down the evolution of antibiotic resistance is by reducing antibiotic use which includes intensive monitoring of antibiotic use in livestock. However, literature search revealed no available study on veterinary drug residue in the Philippines. (Baldias, L., Divina, B., & Ducusin, R. J. 2013)

II. METHODOLOGY

A. Materials and Equipment

Oxytetracycline and doxycycline standards were supplied by a veterinary drug store with 98% purity. Methanol (CH₃OH) and acetonitrile (C₂H₃N) were of HPLC-grade by RCI labscan. Citric acid monohydrate (HOC(COOH)(CH₂COOH)₂ H₂O), disodium phosphate (Na₂HPO₄), oxalic acid (C₂H₂O₄) and ethylenediaminetetraacetic acid disodium salt (Na₂EDTA) were obtained from Sigma-Aldrich and were of analytical grade. Water used was Wilkins distilled water. Solid phase extraction (SPE) of Agilent bond elute C18 cartridge was used for the cleanup of extracts. The HPLC system of Agilent technologies 1200 series equipped with an auto-sampler set at 15- μ L, quaternary pump and a diode array detector was used. A reversed-phase analytical column, Restek pinnacle II C18. (250x4.6mm, 5 μ m) was used. The oven temperature was set at 25°C and UV wavelength was set to detect at 350nm. A flow rate of 1-mL/min was used. Clay Adam compact II centrifuge (3400 rpm), ultrasonic bath, and blender were used for sample preparation.

B. Optimization of Parameters

Parameters used for the HPLC analysis were based from a previous validation study done on HPLC parameters for tetracyclines (Shalaby, Salama, Abou-Raya, Emam, & Mehaya, 2011) and was modified and optimized by changing column from a Nuclosil 100 C18 to a Restek pinnacle II C18 and injection volume from 6 μ L to 15 μ L.

C. Sample Preparation

Chicken meat samples were cut and weighed at 5.00 ± 0.05 g into a 50-mL beaker. Two blank chicken samples were spiked with a combined working tetracycline stock solution 100- μ L of a 25ppm. Twenty milliliters McIlvaine Buffer-EDTA

solution were added into the beaker. Contents were homogenized in a blender for 30 seconds, then transferred back into the beaker plus washings. Samples were then transferred into labeled 15-mL centrifuge tubes. Each sample only used one centrifuge tube. Capped tubes were then centrifuged for 10 mins at 3200 rpm. The supernatant was poured into a second beaker. The process was repeated until all the contents from the initial homogenization had been centrifuged.

D. Solid Phase Extraction

Agilent bond elute C18 cartridge was used for the cleanup of extracts. The SPE cartridge was conditioned with 20-mL methanol followed by 20-mL water then the test samples. The eluate was discarded and the flash was cleaned. The set up was prepared again to elute the tetracyclines. 6.0-mL 0.01M methanolic oxalic acid was added in to the cartridge. The eluate was then transferred into a 10-mL volumetric flask and diluted to 10-mL with water.

III. RESULTS AND DISCUSSIONS

Doxycycline and oxytetracycline are antibiotics belonging to the tetracycline group which have found widespread use in the veterinary practice especially in poultry. Doxytetracycline can penetrate the tissue of poultry efficiently and its rate of elimination from the poultry's system is slow. Allowing poultry farmers to only administer the antibiotic once a day, making poultry production cost efficient.

Oxytetracycline was discovered in the 1940s along with chlortetracycline, and is considered one of the first generation tetracyclines. However, as time went by and the emergence of resistant bacteria increased, the use of oxytetracycline in veterinary practice slowly declined. (Ziolkowski, H., Grabowski., & *et.al.*, 2015)

A. Calibration Curve

Doxycycline registered a single strong signal with a retention time of 14.0 minutes Standard calibration curve was done for doxycycline using concentrations of 100ppm, 80ppm, 40ppm, 10ppm, 1ppm, 100ppb, and 10ppb. R² value obtained from doxycycline standards is 0.9995, revealing a satisfactory calibration curve for determining the concentrations in unknown samples. Equation of the line of the standard curve is $y = 35.513x - 7.9314$. Fig 1 shows the doxytetracycline calibration curve.

Oxytetracycline was discovered in the 1940s along with chlortetracycline, and is considered one of the first generation tetracyclines. However, as time went by and the emergence of resistant bacteria increased, the availability of oxytetracycline as a veterinary slowly faced out of poultry production. (Ziolkowski, H., Grabowski., & *et.al.*, 2015)

Oxytetracycline chromatogram showed a strong single peak with a retention time of 7.4 minutes. A standard calibration curve was likewise established for oxytetracycline which gave an excellent linearity R² value of 0.9994. The standard calibration curve yielded the equation of the line, $y = 22.212x - 1.8644$. Repeated analyses of the meat samples did not yield

any residues of oxytetracycline. These findings coincide with the fact that this antibiotic is hardly ever used these days.

