Standard Operating Procedure

for

Detection of Oxytetracycline in Poultry Tissue by ELISA and HPLC

Copy No.	**01**
Issued to	**Lab Manager (LM)**

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1. INTRODUCTION

Tetracyclines are most commonly used antibiotics that inhibit protein synthesis in both bacterial and human cells; the first members of 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. Tetracyclines when injected administered, after absorption are bound to plasma protein to a limited extend. Literature shows absorption in animals is chlortetracycline (46–51%), tetracycline (28–41%), oxytetracycline (21–76%), and doxycycline (84–92%)

In the veterinary medicines tetracyclines are used for the treatment of skin bacterial, gastrointestinal, respiratory and systematic infections and locomotive organ diseases. Unfortunately veterinary drugs are misused by non-professionals due to lack of knowledge, poor animal health delivery systems, unsatisfactory extension activities, easy access to antibiotics like oxytetracycline and failure to observe the withdrawal period. These actions may contribute to the presence of high levels of antibiotic residues in meat, milk and environment. If the meat containing tetracyclines residues is consumed it can produce certain harmful effects in human such as fetus dysfunction, teeth discoloration, nails discoloration, exfoliative dermatitis, stomatitis, mutagenicity, carcinogenicity, bone marrow toxicity and increased bacterial resistance.

The World Health Organization (WHO) reported public health problems emerging from microbial resistance due to excessive use of antibiotics. The Food and Drug Administration (FDA) also set criteria for the approval of new antibiotics to

perform risk assessments. Codex, EU and FAO Maximum residue levels (MRL) set by FAO/WHO experts for OTC 0.2, 0.6, 1.2 µg/g, OTC residues exceeding this limit are dangerous to edible.

2. SCOPE

These standard Operating Procedures (SOP) describes the procedure for screening of tetracyclines in beef by Enzyme Linked Immunosorbent Assay, High-Performance Liquid Chromatography-Ultraviolet Detection (HPLC-UV) and to determine the withdrawal period of the chicken.

3. OBJECTIVE/PURPOSE

To ensure the accuracy, quality and reproducibility of the procedure / protocol.

4. DEFINITIONS AND ABBREVIATIONS

4.1. TC: Tetracycline

4.2. OTC: Oxytetracycline

4.3. TCs: Tetracyclines

4.4. HPLC: High-Performance Liquid Chromatography

4.5. ELISA: Enzyme Linked Immunosorbent Assay

4.6. TCA: Trichloroacetic acid

4.7. MeOH: Methanol

5. PRINCIPLE OF THE METHOD

5.1. ELISA

Samples were prepared by chopping, homogenizing and centrifugation with solvent. The microtiter plate based tetracycline ELISA consists of one plate (12 strips, 8 wells each) pre-coated with a specific antibody to tetracycline. Horseradish peroxidase labelled tetracycline (tetracycline-HRP conjugate), tetracycline (standard solution or sample) are added to the pre-coated wells. Tetracycline and the tetracycline-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation step of 1 hour, the non-bound reagents are removed in a washing step. The amount of bound tetracycline-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound tetracycline HRP conjugate transforms the colourless chromogen into a coloured product. The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the tetracycline concentration in the sample

5.2. HPLC

Samples were chopped, homogenized, vortexed, shacked and centrifuged with solvent for collection of supernatant. The extract was filtered by filter paper and shacked with n-Hexane to remove Fats for clean-up. After clean-up the solid phase extraction of extract was done with C18 column for separation of tetracyclines from other compounds. The extract was collected in Evaporation tube and then placed in evaporatory system for separation of tetracyclines from solvent. The extract was

reconstituted with 400 µL of mobile phase and the mixture was vortexed and filtered with 0.45 µm (syringe filters) nylon membrane for injection to HPLC. Extract was injected into the HPLC and analyzed. Tetracycline molecules are separated according to their polarities towards mobile phase and stationary phase.

6. SAFETY CONSIDERATIONS AND PRECAUTIONS

The chemicals used in these procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, TCs are considered a possible carcinogen to humans. Suitable gloves must be worn when chemicals/ TCs standards are handled, and the work shall be performed in a fume hood at all possible time.

7. MATERIALS AND EQUIPMENT

7.1. WATER

Unless stated otherwise, use only ultrapure water double distilled water or water of equivalent purity.

