INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0872/2021/26–6–741–749
DOI: 10.17957/IJAB/15.1890
http://www.fspublishers.org

Full Length Article

Depletion and Distribution Studies for Oxytetracycline in Broiler Chicken using Commercial ELISA with Subsequent Confirmatory Analysis by HPLC-UV

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Received 07 September 2021; Accepted 22 November 2021; Published 15 December 2021

Abstract

Tetracyclines are commonly used in livestock for the treatment of various diseases. Their residues above the maximum residual limit-MRL (200 μg kg^{-1}) can cause health hazard issues not only in humans but also in animals. For this purpose, efforts were made to study distribution and depletion profile of Oxytetracycline in broiler chicken by ELISA and HPLC. Regarding this, an experiment was conducted on healthy chickens of average weight 600 ± 20 g. Six broiler chickens were treated with formulation (OXTRA L.A.) containing Oxytetracycline dehydrate @20 ppk solution (150 μL) equivalents to recommended dose 20 mg kg^{-1} through intramuscular route, while control birds were untreated. Treated chickens were slaughtered at an interval of 1, 8, 16, 32, 64 and 120 h. Different tissue samples including liver, kidney, thigh and chest muscles were collected and screened by ELISA (Cat No. 5091TC, Euro Proxima). Lowest detection limit (IC_{20}) was calculated as 0.12 ng mL^{-1} and middle of the test (IC_{50}) 0.5 ng mL^{-1} with recovery 75 to 86%. Reverse phase liquid chromatography (HPLC-UV) method was also standardized by using range of standards from 25 to 200 μg kg^{-1}. Calibration curve showed good response with correlation coefficient (R^2) 0.9727 and recovery 85 to 93%. Overall, withdrawal period (< MRL) in all tissue samples was calculated as 117 h (4.8 days) by ELISA while 128 h (5 days) by HPLC-UV. Both validated methods can be further utilized to generate reliable data for food safety measures in Pakistan to enhance international trade. © 2021 Friends Science Publishers

Keywords: Oxytetracycline residues; Withdrawal period; Validation studies; ELISA; Liquid chromatography

Abbreviation: HPLC-UV: High Pressure Liquid Chromatography, Ultra Violet, ELISA: Enzyme-Linked Immunosorbent Assay, WHO: World Health Organization, MRM: Maximum Residue Limit, FDA: Food and Drug Administration, CODEX: Alimentarius Collection of Food Standards, JECFA: Joint FAO/WHO Expert Committee on Food Additives

Introduction

Food safety is of great importance these days, especially in a world where monitoring studies is favorable in search of safe food. Local people are unaware of ill practices used in antibiotics in livestock sector, several efforts are being made to reduce antibiotics use (Rana *et al*. 2019). This is due to non-existence of strict regulations and lack of food safety awareness to public. Antibiotics are most commonly used in poultry but hardly examined (Khatun *et al*. 2018). Their illegal use can increase the chances of food contamination instead of their benefits, *e.g*., creates resistance in human pathogens (Al-Gendy *et al.* 2014).

Antibiotics are widely used in animals to control diseases as well as growth promoter to increase meat, milk and egg production (Wang *et al.* 2011). The tetracyclines have applications for the treatment of infections in poultry, cattle, sheep, and swine. In some cases, for therapeutic treatment of large numbers of poultry reared on commercial farms, the antibiotics are added directly to feed or water or can be administered (Khatun *et al*. 2018). The presence of drug residues in animal-derived food can cause serious health issues. Due to this reason, food safety has become a serious concern all over the world (Biswas *et al.* 2007). The inappropriate and overuse of veterinary drugs has become a common practice in recent years (CODEX 2012).

The main group of drugs used in veterinary medicine include tetracyclines, amphenicols, aminoglycosides, macrolides, nitrofurans, nitromidazoles, sulfonamides and quinolones (Ang *et al*. 2017).

Tetracyclines are effective against Gram-positive and Gram-negative bacteria, including some anaerobes. Susceptible organisms include *Escherichia coli*, *Klebsiella* species, *Pasteurella* species, *Salmonella* species, and *Streptococcus* species. Tetracyclines are also active against chlamydia, mycoplasmas, some protozoan and several

To cite this paper: Mumtaz M, U Maqbool, MI Chughtai, MS Shah, G Hussain (2021). Depletion and distribution studies for oxytetracycline in broiler chicken using commercial ELISA with subsequent confirmatory analysis by HPLC-UV. *Intl J Agric Biol* 26:741–749

rickettsia. Drugs are mostly used in broiler chickens to treat chronic respiratory disease and infectious sinusitis, it is also suitable to enhance immune system in farm produce animals (Shahid *et al*. 2007; Wang *et al.* 2011).