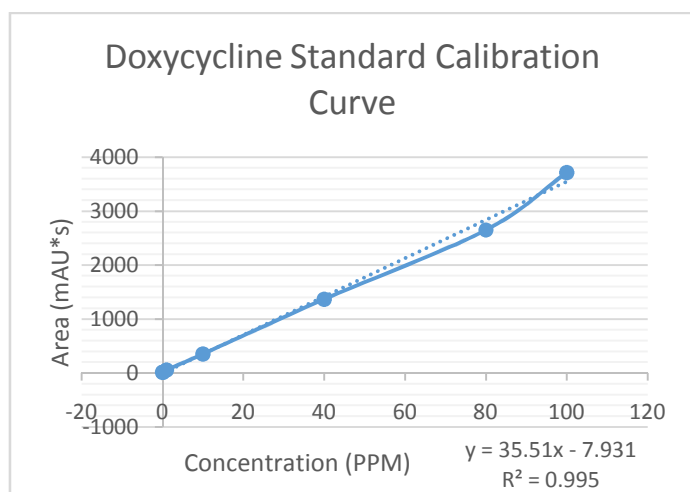


Figure 1. Standard Calibration Curve for doxycycline

B. Analysis of Chicken Meat Samples

A total of 19 chicken samples were analyzed for doxycycline content. Among the 19 samples, two were analyzed as blanks (chicken sources were not injected with antibiotics), two were spiked with doxycycline and the remaining 15 samples were analyzed via standard calibration.

The blank samples had peaks in the chromatogram with an average retention time of 14.3605 minutes and an average peak area of 11.8369. Considering the complex and selective separation method employed in the extraction of the analyte, these results suggest that the blank samples are positive for doxycycline despite the fact that they were not injected with the antibiotics. One possible explanation is that there are other avenues by which the antibiotic doxycycline reached the blank samples other than by injection. Certain food and vitamin preparations fed to the chicken could possibly contain certain amounts of the antibiotic. Invariably, the widespread use of the drug could have contaminated the farm which houses the stock. If this assumption is correct, then the amount of the analyte found in the blank corresponds to 0.5566 ppm doxycycline.

Table 1 summarizes all the important doxycycline results from the HPLC chromatogram with blanks that are assumed positive for doxycycline. The average retention time observed from the samples is 13.98 minutes, with a standard deviation of 0.1346, which implies that the retention time for all samples are consistent and are close to the expected retention time of doxycycline standard. The average peak area of the samples is 33.6958 mAU*s and the average concentrations of the unknown samples is 1.1722 ppm.

Comparing the results of this study with the Maximum Residue Limit n(MRL) of 0.1 ppm as set by the EU International Regulation Standard, the present study suggests that the samples used herein contain doxycycline in concentrations well beyond the acceptable residue limit for doxycycline in chicken muscle.

Table 1. Analysis of Doxycycline via HPLC

Samples	Retention Time Doxycycline (mins)	Area (mAU*s)	Doxycycline Concentration (ppm)
1 (spiked)	13.721	191.11278	5.604825838
2 (Spiked)	13.81	36.34571	1.246785966
3	13.769	45.25377	1.497625377
4	13.802	45.18587	1.495713401
5	13.808	44.958	1.489296877
6	13.835	44.787	1.484481739
7	13.877	45.11729	1.493782277
8	13.885	46.73358	1.539294906
9	14.042	47.52	1.561439473
10	14.086	16.51263	0.688312167
11	14.091	25.26919	0.934885535
12	14.097	25.54291	0.942593135
13	14.083	22.6	0.859724608
14	14.096	25.0442	0.928550108
15	14.106	23.32308	0.880085602
16	14.094	23.18507	0.87619942
17	14.096	24.40367	0.910513615
Average	13.98446667	33.69575067	1.172166549
Standard Dev.	0.134597743	11.77806982	0.331655163

IV. CONCLUSION

In summary, the results showed the effectiveness of the optimized SPE conditions in isolating the analytes as well as the optimized HPLC conditions in analyzing tetracycline antibiotic residues. The results of the study suggests that local chicken meat (which are available in our markets) can possibly contain large amounts of doxycycline residues in amounts much larger than the limits set by international standards.

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