7.2. CHEMICALS

Unless stated otherwise, use only the reagents of recognized analytical grade

7.2.1.Methanol (CH_3OH), HPLC and LC-MS grade, VWR International

7.2.2.n-Hexane (C_6H_{14}), HPLC grade, VWR International, America

7.2.3.Sodium Dibasic (Na_2HPO_4), Sigma-Aldrich, Germany, ACS reagent

7.2.4.Oxalic Acid ($C_2H_2O_4$), Sigma-Aldrich, reagent grade

7.2.5.Trichloroacetic Acid (CCl_3COOH), Sigma-Aldrich, reagent grade

7.2.6. Double Distilled water (Fistreem International LTD, WSC044.MH3.7)

7.2.7.Trifloroacetic Acid (CF_3COOH), Sigma-Aldrich, Reagent Plus

7.2.8. Acetonitrile (CH_3CN), Sigma-Aldrich, Reagent grade

7.2.9. Oxytetracycline hydrochloride, Dr. Ehrenstorfer GmbH, Germany

7.2.10. Tetracycline hydrochloride, Dr. Ehrenstorfer GmbH, Germany

7.3. EQUIPMENT / APPARATUS

7.3.1. Bench top Centrifuge 5430 R (Eppendorf, Germany)

7.3.2. ELISA washer (BioTek ELx50)

7.3.3. ELISA reader (BioTek ELx808)

7.3.4. High Performance Liquid Chromatograph (Hitachi L- 2000 series, Agilent Open Lab software)

7.3.5. Water distillation unit (Fistreem International LTD, WSC044.MH3.7)

7.3.6. Electric Balance (Shimazdu, ATX224)

7.3.7. Vacuum pump (GAST, DOA-P504-BN)

7.3.8. Sonicator (Kerry-ultrasound LTD)

7.3.9. Vortex mixer (Wisd Vortex Mixer VM-10)

7.3.10. Solid phase extraction system (Discovery DSC 18-52603-U Supelco, USA)

7.3.11. Water bath (Buchi B-480 Water Bat)

7.3.12. Incufridge (Revsci, RS-IF-233 basic incufridge)

7.3.13. Tube shaker (Dream SI-300)

7.3.14. Homogenizer (Wisd HG-15D)

7.3.15. pH meter (Horiba, 502-S)

7.3.16. Fume Hood (Abaseen Fume Hood, 25/A)

7.3.17. Evaporator system (Romer EVAP Manual, EVAP 01.02)

7.3.18.Microwave oven (Dawlance, DW-B1HP)

7.3.19.Freezer (Sanyo Biomedical Freezer, MDF U-333)

7.3.20.Graduated cylinder (50, 250 and 1000mL, glass), Borosil.

7.3.21.Separating funnel (100mL, glass), Pyrex Japan

7.3.22.petri dishes (90×20 mm, glass)

7.3.23.Beakers (50-1000mL, glass), Pyrex Indonesia

7.3.24.Centrifuge tubes with caps (50ml) , VWR USA

7.3.25.Test tubes (13 x 99, round bottom) , Charm Sciences INC USA

7.3.26.Conical flasks (Erlenmeyer , Pyrex)

7.3.27.Micropipettes (100-1000µL) Eppendorf Reasearch Plus, Germany

7.3.28.Filter paper (Whatman)

7.3.29.SPE Cartridges (Supelco, Discovery DSC-18)

7.3.30.Syringe Filters, (Phenex, AFO-3102-12)

7.3.31.Screw cap tubes (15mL)

7.3.32.Spatula

7.3.33.Gloves

7.3.34.Scalpel

7.3.35.Tetracycline ELISA kit, Europroxima (5091TC), Netherland

7.3.36.Oxytetracycline ELISA kit, Europroxima (5091OTC), Netherland

7.3.37.Eppendorf tubes (l.5 mL) Eppendorf AG, Germany

8. SOLUTIONS

8.1. ELISA

8.1.1. Sodium Dibasic Solution

Sodium Dibasic solution (0.2 M) was prepared by mixing 28.4 g of Sodium Dibasic in 1 liter distilled water.

8.1.2. Dilution Buffer

Dillution buffer was 4x concentrated it was diluted 1:4 (1 mL buffer + 3 mL distilled water).

8.1.3. Sample Dilution Buffer

Sample dilution buffer was prepared by mixing 18 mL of dilution buffer (provided in oxytetracycline ELISA kit) with 2 mL methanol.