Tetracyclines inhibit protein synthesis in both bacterial and human cells, the first members which were derived from the *Streptomyces* genus of *Actinobacteria*. Oxytetracycline and chlortetracycline show moderate lipid solubilities while doxycycline and minocycline show higher, so that they are able to traverse cell membranes moderately or readily (Wang *et al.* 2011). Tetracyclines after absorption bound to plasma protein to a limited extend, absorption percentage of tetracycline's in animal body is chlortetracycline 46–51%, tetracycline 28–41%, oxytetracycline 21–76% and doxycycline 84–92% (Riviere and Papich 2018).

Chlortetracycline and oxytetracycline both discovered in the late 1940s, were the first members of the tetracycline group to be described, are commonly used in livestock sector to control diseases. If their depletion profile studies are not conducted then their residues will remains in edible tissues (Widiastuti and Anastasia 2015). Overuse of these drugs can cause microbial resistance. Hence, non-susceptible organisms grow rapidly; this may result in colitis and severe diarrhea (Wang *et al.* 2011). About 20 to 90% of the drugs are not adsorbed and excreted (EU Regulation 2010; Muaz *et al*. 2018).

Local farmers are unaware of actual withdrawal period of the antibiotics so the residues persist in the meat beyond safe limit (Mund *et al.* 2017; Xu *et al.* 2019). The continual use of the antibiotics in poultry also increases resistance against antibiotics not only in animals but also in consumers (Khatun *et al.* 2018; Muaz *et al.* 2018). This leads to rapid change in genetic variation of certain beneficial bacteria which become dangerous for the human life and their presence continued in environment (Agha *et al*. 2003).

The World Health Organization (WHO) reported public health problems emerging from microbial resistance due to excessive use of antibiotics (Cinquina *et al*. 2003). The Food and Drug Administration (FDA) also set criteria for the approval of new antibiotics to perform risk assessment (Biswas *et al.* 2007; Khatun *et al.* 2018). Codex and FAO/WHO experts set maximum residue levels for tetracyclines are 0.2, 0.6 and 1.2 μg g^{-1} for poultry muscle, liver and kidney; respectively. In order to ensure the customers food safety, plans for monitoring of contaminants in food are set by Codex (Al-Gendy *et al*. 2014; CODEX 2018). Similarly, tetracyclines MRL is described as the combination of parent compounds and its metabolites in poultry products, set as 200 ng g^{-1} for meat, 600 ng g^{-1} for liver and 400 ng g^{-1} for eggs. A group ADI for tetracycline, oxytetracycline, and chlortetracycline has been allocated by JECFA. The MRL value is also set by CAC for tetracycline, oxytetracycline and chlortetracycline applicable to cattle, sheep, pigs, poultry and fish while for giant prawn only oxytetracycline (Wang *et al*. 2011).

According to EU MRL limits for tetracyclines is set as 100 μg kg^{-1} for muscles, 300 μg kg^{-1} for liver and 600 μg kg^{-1} for kidney (Commission Decision 2002; CODEX 2012). A strict surveillance system exists in the European Union by Council Directive 96/23/EC for screening of veterinary drug residue. Rapid alerts due to residues of antibiotics in broiler chicken and its products have been appeared in the market of Bangladesh, Indonesia, Oman and Philippines as their consignments are rejected by EU member states. Indonesia has also sets the MRL for oxytetracycline as 100 ng g^{-1} for meat and 50 ng g^{-1} for eggs through SNI No. 01–6366 2000 (Widiastuti and Anastasia 2015).

In Pakistan, oxytetracycline residues monitoring in Rawalpindi, Islamabad and Faisalabad regions was reported as 44.8% broiler chicken samples found positive as per recommendations of Joint FAO/WHO Committee of food additives (Shahid *et al*. 2007). Punjab Food Authority of Pakistan has also established a document for setting MRPL and MRL to control antibiotics by regular monitoring through different ministries. Screening of antibiotics through like commercial and in-house ELISA and subsequent confirmatory analysis of positive or selected samples by HPLC-UV is cost-effective to screen large number of samples (Cinquina *et al*. 2003). The present studies depict the procedure to determine withdrawal period of oxytetracycline in broiler chicken and drug distribution in different body parts of broiler chicken. The generated data can be applied to set withdrawal period of oxytetracycline. It can further contribute in giving awareness to the farmers and other stakeholders involved in this business as well as consumption.

Materials and Methods

Chemicals and reagents used

Trichloroacetic acid (Merck), Methanol (VWR), Oxalic acid (Sigma), nylon membrane PTFE membrane (0.45 µm), syringe filter (4 mm), PP housing, double distilled water, (from Cyclon Firsteem Automatic Ultrapure Water Still) Filtration Assembly, Sartorius, Ethyl acetate (VWR), n-Hexane (VWR), Polycons (50 mL capacity, VWR), Glass test tubes (Kimax), Separatory funnels, Erlenmeyer flask, Commercial ELISA Kits (Cat. # 5091TC, Europroxima, Netherlands), DMSO (Sigma), Trifloroacetic acid (Sigma) and Acetonitrile (VWR).