8.1.4. Oxytetracycline Standards (2.5 ng/mL)

Sample dilution buffer (2 mL) was added to oxytetracycline standards (provided in oxytetracycline ElISA kit) and mixed to prepare standard solution containing 2.5 ng of oxytetracycline per mL. Then dilutions of 1.25, 0.625, 0.3125, 0.156, and 0.078 ng/ mL were made.

8.1.5. Oxytetracycline Spiking Solution

Sample dilution buffer (1 mL) was added to oxytetracycline standards (provided in oxytetracycline ElISA kit) and mixed to prepare standard solution containing 100 ng of oxytetracycline per mL. The solution was used for spiking.

8.1.6. Tetracycline Standards (2 ng/mL)

Sample dilution buffer (2 mL) was added to tetracycline standards (provided in tetracycline ElISA kit) and mixed to prepare standard solution containing 2 ng of tetracycline per mL. Then dillutions of 1.0, 0.5, 0.25, 0.125, and 0.0625 ng/mL were made.

8.1.7. Tetracycline Spiking Solution

Sample dilution buffer (1 mL) was added to oxytetracycline standards (provided in oxytetracycline ElISA kit) and mixed to prepare standard solution containing 1000 ng oxytetracycline per mL. The solution was used for spiking.

8.1.8.Enzyme Conjugate Solution

The vial of lyophilized conjugate (horseradish peroxidase, HRP) was reconstituted with 6 ml of dilution buffer to prepare enzyme conjugate solution.

8.1.9.Rinsing Buffer Dilution

Rinsing buffer was 20 times concentrated (provided in ElISA kit) and it is diluted by mixing 2 mL of rinsing buffer with 38 mL of distilled water.

8.2. HPLC

8.2.1. Trichloroacetic Acid solution

Trichloroacetic Acid (5%) solution was prepared by mixing 50 gram TCA in 1000 mL double distilled water.

8.2.2. Oxalic Acid Solution

Oxalic acid solution (0.01 M) was prepared by mixing 0.63035 g oxalic acid in 500 mL MeOH.

8.2.3. Trifloroacetic Acid Solution

TFA solution (0.1%) was prepared by mixing 1.49 g of TFA in 1000 mL water.

8.2.4. Mobile Phase

Mobile phase was prepared by mixing 0.1% TFA and acetonitrile in a ratio of (75:25).

8.2.5. Standard Solutions

Stock standard solutions of TC and OTC compound were prepared by dissolving 10 mg of the compound in 10 mL of methanol to obtain a final concentration of (1 mg/ mL). Stock standard solutions were prepared in amber glasses to prevent photo-degradation and stored at – 20C and left to stabilize for at least 4 weeks. They were diluted with methanol to give a series of working standard solutions on the requirement. Chromatographic solutions for tetracyclines compound were prepared by dilution of the combined working solution with mobile phase.

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9. PROCEDURE

9.1. ELISA

Preliminary screening of beef samples (liver, kidney and muscle) for presence or absence (+/-) of tetracyclines was done by ELISA. Two separate kits of ELISA for tetracycline and oxytetracycline screening were used. The samples were prepared according to sample preparation protocol of tetracycline given in Europroxima (5091TC), Netherland booklet and sample preparation protocol of Oxytetracycline given in Europroxima (5091OTC), Netherland booklet. In brief, each sample (1 g) was taken in centrifuge tube and Mcllvain Buffer (3 ml) for TC and distilled water (0.5mL) and methanol (1.5 mL) for OTC was added to the tube. The tissue was homogenized, vortex it (10 min) and centrifuged at 2000 x g for 10 minutes (20-25°C). The supernatant (50 µL) was pipetted to Sample dilution buffer (200 µL) in Eppendorf tube and vortex it. This solution (50 µL) was used per well in ELISA. One sample of each (kidney, liver and muscle) was spiked with 600 µg/g, 300 µg/g and 100 µg/g TC and OTC.

Analysis of tetracycline and oxytetracycline was done according to manufacturer's instructions. The absorbance value was read at 450 nm immediately after adding the stop solution. The concentrations of TCs in the samples were calculated according to the percentage of their mean absorbance divided by the absorbance of the maximum binding (B/B0%) using the standard curve. The absorbance was expressed as percentage (%) and calculated using the following formula

$$\frac{\text{O.D. standards (or samples)}}{\text{O.D. Sample dilution buffer}} \times 100\% = \%\ \text{Maximal absorbance } (\%\beta_0)$$

The values were multiplied by dilution factors 24 to obtain the TC and OTC contents respectively in tissue samples samples. Distribution of standards and samples on kit is represented in form of kit layout Figure no. 1.