Experimental planning and execution

Experiments were conducted to determine drug profile *in vitro*. Regarding this, all requirements were fulfilled as mentioned in ARRIVE guidelines with the recommendations of NIAB animal house committee. Special considerations were taken to minimize the stressful conditions of the animals. A group of seven broiler chicken was grown under controlled conditions in NIAB Animal Farm House. Injectable Oxytetracycline products are stable as shown by the retention

of more than 90% potency for at least 24 month storage (Ang *et al.* 2017). In group-I (n = 6), which served as the treated or positive control, each bird received antibiotic-free water and feed until the end of the experiment. In the fifth week of the chickens' lives, a veterinary drug containing oxytetracycline dihydrate (OXTRA L.A. E.U.P 21.978 g equivalent to oxytetracycline 20 g-excipients q.s. 100 mL (Bologna, Italy) was administered @ 0.15 mL per kg body weight. One bird was taken as negative control that was non-treated. Drug was administered to each broiler chicken at right thigh and breast side through intramuscular route. Five treated chickens were slaughtered after 1, 8, 16, 32, 64 h while 6th after one week along with control. Selected tissue samples were collected and transferred to Food Safety Labs (ISO/IEC 17027:2017 accredited) of NIAB Faisalabad and stored at -20°C till further analysis.

Collection of samples

For distribution and depletion profile studies of Oxytetracycline, different tissue organs (liver, kidney, thigh and chest) were selected. From these selected organs, total 42 treated including 6 control samples were collected for analysis. Detail of samples is given in Table 1:

Preparation of standards and buffers

Stock and working standards: Lyophilized stock standard (2 μg kg^{-1}) was provided with ELISA kit. Tetracycline working dilutions were prepared including 0, 0.025, 0.125, 0.25, 0.5, 1.0 and 2.0 μg kg^{-1} with dilution buffer and stored at -20°C. A standard of 1000 μg kg^{-1} was also provided for spiking of negative control samples.
Dilution buffer: Prepared from 4X concentrate buffer by adding three times double distilled water.
Rinsing buffer: Prepared from 20X concentrate by dilution of 2 mL of buffer with 38 mL of double distilled water.
Sample dilution buffer: Prepared by adding 2 mL 100% methanol in 18 mL dilution buffer (mix and store at 4˚C).
Enzyme-linked conjugate: Concentrate centrifuged at 1000 rcf for 1 min before use, diluted with dilution buffer in proportion (10 μL:1 mL) and kept in dark until use.

Preparation of tissue samples

A sample weighing 1 g + 0.01 g was taken in 50 mL polycon tube by using analytical balance (ATX224, Schmadzu). Added 0.5 mL double distilled water and 1.5 mL of 100% methanol. After mixing for 15 min, samples were homogenized using tissue homogenizer (HG-15D, DAIHAN Scientific). Centrifuged (5430R, Eppendorf) for 5 min at 2000 rcf at room temperature. Supernatant 50 μL was transferred in glass tubes and mixed with 350 μL of sample dilution buffer. After vortex for 1 min, 50 μL of diluted samples was further used for assay development.

Assay development

As per layout plan (Table 2), ELISA was performed in the polystyrene 96 well microtiter plates (provided along the kit). For assay preparation, 50 μL of each standard and/or samples and enzyme conjugate were added. After rocking and mixing, sealed the microtiter plate and incubate for 1 h at 20–25ºC. After incubation, microtiter plates were washed three times with washing buffer using Microplate ELISA Washer (BioTek, ELx50). After washing, added 100 μL of substrate solution in all wells and incubate for 30 min at 20–25ºC in dark to develop colour. After incubation, added 100 μL stop solution (provided along kit) in each well, mixed gently by rocking the plate manually on top of the working bench and optical density was measured at 450 nm using Microplate ELISA Reader (BioTek. ELx808).

Calculations

The relative absorbance (RA) was calculated for both standards and samples by using the formula given below and Microsoft Excel was used to construct standards curve point by point. The RA of unknown samples was interpolated in standards curve to calculate the concentration of unknown samples.

$$\text{Relative absorbance (\%)} = \frac{\text{Absorbance of standards (or samples)}}{\text{Absorbance of zero standard}} \times 100$$

Confirmatory analysis

An HPLC system (HITACHI L-2000 series) equipped with UV-Vis detector (L-2420), column oven (L-2300) and auto-sampler (L-2200) was used for standardization and validation of oxytetracycline detection in tissue for analysis of selected positive tissue samples.

HPLC conditions

Agilent Zorbax Rx C8 Column (5 μm, 4.6 mm and 150 mm) was used with mobile phase 0.1% TFA: ACN (75:25 v/v and filtered by filtration assembly using filter AG 0.45 μm, Sartorius) at 252 nm wavelength and 1.0 mL per min flow rate. Column oven temperature was set at 26ºC.

Extraction and cleanup of samples

Sample weighing 5 g + 0.05 g muscle/tissue was transferred in 50 mL centrifuge tube and homogenized by for 30 s at 1000 rpm. Added 20 mL of 5% Trichloroacetic acid (TCA, Sigma), mixed it at 120 rpm for 20 min in shaker (Julabo, SW(22), Germany) and centrifuged at conditions 4000 rpm, 25ºC, 10 min. Took the supernatant without disturbing the tissue and filtered it using cellulose filter paper, repeated extraction in twice using 10 mL of 5% TCA. Supernatant from 3 extractions were collected and filtered through

Table 1: Details of Oxytetracycline treated selected tissue samples

Sampling matrix	No. of samples	Identification code	Sample quantity
Left Thigh Muscle	07	CHK_{LT}-18-001 to CHK_{LT}-18-007	50 g
Kidney	07	CHK_{K}-18-001 to CHK_{K}-18-007	50 g
Liver	07	CHK_{L}-18-001 to CHK_{L}-18-007	20 g
Right Thigh Muscle	07	CHK_{RT}-18-001 to CHK_{RT}-18-007	50 g
Right Breast Muscle	07	CHK_{RB}-18-001 to CHK_{RB}-18-007	50 g
Left Breast Muscle	07	CHK_{LB}-18-001 to CHK_{LB}-18-007	50 g

RT = Right Thigh, RB = Right Breast, LT = Left Thigh, LB = Left Breast, L= liver, K = kidney, SP = Spiked Broiler chicken

Table 2: Microtiter plate layout for tetracycline analysis by ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	$CK\text{-}1_{RT}$	$CK\text{-}1_{RT}$	$CK\text{-}1_{RB}$	$CK\text{-}1_{RB}$	$CK\text{-}1_{LT}$	$CK\text{-}1_{LT}$	$CK\text{-}1_{LB}$	$CK\text{-}1_{LB}$	$CK\text{-}1_{L}$	$CK\text{-}1_{L}$
B	S_0	S_0	$CK\text{-}2_{RT}$	$CK\text{-}2_{RT}$	$CK\text{-}2_{RB}$	$CK\text{-}2_{RB}$	$CK\text{-}2_{LT}$	$CK\text{-}2_{LT}$	$CK\text{-}2_{LB}$	$CK\text{-}2_{LB}$	$CK\text{-}2_{L}$	$CK\text{-}2_{L}$
F	$S_{0.0625}$	S_1	$CK\text{-}3_{RT}$	$CK\text{-}3_{RT}$	$CK\text{-}3_{RB}$	$CK\text{-}3_{RB}$	$CK\text{-}3_{LT}$	$CK\text{-}3_{LT}$	$CK\text{-}3_{LB}$	$CK\text{-}3_{LB}$	$CK\text{-}3_{L}$	$CK\text{-}3_{L}$
D	$S_{0.125}$	S_2	$CK\text{-}4_{RT}$	$CK\text{-}4_{RT}$	$CK\text{-}4_{RB}$	$CK\text{-}4_{RB}$	$CK\text{-}4_{LT}$	$CK\text{-}4_{LT}$	$CK\text{-}4_{LB}$	$CK\text{-}4_{LB}$	$CK\text{-}4_{L}$	$CK\text{-}4_{L}$
E	$S_{0.25}$	S_3	$CK\text{-}5_{RT}$	$CK\text{-}5_{RT}$	$CK\text{-}5_{RB}$	$CK\text{-}5_{RB}$	$CK\text{-}5_{LT}$	$CK\text{-}5_{LT}$	$CK\text{-}5_{LB}$	$CK\text{-}5_{LB}$	$CK\text{-}5_{L}$	$CK\text{-}5_{L}$
F	$S_{0.5}$	S_4	$CK\text{-}6_{RT}$	$CK\text{-}6_{RT}$	$CK\text{-}6_{RB}$	$CK\text{-}6_{RB}$	$CK\text{-}1_{K}$	$CK\text{-}1_{K}$	$CK\text{-}4_{K}$	$CK\text{-}4_{K}$	$CK\text{-}6_{L}$	$CK\text{-}6_{L}$
G	$S_{1.0}$	S_5	$SP\text{-}6_{RT}$	$SP\text{-}6_{RT}$	$SP\text{-}6_{RB}$	$SP\text{-}6_{RB}$	$CK\text{-}2_{K}$	$CK\text{-}2_{K}$	$CK\text{-}5_{K}$	$CK\text{-}5_{K}$	$SP\text{-}6_{L}$	$SP\text{-}6_{L}$
H	$S_{2\cdot0}$	$S_{2.0}$	$SP\text{-}6_{LT}$	$SP\text{-}6_{LT}$	$SP\text{-}6_{LB}$	$SP\text{-}6_{LB}$	$CK\text{-}3_{K}$	$CK\text{-}3_{K}$	$CK\text{-}6_{K}$	$CK\text{-}6_{K}$	$SP\text{-}6_{L}$	$SP\text{-}6_{L}$