TC	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	$CKN\text{-}1_{RL}$	$CKN\text{-}1_{RL}$	$CKN\text{-}1_{RC}$	$CKN\text{-}1_{RC}$	$CKN\text{-}1_{LL}$	$CKN\text{-}1_{L}$	$CKN\text{-}1_{LC}$	$CKN\text{-}1_{LC}$	$CKN\text{-}1_{L}$	$CKN\text{-}1_{L}$
B	S_0	S_0	$CKN\text{-}2_{RL}$	$CKN\text{-}2_{RL}$	$CKN\text{-}2_{RC}$	$CKN\text{-}2_{RC}$	$CKN\text{-}2_{LL}$	$CKN\text{-}2_{L}$	$CKN\text{-}2_{LC}$	$CKN\text{-}2_{LC}$	$CKN\text{-}2_{L}$	$CKN\text{-}2_{L}$
F	$S_{0.0625}$	S_1	$CKN\text{-}3_{RL}$	$CKN\text{-}3_{RL}$	$CKN\text{-}3_{RC}$	$CKN\text{-}3_{RC}$	$CKN\text{-}3_{LL}$	$CKN\text{-}3_{R}$	$CKN\text{-}3_{LC}$	$CKN\text{-}3_{LC}$	$CKN\text{-}3_{L}$	$CKN\text{-}3_{L}$
D	$S_{0.125}$	S_2	$CKN\text{-}4_{RL}$	$CKN\text{-}4_{RL}$	$CKN\text{-}4_{RC}$	$CKN\text{-}4_{RC}$	$CKN\text{-}4_{LL}$	$CKN\text{-}4_{L}$	$CKN\text{-}4_{LC}$	$CKN\text{-}4_{LC}$	$CKN\text{-}4_{L}$	$CKN\text{-}4_{L}$
E	$S_{0.25}$	S_3	$CKN\text{-}5_{RL}$	$CKN\text{-}5_{RL}$	$CKN\text{-}5_{RC}$	$CKN\text{-}5_{RC}$	$CKN\text{-}5_{LL}$	$CKN\text{-}5_{L}$	$CKN\text{-}5_{LC}$	$CKN\text{-}5_{LC}$	$CKN\text{-}5_{L}$	$CKN\text{-}5_{L}$
F	$S_{0.5}$	S_4	$CKN\text{-}6_{RL}$	$CKN\text{-}6_{RL}$	$CKN\text{-}6_{RC}$	$CKN\text{-}6_{RC}$	$CKN\text{-}1_{K}$	$CKN\text{-}1_{K}$	$CKN\text{-}4_{K}$	$CKN\text{-}4_{K}$	$CKN\text{-}6_{L}$	$CKN\text{-}6_{L}$
G	$S_{1.0}$	S_5	$SCK\text{-}6_{RL(100)}$	$SCK\text{-}6_{RL(TC)}$	$SCK\text{-}6_{RC(50)}$	$SCK\text{-}6_{RC(TC)}$	$CKN\text{-}2_{K}$	$CKN\text{-}2_{K}$	$CKN\text{-}5_{K}$	$CKN\text{-}5_{K}$	$SCK\text{-}6_{L50}$	$SCK\text{-}6_{L50}$
H	$S_{2.0}$	$S_{2.0}$	$SCK_{LL(150)}$	$SCK_{LL(OTC)}$	$SCK_{LC(100)}$	$SCK_{LC(OTC)}$	$CKN\text{-}3_{K}$	$CKN\text{-}3_{K}$	$CKN\text{-}6_{K}$	$CKN\text{-}6_{K}$	$SCK\text{-}6_{L2}$	$SCK\text{-}6_{L2}$

Layout for tetracycline analysis by ELISA
RL = Right Leg, RC = Right Chest, LL = Left Leg, LC = Left Chest, L= lever, K = kidney, SCK = Spiked Broiler chicken

HPLC

HPLC was performed for the quantification and confirmation of positive sample from ELISA.