cellulose filter paper in the separatory funnel. To remove fat from supernatants, mixed with n-hexane (VWR), then transfer lower layer in Erlenmeyer flask (Pyrex) and kept for sample loading. SPE cartridge assembly (Phenomenex) was used for cleanup of samples using cartridges, lower layer was passed over C18 SPE Cartridges (Strata C18-E, (55 μm, 70A, Phenomenex, 500 mg/6 mL). Cartridges were conditioned with 20 mL methanol and 20 mL double distilled water, the combined supernatant (treated sample) were loaded and desired analyte were eluted with 4 mL of 0.01 *M* Oxalic acid (Merck) in MeOH (VWR). The sample was dried using Evap. system having capacity 24 samples at a time (Romer labs, USA) at 35–40°C under vacuum. Re-constitute extract with 400 μL mobile phase 0.1% TFA: ACN (75:25) v/v Vortex and filter with 0.45 µm nylon membranes (PTFE membrane syringe filters, 4 mm, 0.45 μm) in sample injection vial (VWR) for injection to Amber glass vial 1.5 mL VWR HPLC grade for determination of oxytetracycline residues.

Preparation of calibration curve

Different oxytetracycline working standards (above and below the EU MRL) including 25, 50, 100 and 200 μg kg^{-1} were used for standardization and validation studies. Calibration curve was prepared between concentration and their respective areas to determine limit of detection (LOD) and limit of quantification (LOQ) for method.

Results

Temperature and weight of each animal was recorded twice a day throughout the experimental duration (one month). When animals gained average weight of 600 ± 20 g, then they were considered for the recommended dose as desired in experiment. Applied drug was also standardized with HPLC method with provided conditions and good response was observed. The declared period for Oxytetracycline in injectable (Hydrochloride salt, 20 ppk) dose was 5 days before slaughtering.

Screening of samples through ELISA

ELISA kit was standardized by calculating inhibition conc. IC_{20} and IC_{50} (criteria for test performance), relative absorbance found inversely proportional to the concentration of the tetracycline. A linear regression was obtained ($y = -20.86\ln(x) + 34.762$) with correlation ($r^2$) of 0.9996 as shown by Fig. 1. To check criteria for test performance, lowest detection limit (IC_{20}) and middle of the test (IC_{50}) were found to be 0.12 μg kg^{-1} and 0.5 μg kg^{-1}, respectively which is well below the maximum residue limit for tissue (200 μg kg^{-1}). According to Fig. 2, average concentrations of oxytetracycline in poultry tissues decreased gradually with time and after 117 h, residues were found well below the MRL value. The detectable residue concentrations were calculated as 4870, 4062, 3354, 3142, 1556 and 101 μg kg^{-1} at 1, 8, 16, 32, 64 and 120 h intervals, respectively. Results showed that declared period in proportion to our findings. These are not in accordance to Bangladesh due to may be difference in environmental conditions (Khatun *et al*. 2018).

In order to check the drug distribution profile, it was found that concentration of the left sides (breast/thigh) was lowered than right side of broiler chicken as shown by Table 3. Most important is that the treated samples were compared with non-treated and concentration was negligible and declared as negative control. It was cleared that the residues found in kidney and liver was more than other parts of body, this is because organs of metabolism and excretion were expected to have higher concentrations of these residues than breast and thigh tissue. In order to calculate recovery (%), known negative samples (tissue, kidney and liver) were spiked with different oxytetracycline concentrations below

Table 3: Oxytetracycline residue depletion in treated poultry by ELISA (n = 3)

Withdrawal time (h)	Animal No.	Concentration (μg kg^{-1}) in different chicken tissues					
		RB	LB	RT	LT	Kidney	Liver
1	1	2120	1680	2304	24	2020	2480
8	2	22000	2256	2200	2340	2600	2400
16	3	2200	1680	2400	2000	2600	2200
32	4	1500	1640	1632	1540	2160	1440
64	5	288	298	500	750	560	1481
120	6	150	97	101	144	540	580

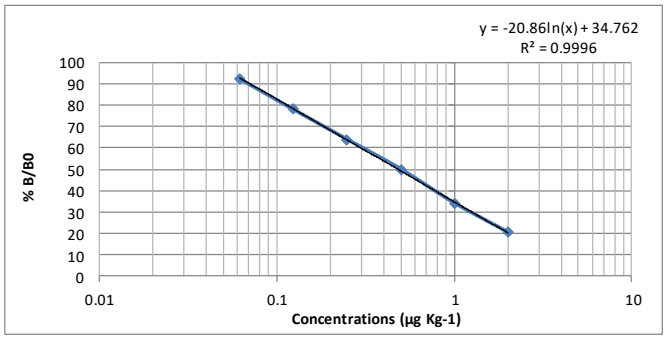

Fig. 1: Standard curve used for Oxytetracycline detection in chicken tissue by ELISA

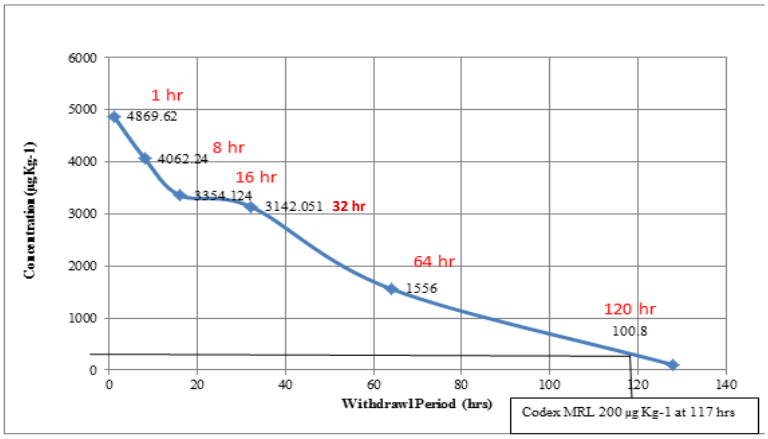

Fig. 2: Withdrawal period of Oxytetracycline in broiler chicken through ELISA

and above the MRL. Overall results indicated that recovery was calculated from 75 to 86% as shown in Table 4.

Standardization and validation by HPLC-UV

Before confirmatory analysis, standardization was done on at four different days by using HPLC UV-Vis detector. Good linearity of calibration curve was found through regression equation y = mx + C with ccorrelation coefficient (R^2) 0.9724 using different oxytetracycline working standards including 25, 50, 100 and 200 μg kg^{-1} (Fig. 3). Retention time was found as 1.85 ± 0.03 min and peaks area was found directly proportional to the standard concentration 25, 50, 100 and 200 μg kg^{-1} (Fig. 4). After 128 h, oxytetracycline residues were found less than MRL *i.e.*, 200 μg kg^{-1} as shown by Fig. 5 and reported values are in Table 5.

Discussion

This study relates to the public safety and awareness to the local farmers for safe handling of drugs. Oxytetracycline is being used as drug to enhance the growth of the poultry (Bosha *et al*. 2019). In order to meet the validation criteria, all parameters were followed as per 657/2002/EC guidelines (GL49 2015). The usage of the commercial drug in the animal then their residues still retained the body even till 120 h. The HPLC validated method employed for analysis is accurate and capable of determining the tetracyclines precisely over the concentration range 25 to 250 ppb.

The LOD value is minimum amount of the analyte detected, calculated as Xn+3SD under optimal conditions.

Table 4: Recovery calculations of different Oxytetracycline concentrations in known negative samples (n = 3)

Matrix	Spiking Conc. (μg kg^{-1})	Analysis by ELISA		Analysis by HPLC	
		Recovery (%)	CV	Recovery (%)	CV
Liver	100	75	5.4	85	3.8
	150	82	3.8	87	2.8
	200	85	4.5	92	3.8
Kidney	100	79	3.5	87	2.8
	150	85	5.4	86	6.7
	200	82	6.5	93	8.6
Muscle	100	82	3.2	86	3.8
	150	86	2.4	92	3.9
	200	75	1.5	85	5.4

CV: Coefficient of variation

Table 5: Oxytetracycline residues depletion in treated poultry by HPLC (n = 3)

Withdrawal time (h)	Animal No.	Concentration (μg kg^{-1}) in different chicken tissues					
		RB	LB	RT	LT	Kidney	Liver
1	1	7164	5844	7195	5150	1076	2786
8	2	5139	6095	4736	4820	763	2819
16	3	3619	2927	5783	4526	620	2848
32	4	1425	4421	4702	4341	995	1800
64	5	680	179	2800	280	890	1785
120	6	210	21	235	80	838	1741