9.1.1. Sample preparation

Extraction, Filtration and Derivatization of meat sample was done by the following procedure**.** Frozen samples were thawed at room temperature for about 1 hour. Finely cut sample (1g) was taken in a centrifuge tube (50mL). 4 ml of 5% TCA was added to tube. The sample was homgenized at 1000 rpm for 20 seconds. 4 ml of 5% TCA was added to tube. The mixture was votexed for 1 minute. The mixture was shaked at 120 rpm for 20 minutes. The mixture was centrifuged at 4000 rpm for 10 minutes at 25C. The supernatant was collected. The step was repeated three times and three supernatants were combined. The supernatants were filtered in a separatory funnel by using a fiter paper. Fats were removed by adding and shaking 5 mL of n-Hexane to filtered supernatant in the separatory funnel. Lower layer was collected from funnel for solid phase extraction.

Clean-up by Solid Phase Extraction.

- C_{18} SPE cartidge was attached to vacuum manifold.
- Conditioning of cartridge was done by 5 mL methanol and 5 mL water.
- Sample was loaded.
- Washing was done with 5 mL ditilled water.
- Elution was done with 4 mL of oxalic acid (0.01 M oxalic acid in MeOH).

The extract was collected in Evaporation tube and then placed in evaporaotry system for evaporation at 35-40 C. The extract was reconstituted with 400 µL of 0.1%

TFA:ACN (75:25). The mixture was vortexed and filtered with 0.45 µm (syringe filters) nylon membrane for injection to HPLC.

9.1.2. Analysis

The mobile phase 0.1 % TFA:ACN (75:25) was used to carry sample and Isocratic Mode of HPLC was used. The Ultraviolet detector was set at 250 nm. Aliquotes of 400 µL of final extract were collected into the HPLC vials, (with capacity of 1.5 mL). 10 µL of extract was injected into the column and analyzed at 1.0 mL/min. Tetracyclines standard solutions of 25, 50, 100, 150 and 200 ppb were run through HPLC and standard curve at (average) 4.88 retention time was obtained. Oxytetracycline standard solutions of 25, 50, 75 and 100 ppb were run through HPLC and standard curve at (average) 1.817 retention time was obtained. Tetracylines residue in chicken samples extract were identified by matching peak retention time with standards retention time. Residues were quantified by comparing the peak area of samples with peak area of standards.

9.3. Experimental Procedure

Experiment conducted in animal farm house, Nuclear Institute for Agricultural and Biology and performed in Veterinary Drug Residues Laboratories (ISO-IEC17025:2005), NIAB (PAEC) Faisalabad. To determine drug distribution profile in broiler Broiler chicken six in numbers were grown under observation. LD vaccine was injected to safe the Broiler chicken. Drug available in local market named as OXTRA L.A., the drug found as parent compounds (e.g., oxytetracycline dihydrate) or as salts (e.g., oxytetracycline hydrochloride) [1] Injectable oxytetracycline

products are also very stable as shown by the retention of more than 90% potency for at least 24 month storage[22].

Oxytetracycline is excreted in form of its parent compounds and does not metabolized rather its 4-epimers are formed. Oxytetracycline is available as OXTRA L.A. each Broiler chicken was given a 0.75 ml injection to right leg and chest side, slaughter time was 1 hr, 8 hr, 16 hr and 36 hr, control and 1 Broiler chicken was slaughter after 1 wk. Samples were taken in polycon tubes and zip bags then properly labeled according to 17025:2005 coding schemes specie-year-no. Applied in lab. To avoid deterioration before analysis and stored at -20 °C in, Bio Medical Freezer SANYO MDF-U333. The samples were further analysed for Tetracyclines residues. Detail of samples is given below:

Sampling Matrix	No. of Samples	Identification Code	Sample Quantity
Broiler chicken Legs	06	CHK_{LL}-18-001 to CHK_{LL}-18-006	50 g
kidney	06	CHK_{K}-18-001 to CHK_{K}-18-006	50 g
Lever	06	CHK_{L}-18-001 to CHK_{L}-18-006	20 g
Right Leg	06	CHK_{RL}-18-001 to CHK_{RL}-18-006	50 g
Right chest muscle	06	CHK_{RC}-18-001 to CHK_{RC}-18-006	50 g
Left chest muscle	06	CHK_{LC}-18-001 to CHK_{LC}-18-006	50 g

REFERENCES

AOAC Official Methods of Analysis. (2012). Appendix F: Guidelines for standard method performance requirements.