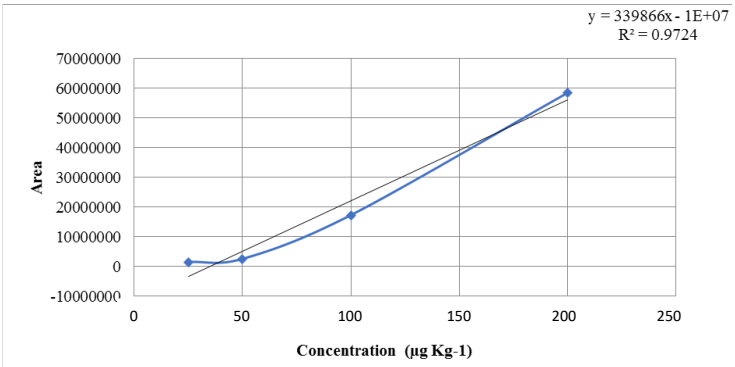

Fig. 3: Standard curve used for Oxytetracycline detection in chicken tissue by HPLC

Similarly, the LOQ is smallest quantity of analyte that can be measured with acceptable accuracy and precision (95% confidence level). The LOQ was found by calculating recovery and precision data *i.e.*, CV, and was defined as the lowest validated spike level meeting the requirements of a recovery was found from 85 to 93% by spiked samples with oxytetracycline by HPLC as shown by Table 6. LOD and LOQ of the method were found to be 16 and 25 μg kg^{-1} respectively. Signal to noise ratio was found to be 3:1 in acceptable range.

Most important findings are that the concentrations of the tetracycline were varied between slaughtered samplings. The possible reason is the reabsorption or recirculation of OTC in the birds' body to other organs or parts. As a target tissue bone tissue (breast/chest) were collected along with lever and kidney, detected OTC concentrations in treated broiler chicken bone tissue. It is also being reported that this were a more complex link takes place between the tissue and the rings of the basic tetracycline structure (GL49 2015; Odore *et al.* 2015). Most important is that the treated samples were compared with non-treated and concentration was negligible and declared as negative control (Khatun *et al.* 2018). It was cleared that the residues found in kidney and liver was more than other parts of body, this is because organs of metabolism and excretion were expected to have higher concentrations of these residues than breast and thigh tissue.

Further, it is very important to consider the interaction of the drug with the body, OTC is lipophilic with a large distribution volume, because of this reason the concentration in edible tissue was found higher (Mestorino *et al.* 2007). Further, it can be evaluated that by passage of time concentration in the body parts decreases but definitely it is continuously deposited in the manure/droppings. Literature provides evidence that droppings may be a route of contamination and dissemination of OTC residues in the environment indeed a great health risk for the soil microflora. Poultry birds were free to move in specified place and as a result dust in the air can result in the dissemination of OTC from treated to untreated birds. The long half-life of OTC and

Table 6: Recoveries of Oxytetracycline spiked matrices collected from control poultry (n = 3)

Matrix	Spiking Concentration (μg kg^{-1})	Analysis by ELISA		Analysis by HPLC	
		Recovery (%)	CV[a] (%)	Recovery (%)	CV[a] (%)
Liver	100	75	5.4	85	3.8
	150	82	3.8	87	2.8
	200	85	4.5	92	3.8
Kidney	100	79	3.5	87	2.8
	150	85	5.4	86	6.7
	200	82	6.5	93	8.6
Muscle	100	82	3.2	86	3.8
	150	86	2.4	92	3.9
	200	75	1.5	85	5.4

[a] Coefficient of variation

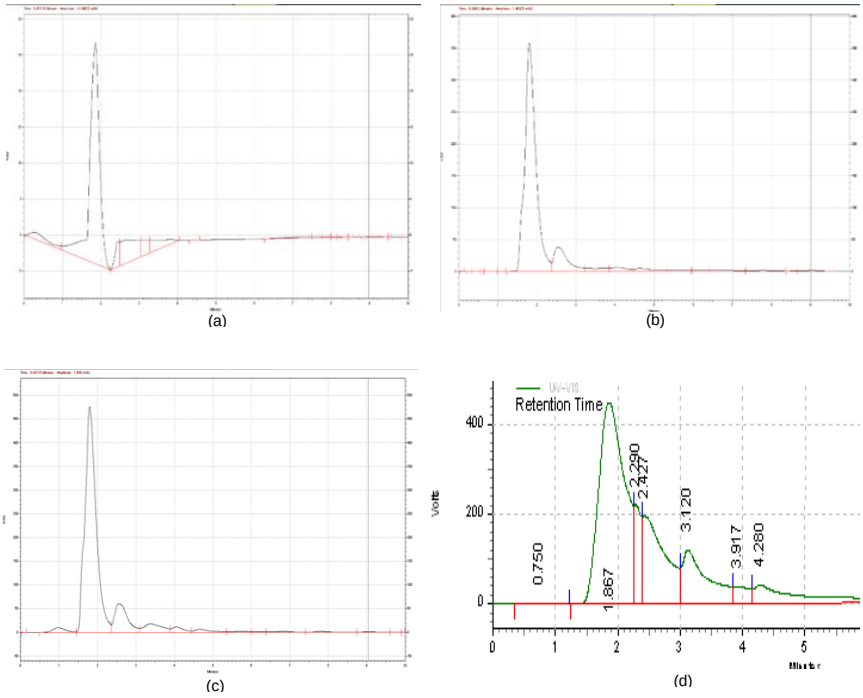

Fig. 4: Oxytetracycline chromatographs of standards and sample (**a**) 50 μg kg^{-1} (**b**) 100 μg kg^{-1} (**c**) 200 μg kg^{-1} and (**d**) Oxytetracycline treated tissue

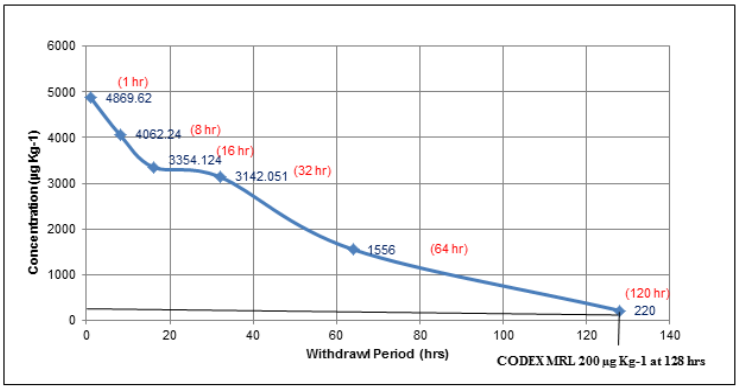

Fig. 5: Withdrawal period of Oxytetracycline in broiler chicken through HPLC

the use of broiler droppings to fertilize soil also present a risk for the transfer and persistence of OTC. It is a threat to the food cycle that excretion of Oxytetracycline bind to soil (Carballo *et al.* 2016; Pokrant *et al.* 2021).

Withdrawal period reported by Bangladesh research article is not resembled to our findings, it is due to difference

in environmental conditions. In comparison to Bangladesh, the withdrawal periods for oxytetracycline in poultry birds were found greater throughout the study period on all visited farms. That's why; they set the different MRP value to control illegal use of oxytetracycline. This means residue value of oxytetracycline can be used in marketed poultry which was found in the build-up relationship study as shown by the research article (Khatun *et al.* 2018). Regression equation was found to be $y=339866x=E^{+07}$ for confirmatory analysis, $R^2=0.9724$ as shown in Fig. 3 and chromatograms Fig. 4. Our findings are in accordance with other studies (Cinquina *et al*. 2003).

So, in our experiment maximum oxytetracycline residue values in marketed poultry were found after one hour followed by drug administration. The present finding is agreed by researchers (Agha *et al*. 2003). According to FARAD digest, withdrawal period in egg laying poultry for tetracycline were approximately more than 5 days in Ireland, Canada, US and Australia (Marmulak *et al.* 2015). Our results are in accordance with Mund *et al.* (2017) as withdrawal period was observed 5 days. However, only trace concentrations of OTC were detected in droppings and litter from sentinel birds. These findings establish the first evidence that there is a low likelihood of the transfer of OTC residues from treated birds to the environment and to untreated birds in adjacent or separate pens, which needs to be further studied.

Conclusion

This study provides the depletion and distribution of oxytetracycline in edible parts of broiler chicken like muscles, liver and kidney. Withdrawal period of oxytetracycline was calculated 117 h by ELISA while 128 h by HPLC. It is of great concern especially for the farmers to wait until the residues of the injected dose becomes less than the MRL. According to the study, it is strongly suggested that the need for more stringer regulatory authorities for the use of antibiotics in the poultry farms, as well as the survey of chicken for drug residues prior to marketing.

Acknowledgments

We are grateful to the International Atomic Energy Commission (IAEA) for providing commercial ELISA kits under Technical Cooperation Project RAS 5078 entitled "Enhancing food safety laboratory capabilities and establishing a network in Asia to control veterinary drug residues and related chemical components" to develop validated protocols for monitoring veterinary drug residues in different food matrices.

Author Contributions

MM planned experiments and conducted sampling, analysis, interpretation of results, and write up. UM contributed in planning of experiment. MIC and MSS contributed in the experiment execution and write up. GH helped during collection of samples and analysis.

Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Experiment was conducted at Animal House, NIAB with proper handling and as per ARRIVE guidelines.

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